IDENTIFICATION AND CHARACTERIZATION OF CLF-1, A NOVEL MEMBER OF THE CYTOKINE TYPE-I RECEPTOR FAMILY

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GREG ELSON

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CONTENTS

ABSTRACT

Cytokines are small soluble proteins regulating cell function via specific transmembrane receptors located on the cell surface. These receptors are grouped into families according to structural criteria. Members of the cytokine type-I receptor family are characterized by a conserved extracellular domain of approximately 200 amino acids known as the cytokine receptor-like domain, which has been shown to play an important role in receptor function.

This report describes the identification, cloning and initial characterization of human and murine cytokine-like factor 1 (CLF-1). The sequences were identified from expressed sequence tags (ESTs) using amino acid sequences from conserved regions of the cytokine type-I receptor family. Human and murine CLF-1 have 96% amino acid identity, and share significant homology with members of the cytokine type-I receptor family within the cytokine receptor-like domain. CLF-1 appears to exist as a soluble protein, suggesting that it is either a soluble subunit within a cytokine receptor complex, such as is exhibited by the soluble form of the IL-6 receptor α chain, or as a subunit of a multimeric cytokine such as the IL-12 p40 subunit.

In human tissues, the highest levels of CLF-1 mRNA were observed in the lymph node, spleen, thymus, appendix, placenta, stomach, bone marrow and fetal lung. In murine tissues, CLF-1 was predominantly expressed in embryonic tissue and adult lung. Constitutive expression of CLF-1 mRNA was detected in a human kidney fibroblastic cell line. In human fibroblast primary cell cultures, CLF-1 mRNA was upregulated by the proinflammatory cytokines TNF-α, IL-6 and IFN-γ.

Western blot analysis of recombinant forms of hCLF-1 showed that the protein had a tendency to form covalently linked complexes. In addition, a counterstructure for CLF-1 was detected on two human cells lines, one of B cell origin and one of fibroblast origin. The counterstructure was also seen to be upregulated on purified monocytes in response to several proinflammatory cytokines, especially IFN-γ.

These results suggest that CLF-1 is a novel soluble cytokine receptor subunit or part of a novel cytokine complex, with evidence suggesting that it plays a regulatory role in the immune system and during fetal development.

DECLARATION

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of the University of Manchester or any other university or institute or learning.

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ABBREVIATIONS

aa	amino acid
Ab	Antibody
ATCC	American Type Culture Collection
βc	common β chain
bp	base pair
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
cDNA	complementary DeoxyriboNucleic Acid
CLF-1	Cytokine Like Factor-1
CNTF	Ciliary NeuroTrophic Factor
CT-1	CardioTrophin-1
DMEM	Dulbecco's Modified Eagle's Medium
dNTP	deoxyNucleoside Tri-Phosphate
d.p.c.	days post coitum
EBI-3	Epstein Barr virus Induced gene-3
EDTA	Ethylene Diamine Tetra-acetic Acid
ELISA	Enzyme Linked ImmunoSorbant Assay
EST	Expressed Sequence Tag
FACS	Fluorescence Activated Cell Sorting
FCS	Fetal Calf Serum
γc	common γ chain
G-CSF	Granulocyte-Colony Stimulating Factor
GH	Growth Hormone
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
GPI	Glycosyl-Phosphatidyl-Inositol
^{125}I	Iodine 125
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
Jak	Janus Kinase

kb	kilobase
kD	kiloDalton
LIF	Luekemia Inhibitory Factor
LPS	LipoPolySaccharide
mAb	monoclonal Antibody
2-ME	2-MercaptoEthanol
MFI	Mean Fluorescence Intensity
mRNA	messanger RiboNucleic Acid
NK	Natural Killer
OD	Opitcal Density
ORF	Open Reading Frame
OSM	OncoStatin M
^{32}**P**	Phosphorous 32
PBL	Peripheral Blood Leukocytes
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PMA	Phorbol Myristate Acetate
RACE	Rapid Amplification of cDNA Ends
rs	recombinant soluble (i.e. rshCLF-1)
s	soluble (i.e. sIL-6Rα)
^{35}**S**	Sulphur 35
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	SDS-PolyAcrylimide Gel Electrophoresis
STAT	Signal Transducer and Activation of Transcription
TAE/TBE	Tris-Acetate-EDTA/Tris-Borate-EDTA
Th	T helper
TNF	Tumour Necrosis Factor
UTR	UnTranslated Region
w/v	weight for volume

CHAPTER 1. INTRODUCTION

(1.1) Cytokines and Their Receptors

Intercellular communication is often mediated by small soluble factors, designated by the terms cytokines or growth factors. These soluble mediators convey a signal to their target cell via high affinity single or multiple chain cell surface receptors. They are expressed by a wide variety of cell types, and play a role in a very broad spectrum of physiological responses, including amongst others immune regulation, neuroregulation, hematopoiesis, angiogenesis and tissue development.

Cellular responses to cytokines typically involve positive or negative regulation of gene expression, subsequently regulating cell proliferation, differentiation, maturation and localization. These cellular responses are often controlled by a number of different cytokines, suggesting that a sophisticated regulatory control exists to achieve precise responses for a given target cell. A good example of this subtle regulatory control is shown by the haematopoetic system, where a fine balance in cytokine signalling controls the survival, self-renewal and differentiation of hematopoietic stem cells (reviewed in Ogawa, 1993; Metcalf, 1989; Metcalf, 1993).

The complexity of the cytokine network is further increased by the observation that many of the cytokine characterized thus far can exhibit pleiotrophy (i.e. have a different effect on the same or two different target cell types) and that two different cytokines can exhibit redundancy (i.e. both having the same effect on a given target cell). These phenomena are perfectly demonstrated by the structurally related cytokines interleukin 6 (IL-6), interleukin 11 (IL-11) leukemia inhibitory factor (LIF), oncostatin M (OSM) and ciliary neurotrophic factor (CNTF). All five soluble factors are capable of promoting the growth of myeloma cells as well as inducing hepatic acute phase proteins (reviewed in Taga and Kishimoto, 1997), thus demonstrating both pleiotrophy and redundancy. In fact, IL-6 has so many seemingly unrelated biological functions that it was originally given names ranging from interferon-β_2 (Weissenbach *et al.*, 1980; Zilberstein *et al.*, 1986) B cell stimulatory factor 2 (BSF-2; Hirano *et al.*, 1985), hybridoma growth factor (Van Snick *et al.*, 1986; Brakenhoff *et al.*, 1987), hepatocyte-stimulatory factor (Gauldie *et al.*, 1987), hematopoietic factor (Ikebuchi *et al.*, 1987) and cytotoxic T cell differentiation factor (Takai *et al.*, 1988).

The classification of this diverse network of soluble mediators and their receptors into families based on structural and sequence similarity has helped in the understanding of some of this complexity. The well defined families involved in hematopoietic and immune regulation include the tumour necrosis factor (TNF) superfamily, immunoglobulin (Ig) family members, chemokines, interferons and the hematopoetic growth factors.

(1.1.1) The TNF Superfamily

The fast-growing family of TNF-related molecules include the rather familiar cytokines tumour necrosis factor-α (TNF-α), lymphotoxin-α (LT-α or TNF-β), lymphotoxin-β (LT-β), CD40 ligand (CD40-L) and FAS ligand (FAS-L). They are all type II transmembrane proteins (i.e. an intracellular N terminus) and are characterized by an extracellular motif of 2 β sheets known as the 'jelly roll β sandwich motif' (Jones *et al.*, 1989, Eck and Sprang, 1989, Eck *et al.*, 1992). They also have the tendency to exist as homotrimers, although LT-α and LT-β can form a heterotrimer, and most members of the family can be processed to give active soluble forms. The members of the family share several overlapping functions, such as signalling for proliferation and apoptosis, although unique activities are exhibited by some. (reviewed in Armitage, 1994).

(1.1.2) The Receptors of the TNF Superfamily

This family of structurally related type I (i.e. intracellular C terminus) glycoproteins are characterized by an extracellular motif of three to four cysteine rich repeats of approximately 40 amino acids. They share a certain level of amino acid sequence identity, especially within the cysteine rich domain where homology between the family members is approximately 25%. The domain is characterized by the presence of either four or six cysteine residues in a conserved pattern, suggesting that they form disulphide bridges that are essential to the overall structure (Mallett and Barclay, 1991). Many of the TNF receptor family members exist in both soluble and membrane bound forms, and similar to their ligands, form tri- or multimeric complexes. It has

been suggested that the ligands and their receptors undergo 'clustering' during signal transduction (discussed in Cosman, 1994 and Gruss and Dower, 1995). Figure 1.1 demonstrates schematically the interaction between TNF-α and its type II receptor (TNFRII) leading to signal transduction. The receptor complex induced by TNF-α in this particular example is typical of this family of cytokines.

(1.1.3) Immunoglobulin Family Members

Probably the best known members of this family are the interleukin 1 (IL-1) receptors, IL-1RI and IL-1RII and the IL-1 receptor accessory protein (IL-1RAcP; Sims *et al.*, 1988; McMahan *et al.*, 1991; Greenfeder *et al.*, 1995). IL-1 is a highly inflammatory multifunctional cytokine, affecting a broad range of cell types. The three IL-1 receptors are type I glycoproteins composed of three immunoglobulin (Ig)-like domains (Williams and Barclay, 1988). The receptors also share significant sequence homology. IL-1RI and IL-1RAcP are responsible for signalling upon complex formation with IL-1, inducing the MAP kinase signalling pathway. IL-1RII binds IL-1, but has no signal transduction capacity, and therefore acts in an antagonistic fashion (reviewed in Dinarello, 1996). The ligand IL-1 itself has three family members, IL-1α, IL-1β and IL-1 receptor antagonist (IL-1Ra). IL-1α and β are receptor agonists, whilst as the name suggests, IL-1Ra has the opposite effect on receptor function. They share both amino acid and structural similarities, making β sheet molecules forming open barrel-like three dimensional structures (Labriola-Tompkins *et al.*, 1993; Grutter *et al.*, 1994; Priestle *et al.*, 1989; Graves *et al.*, 1990; Vigers *et al.*, 1994,). The complex model for IL-1/IL-1 Receptor component interactions is outlined in Figure 1.2.

The fibroblast growth factor (FGF) receptors are type I glycoproteins belonging to this family but are involved in signal transduction during different stages of development (reviewed in Wilkie *et al.*, 1995). The receptors interact with a family of 9 FGFs as well as with heparin sulphate oligosaccharides, and are composed of three Ig-like domains in their extracellular portion with an intracellular tyrosine kinase domain. The intrinsic receptor kinase activity of the FGF receptors is the principal difference between the FGF receptors and the IL-1 receptor signalling complex. The FGFs themselves predictably share structural homology with the IL-1 molecules,

making β sheet molecules which form open barrel-like three dimensional structures (Murzin *et al.*, 1992). In addition, both the FGFs and IL-1s lack signal peptides. It is therefore believed that IL-1 and FGF may have evolved from a common ancestral gene.

(1.1.4) Chemokines

The chemokines are a rapidly expanding family of small (8-10 kD), inducible proinflammatory cytokines acting mainly as chemoattractants and specific leukocyte activators (reviewed in Baggiolini, *et al.*, 1997; Mackay, 1997). They are grouped into 4 subfamilies depending upon the conservation of the cysteine residues within their amino acid sequence. The CC chemokines have their first two cysteine residues adjacent, whilst the CXC chemokines have an amino acid separating the first two cysteines. These chemokines can be further subdivided depending on the presence or absence of an ELR motif directly upstream of the first conserved cysteine residue. The CX_3C chemokines have three amino acids between the first two cysteines, whilst the C chemokines lack the first and third of the four conserved cysteines. In general, CC chemokines are chemotactic for monocytes, T cells, B cells, NK cells, dendritic cells, eosinophils and basophils. The ELR/CXC family members are chemoattractants for neutrophils, whilst the non-ELR/CXCs are chemoattractants for lymphocytes (reviewed in Baggiolini *et al.*, 1997).

(1.1.5) Chemokine Receptors

Chemokine receptors belong to the large family of G protein-coupled, 7 transmembrane receptors (Power and Wells, 1996). Not suprisingly, the receptors are grouped into families according to their ligand binding capacities. The CC chemokine receptors (1 to 8) bind the CC chemokines whilst the CXC chemokine receptors (1 to 4) bind the CXC chemokines. To date, only one CX_3C chemokine receptor has been identified and only one receptor, the duffy blood group antigen, has been shown to bind both CC and CXC chemokines. The large amount of redundancy between different chemokines in the same subfamily is accounted for by overlapping ligand specificity within the family of chemokine receptors. This is particularly evident

amongst the CC chemokines. Rantes for example, is known to bind and signal through CCR-1, CCR-3, CCR-4 and CCR-5 whilst MIP-1α uses CCR-1, CCR-4 and CCR-5. This is less evident amongst CXC chemokines, although interleukin 8 (IL-8) signals through both CXCR-1 and CXCR-2. A generalized structure for chemokine receptors is shown in Figure 1.3.

(1.1.6) Interferons

The interferon (IFN) family of cytokines are subdivided into the type I IFNs (IFN-α, IFN-β and IFN-ω), and type II IFN (IFN-γ). The type-I interferons, defined by their ability to induce a cellular antiviral, antiproliferative state, are comprised of 13 IFN-α subtypes, with a single IFN-β and IFN-ω species. They have 20% overall sequence homology, giving an identical secondary and tertiary folding pattern for each protein. Three dimensionally, they form a bundle of 5 α-helices. Although the type I IFNs share the same receptor complex (described below), the different subtypes have different biological effects, accounted for by subtle sequence heterogeneity leading to structural individuality (reviewed in Kontsek, 1995).

Type II IFN, or IFN-γ, is a general immunoregulator expressed under certain conditions by T helper cells of the Th1 subset, activated CD8+ cytotoxic T cells and natural killer (NK) cells. It serves as an antiviral and antibacterial agent, controls cell proliferation and apoptosis, regulates immunoglobulin production and class switching and also induces the expression of a multitude of genes including the major histocompatibility complex (MHC) class I and II molecules, Fc receptors and several cytokine and cytokine receptor genes (reviewed in Boehm *et al.*, 1997). The mRNA for IFN-γ encodes a 16 kD glycoprotein expressed as a non-covalently linked homodimer. Each dimer contains 6 parallel α-helices (Ealick *et al.*, 1991).

(1.1.7) Interferon Receptors

IFN-α, β and ω share the same receptor, comprised of the signalling and binding subunits, IFNαRI and II. The 51 kD type I glycoprotein IFNαRII is the major ligand binding component, whilst the 115 kD type I glycoprotein IFNαRI is essential but not sufficient for a type I IFN response. Signalling occurs when the two chains are associated in the presence of the ligand, and is suspected to involve the Jak1 and Tyk2 kinases (Cohen *et al.*, 1995), activating the transcription factors STAT 1α and STAT1β, as well as STAT 2 (Fu, 1992a, Fu *et al.*, 1992b; Schindler *et al.*, 1992a; Schindler *et al.*, 1992b).

The IFN-γ receptor also comprises two subunits, IFNγRα and IFNγRβ. The α chain is a 90 kD type I glycoprotein and has high affinity binding properties with IFN-γ and is associated with Jak1. The 314 aa β chain is essential for signalling, and associates with Jak2. For signalling to occur, the IFN-γ homodimer binds to two α chains, which in turn dimerize (Fountoulakis *et al.*, 1992; Greenlund *et al.*, 1993), leading to β chain association followed by Jak activation. This in turn causes STAT 1α homodimerization (Greenlund *et al.*, 1993) which translocates to the cell nucleus.

Although there exists no striking homology between the receptor subunits, JF Bazan has demonstrated that through conservation of amino acid positions and structural alignments, the interferon receptor subunits show significant architectural homology in their binding domains, suggesting that a family of receptor subunits exists, now known as the cytokine type II receptors. They are characterized by a domain of approximately 250 aa residues, containing four conserved cysteine residues, having the potential to form two critical disulphide bridges in the N and C termini of the domain. The domains also appeared to be composed principally of amphiphatic β strands (Bazan, 1990b). A certain amount of similarity was also found between the type II receptor extracellular domain and the analogous domain of the type I cytokine receptors (Bazan, 1990a; Bazan, 1990c). This will be discussed below. The interaction between IFN-γ and its receptor complex is represented in Figure 1.4.

(1.1.8) The Hematopoeitic Growth Factors

This is the most diverse cytokine family, but owing to a very limited amount of sequence homology, they were originally classified in terms of their function, with the family beginning to be redefined owing to homology identified within the receptors (discussed below). Members of the family have an incredibly diverse and complex array of physiological functions, ranging from embryonic development to neuroregulation and immunoregulation. Upon close inspection however, most of these growth factors do actually show a remarkably similar structure. Three dimensional studies of several of these cytokines including growth hormone (GH; Abdel-Meguid *et al.*, 1987), interleukin 2 (IL-2; Brandhuber *et al.*, 1987), interleukin 4 (IL-4; Redfield *et al.*, 1991; Powers *et al.*, 1992; Walter *et al.*, 1992; Wlodaver *et al.*, 1992), granulocyte macrophage-colony stimulating factor (GM-CSF; Diederichs *et al.*, 1991) and interleukin 5 (IL-5; Milburn *et al.*, 1993) have revealed that they contain a common fold. This consists of a so called four α-helix bundle in which the first and second helices and third and fourth helices run parallel to one another and are connected by long overhanging loops (Figure 1.5). It has been proposed that the two helical faces of the protein are responsible for receptor engagement. This has been demonstrated by the co-crystalization of GH and its receptor, and the solution of its structure. It was seen that two GH receptor chains simultaneously contact distinct sides of the monomeric GH (Figure 1.6b; de Vos *et al.*, 1992).

(1.1.9) Hematopoietic Growth Factor Receptors

When taking into account the considerable structural homology evident amongst the hematopoietic growth factors, it comes as no suprise that the extracellular domain of their receptors also share architectural similarity and a significant degree of sequence homology. A family of related type I transmembrane glycoproteins with the potential to bind hematopoietic growth factors was first described by JF Bazan (Bazan, 1989). A common binding domain was identified in the extracellular domain of the receptors for GH, prolactin, erythropoietin (EPO), IL-6 and the IL-2 R β chain where the absolute conservation of four cysteine and one tryptophan residue, in a C_1- $X_{9/10}$-C_2 - X-W-$X_{26/32}$-C_3-$X_{10/15}$-C_4 motif was identified. A year later, Bazan (Bazan, 1990a;

Bazan, 1990c) had further characterized the binding domain into a 200 aa region, dividing it into a N-terminal (membrane distal) half containing the four cysteine motif and a C terminal (membrane proximal) half containing another highly conserved motif containing the residues W-S-x-W-S. He also noted that each half of the domain, now known as the N-terminal and C-terminal cytokine receptor-like domain, was related to a common 90 amino acid structure known as a fibronectin type III domain (Ruoslahti, 1988).

Certain structural similarities between the binding domains of the type I and type II receptor families was also identified. A canonical receptor binding segment was predicted for both receptor types, with each of the two extracellular domain folds comprising an antiparallel β-sandwich comprising seven β strands (Figure 1.6a). This configuration was confirmed for the type I receptors by the determination of the crystal structure of the GH/GH receptor (GHR) complex (Figure 1.6b; de Vos *et al.*, 1992), demonstrating the importance of the cytokine receptor-like domain in ligand binding. The highly conserved four cysteine and W-S-x-W-S residues are though to be essential for correct folding and functioning of the cytokine receptor-like domain (Yawata *et al.*, 1993). This was probably best demonstrated by the finding that a dwarf chicken strain (Leghorn) which has a phenotype similar to the human growth hormone (GH) deficiency condition Laron dwarfism, has a deficiency in GH receptor (GHR) function. Sequencing of the Leghorn chicken GHR gene revealed a point mutation changing a serine to an isoleucine in the W-S-x-W-S motif. Cloning and expression of the mutant cDNA revealed impaired plasma membrane expression and ligand binding (Duriez *et al.*, 1993).

Many of the type I cytokine receptors also possess an Ig-like domain situated at the N-terminus of the protein. Although it has been established that the cytokine receptor-like domain plays an essential role in ligand binding, the role of the Ig-like domain is unclear, with different results obtained from studies with different receptors. Two examples of such contradictory evidence have been reported for the granulocyte-colony stimulating factor (G-CSF) receptor (G-CSFR) and the IL-6 receptor α chain (IL-6Rα). Hiraoka *et al* (Hiraoka *et al.*, 1995) and Layton *et al* (Layton *et al.*, 1997) reported that the Ig like domain was important in both receptor oligomerization and

ligand binding. The approach of Hiraoka *et al* was to generate portions of the extracellular region of the receptor corresponding to the Ig-like domain and the N-terminal cytokine receptor-like domain (called Ig-BN) or the N-terminal and C-terminal cytokine receptor-like domains (called BN-BC). They saw that BN-BC/G-CSF complex binding ratio was 1:1 and not 2:1 as with the BN-BC/GH complex (de Vos *et al.*, 1992) and that binding between G-CSF and Ig-BN was also 1:1. In addition to this, they saw a 1:1:1 Ig-BN/G-CSF/BN-BC binding. From these observations, they concluded that Both the Ig-like and cytokine receptor like domains were important for receptor oligomerization, G-CSF had two binding sites on the receptor, and that both the Ig-like and cytokine receptor-like domains had a binding site for G-CSF. Layton *et al.* essentially came to the same conclusions using neutralizing antibodies in different regions of the G-CSF receptor to inhibit G-CSF binding and inhibit proliferation of the cell line BA/F3.

Conversely, Yawata and co-workers (Yawata *et al.*, 1993) demonstrated the redundancy of the Ig-like domain present at the N terminus of IL-6Rα in IL-6 binding and subsequent signal transduction. Their approach was to demonstrate the viability of a eukaryote cell transfected deletion mutant, IL6RΔIg, lacking the Ig-like domain, to bind a biotinylated IL-6. Binding was revealed by flow cytometry using fluorescin-labeled avidin. Binding was seen to be comparable to that of wild type IL-6, and completely inhibited by the addition of an excess of non-biotinylated IL-6. Another mutant lacking the cytokine receptor-like domain (IL6RΔFam) had no capacity to bind IL-6. The IL6RΔIg was also able to increase the sensitivity of the cell line M1 to IL-6 when expressed on its cell surface, thus the Ig mutant was capable of mediating IL-6 signalling.

As seen with sequence motif and secondary structure, the cytokine type-I receptor gene structure is also well conserved. A pattern was first observed by Nakagawa *et al* (Nakagawa *et al.*, 1994) who saw that the exon/intron structure could help to explain the evolution of this receptor superfamily. Although the size of the genes in this family tends to vary enormously (due to differences in the size of non-coding introns), the size and placement of the exons is very conserved. In general, it has was observed that the signal peptide and transmembrane domains are each encoded by single exons.

The N-terminal Ig-like domain, if present, is encoded by a single exon, and cytokine receptor-like domains are encoded by four exons, two for the N-terminal region (with each CC pair encoded by a different exon) and two for the C-terminal region. The general rule is that each fibronectin type III module is encoded by two exons. Exons encoding the cytoplasmic tail (if present), are less well defined, but the general rule is one exon for the membrane proximal region and a single large exon encoding the carboxy-terminus of the protein and 3' UTR. The exons containing the 5' UTR are more varied and less well defined (Figure 1.7a).

The ordering of the 'intron phases' is also a very interesting feature of the cytokine type-I receptor genes. Intron phases (Sharp, 1981) are defined as follows. Phase 0 introns interrupt the reading frame between codons. Phase 1 and 2 introns interrupt codons after the first and second nucleotide, respectively. As Nakagawa *et al* (1993) explain, the most important feature of intron phases is that exons surrounded by introns of the same phase can be inserted and duplicated without any frameshift to the neighbouring coding region. The fibronectin type III modules encoding the cytokine receptor-like domain within this receptor family show very well conserved intron phases. The cytokine receptor-like domain is composed of four exons, two for the N-terminal region and two for the C-terminal region. and the outer boundaries of these two regions are defined by phase 1 introns. The exons encoding the N-terminal region are interrupted by a phase 2 intron whilst the C-terminal region exons are interrupted by a phase 0 intron (Figure 1.7a). In addition, cytokine type-II receptors (IFN receptors) show a similar pattern. Nakagawa *et al* (1993) therefore propose that the extracellular domain of these cytokine receptors derive from a common ancestral gene, probably encoding a prototype fibronectin type III domain. The gene segment, being surrounded by phase 1 introns and interrupted by intron insertion, is capable of recombining without a frameshift, thus allowing insertion and tandem duplication. A simplified representation of this model is outlined in Figure 1.7b.

In addition to gene structure homology, cytokine type-I receptor genes are also seen to form gene clusters. For example, LIFR is found to be present within a cluster of genes encoding cytokine receptors on chromosome 5 in humans and 15 in mice (Gearing *et al.*, 1993). IL-3Rα and GM-CSFRα are also co-localized within the

pseudoautosomal region (PAR) of the sex chromosomes. In some cases, these clusters also contain the genes for cytokines. Chromosome 5 for example also contains the genes for GM-CSF, IL-3, IL-4, IL-5, IL-9 and IL-13 (Warrington *et al.*, 1992). The precise significance for these clusters in not precisely known. It could simply be the result of recent gene duplication and serve no additional role. On the other hand selective pressure could be maintained for a close association if these genes share promoter or enhancer elements, such as for the IL-3 and GM-CSF genes (Cockerill *et al.*, 1993) where presumable there is an importance in the related expression pattern of the two genes.

As mentioned earlier, a complex degree of redundancy and pleiotrophy is demonstrated by cytokines in general. This is especially true for the different members of the hematopoietic growth factors. Much of this apparent complexity, however has been resolved by the understanding of the mechanisms involved in complex formation and signalling through the receptors for these growth factors, the cytokine type-I receptors. Type-I receptor function is detailed in the next section.

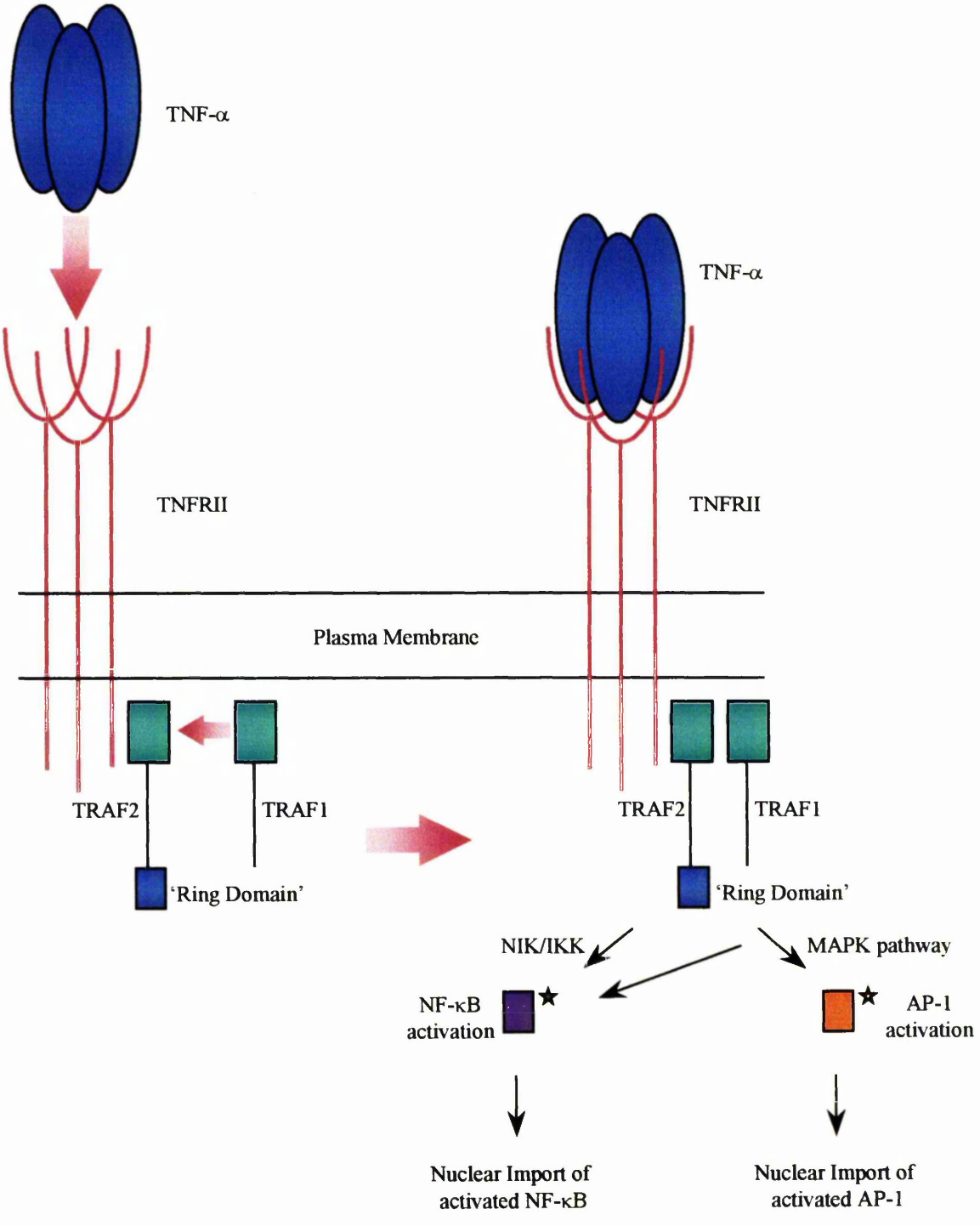


Figure 1.1. TNF-α Signaling through TNFRII.

Ligation of trimeric TNF-α leads to TNFRII oligomerization followed by TRAF1 and TRAF2 binding and homotypic interaction of the two proteins through their TRAF domains. TRAF2 subsequently activates both NF-κB and activating protein-1 (AP-1) which are transported into the nucleus to promote transcription.

NIK, NF-κB inducing kinase; IKK, IκB kinase; MAPK, mitogen-activated protein kinase.

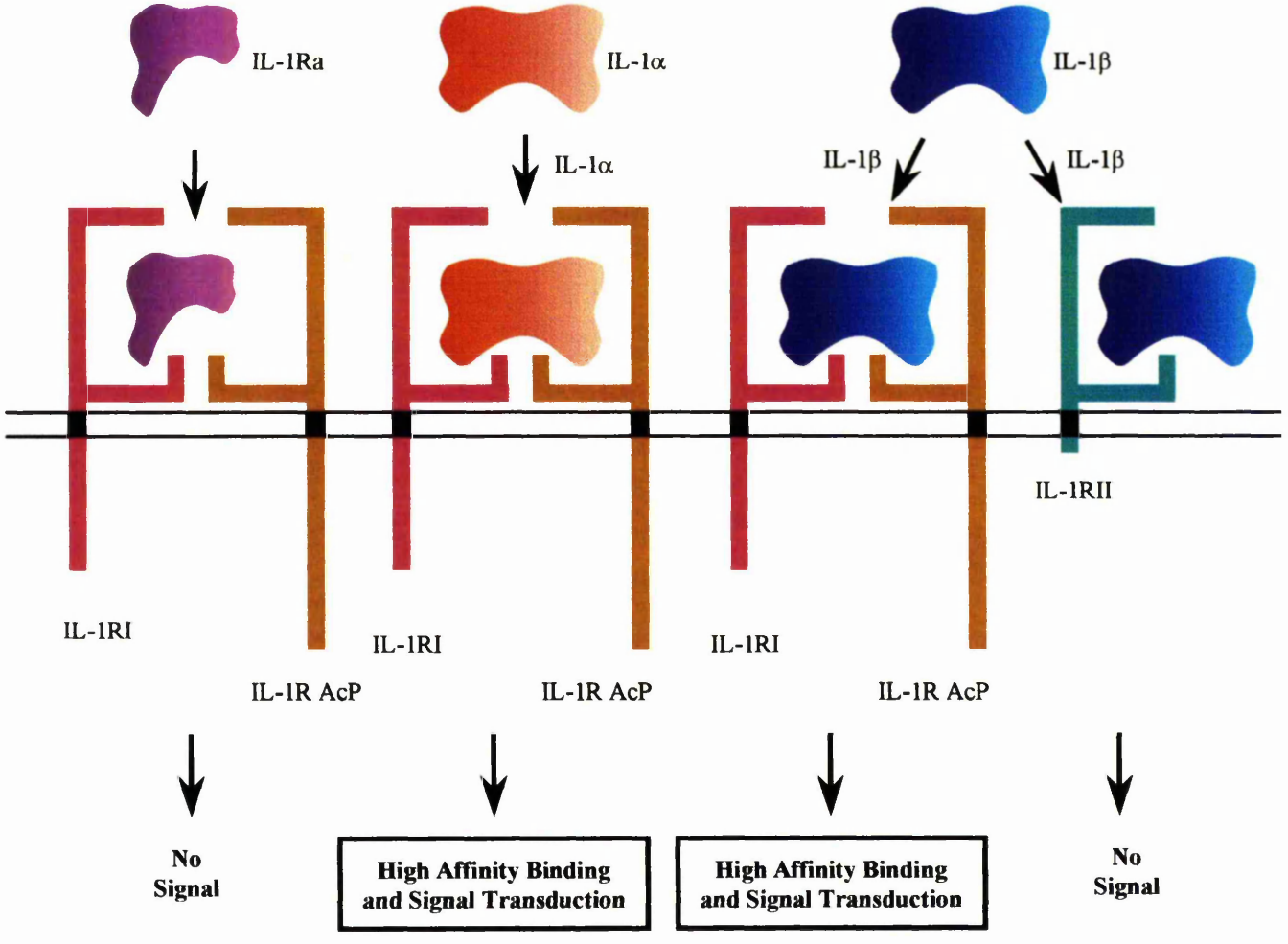


Figure 1.2. Interactions between IL-1/IL-1Ra and IL-1 Receptor Components

IL-1Ra binds to IL-1RI but does not allow it to interact with IL-1R AcP, thus not permitting signal transduction. IL-1α and β bind to IL-1RI and allow it to interact with IL-1R AcP resulting in a biological response. IL-1RII preferentially binds IL-1β but does not transduce a signal, thus acting as a 'sink' for IL-1β. (Adapted from Dinarillo, 1996).

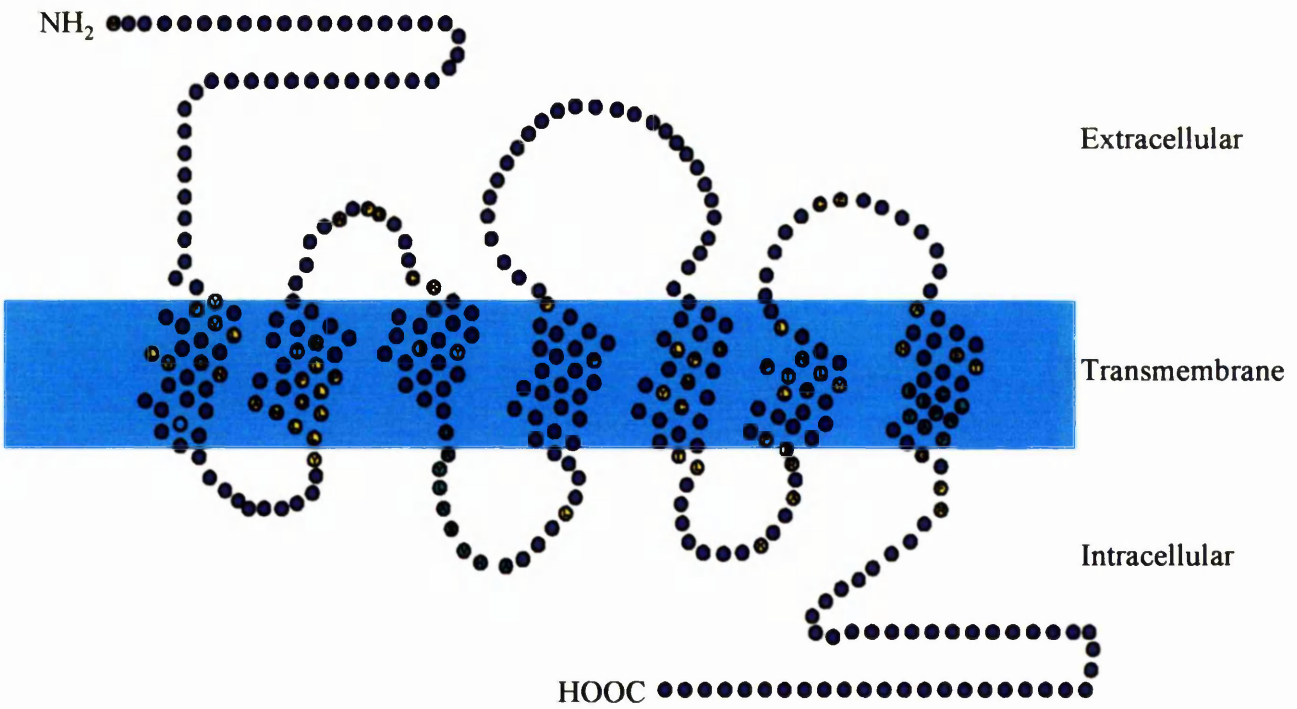


Figure 1.3. The Generalized Structure of Chemokine Receptors

The chemokine receptors are characteristic of the 7 transmembrane domain (7 TM) G protein-coupled receptors. Residues shown in yellow are conserved throughout the family. Regions shown in green are highly conserved and have been used to design degenerate oligonucleotides for the cloning of novel chemokine receptors by PCR (Power, 1996).

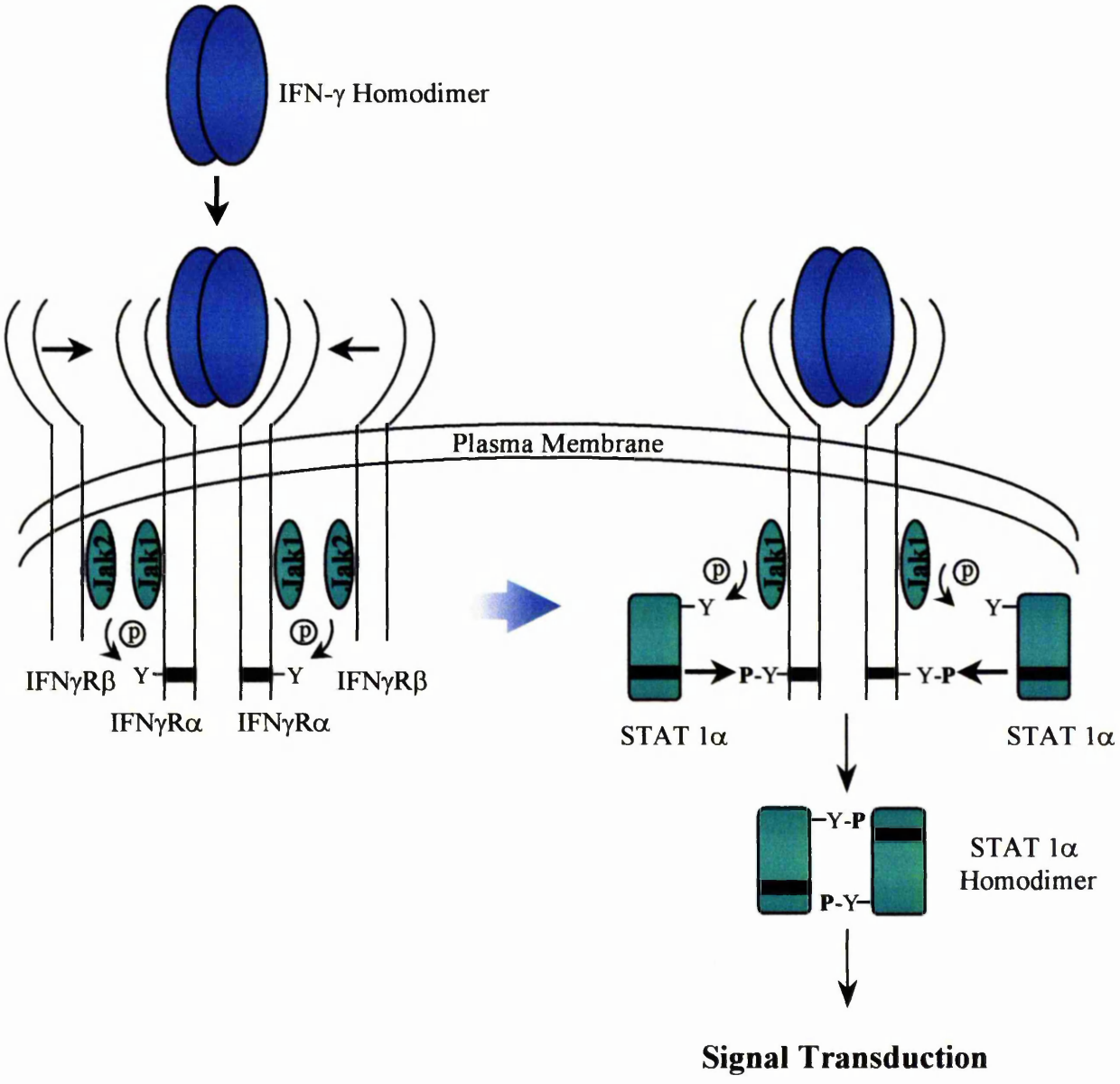


Figure 1.4. The Interaction between IFN-γ and its Receptor Components

Homodimeric IFN-γ binds to two α chains associated with Jak1 leading to dimerization and association of β chains associated with Jak2. Trans-phosphorylation of Jak kinases leads to tyrosine residue phosphorylation on the α chain, resulting in the juxtapositioning of the STAT 1α SH2 domain to the α chain cytoplasmic tail and phosphorylation of the STAT 1α subunit by the Jak kinase. Phosphorylated STAT 1α subsequently homodimerizes, and translocates into the nucleus in an active form to promote transcription.

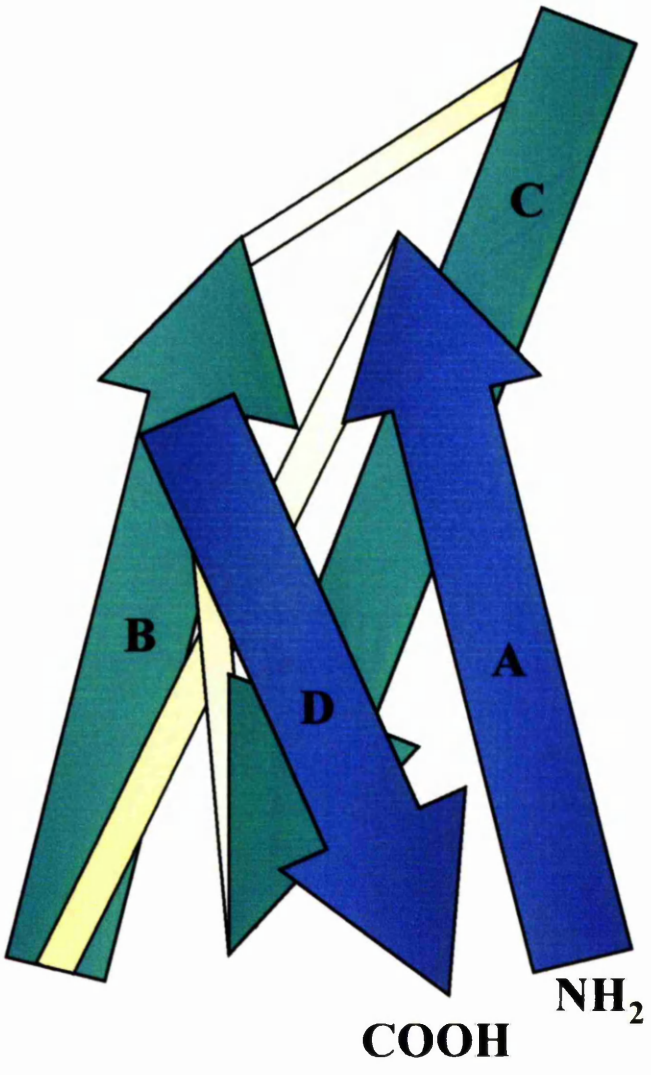
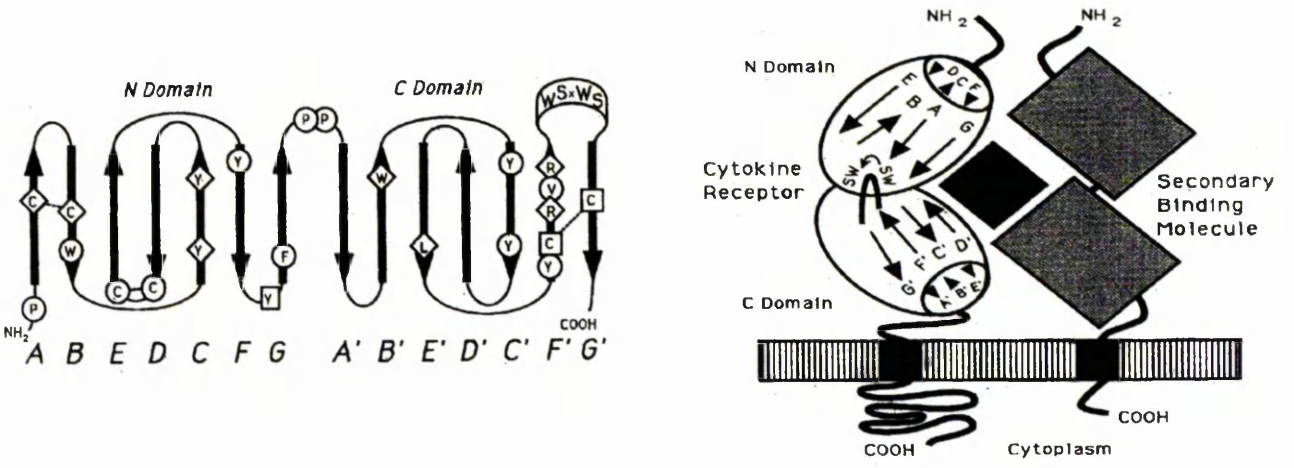


Figure 1.5. Schematic Drawing of the Conserved Structure of the Hematopoietic Growth Factors.

α helices are depicted as arrows, with yellow lines representing connecting loops. Arrows with the same colour represent helices found on the same side of the α helix bundle. The first two helices (A and B) run parallel, although found on different sides of the bundle, with helices C and D also running parallel but in an opposite direction to A and B. Like A and B, C and D are found on opposite sides of the bundle (reviewed in Wells and De Vos, 1996).

A



B

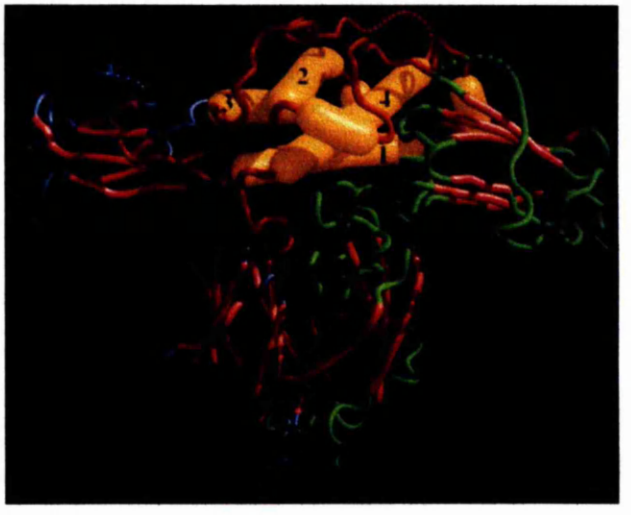


Figure 1.6. The Structure of the Cytokine Receptor-Like Domain

(A) The tertiary folding of β strands within a cytokine receptor-like domain. *(Left)* β-strand topology map with conserved residues depicted. The W-S-x-W-S motif is shown in the F'-G' loop. (*Right*) Predicted configuration of the cytokine receptor-like domain. The two domains form a V-shaped groove lined by β sheet surfaces, allowing the docking of a specific cytokine shown in black. The receptor/receptor complex is associated with a secondary binding molecule (typically a cytokine receptor family member) that recognizes the free surface of the cytokine as well as selected receptor loops distal to the binding pocket (from Bazan, 1990b).

(B) Backbone structure of the GH-$(GHR)_2$ complex. The cytokine α helices are shown as yellow cylinders connected by red tubes. The β strands of the cytokine receptor-like domain are also shown in red, connected with green (GHRI) or blue (GHRII) loops. From this view, the cell membranes are at the bottom of the picture, thus the N-terminal cytokine receptor-like domains are above and the C terminal cytokine-receptor-like domains are below (from De Vos, 1992).

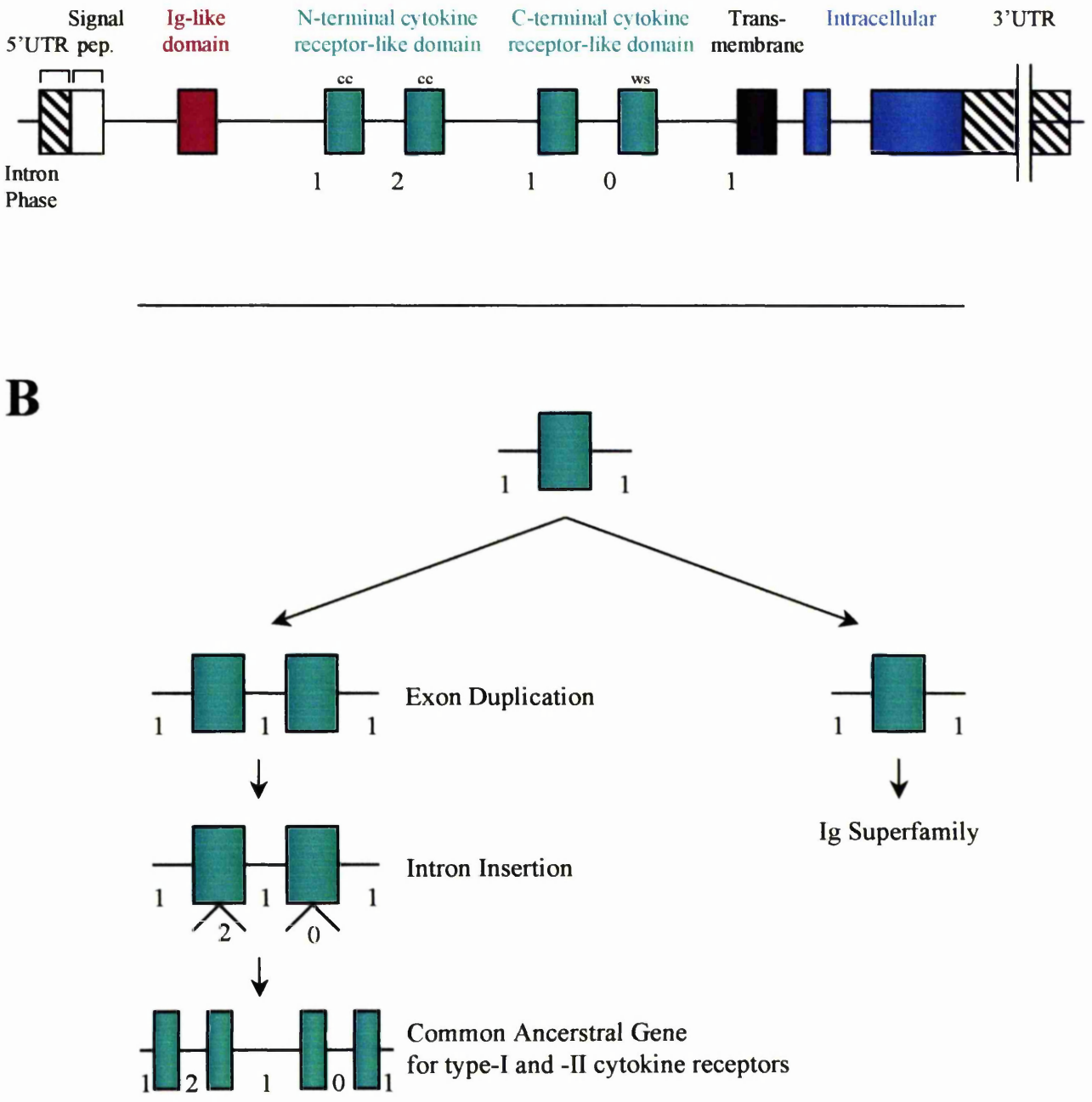


Figure 1.7. Gene Structure of the Cytokine Receptor Superfamily

(A) Generalized structure of cytokine type-I receptor genes. Each box represents an exon while lines represent introns. Green boxes represent fibronectin type III domains. Conserved cysteine and WSXWS motifs are denoted by 'cc' and 'ws', respectively.

(B) Putative model for the evolution of the cytokine receptor-like domain. Boxes and lines represent exons and introns, respectively. The model suggests that the common ancestral gene for the family was generated by exon duplication and intron insertion (adapted from Nakagawa et al, 1994).

(1.2) The Biology of Cytokine Type-I Receptors

The characterization of these receptors into a family has helped to group together a collection of ill-defined soluble mediators with redundant and pleiotrophic activities, now known to be functionally and genetically related and called the hematopoietic growth factors. This ever growing family of type I glycoproteins includes at present the receptors for the interleukins IL-2, IL3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11, IL-12, IL-13 and IL-15 as well as G-CSF, GM-CSF, thrombopoietin (TPO), erythropoietin (EPO), LIF, OSM, CNTF, cardiotrophin-1 (CT-1), GH, prolactin (PRL) and leptin. There are other soluble factors also sharing homology to these receptors in the cytokine receptor-like domain which can also be included in this family, such as the IL-12 p40 subunit (Gearing and Cosman, 1991) and the Epstein-Barr virus induced protein, EBI-3 (Devergne *et al.*, 1996). Figure 1.8 represents schematically the members of this family of cytokine receptors.

As observed with the cytokine type II (interferon) receptors, the general structure of the receptor complexes within this family is that of a ligand binding subunit which interacts with a signal transducing component in the presence of the ligand, forming a high affinity heteromeric complex giving a signal transposed to the target cell nucleus. Although some of the members of this family act as homomeric receptors fulfilling the duel role of ligand binder and signal transducer (for example, the receptors for EPO, TPO, G-CSF and GH), much of the observed redundancy within the hematopoietic growth factor family is explained by the fact that subfamilies of the hematopoietic growth factors share signalling chains within their receptor complex along with their specific ligand binding chain. The three well defined receptor subfamilies utilizing shared signalling subunits within the cytokine type-I receptor family are the common γ (γc) chain sharing receptors, the common β (βc) chain sharing receptors and the LIFR/gp130 sharing receptors. This section will give an overview of the γc and βc sharing receptor families, as well as describing in detail the cytokine receptors pertaining to the subfamily sharing LIFR and gp130 as their signalling subunits. The implications of receptor subunit sharing in terms of cytokine redundancy will also discussed.

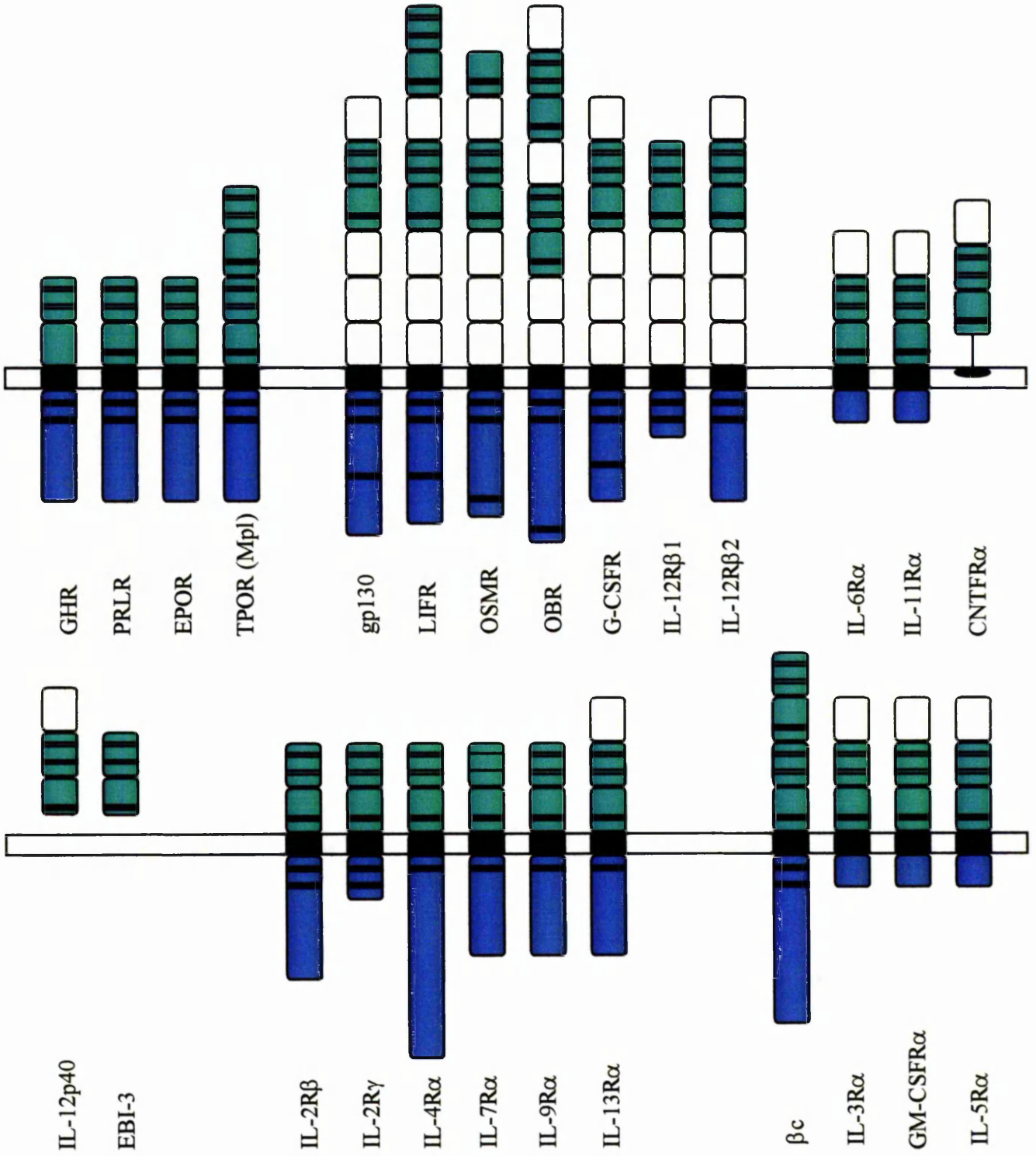


Figure 1.8. Human Cytokine Type-I Receptor Family Members.

Each block in the extracellular domain represents either a fibronectin type III or an Ig-like domain. Each receptor contains at least one cytokine receptor-like domain (shown in green). Conserved cysteines found in the N-terminal cytokine receptor-like domain are represented by thin lines, whilst the W-S-x-W-S motif in the C-terminal domain is shown by a thick line. The cytoplasmic domain is shown in blue, and the conserved 'Box' motifs within this domain are represented by thick lines. Trans-membrane domains are shown as black blocks.

(1.2.1) The γc Sharing Receptors

Figure 1.9 gives an overview of the receptor complexes pertaining to this family

Although now known to be a component within the functional receptors for IL-4, IL-7, IL-9 and IL-15, γc was originally identified as a component of the functional IL-2 receptor. The IL-2 receptor is composed of 3 polypeptide subunits, α, β and γ. The α chain was the first to be cloned (Leonard *et al.*, 1982; Leonard *et al.*, 1984; Cosman *et al.*, 1984) and was identified as a 251 aa residue protein with a very truncated (13 aa residue) cytoplasmic tail. It does not contain the domains typical of the cytokine type-I receptors. The β chain was identified by expression cloning and found to be a 525 aa residue protein with a more substantial cytoplasmic tail than the α chain, involved in cytoplasmic signalling. It also contained sequences characteristic of the cytokine type-I receptor family (Sharon *et al.*, 1986; Tsudo *et al.*, 1986; Teshigawara *et al.*, 1987; Hatakeyama *et al.*, 1989). The γ chain (γc) was identified by coimmunoprecipitation from lymphoid cells with the IL-2Rβ chain using a mAb specific for the β chain (Takeshita *et al.*, 1992). It is a 64 kD glycoprotein and also contains the type I receptor motifs in its extracellular domain. The 86 residue cytoplasmic domain also plays an important role in IL-2 signal transduction. Three types of IL-2 receptor were found to exist involving the three chains. A low affinity receptor (α chain alone), an intermediate affinity receptor (β and γ chains) and a high affinity receptor (α, β and γ chains). Signal transduction is only permissible through the intermediate and high affinity receptors. Chimeric receptor experiments showed that heterodimerization of the β and γ chain cytoplasmic tails was a prerequisite of receptor signalling (Nakamura *et al.*, 1994; Nelson *et al.*, 1994). STATs 3 and 5 have been implicated in IL-2 signal transduction, being phosphorylated by Jak1 and/or Jak3 (Hou *et al.*, 1995; Beadling *et al.*, 1994; Wakao *et al.*, 1995; Nielsen *et al.*, 1994; Fujii *et al.*, 1995).

The IL-15 receptor is very similar to the IL-2 receptor, accounting for the overlapping functions observed between their respective cytokines. In fact the IL-15 receptor is composed of the IL-2 receptor β and γ chains, in addition to an IL-15 specific α chain. The presence of the β and γ chains within the receptor complex suggested that IL-15 utilizes the same signalling components as IL-2 (Giri *et al.*,

1994). The IL-15Rα chain cloned by Giri *et al* (Giri *et al.*, 1995) permits high affinity IL-15 binding, shares homology with IL-2Rα (thus not belonging to the cytokine type-I receptor family) and their genes are closely linked in both human and mouse (Anderson *et al.*, 1995). Like IL-2Rα, IL-15Rα plays no part in signal transduction. The observation that IL-15, but not IL-2 had an effect on mast cells suggested that IL-15 could signal through a pathway independent of IL-2Rβ and γc. Cross linking experiments demonstrated that IL-15 bound to a 60-65 kD protein on these cells, designated IL-15RX. The receptor was also shown to recruit Jak2 and STAT 5, leading to cell proliferation (Tagaya *et al.*, 1996), thus demonstrating an alternative IL-15 signalling pathway distinct from that of the used by IL-2.

IL-4 has been shown to signal through a functional high affinity receptor complex consisting of γc and an IL-4 binding chain (IL-4Rα). The IL-4Rα chain is an 800 aa residue 140 kD molecule demonstrating homology in its extracellular domain with the cytokine receptor-like domain pertaining to the cytokine type-I receptor family (Mosley *et al.*, 1989). It demonstrates low affinity binding for IL-4, the high affinity binding provided by the involvement of γc (Kondo *et al.*, 1993; Russell *et al.*, 1993). Interestingly, this IL-4R has been implicated in signal transduction via Jak1, Jak3 and STAT 6 activation, with the IL-4Rα chain associating with Jak1 and STAT 6 (Hou *et al.*, 1994; Quelle *et al.*, 1995), whilst the γc chain associates with Jak3. This is in contrast to the IL-2R and IL-15R systems, where Jak1 and Jak3 phosphorylate STAT 3 and STAT 5, suggesting that specificity is determined by the cytokine receptor directly interacting with the STATs, and that Jak activation of STATs is less specific. A model for such 'triggering' by non-specific Jaks of specific 'driver' subunits is discussed by Lai *et al* (Lai *et al.*, 1996).

More recently, a second IL-4 receptor has been proposed which appears to be shared by IL-13. It is well documented that most if not all of the functions of IL-13 can be mimicked by IL-4. The reverse however seems not to be true. Suspicions of such a IL-4/IL-13 shared receptor (IL-4RII or IL-13R) were aroused when both IL-4 and IL-13 induced responses in cells not expressing the γc chain or lymphocytes from severe combined immunodeficiency (SCID) patients, who are deficient for γc (Schnyder *et*

al., 1996a; Obiri *et al.*, 1995; Lin *et al.*, 1995; Matthews *et al.*, 1995; Izuhara *et al.*, 1996). The proposed receptor would contain the IL-4Rα chain and an IL-13R specific chain (reviewed in Callard *et al.*, 1996). Two IL-13 binding proteins of both murine and human origin have recently been described, named IL-13Rα1 (Hilton *et al.*, 1996; Aman *et al.*, 1996; Miloux *et al.*, 1997; Gauchat *et al.*, 1997) and IL-13Rα2 (Caput *et al.*, 1996; Zhang *et al.*, 1997) both of which showed the characteristic extracellular homology to members of the cytokine type-I receptor family. The IL-13Rα1 chain was been shown to be implicated in a functional IL13 and IL-4 receptor complex, thus demonstrating that the IL-4Rα chain is also a shared or common chain within the cytokine type-I receptor family. The human IL-13Rα2 chain cloned by Caput et al (Caput *et al.*, 1996) has also been implicated in an IL-4/IL-13 receptor complex through its interaction with IL-4Rα but due to the very short cytoplasmic tail of IL-13Rα2, the signalling capacity of this complex has yet to be established.

IL-7 signals through a receptor complex composed of an IL-7 binding protein (IL-7Rα) and γc. The IL-7Rα chain was first identified by Goodwin *et al* (Goodwin *et al.*, 1990) using a direct expression cloning strategy. The cDNA encodes a 439 aa residue, 49.5 kD type I membrane glycoprotein, with the extracellular domain sharing the features typifying the cytokine type-I receptor family. Although IL-7 bound the IL-7Rα chain directly with principally low affinity (with a small number of high affinity binding sites, suspected to be formed of IL-7Rα homodimers), a second chain was implicated in the IL-7 receptor complex by the observation that lymphoid cells bound IL-7 with high affinity. The γc chain was first suspected to be the high affinity converting chain when it was observed that γc chain blocking Abs suppressed IL-7 dependent proliferation of the mouse pre-B cell line IxN/2b. This was confirmed by chemical cross-linking of IL-7 to the γc chain (Kondo *et al.*, 1994). The IL-7R has been implicated in the Jak/STAT signalling pathway, specifically utilizing Jak1, Jak3 kinases (Sharfe *et al.*, 1995) and STAT 1, STAT 5 with the possible involvement of STAT 3 (van der Plas *et al.*, 1996; Foxwell *et al.*, 1995).

Like the IL-4Rα chain, the IL-7Rα chain has been implicated in a receptor complex independent of the γc chain. It was observation that the IL-7Rα knock-out mouse had a more severe lymphopenia than the IL-7 deleted mouse. This was attributed to the finding that a second cytokine, thymic derived stromal lymphopoietin (TSLP) which can replace the activity of IL-7 in certain situations, can also bind IL-7Rα (Candeias *et al.*, 1997). It is not clear whether TSLP uses the γc chain, but the fact that T and B cell phenotypes in the γc knock-out mouse resemble those of the IL-7 knockout mouse give weight to the hypothesis that TSLP uses a second novel receptor distinct from γc. It unknown whether IL-7 can signal through a second such receptor.

IL-9 signals through a complex comprising the IL-9Rα and γc chains. The IL-9Rα chain was first identified by expression cloning (Renauld *et al.*, 1992) as a 483 aa residue 52 kD type I membrane glycoprotein belonging to the cytokine type-I receptor family, which binds IL-9 with low affinity. Due to the fact that the cytoplasmic tail of the IL-9Rα chain shared significant homology with the cytoplasmic tail of the IL-2Rβ chain, Kimura and co-workers (Kimura *et al.*, 1995) investigated the possibility of an involvement of γc in the formation IL-9R complexes capable of signal transduction. They demonstrated that the IL-9 dependent proliferation of the cell lines MC/9 and CTLL-2 was inhibited by a blocking Ab to the γc chain. Although the Ab did not affect the high affinity binding of IL-9 to the cell surface, it was shown to interact with γc using chemical cross linking experiments. In terms of signalling, the kinases Jak1, Jak3 and STAT 3 have been implicated (Yin *et al.*, 1994a; Yin *et al.*, 1995).

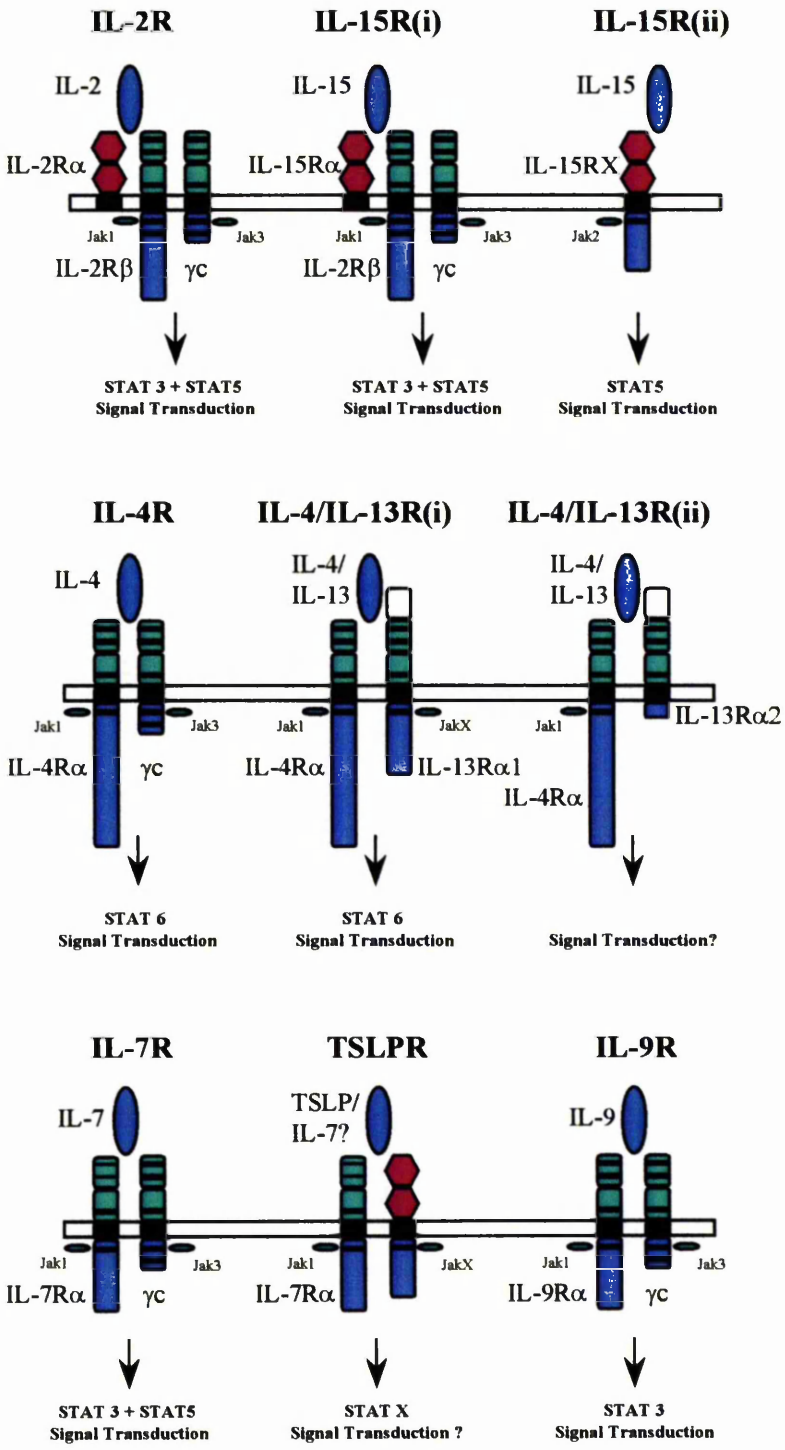


Figure 1.9. Receptor Signaling Complexes for Subunits which Associate with γc.

Members of the cytokine type-I receptor family have their cytokine receptor-like domains coloured in green, with each block representing an Ig-like or fibronectin type III domain. Non cytokine type-I or unknown receptor subunits have their extracellular regions coloured in red. All receptor intracellular domains are shown in blue. Transmembrane domains are shown as black blocks. Cytokines are represented by blue ovals, with receptor associated Jaks shown as small green ovals.

(1.2.2) The βc Sharing Receptors

Figure 1.10 gives an overview of the receptor complexes pertaining to this family

The common β (βc) chain was first identified as a subunit of the GM-CSF receptor (Hayashida *et al.*, 1990). Gearing and co-workers (Gearing *et al.*, 1989) used an expression cloning strategy to clone a receptor for GM-CSF (GM-CSFRα), now known to be the binding chain of the receptor complex. The 400 amino acid residue, 80-85 kD type I membrane glycoprotein contains the extracellular domains and sequence motifs required for membership into the cytokine type-I receptor family. The receptor showed a single class of affinity and specificity for GM-CSF. A year later, Hayashida and colleagues (Hayashida *et al.*, 1990) found that another member of the cytokine type-I receptor family, KH97 (which had been cloned using the mouse IL-3R cDNA as a probe) formed a high-affinity receptor complex for GM-CSF when co-transfected in the murine NIH 3T3 cell line with the cDNA for GM-CSFR, although KH97 alone did not bind GM-CSF. It was confirmed as a component of the GM-CSF receptor complex by cross-linking experiments. KH97, now known as the human βc chain, is an 800 aa residue, 120 kD type I membrane protein containing two consecutive cytokine receptor-like domains in its extracellular region. It is unclear what the role of each individual domain has on βc function, although it is thought that the membrane proximal domain is better positioned for interaction with the ligand and receptor binding chain (discussed in Bagley *et al.*, 1997).

IL-3 also utilizes a receptor complex containing a ligand binding chain and βc for signal transduction. A novel expression cloning strategy was exploited to identify a human IL-3 binding protein (IL-3Rα) and to demonstrate that this subunit interacted with the βc chain to form a high affinity IL-3 binding site. Kitamura and colleagues (Kitamura *et al.*, 1991) transiently co-transfected an IL-3-stimulated TF-1 cell cDNA expression library in parallel with an expression vector containing cDNA encoding the βc chain. Cells were screened for binding of ^{125}I-IL-3 and a clone was identified which bound IL-3 with high affinity. A cDNA was identified which bound IL-3 with low affinity in transfection experiments and which gave high affinity binding upon co-expression with βc. This cDNA encoded the human IL-3Rα chain, also a member of

the type I cytokine receptor superfamily. Therefore, the βc chain was implicated in the IL-3 receptor complex, and this was confirmed by cross-linking experiments.

In the murine system, two βc chains have been identified. The first, originally named AIC2A was identified by expression cloning using a mAb capable of partially blocking IL-3 binding (Itoh *et al.*, 1990). The second, named AIC2B, was identified due to its high homology (95%) with AIC2A (Gorman *et al.*, 1990). Both βc chains are capable of forming high affinity functional receptors for IL-3, and both share the same IL-3Rα chain (Hara and Miyajima, 1992). AIC2B is the now called the murine βc chain due to the fact that it is a shared subunit for the IL-3, GM-CSF and IL-5 receptors, similar to the human βc chain (reviewed in Miyajima *et al.*, 1993). AIC2A however appeared to be IL-3 specific, a finding confirmed by the phenotype of mutant mice lacking either the βc chain or the β_{IL-3} chain (Nishinakamura *et al.*, 1995; reviewed in Miyajima *et al.*, 1997) and has therefore been named β_{IL-3}.

Like GM-CSF and IL-3, IL-5 signals through a receptor complex consisting of an α chain and βc. Tavernier *et al.* (Tavernier *et al.*, 1991) isolated the murine IL-5Rα by immunoaffinity chromatography using a mAb against the mouse α chain (Devos *et al.*, 1991) and used the amino acid sequence to design oligonucleotides to screen a human stimulated HL60 cDNA library. The 41 clones encoding the human α chain cDNA were subsequently cloned, all of which encoded a 315 aa residue soluble receptor (sIL-5Rα) which bound IL-5 with low affinity. Co-transfection of a membrane-bound IL-5Rα fusion construct with the βc chain resulted in higher affinity binding of IL-5. Cross-linking experiments confirmed the presence of βc in the IL-5 receptor complex. sIL-5Rα has antagonistic effects in terms of IL-5 receptor binding and signalling. It binds IL-5 in solution and does not associate with βc (Tavernier *et al.*, 1991). It was therefore speculated that a membrane-bound form of IL-5Rα must exist.

The cDNA for membrane-bound IL-5Rα was cloned a year later by Tavernier and co-workers (Tavernier *et al.*, 1992) using a 3'extension PCR technique. The mRNA

was found to be many times less abundant than that of the soluble receptor and the differences between the two transcripts seen to be the result of alternative splicing. It could be hypothesized that such an abundance of an antagonistic soluble receptor could have a systemic role acting as a 'sink' *in vivo* preventing the spread of this proinflammatory cytokine as well as a negative regulator in dampening local inflammatory responses.

Owing to the small cytoplasmic tails exhibited by the ligand binding α chains within this family, it comes as no suprise that the βc chain is responsible for signal transduction. It has been demonstrated using chimeric receptors where the extracellular domain of the βc chain has been replaced by the extracellular domain of a ligand binding α chain, that aggregation of the βc chain is essential for signal transduction. The chimeric proteins are mitogenically active upon ligand binding, the ligand having the effect of juxtaposing homomerically the chimeric proteins. It has therefore been proposed that the role of the ligand binding α chains within this family is to associate with and facilitate aggregation of the βc chain. It is thought that this aggregation leads to higher affinity binding and association of Jaks, resulting in transphosphorylation and autophosphorylation and the subsequent propagation of the signal. This model of subunit oligomerization is thought to be valid for all receptor complexes involving the cytokine type-I receptor family (discussed in Ihle *et al.*, 1995). Jak2 has been implicated in signalling through this family of receptors along with STAT 5 (reviewed Miyajima *et al.*, 1997) and shown to interact with βc (Quelle *et al.*, 1994; Silvennoinen *et al.*, 1993). GM-CSF, IL-3 and IL-5 signalling pathways are more closely related than those cytokines sharing the γc signalling subunit. This is invariably due to the limited involvement of the cytoplasmic domain of the α chains within this family, whose role is simply to induce the homomeric aggregation of βc. In contrast, α chains of γc sharing receptors generally participate in signalling via interactions of their cytoplasmic domains with that of γc and the recruitment of specific STATs interacting with their cytoplasmic domain.

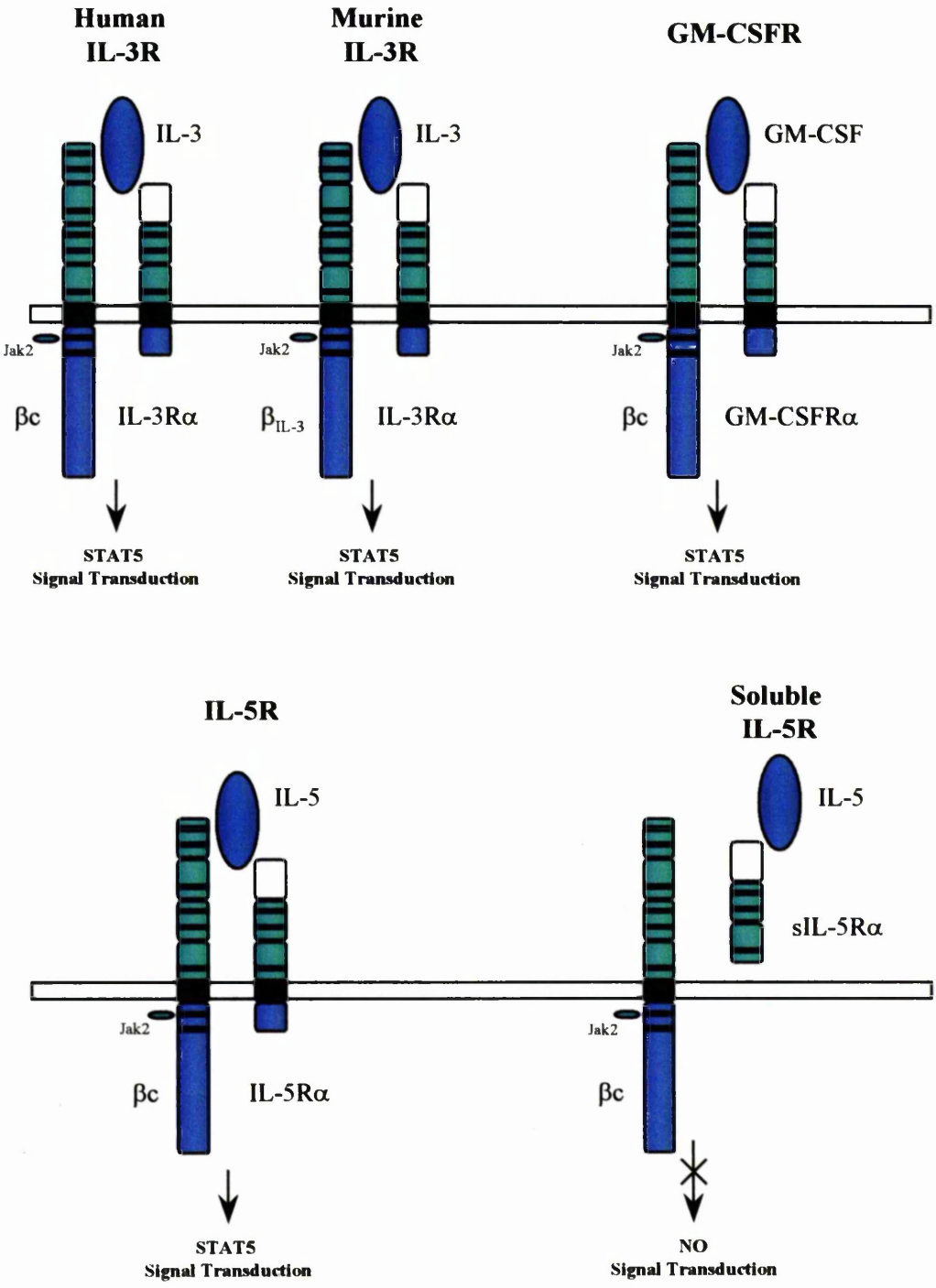


Figure 1.10. Receptor Signaling Complexes for Subunits which Associate with βc.

Cytokine receptor-like domains are coloured in green, with each block representing an Ig-like or fibronectin type III domain. All receptor intracellular domains are shown in blue. Transmembrane domains are shown in black. Cytokines are represented by blue ovals, with receptor associated Jaks shown as small green ovals.

(1.2.3) The LIFR/gp130 Sharing Receptors

The family of cytokines signalling through LIFR and gp130 show not only a high degree of redundancy, but they are also probably the family of cytokines showing the most pleiotrophy. Unlike the cytokines signalling through γc and βc, their biological effects extending beyond hematopoietic and lymphoid tissues. Table I below outlines the broad range of functions exhibited by this group of cytokines.

Table 1.1. Overlapping Biological Activities of the IL-6-Type Cytokines

Factor	*Identified by*	*Effect on Heaptocytes*	*Effect on ES Cells*	*Hematopoietic Functions*	*Neuroregulatory Functions*
IL-6	*Induction of Ig prod^n in B cells*	*Acute phase protein product^n*	*None*	*Myeloma growth, progenitor prolif^n, thrombopoiesis, growth inhibit^n and differentiat^n of M1 cells*	*PC12 cell differentiat^n, cholinergic neuron survival, pituitary hormone secretion*
IL-11	*Plasmacytoma growth*	*Acute phase protein product^n*	*None*	*Myeloma growth, progenitor prolif^n, thrombopoiesis, osteoclast formation*	*Hippocampal progenitor differentiat^n*
LIF	*Inhibit^n of M1 cell growth*	*Acute phase protein product^n*	*Pluripotential maintenance*	*Myeloma growth, progenitor prolif^n, thrombopoiesis, growth inhibit^n and differentiat^n of M1 cells, osteoclast formation*	*Cholinergic nerve differentiat^n, motor neuron survival, sympathetic neuron survival*
OSM	*Inhibit^n of growth of melanoma cells*	*Acute phase protein product^n*	*Pluripotential maintenance*	*Myeloma growth, progenitor prolif^n, thrombopoiesis, growth inhibit^n and differentiat^n of M1 cells, osteoclast formation*	*Cholinergic nerve differentiat^n*
CNTF	*Ciliary Neuron Survival*	*Acute phase protein product^n*	*Pluripotential maintenance*	*Myeloma growth*	*Cholinergic nerve differentiat^n, cholinergic neuron survival, motor neuron survival, astrocyte differentiat^n*
CT-1	*Hypertrophy in cardiomyocytes*	*Acute phase protein product^n*	*Pluripotential maintenance*	*Growth inhibit^n and differentiat^n of M1 cells*	*Cholinergic nerve differentiat^n, cholinergic neuron survival, dopaminergic neuron survival*

(a) The IL-6 Receptor

The gp130 signal transducer was first identified as component of the IL-6 receptor complex. IL-6, originally described as a factor inducing immunoglobulin production in B cells is now known to exert its wide ranging biological effects both within and outside of the immune system. The cDNA of an IL-6 binding protein was cloned by Yamasaki *et al* (Yamasaki *et al.*, 1988) by expression cloning. They used FACS to identify and select transiently transfected COS7 cells expressing proteins capable of binding a biotinylated IL-6. The protein, now known as IL-6Rα had a molecular weight of 80 kD and contained 449 aa residues, the sequence of which suggested it was a member of the cytokine type-I receptor family. The extracellular region of the protein has an N-terminal Ig-like domain followed by 2 fibronectin type III modules housing the cytokine receptor-like domain. The cytoplasmic region was found to be very truncated, containing 87 aa residues suggesting that the receptor has limited signal transducing capabilities.

When expressed alone, IL-6Rα bound IL-6 with mainly low affinity, but it was established that IL-6 bound to responsive cells with both low and high affinity. It was therefore widely suspected that IL-6 would therefore employ a second receptor chain for signal transduction. Taga and colleagues (Taga *et al.*, 1989) demonstrated firstly by deletion mutagenesis that the cytoplasmic chain of the IL-6Rα chain was dispensable for IL-6 signalling, and secondly that in the presence of IL-6, the IL-6Rα chain co-immunoprecipitated with a membrane glycoprotein having a molecular mass of 130 kD. The cDNA encoding the glycoprotein, named gp130, was subsequently cloned by immunoscreening a human λ library using mAbs raised against gp130 (Hibi *et al.*, 1990). The 918 aa residue protein, a member of the cytokine type-I receptor family, had a predicted cytoplasmic tail of 277 aa residues, making it a candidate for signal transduction. The extracellular portion of the receptor chain contained six fibronectin type III modules, the second and third of which from the N-terminus contained a cytokine receptor like domain. The mRNA distribution of gp130 has also shown the protein to be ubiquitous. Biochemical analysis suggests that the IL-6/IL-6

receptor complex is hexameric, containing two each of IL-6, IL-6Rα and gp130 (Ward *et al.*, 1994; Paonessa *et al.*, 1995).

It is now well documented that IL-6Rα exists in soluble form (sIL-6Rα), and this soluble form has an agonistic effect on IL-6 signalling, in contrast to the antagonistic properties described for the sIL-5Rα, for example. The ability of sIL-6Rα to permit IL-6 signalling in the presence of gp130 was first described by Taga *et al.* (Taga *et al.*, 1989). They generated a mutant IL-6Rα cDNA truncated at the aa residue 322 and therefore encoding the IL-6Rα protein lacking the transmembrane domain and cytoplasmic tail. The protein was expressed in COS7 cell supernatants and subsequently shown not only to bind IL-6, but also to associate with gp130 by immunoprecipitation in the presence of IL-6 and to increase the sensitivity of M1 cells to IL-6 mediated growth inhibition, thus demonstrating that sIL-6Rα retained the biological properties exhibited by membrane-bound IL-6Rα. Since then, a number of groups have demonstrated the effect of recombinantly generated sIL-6Rα on IL-6-dependent cell regulation. These effects are solely dependent on expression of gp130 on the target cell surface (Yoshida *et al.*, 1994; Murakami-Mori *et al.*, 1996; Romano, 1997; Franchimont *et al.*, 1997). It also comes as no suprise that transgenic mice expressing sIL-6Rα are hypersensitized to IL-6 (Hirota *et al.*, 1995; Peters *et al.*, 1996).

Naturally occuring sIL-6Rα has also been detected in the certain body fluids. In addition, increased levels have been found during inflammation (Narazaki *et al.*, 1993; Novick *et al.*, 1989; Honda *et al.*, 1992; Suzuki *et al.*, 1993). There appears to be two mechanisms by which naturally-occurring sIL-6Rα can be generated. Müllberg *et al* (1992, 1993a and b, 1994) demonstrated that membrane-bound IL-6Rα was proteolytically cleaved (shedded) from the surface of transfected COS7 cells, especially when treated with PMA. This process appeared to be regulated by protein kinase C. In addition, point mutations or deletions in the extracellular region near the transmembrane domain severely altered or abolished the rate of shedding. In contrast, two groups have identified cDNA encoding a soluble form of sIL-6Rα. They used RT-PCR on mRNA derived from human myeloma cell lines, bone marrow from

patients with multiple myeloma, T and granulocyte/ macrophage cell lines and peripheral blood mononuclear cells (Lust *et al.*, 1992; Horiuchi *et al.*, 1994). Although there is good evidence *in vivo* to show that proteolysis leads to a soluble form of sIL-6Rα, Müller-Newen *et al* (Muller-Newen *et al.*, 1996) rather significantly showed that not only did human plasma contain a certain amount of sIL-6Rα generated by alternative splicing, but in addition that it was physiologically active, binding IL-6 and inducing IL-6 dependent α_1-antichymotrypsin synthesis in HepG2-IL-6 cells. The observation that sIL-6Rα can facilitate ligand-mediated signalling in the presence of gp130, and that biologically active sIL-6Rα is present in human serum does have certain implications. Gp130 is a ubiquitously expressed protein, whereas IL-6Rα is not. The fact that a given cell may be capable of secreting sIL-6Rα and IL-6 simultaneously leads to the prospect that a 'bystander' cell expressing gp130 may be sensitised to IL-6-dependent signalling through gp130. This in turn could lead to a loss of regulation of IL-6 mediated effects. Indeed, elevated levels of sIL-6Rα have been detected in certain disease states such as multiple myeloma (Klein *et al.*, 1995).

Soluble forms of the two signal transducing components in this receptor subfamily have also been identified. Soluble gp130 (sgp130) has been identified in biological fluids (Narazaki *et al.*, 1993) and evidence exists that it generated both by shedding and alternative splicing (Mullberg *et al.*, 1993; Mullberg *et al.*, 1993; Diamant *et al.*, 1997). As gp130 serves as a signal transducer, it is not suprising that soluble gp130, lacking a transmembrane and cytoplasmic domain, is a natural antagonist of cytokine signalling through gp130 (Narazaki *et al.*, 1993; Montero-Julien *et al.*, 1997). Similarly, a naturally occurring soluble form of the other signalling component in this family, LIFR, has been detected as a cDNA clone (Gearing *et al.*, 1991) and in mouse serum, being shown to block LIF-mediated responses (Layton *et al.*, 1992).

The receptor complexes involved in IL-6 signalling are shown in Figure 1.11.

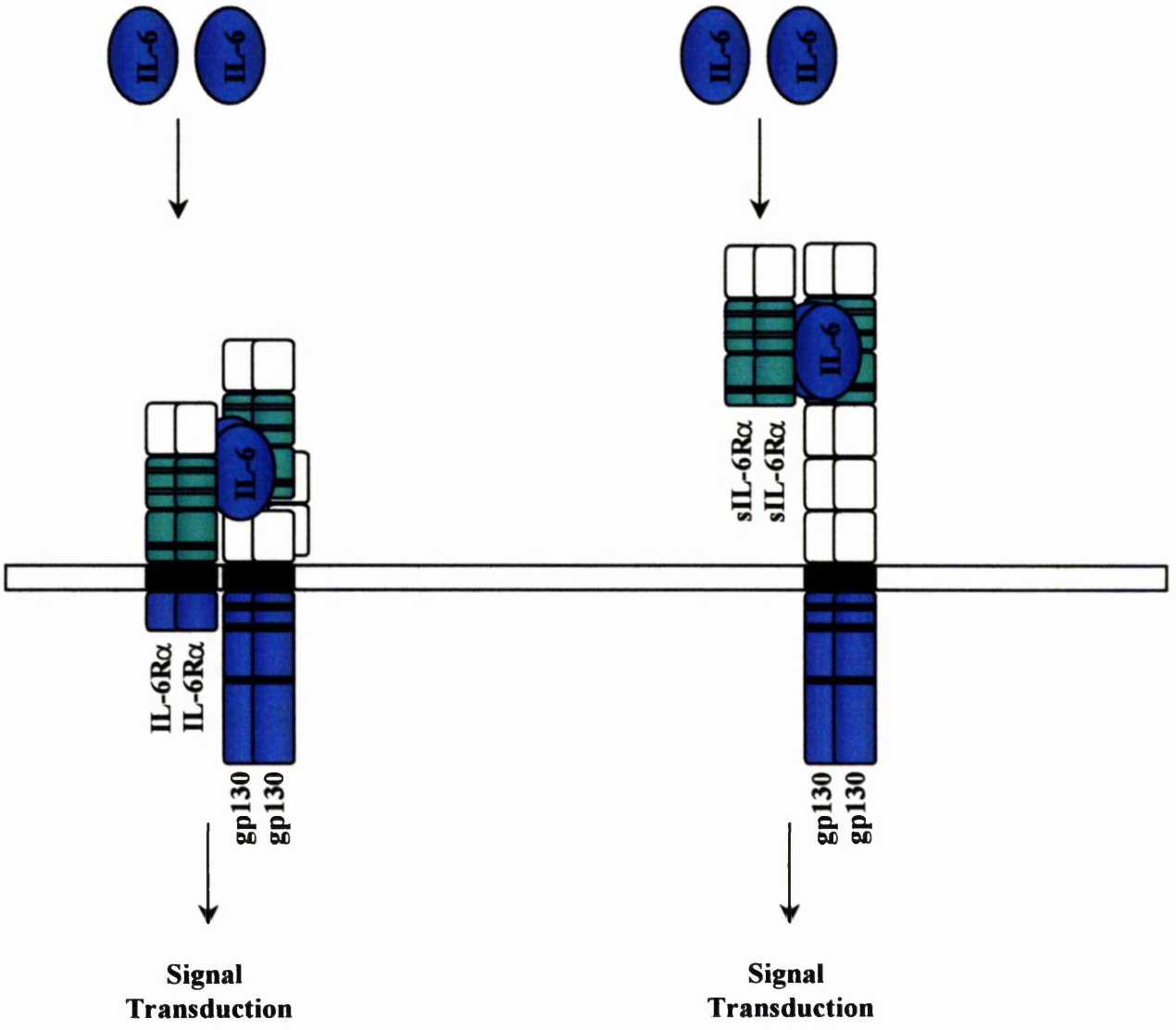


Figure 1.11. Functional IL-6 Receptors.

Each block in the extracellular domain represents an Ig-like or fibronectin III domain. The cytokine receptor-like domain is shown in green, and the receptor intracellular domain shown in blue. IL-6 is represented by the blue oval. Transmembrane domains are shown as black blocks.

(Left) The hexameric complex formed by two IL-6, two gp130 and two membrane-bound IL-6Rα molecules, following an initial interaction between IL-6 and IL-6Rα molecules.

(Right) The hexameric complex formed by two IL-6, two gp130 and two soluble IL-6Rα molecules, following an initial interaction between IL-6 and sIL-6Rα molecules. The above complexes are based on the model proposed by Ward *et al* (1994) and Paonesa *et al* (1995).

(b) The IL-11 Receptor

IL-11 was initially identified by its ability to stimulate proliferation of the IL-6 dependent cell line T1165 (Paul *et al.*, 1990). It shares overlapping biological actions with IL-6 as well as other cytokines signalling through LIFR/gp130. An IL-11 binding protein was first cloned by Hilton and colleagues (Hilton *et al.*, 1994). They screened an adult mouse liver cDNA library using an oligonucleotide corresponding to the highly conserved W-S-x-W-S motif characteristic of the cytokine type-I receptors. They found that one of the proteins isolated by this random screen bound IL-11 with low affinity. This 432 aa residue contained an Ig-like and cytokine-receptor-like domain in its extracellular region and only a short cytoplasmic tail (38 aa residues). The receptor showed 24% amino acid identity to the IL-6Rα chain. As well as identifying the IL-11 binding protein (now known as IL-11Rα), Hilton *et al* demonstrated that high affinity IL-11 binding sites were created on Ba/F3 cells stably transfected with IL-11Rα and gp130, whilst only low affinity sites were generated after co-transfection of IL-11Rα and LIFR. In addition, co-transfection of IL-11Rα and gp130 in Ba/F3 cells resulted in a proliferative response of the cells to IL-11. This suggested that the IL-11 receptor complex consisted of a ligand-binding IL-11Rα and the high-affinity converter and signal transducer gp130. This confirmed the finding by Yin *et al* (Yin *et al.*, 1993) who saw that gp130 neutralizing Abs inhibited the IL-11 induced proliferation of TF-1 cells.

Chérel and co-workers (Cherel *et al.*, 1995) utilized PCR amplification with redundant oligonucleotides corresponding to the W-S-x-W-S motif on a human placental cDNA expression library to isolate the human IL-11Rα. A cDNA clone encoding a protein with 82% homology to the murine IL-11Rα was identified. The 422 aa residue protein contained an Ig-like and cytokine receptor-like domain in its extracellular domain and a short (32 aa residue) cytoplasmic tail, similar to its murine counterpart. They also identified an alternatively spliced membrane-bound isoform which basically lacked a cytoplasmic domain, suggesting that the cytoplasmic tail could be of reduced importance.

Similar to the IL-6Rα chain and IL-6, recombinant soluble forms of IL-11Rα (sIL-11Rα) have been shown to bind IL-11 and possess agonistic properties in terms of IL-11 mediated signalling (Baumann *et al.*, 1996; Karow *et al.*, 1996; Neddermann *et al.*, 1996). Conversely, Curtis *et al* (Curtis *et al.*, 1997) have recently reported that on cells expressing both gp130 and the IL-11Rα chain as membrane-bound proteins, sIL-11Rα was able to antagonize IL-11 activity, speculating that sIL-11Rα antagonism may be due to limiting numbers of gp130 molecules on the cell surface. Whether soluble forms of IL-11Rα occur naturally is open to speculation, but given that closely related cytokine receptors do have such soluble isoforms, it seems probable.

Neddermann *et al* (Neddermann *et al.*, 1996) looked at the stoichiometry of IL-11 receptor complexes *in vitro* using a recombinant soluble form of IL-11Rα. They demonstrated that their soluble IL-11Rα was biologically active and bound IL-11 (the binding being enhanced by recombinant sgp130). By immunoprecipitation, they found evidence that a pentameric structure was formed, consisting of two IL-11 molecules, two sIL-11Rα molecules and one gp130. This finding was suprising because the IL-6 receptor complex is hexameric, comprising two each of IL-6, IL-6Rα and gp130, the gp130 molecules therefore homodimerizing (Ward *et al.*, 1994; Paonessa *et al.*, 1995). The CNTF receptor complex has also been studied (described below; De Serio *et al.*, 1995) and is also hexameric, consisting of two CNTFs, two CNTFRα chains and a heterodimer between gp130 and LIFR. As LIFR is known not to be involved in IL-11 receptor signalling, it was expected that two gp130 molecules would associate in the complex. As signal transducer chain dimerization is essential for signalling (discussed later), it was proposed that an as yet unidentified signal transducing chain is involved in the IL-11 receptor complex. This suggestion is still awaiting confirmation.

The receptor complexes involved in IL-11 signalling are shown in Figure 1.12.

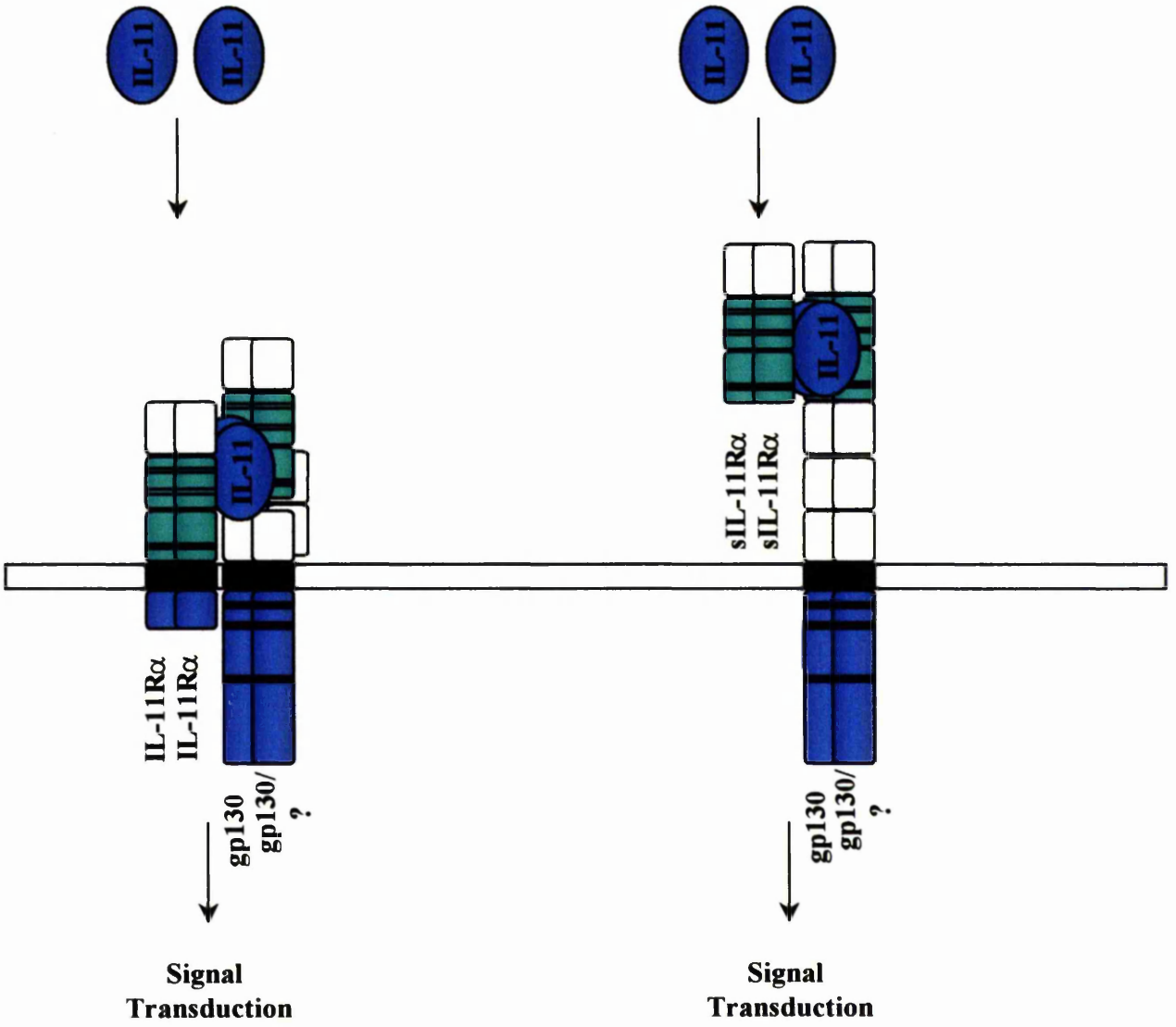


Figure 1.12. Functional IL-11 Receptors.

Components of the receptor complexes are coloured as in Figure 11. *(Left)* The complex formed by two IL-11, two gp130 (or gp130 and an as yet unidentifed signal transducer) and two membrane-bound IL-11Rα molecules, following an inital interaction between IL-11 and IL-11Rα molecules.

(Right) The complex formed by two IL-11, two gp130 (or gp130 and an as yet unidentifed signal transducer) and two soluble IL-11Rα molecules, following an inital interaction between IL-11 and sIL-11Rα molecules.The above complexes are based on the model proposed by Neddermann *et al* (1996).

(c) The LIF Receptor

LIF is a cytokine probably best known for its ability to inhibit embryonic stem (ES) cell differentiation (Smith *et al.*, 1988; Williams *et al.*, 1988). It is also a regulator of the hematopoietic and immune systems, as well as being active in hepatocyte stimulation, bone remodeling, embryonic development and neuronal differentiation (reviewed in Hilton, 1992).

High and low affinity LIF binding sites had been described on certain cell types prior to the cloning of LIFR (Hilton *et al.*, 1991). The cDNA encoding human LIFR was first identified by Gearing *et al* (Gearing *et al.*, 1991) using an expression cloning strategy with radioiodinated recombinant LIF on a human placental cDNA expression library. The open reading frame encoded a 190 kD protein of 1097 aa residues. The extracellular region of human LIFR shows homology with the cytokine type-I receptor family, actually containing 2 cytokine receptor-like domains separated by an Ig-like domain. Three fibronectin type III modules are found C terminal to the membrane proximal cytokine receptor like domain, similar to gp130, although they are not suspected to be involved in ligand binding (Horsten *et al.*, 1995). The 238 aa residue cytoplasmic domain also has similarity to other members of the cytokine type-I receptor, with regions rich in serine and threonine, proline and acidic residues. Certain regions of the cytoplasmic tail were found to be especially homologous with gp130, sharing 65% amino acid similarity. Human LIFR transfected onto the surface of COS7 cells was found to increase the number of low affinity binding sites for LIF (COS7 cells already containing high and low affinity binding sites for LIF), suggesting that it bound LIF with low affinity. The human LIFR cDNA was then used to identify cDNA clones expressing murine LIFR from a mouse adult liver cDNA library. Two clones were identified, both of which expressed a soluble form of the murine receptor and shared 76% amino acid identity with the human receptor. Full length cDNA encoding a membrane-bound form of the murine LIFR was later cloned and found to have a similar structure to the human LIFR.

It was subsequently observed that human LIF binds to murine and human LIFR, whilst murine LIF binds only murine LIFR. In fact, human LIF binds to murine LIFR

with a higher affinity than it does to human LIFR and murine LIF does to murine LIFR. By generating a series of interspecies chimeric receptors, Owczarek and colleagues (Owczarek *et al.*, 1997) recently showed that the Ig-like domain of murine LIFR was important for ligand-receptor interactions, irrespective of the origin of the cytokine receptor-like domains. It has been proposed that the Ig-like domain functions as a hinge, allowing binding to occur at two contact sites on the receptor (the two cytokine receptor-like domains). This receptor isomerization is efficient for human LIF and mouse LIFR, but less efficient for human LIF and human LIFR or mouse LIF and mouse LIFR. Owczarek *et al* have proposed that if this is the case, humanized mouse receptors can be generated by inserting the essential residues of the murine Ig-domain to allow efficient receptor isomerization into a soluble form of the human receptor. They suggested a use for such a chimeric receptor, being a potent antagonist of human LIF, as a therapeutic agent in diseases where LIF levels are expected to be elevated.

Gp130 was found to be an affinity converting co-receptor for LIF soon after the cloning of LIFR (Gearing *et al.*, 1992b), raising the possibility that gp130 could be a shared signal transducer for a family of cytokines, similar to the γc and βc chains. It was observed that when COS7 cells were transfected with gp130 and LIFR together, the affinity of LIF binding increased compared to that seen with LIFR alone, without increasing the number of LIF binding sites. Interestingly, the affinity of binding with both chains together was not as high as the high affinity LIF binding observed on LIF responsive cells, suggesting the possible participation of a third receptor component. In addition, Heymann and co-workers (Heymann *et al.*, 1996) more recently saw by cross-linking experiments that high affinity LIF binding on the surface of CHO cells transfected with gp130 and LIFR implicated a third chain of 140-150 kD. The identification and characterization of such a participating chain at the molecular level is presumably ongoing.

As mentioned above, a naturally occurring soluble form of LIFR has been identified in mouse serum (Layton *et al.*, 1992). They purified a so called LIF binding protein (LBP) by affinity chromatography using recombinant LIF, showed that it bound LIF and blocked LIF induced differentiation of M1 cells. It was seen to be present at fairly

high concentrations (1 μg/ml) in normal mouse serum, reduced in neonatal serum (2 to 4 fold) and elevated in pregnant mice (20 to 30 fold). The generation of mRNA encoding sLIFR was first described by Tomida *et al* (Tomida *et al.*, 1993) and more recently by Owczarek *et al* (Owczarek *et al.*, 1996), both groups demonstrating that the soluble form was encoded by a 3Kb mRNA transcript independent of the 9.5 kb mRNA transcript encoding the membrane-bound form of LIFR. The mouse LIFR gene structure has recently been reported, and been shown to have the potential to generate a splice alternative encoding a soluble form of the protein under different transcriptional control from the membrane-bound form (Chambers *et al.*, 1997).

It was speculated by Layton *et al* (Layton *et al.*, 1992) that the presence of sLIFR in the blood at fairly high concentrations could have the effect of blocking potentially harmful and undesired systemic actions of locally produced LIF, pointing out that LIF is actively produced prior to blastocyst implantation and it could be desirable to avoid translocation of LIF into other tissues. In accordance with this, elevated levels of sLIFR are found in the blood of pregnant mice. In addition, Tomida *et al* (Tomida *et al.*, 1993) saw that mRNA encoding sLIFR was upregulated in the liver of pregnant mice. Conversely, the low levels of sLIFR found in neonatals could allow the systemic beneficial effects of LIF such as neuron and muscle cell growth. Interestingly, soluble forms of LIFR have not been identified in human serum, although Tomida (Tomida, 1997) has recently reported the isolation of cDNAs encoding soluble forms of human LIF derived from a liver cDNA library, capable of expressing soluble human LIFR with the ability to bind LIF when transiently transfected in COS7 cells. In addition, a 160 kD native soluble receptor which bound LIF was detected in the supernatant of human chorio-carcinoma cells.

The receptor complex involved in LIF signalling is shown in Figure 1.13.

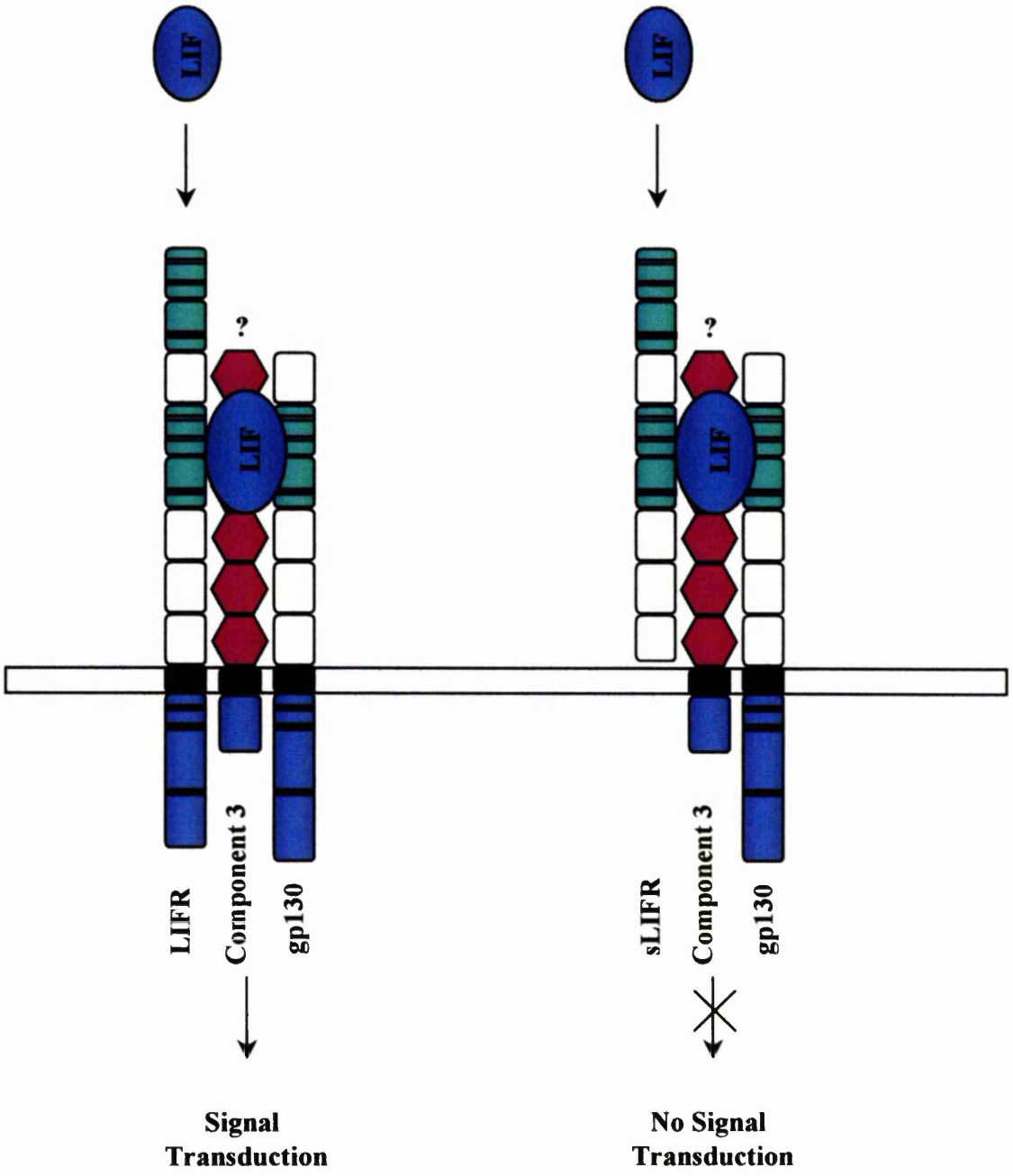


Figure 1.13. The LIF Receptor.

Components of the receptor complexes are coloured as in Figure 1.11.

(Left) The functional complex formed by two LIF and the signal transducing components gp130 and LIFR, following an initial interaction between LIF and LIFR.

(Right) The non-signaling complex formed by LIF, sLIFR and gp130. Although sLIFR presumably interacts firstly with LIF and subsequently recruits gp130, the inability of gp130 to undergo dimerization prevents signal transduction. In both cases, the as yet uncharacterized third putative subunit of the LIF receptor complex (Gearing, 1992; Heymann, 1996) is shown with red hexagonal modules within its extracellular region.

(d) The OSM Receptor

The cytokine OSM was first identified for its ability to inhibit the growth of the melanoma cell line A375 and augment the growth of normal fibroblasts. It was subsequently purified from a PMA stimulated lymphoma cell line, U937 and found to be a 18 kD polypeptide (Zarling *et al.*, 1986) expressed in monocytic and lymphocytic cell lines as well as normal adherent macrophages (Malik *et al.*, 1989; Brown *et al.*, 1987). It shares significant sequence and predicted secondary structure homology with LIF, G-CSF and IL-6 (Rose and Bruce, 1991).

The high affinity LIFR complex was first identified as a high affinity receptor for OSM (Gearing and Bruce, 1992a). It was seen that OSM was capable of binding the high affinity (LIFR and gp130) but not low affinity (LIFR) LIF receptor, possibly explaining the many overlapping functions of LIF and OSM. They did however speculate that this was not the only high affinity receptor for OSM, as A375 melanoma cells and H2981 carcinoma cells bound OSM with high affinity but not LIF. This would explain a number of OSM specific functions not shared by LIF. The differences found in signalling induced by the two cytokines confirmed suspicions that a second high affinity OSM receptor existed (Thoma *et al.*, 1994).

Gearing and Bruce, (1992a) showed that LIF and OSM shared a high affinity receptor by transfecting LIFR (which doesn't bind OSM) on the surface of COS7 cells in conjunction with a placental cDNA expression library. They screened cells for high affinity OSM binding and subsequently identified gp130 as the affinity converter. Co-transfection of LIFR and gp130 was found to confer high affinity binding to both LIF and OSM, thus demonstrating in parallel that gp130 was a second subunit in the high affinity LIFR complex and that the high affinity LIFR is shared by both LIF and OSM. They also saw that OSM bound gp130 with a low affinity comparable to the affinity found on low affinity binding sites on H2981 cells. They therefore speculated that gp130 was also a component in the OSM specific high affinity receptor. Liu *et al* (Liu *et al.*, 1992) also saw that gp130 bound OSM with low affinity, but this interaction alone was incapable of inducing an OSM specific response.

Heymann and colleagues (Heymann *et al.*, 1996) subsequently confused the model of OSM binding to LIFR/gp130 by demonstrating that OSM does not bind to LIFR and gp130 transfected CHO cells. One must assume from these results that a third chain is also required for high affinity binding of OSM to LIFR and gp130, not expressed endogenously on the CHO cell surface. Furthermore, we also must assume that this subunit is present on the surface of COS7 cells, as Gearing and Bruce (1992a) were capable of creating high affinity OSM binding sites on COS7 cells by co-transfection of LIFR and gp130 alone. These conclusions obviously await future validation.

The identification of an OSM-specific receptor gathered pace in 1994 (Liu *et al.*, 1994) when it was found by cross linking experiments on the H2981 cell line (which has both high affinity and low affinity OSM binding sites but does not bind LIF) identified either a 180 kD complex and a 280 kD complex. It was proposed that the 180 kD complex was the low affinity OSM /gp130 interaction, whilst the 280 kD complex represented a high affinity interaction between OSM, gp130 and the putative OSM specific chain of the high affinity OSM receptor complex. Murakami-Mori *et al* (Murakami-Mori *et al.*, 1995) reported the presence of an OSM specific receptor on the surface of AIDS-associated Kaposi's Sarcoma (KS) cells with the absence of the high affinity OSM/LIF shared receptor. They saw that the OSM induced KS cell growth (Radka *et al.*, 1993; Nair *et al.*, 1992; Miles *et al.*, 1992) and OSM binding was completely blocked by α-gp130 Abs, again suggesting that gp130 was a component of the high affinity OSM specific receptor.

The OSM specific subunit in the human high affinity receptor complex was cloned by Mosley *et al* (Mosley *et al.*, 1996) using degenerate PCR with primers designed from sequences conserved between LIFR, G-CSFR and gp130. PCR fragments showing novel sequence were used to screen cDNA libraries, and a cDNA clone of 3096 bp was identified with on ORF of 979aa residues. Sequence analysis showed the cDNA to encode a member of the cytokine type-I receptor family. The extracellular sequence showed a familiar structure with (from the N-terminus) the second half of a cytokine receptor-like domain (with a W-S-x-W-S motif), an Ig-like domain, a complete cytokine receptor-like domain and 3 fibronectin type III modules. The

structure is therefore very similar to the LIFR except that it is lacking the first half of the first cytokine receptor-like domain. The protein also had a relatively long (218 aa residue) cytoplasmic domain, with conserved sequences found throughout signalling subunits of the cytokine type-I receptor family. It was therefore hypothesized that the receptor subunit played a role in signal transduction. Transfection experiments in the cell line BAF-BO3 cell line showed that the novel receptor chain, in conjunction with gp130 conferred a response to OSM but not LIF. The response was blocked with Abs against the extracellular domain of the OSM specific receptor subunit (OSMR). Interestingly, OSM was found to bind to gp130 but not OSMR, indicating that OSMR and not gp130 is the high affinity converter.

Differences in the structure and mode of action of certain cytokine type-I receptor complexes between different species is not uncommon, and a recent publications suggests that the OSM and LIF receptor complexes are no exception. The murine cDNA encoding OSM (mOSM) was cloned (Yoshimura *et al.*, 1996) and used to produce recombinant OSM (Ichihara *et al.*, 1997). This allowed the characterization of biological function of mOSM as well as its functional receptors. mOSM, like mLIF and hOSM inhibited M1 cell proliferation and inhibited their differentiation, although it was significantly less potent. mOSM did not however induce proliferation of the cell line DA1.a whereas mLIF did. In addition, mOSM was much less efficient at promoting ES cell differentiation than mLIF or hOSM. It therefore appears that mOSM does not transmit signals through the functional mLIF receptor. This was confirmed by co-transfection of Ba/F3 cells with murine gp130 and LIFR. The cells proliferated in response to mLIF but not mOSM. It was however shown that mOSM binds to the cell line NIH3T3 with high affinity and inhibits growth of these cells. mLIF was not active on these cells. mOSM also bound gp130 with low affinity, suggesting that this is part of the OSM specific high affinity complex in the murine system as well. These results indicate that OSM and LIF have separate high affinity receptors in the mouse, and OSM does not act through the high affinity LIFR. The cloning of a murine OSMR specific subunit will help clarify these differences.

The reecptor complexes for OSM are shown in figure 1.14.

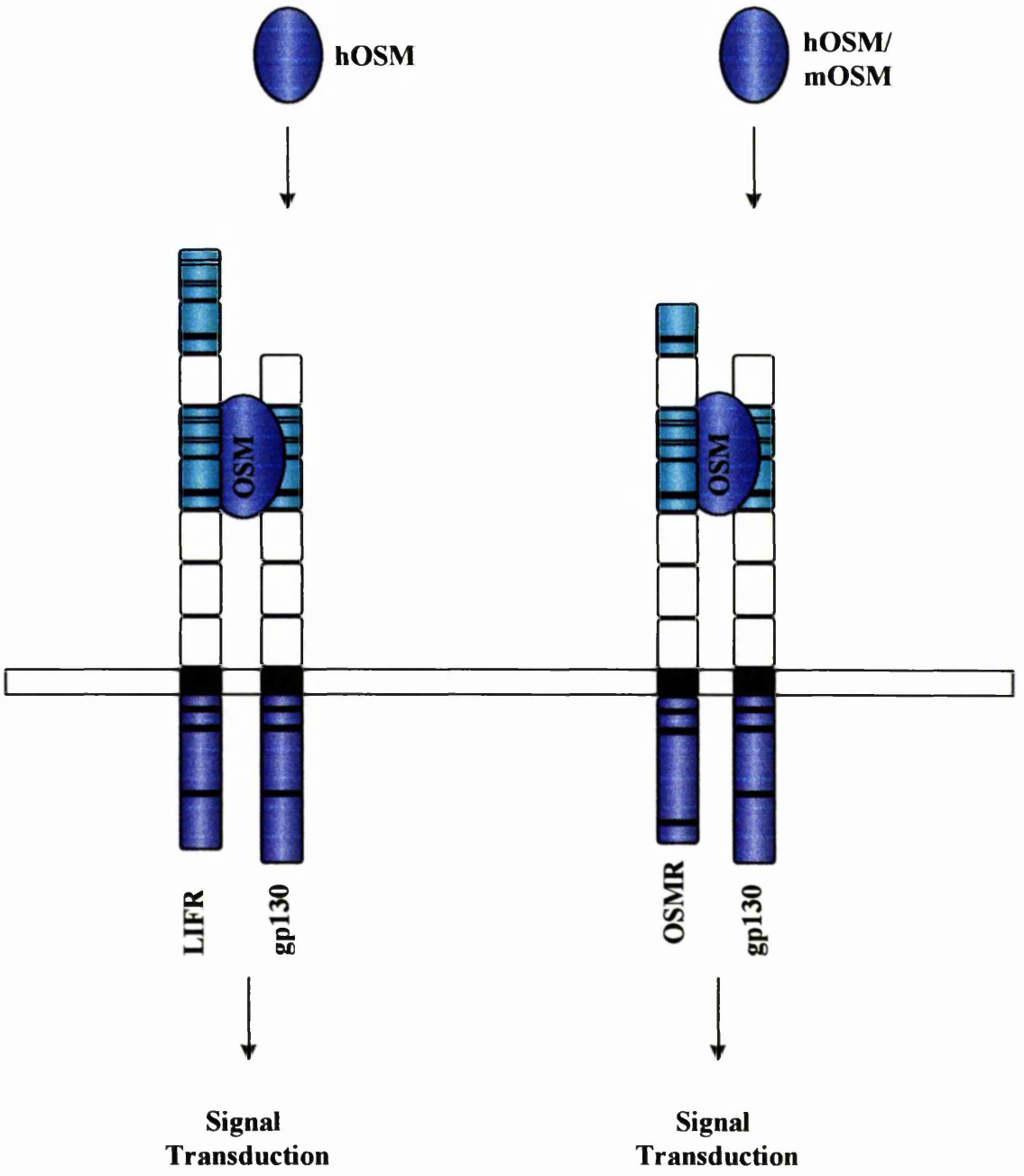


Figure 1.14. Functional OSM Receptors.

Components of the receptor complexes are coloured as in Figure 1.11.

(Left) Human OSM (hOSM) binds initially to gp130 followed by the recruitment of LIFR leading to signal transduction.

(Right) hOSM or murine OSM (mOSM) binds initially to gp130 followed by the recruitment of OSMR leading to signal transduction.

The above model is in accordance with the findings of Ichihara *et al* (1997), who showed that in mice, OSM does not use a receptor complex comprising gp130 and LIFR.

(e) The CNTF Receptor

CNTF was first identified as a neuronal survival factor as far back as 1979 (Adler *et al.*, 1979) with a molecular weight of 20-24 kD. CNTF has been found to promote the survival of a wide range of cultured neurons (reviewed in Manthorpe *et al.*, 1985; Sendtner *et al.*, 1991) as well as inducing certain types of neuronal differentiation and survival. Outside of the nervous system, CNTF also acts on skeletal muscle, liver, bone marrow and ES cells (reviewed in Sendtner *et al.*, 1994).

The molecular cloning of CNTF (Lin *et al.*, 1989; Stockli *et al.*, 1989) revealed that it was not a member of the neurotrophin gene family but showed more similarity to IL-6, LIF, G-CSF and OSM (Bazan, 1991). This observation has was underscored by the cloning of a CNTF binding protein, CNTFRα. The receptor was first detected using a genetically engineered recombinant CNTF with an epitope 'tag' allowing Abs against the tag to detect the receptor on neuronal cells (Squinto *et al.*, 1990). An expression cloning approach with COS7 cells using the same tagged CNTF captured with immobilized α-tag Ab was then utilized to clone the cDNA for the receptor (Davis *et al.*, 1991). The cDNA encoded a 372 aa residue 72 kD protein sharing homology with IL-6Rα, sharing 30% aa identity within the extracellular region. Like IL-6Rα and IL-11Rα, CNTFRα has an extracellular region containing an Ig-like domain and a cytokine receptor-like domain.

CNTFα appeared to be relatively novel within this family however in that it contains a hydrophobic region C terminal of the cytokine receptor-like domain and no cytoplasmic domain, reminiscent of many proteins anchored to the membrane by glycosyl-phosphatidylinositol (GPI) linkage. The hydrophobic region of such proteins are invariably cleaved during processing and absent from the mature protein (Ferguson and Williams, 1988; Englund, 1993). CNTFRα was confirmed to be GPI-anchored by treatment of the cell line SH-SY5Y, expressing the CNTFRα, and COS cells transfected with the cloned cDNA for CNTFRα, with GPI-specific phospholipase C (PI-PLC). GPI linkage is sensitive to PI-PLC, and in the cells lines mentioned above, ^{125}I-CNTF binding was reduced by 50% following treatment with

PI-PLC. Epitope-tagged receptor could also be detected in the supernatant by Western blot analysis following PI-PLC treatment. In accordance with the biological effects of CNTF, CNTFRα expression was found predominantly in the nervous system and skeletal muscle.

The cytoplasmic domain of CNTFRα was absent, thus excluding this chain from participating in signal transduction. Taking this into account as well as the homology between CNTFRα and IL-6Rα, it was widely suspected that CNTF required a second receptor component for signalling. The observation that CNTF and LIF had overlapping functions on neurons (Rao *et al.*, 1990) as well as signalling pathways implicated one or more of the LIF signal transducers (LIFR and gp130) in CNTF signalling. Ip *et al* (Ip *et al.*, 1992) were the first to implicate gp130 in CNTF signalling. They demonstrated the overlapping functions of LIF and CNTF in the rat sympathoadrenal progenitor cell line MAH in terms of blocking cell proliferation and increasing choline acetyltransferase (ChAT) activity. In addition, similar patterns of tyrosine phosphorylation were detected in response to the two cytokines in MAH and Ewing's sarcoma cell lines as well as a neuroepithelioma (SK-N-LO).

Three proteins named CLIP 1 (approximately 190 kD), CLIP 2 (approximately 145 kD) and CLIP 3 (approximately 75 kD) were seen to be tyrosine-phosphorylated in response to both LIF and CNTF, but not FGF (to which the cells were also responsive). CLIP 3 was found to be both variable and difficult to detect by immunoprecipitation with α-phosphotyrosine Ab. CLIP 1 and CLIP 2 however were found to be cell surface proteins making up part of the receptor complexes. Immunoprecipitation with biotinylated CNTF revealed proteins with a size corresponding to CLIP 1 and CLIP 2, demonstrating that they were part of the CNTF receptor complex. CLIP 2 was subsequently identified as gp130 by depleting it from cell lysates prior to immunoprecipitation with an α-gp130 Ab. In addition, α-gp130 Abs blocked CNTF- and LIF-induced signalling in responsive cells. CLIP 1 was suspected to actually be LIFR, sharing the same molecular weight.

LIFR was subsequently confirmed as a CNTFR component by Davis *et al* (Davis *et al.*, 1993b) and Baumann *et al* (Baumann *et al.*, 1993). Davis *et al* generated epitope tagged gp130, LIFR and CNTFRα and reconstituted different combinations of the receptor subunits on COS cells. Cells were treated with LIF or CNTF, and ligand-dependent co-immunoprecipitation was assayed. Functional activation of the complexes were also assayed by the induction of gp130 and LIFR tyrosine phosphorylation. As expected, LIF triggered gp130/LIFR association and activation but CNTF did not. CNTF did however induce the association of gp130, LIFR and CNTFRα as well as gp130 and LIFR tyrosine phosphorylation. In the absence of LIFR, CNTF induced CNTFRα/gp130 association, but failed to induce tyrosine phosphorylation of gp130. The proposed model for receptor complex constitution was as follows: (1) CNTF interacts with its ligand specific receptor subunit, CNTFRα. (2) a single gp130 molecule is recruited to give a CNTF/CNTFRα/gp130 intermediate. (3) A LIFR subunit is engaged, with LIFR/gp130 heterodimerization leading to signal transduction. This model has since been refined by De Serio et al (De Serio *et al.*, 1995). They used epitope tagged recombinant soluble molecules to demonstrate by immunoprecipitation that, similar to the IL-6/IL-6R complex, CNTF also forms a hexameric complex with its high affinity receptor. It is proposed that two CNTF molecules associate with two CNTFRα molecules along with one gp130 molecule and one LIFR molecule.

CNTF and LIF share the same signal transducing receptor chains and therefore the same signalling pathways. CNTF however has a more limited spectrum of biological activities than LIF. This is accounted for by the relatively ubiquitous expression of LIFR and gp130 with the more regulated and specific expression of CNTFRα. Similar to the other ligand-binding, non-signalling receptor chains (IL-6Rα and IL-11Rα), CNTFRα exists naturally in agonistic soluble form. This form was initially identified and characterized by Davis *et al* (Davis *et al.*, 1993a). Soluble CNTFRα (sCNTFRα) generated either in E. *coli* or generated by PI-PLC cleavage from mammalian cells was capable, in concert with CNTF, of inducing LIF-like responses (substrate attachment, cell flattening and elongation) in the normally CNTF unresponsive M1 cell line. CNTF or sCNTFRα alone had no effect on the cells. sCNTFRα also made

CNTF almost as potent an effector as LIF on TF1 cells. On cell lines unresponsive to LIF, CNTF and sCNTFRα were also unable to induce an effect. In addition to these findings, large amounts of sCNTFRα (up to 10ng/ml) were detected in human cerebrospinal fluid as well as detectable levels in explants derived from denervated muscle. The latter was proposed to coincide with the release of CNTF from injured nerves, thus allowing the two to combine and evoke *in vivo* responses. The sCNTFRα could increase the neuronal sensitivity to CNTF. Such interactions could play an important part in the regeneration response following injury. LIF responsive cells would also become a target for circulating CNTF and sCNTFRα, thus allowing CNTF to become an effector in the hematopoietic and other systems.

The receptor complexes involved in CNTF signalling are shown in Figure 1.15.

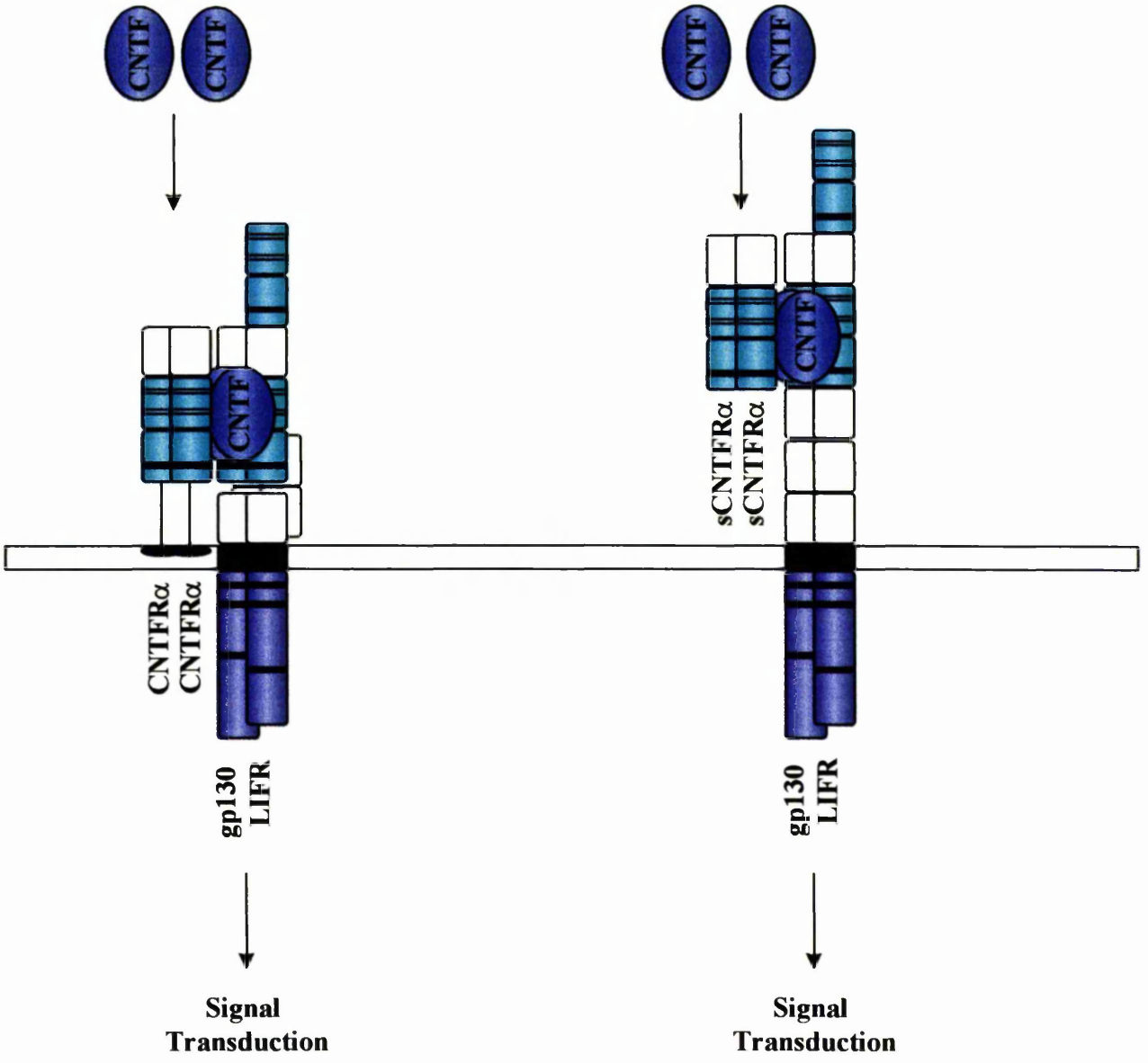


Figure 1.15. Functional CNTF Receptors.

Components of the receptor complexes are coloured as in Figure 1.11. The GPI-linkage (if present) is represented by a thin line between the cytokine receptor-like domain of CNTFRα and the cell membrane.

(Left) The complex formed by two CNTF, two membrane-bound CNTFRα, one LIFR and one gp130 molecule following an inital interaction between CNTF and CNTFRα molecules.

(Right) The complex formed by two CNTF, two soluble CNTFRα, one LIFR and one gp130 molecule following an inital interaction between CNTF and soluble CNTFRα molecules.

The above complexes are based on the model proposed by De Serino *et al* (1995).

(f) The CT-1 Receptor

CT-1 is the most recent addition to the family of cytokines sharing the LIFR/gp130 signal transducers, first identified by Pennica et al (Pennica *et al.*, 1995a) using an expression cloning strategy. This group set out to identify fetal or embryonic growth factors mediating the onset of cardiac hypertrophy, a condition that ultimately leads to heart failure. They utilized an *in vitro* assay system for myocardial hypertrophy based on embryonic stem cells coupled to an expression cloning strategy. A cDNA library derived from differentiated mouse embryonic stem cells was transfected into HEK 293 cells and conditioned media from pools of clones tested for their hypertrophic activity. Two cDNA clones were identified encoding a 22 kD, 203aa residue protein, subsequently named CT-1 with structural homology to LIF and CNTF.

The known biological activities of CT-1 have progressively expanded and it is now known to share many of the overlapping biological activities exhibited by cytokines signalling through LIFR/gp130 (Pennica *et al.*, 1995b; Peters *et al.*, 1995; Arce *et al.*, 1998; Pennica *et al.*, 1996; Richards *et al.*, 1996). Evidence for CT-1 utilizing a LIFR/gp130 receptor complex resulted from the demonstrated that CT-1 could not only compete LIF for binding to M1 cells, but this binding was inhibited by up to 80% with α-gp130 mAbs. In addition, both CT-1 and LIF could be cross-linked to a protein with a molecular weight resembling that of LIFR (approximately 200 kD) and CT-1 directly bound recombinant sLIFR with relatively low affinity, this affinity being increased by the addition of recombinant sgp130. It did not however bind to sgp130 alone (Pennica *et al.*, 1995b).

A more recent publication however has hinted at the involvement of a third receptor component involved in the functional CT-1 receptor. Robledo *et al* (Robledo *et al.*, 1997) confirmed the involvement of gp130/LIFR in the CT-1 receptor complex by analyzing tyrosine phosphorylation, using α-gp130 neutralizing Abs and reconstituting gp130 and LIFR on the surface of COS7 cells. Cross-linking experiments on the surface of SK-N-MC cells (which express high and low affinity binding sites for CT-1) with ^{125}I labeled CT-1 did however reveal not only bands of

molecular weight 190-210 kD and 130-150 kD (presumably representing LIFR and gp130 respectively) but an additional band of 80 kD. Immunoprecipitation experiments with Abs raised against gp130 or LIFR confirmed that gp130 and LIFR were components of the cross-linked products. The three components (gp130, LIFR and the 80 kD protein) were also shown to remain tightly associated suggesting their participation in a tripartite receptor complex. In addition, bands were competed by an excess of unlabeled CT-1 or LIF. When carbohydrates were removed from the protein backbone, the third receptor component had an apparent molecular mass of 45kD. When COS7 cells were used for the same cross-linking experiments, an 80 kD protein was again identified (presumably the endogenous protein), but only when both gp130 and LIFR were co-transfected on the cell surface, with no cross-linking observed on untransfected cells, suggesting that CT-1 did not bind directly to the 80kD component.

Robledo *et al* then demonstrated in the same paper that the 80 kD protein (the putative CT-1Rα) was required for a fully functional CT-1 response. It was observed that the murine hematopoietic cell line Da1.a only bound CT-1 with low affinity, although the cell line displays high affinity binding sites for both human and mouse LIF, and strongly proliferates in the presence of LIF. This suggested that both LIFR and gp130 were present on the surface of this cell line. Cross-linking of murine ^{125}I labeled CT-1 to the cell surface revealed only one band of 170 kD corresponding to murine LIFR. This indicated that the cells did not express CT-1Rα. Although a superficial similarity of this proposed CT-1 receptor model to that of the CNTFR complex does seem apparent, the fact that the putative CT-1Rα does not directly bind CT-1 would seem to point towards an initial complex forming between CT-1, LIFR and CT-1Rα followed by recruitment of gp130 and subsequent signalling. It therefore appears that contrary to the other α chains in this family, CT-1Rα acts as an affinity converter as opposed to a ligand binder.

The proposed receptor complex involved in CT-1 signalling is shown in Figure 1.16.

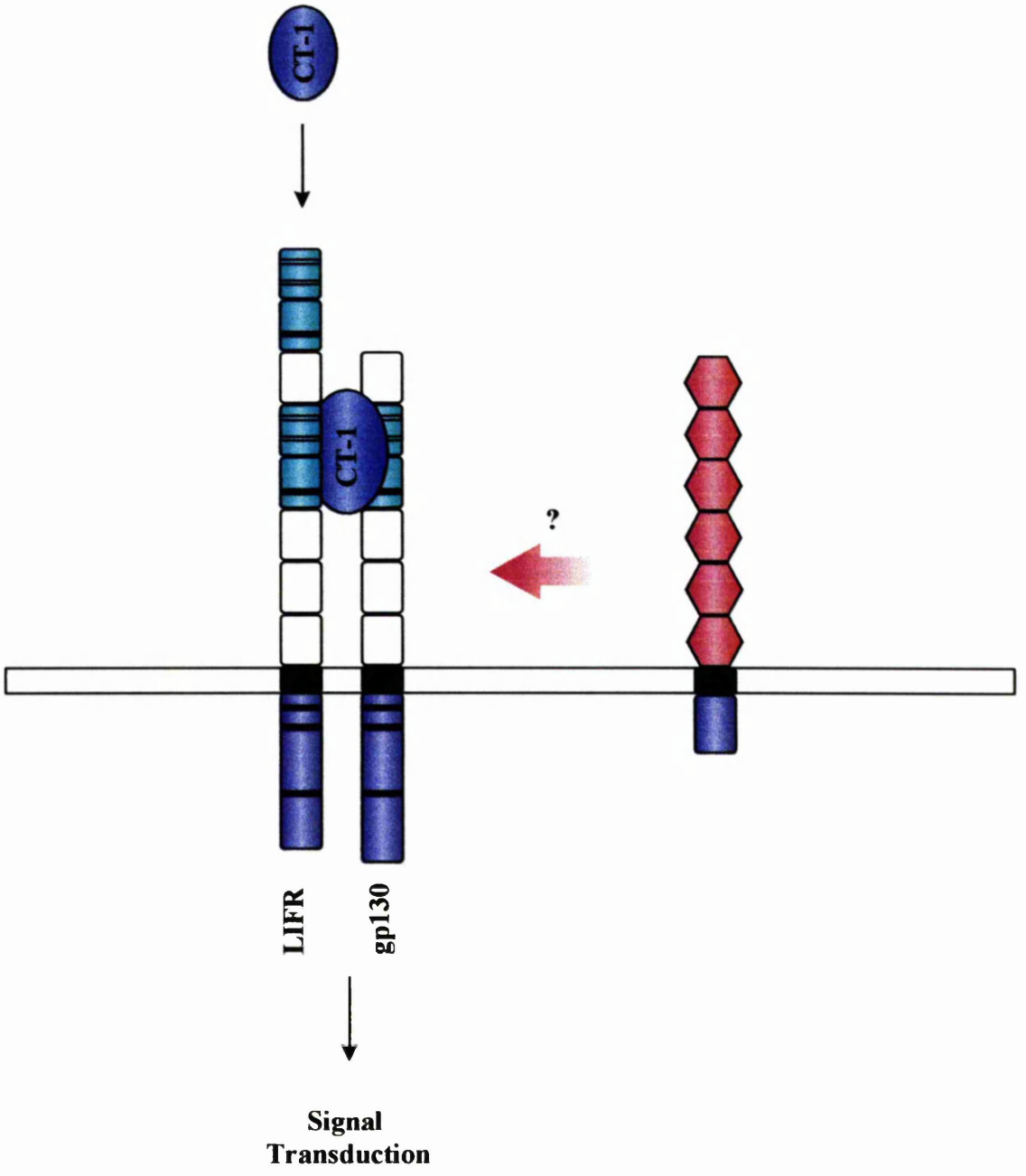


Figure 1.16. The Functional CT-1 Receptor.

Components of the receptor complex are coloured as in Figure 1.11.

CT-1 binds initially to LIFR followed by the recruitment of gp130 leading to a high- affinity receptor complex capable of signal transduction (Pennica, 1995b). The 80 kD putative third component of this receptor complex, reported by Robledo et al (1997), is shown with red hexagonal modules.

(g) LIFR/gp130 Shared Receptor Signalling

As a direct consequence of shared signalling subunits, cytokines utilizing this family of receptors exhibit similar cytoplasmic signalling. Ligand binding (or high affinity converting in the case of the CT-1 receptor) α chains within this family typically have short cytoplasmic tails and hence they are not involved in signalling. They generally serve to convey cellular responsiveness to their respective cytokines by facilitating the formation of a functional high affinity receptor complex between the ligand and the signalling subunits. The known signalling subunits within this family are gp130, LIFR and OSMR. Signalling is suspected to occur upon homodimerization of two gp130 subunits in the case of IL-6 and IL-11, heterodimerization of LIFR and a gp130 subunits in the case of LIF, OSM, CNTF and CT-1 or heterodimerization of OSMR and gp130 subunits in the case of OSM.

As seen with other cytokine type-I receptors, ligand binding causes the signal transducing receptor components to undergo tyrosine phosphorylation. In terms of gp130, homo- or heterodimerization triggers the activation of cytoplasmic tyrosine kinases. The Janus kinase family members Jak1, Jak2 and Tyk2 associate with gp130 and are activated in response to cytokines signalling through gp130 (Lutticken *et al.*, 1994; Stahl *et al.*, 1994; Stahl *et al.*, 1995; Narazaki *et al.*, 1994). The cytoplasmic region of gp130 contains three regions of homology with other cytokine type-I receptors, called box 1, box 2 and box 3. Box 1 and box 2 motifs are found and shown to be necessary for signalling in many of the cytokine type-I receptor signalling subunits (Murakami *et al.*, 1991; Ziegler *et al.*, 1993), with the box 3 motif restricted to a subfamily of receptors showing closer homology to gp130 (LIFR, OSMR, G-CSFR and OBR, the receptor for leptin). Mutational analysis has showed the box 1 region of gp130 to be important for Jak activation (Tanner *et al.*, 1995). STAT 3 has also been implicated in cytokine signalling through gp130 (Akira *et al.*, 1994; Nakajima *et al.*, 1996; Minami *et al.*, 1996; Yamanaka *et al.*, 1996) with the possible involvement of STAT 1α as well as STAT 5 (Lai *et al.*, 1995b; Lai *et al.*, 1995c; Piekorz, 1997). All the receptors pertaining to the gp130 subfamily, with the exception of IL-12R, contain a box 3 motif and can utilize STAT 3 for signalling. The

IL-12 receptor lacks the box 3 domain and has an impaired STAT 3 activation (Jacobson *et al.*, 1995; Bacon *et al.*, 1995) when compared to other members of the subfamily. This seems to imply an involvement for box 3 in STAT 3 activation.

A recently proposed model of signal transduction through gp130 (Taga and Kishimoto, 1997) is as follows. Jaks associate with gp130 and become activated upon its homo-/heterodimerization, leading to phosphorylation of tyrosine residues on the cytoplasmic tail of gp130, including site(s) within box 3. This gives a docking site for the SH2 domains of STAT 3 (Heim *et al.*, 1995), the STAT 3 then becoming a substrate for the resident Jak kinases. Phosphorylated STAT 3 can then dissociate from gp130 and homodimerize via two intermolecular SH2-phosphotyrosine bonds. STAT 3 can then interact directly with specific DNA binding sites to activate target gene transcription (Wegenka *et al.*, 1993). It therefore appears that specificity of the signal is entirely dependent on the specificity of the STAT docking site present on the cytoplasmic tail of receptor subunits. The box 2 motif of gp130 is also suspected to play a role in signal transduction, as demonstrated by its role in gp130-mediated upregulation of DNA synthesis in murine BAF-BO3 cells (Murakami *et al.*, 1991), although its precise contribution remains unclear.

It should be mentioned that both LIFR and OSMR associate with members of the Jak family of kinases and contain box 3 motifs. They are therefore potentially capable of recruiting and activating identical STAT signalling pathways to those of gp130. Interestingly, it was recently shown that although LIFR and OSMR are equally effective in inducing STAT 3 activation, they are many times more effective at inducing STAT 5 than is the gp130 homodimer (Kuropatwinski *et al.*, 1997). Such a proposed difference in signalling between a gp130 homodimer and a LIFR/gp130 or OSMR/gp130 heterodimer could explain differences observed between the effects of LIF, OSM and IL-6 on hepatic cells (Richards *et al.*, 1992; Lai *et al.*, 1995a).

In addition to STAT signalling pathways, signalling through gp130 and LIFR is also reported to involve a number of other non-receptor protein kinases, most notably mitogen-activated protein kinase (MAPK). IL-6 has been shown to increase the ratio of GTP- to GDP-bound RAS (Nakafuku *et al.*, 1992) whilst c-Raf-1 and MAPK are

also reported to have been phosphorylated (Boulton *et al.*, 1994; Daeipour *et al.*, 1993). Similarly, Signalling through LIFR has also been shown to activate several components of the MAPK cascade (Schiemann and Nathanson, 1994; Thoma *et al.*, 1994; Yin and Yang, 1994b). The nuclear factor NF-IL-6 (which binds to acute phase gene promoter regions to induce their transcription) is phosphorylated and activated by MAPK following IL-6 stimulation (Nakajima *et al.*, 1993).

gp130/LIFR/OSM signalling pathways are summarized in figure 1.17.

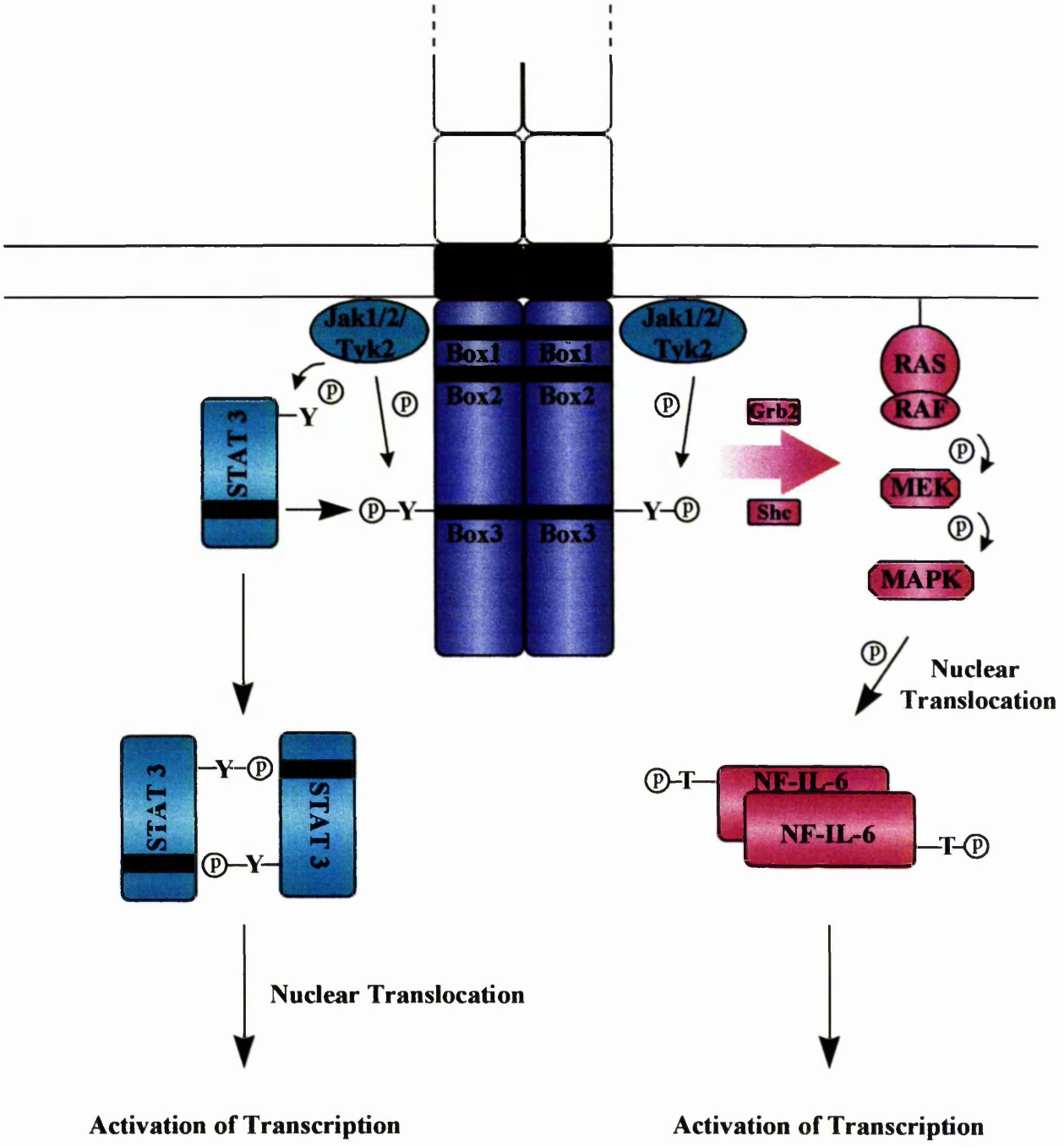


Figure 1.17. gp130 Mediated Activiation of STAT 3 and NF-IL-6.

(Left) Ligand- induced gp130 homodimerization results in activation of Jaks (Jak1 and 2 and Tyk2), which are associated at the box 1 motif, leading to the phosphorylation of tyrosine residues in cytoplasmic region of gp130. STAT3 is subsequently recruited to the phosphotyrosine of the box 3 motif via its SH2 domain (thick black line), where it too is tyrosine phosphorylated by the gp130 associated Jaks. Phosphorylated STAT3 then homodimerizes, via SH2/phosphotyrosine interactions, translocates into the nucleus and activates gene transcription.

(Right) gp130 dimerization leads to activation of NF-IL-6 by threonine phosphorylation through the MAP Kinase activation pathway. (MAPK, MAP kinase; MEK, MAPK Erk kinase). This schematic representation of gp130 induced signaling was adapted from the review of Taga (1996).

(1.2.4) Implications of Shared Receptor Subunits

The best demonstrations of functional cytokine specificity or redundancy comes from the study of mice where the cytokine in question or its ligand binding or signalling subunit has been inactivated by targeted disruption or a naturally occuring mutation. The phenotype given by mice deficient in certain cytokines can be remarkably normal when taking into account the biological functions of a given cytokine, indicating a large degree of functional redundancy.

Within the family of cytokines sharing LIFR/gp130, the large degree of pleiotrophy exhibited could lead one to believe that inactivation of a given cytokine could lead to a rather dramatic phenotype. Mice with a targeted disruption of either IL-6, LIF or CNTF do not exhibit overt developmental abnormalities however, and their phenotypes are relatively mild (Kopf *et al.*, 1994; Stewart *et al.*, 1992; Masu *et al.*, 1993). The fact that all these cytokines signal through gp130 and LIFR suggests that any one of the ligands can substitute for another which may be lacking. This is however dependent on the fact that a substituting ligand must be present in the correct tissue, and it must have its own ligand binding chain (in the case of IL-6, IL-11, CNTF and possibly CT-1) expressed on the target cell of the ligand it is to replace. The presence of soluble receptors could however reduce the necessity of membrane-bound ligand-binding chains however. The impaired production of Abs and acute phase proteins following the appropriate treatment in IL-6 deficient mice (Kopf *et al.*, 1994) could reflect a deficiency in compensatory cytokines or their ligand binding chain/functional receptor in a specific microenvironment.

The functional high affinity receptor for LIF in humans is also utilized by OSM and possibly CT-1. It is therefore probable that in the absence of LIF, its functions on a given cell could be compensated for simply by the local presence of OSM and/or CT-1, (although the signalling of OSM through LIFR in the murine system needs to be clearly defined; Ichihara *et al.*, 1997) as well as cytokines signalling through a gp130 homodimer. LIF deficient mice do however show certain altered phenotypes, like a deficiency in the proliferation of thymocytes following allogenic stimulation (Escary *et al.*, 1993). In addition, LIF was seen to be essential for blastocyst implantation in

pregnant female mice, although the blastocysts in LIF deficient mice were themselves viable (Stewart *et al.*, 1992). The apparent phenotype observed could again be due to the absence of the compensatory cytokines or their ligand binding receptor components in a local environment.

CNTF deficient mice also displayed neuronal problems although the phenotype was mild, came in adulthood and mice showed no neurological abnormalities (Masu *et al.*, 1993). These findings would appear to suggest that other LIFR/gp130 signalling cytokines are capable of replacing CNTF. In has also been seen that 2.5% of the Japanese population are homozygous for a null mutation in CNTF and appear quite normal even in old age (Takahashi *et al.*, 1994). This would also imply that CNTFRα disruption should lead to the same phenotype. Strikingly, mice deficient for CNTFRα showed a rather dramatic phenotype (DeChiara *et al.*, 1995). Newborn mice did not initiate the feeding process, died shortly after birth and showed a dramatic loss in all motor neuron populations examined. These findings clearly point to a second factor signalling through a receptor complex utilizing CNTFRα. This second factor also appears to be more critical for normal development that CNTF. The existence of such a second ligand awaits confirmation.

Unsuprisingly, mice deficient in the receptor signalling chains gp130 and LIFR have much more severe phenotypes. Disruption of gp130 should, in theory, lead to a total deficiency in signalling of the cytokines IL-6, IL-11, LIF, OSM CNTF and CT-1. Indeed, mice lacking gp130 had a lethal phenotype with death occurring at 12.5 days post coitum (d.p.c.; Yoshida *et al.*, 1996). A striking feature was the hypoplastic development of the ventricular myocardium. It has been suggested that cardiomyocytes within the affected area lack the proliferative and/or anti-apoptotic signals provided by gp130 dimerization. These findings correspond to the biological effects of CT-1 mediated through gp130, as well as the observation that IL-6 and sIL-6Rα upregulate DNA synthesis in cultured mouse cardiomyocytes. In addition, mice transgenic for IL-6 and IL-6Rα, demonstrate hypertrophy in the ventricular wall as well as hypertonic change in neonatal cardiomyocytes *in vitro* (Hirota *et al.*, 1995). These results clearly demonstrated the importance of gp130 signalling in

cardiomyocyte regulation. In addition this phenotype, a greatly reduced number of stem cells were seen in the spleen of fetuses at 13.5 d.p.c. (Yoshida *et al.*, 1996), going along with the observation that in the IL-6/IL-6Rα transgenic mice, progenitor cells were dramatically increased and the mice showed a number of associated hematological disorders (Hirota *et al.*, 1995).

As is expected, LIFR deficient mice are also severely impaired, but to a lesser extent than gp130 deficient mice. This is probably explained by the fact that LIFR is not implicated in all the receptor complexes that gp130 participates in. Disruption of LIF, CNTF and CT-1 signalling would be predicted, along with the signalling involved in a subset of OSM responses, although in the murine system, this remains unclear (discussed in Ichihara *et al.*, 1997). The LIFR was disrupted by homologous recombination by Ware *et al* (Ware *et al.*, 1995), where it was observed that some LIFR-deficient mice did survive to term, especially if delivered by Cesarean section, although all surviving pups died within the first day after birth. The major phenotypes were firstly a reduction in the number of -/- fetuses expected, a disrupted placental architecture with -/- fetuses, profound mineralized bone loss, an increase in liver glycogen and an reduction in numbers of spinal cord and brain cell astrocytes. In contrast, fetal hematopoiesis appears to be fairly normal. Such a phenotype is more that likely a direct consequence of the ability of the cytokines signalling through gp130 homodimers (IL-6 and IL-11) to compensate for the loss of function of cytokines signalling through the LIFR. One could argue that in respect to fetal hematopoiesis, the gp130 homodimer and/or the OSMR/gp130 heterodimer plays a dominant role over the LIFR/gp130 heterodimer.

One interesting observation in LIFR deficient mice is the normal ventricular myocaridum development, in contrast to that of gp130 deficient mice. It is known that CT-1 utilizes a LIFR/gp130 heterodimer, therefore raising two possibilities. Firstly, CT-1 signalling is compensated for by signalling through a gp130 homodimer (i.e. by IL-6 or IL-11). It could also be possible that CT-1 utilizes a second receptor independent of LIFR/gp130, containing a CT-1 specific chain and gp130. A CT-1 specific chain has been reportedly identified by cross-linking experiments (Robledo *et al.*, 1997), although this is thought to complex with both LIFR and gp130. This

anomaly between the two knockout mice is open to clarification. A second publication has described the importance of LIFR for the development and survival of motor neurons, a similar finding to that seen for the CNTFRα deficient mice. This could suggest that the putative second factor for CNTFRα (discussed earlier) signals through LIFR, thereby utilizing the same functional receptor complex as CNTF. The third signalling subunit within this family of receptors is OSMR, forming an OSM specific functional receptor with gp130. This functional receptor is responsible for the signalling involved in a subset of responses for human OSM, and possibly all of the responses for murine OSM (Ichihara *et al.*, 1997). OSMR deficient mice have yet to be generated, but if the paper of Ichihara *et al* proves to be accurate, such mice will be completely deficient in their responses to OSM. It will be interesting to see how redundant, if at all OSM responses are in the murine system.

The study of cytokine functions within the family signalling through LIFR/gp130 by analyzing the physiological effects of their absence has demonstrated conclusively that they share a large degree of redundancy. Many of the known responses attributed to the individual cytokines can in fact occur at normal or only reduced levels in their absence. These studies do not exclude a dominant role for individual cytokines within this family. As target cell cytokine specificity is conveyed through the ligand specific receptor components IL-6Rα, IL-11Rα, CNTFRα, OSMR and LIFR, intricate compensatory mechanisms may exist whereby the absence of one cytokine may lead to the upregulated expression of a compensatory cytokine and/or its specific binding subunit on the target cell surface. It should also be mentioned that within this family, the upregulated production of soluble binding subunits is also a possibility. In humans, LIF deficiency could be compensated for directly by a local production of OSM.

The above section has described in some detail the involvement of cytokine type-I receptors in cytokine mediated-effects on target cells and signal transduction to the cell nucleus. The following section deals with members of the cytokine type-I receptor family which actually behave as cytokines themselves.

(1.3) Cytokine Type-I Receptors as Cytokines

(1.3.1) IL-12

(a) Biological Effects of IL-12

IL-12 was first identified as an active component in the supernatant of phorbol diester stimulated EBV-transformed cell lines capable of inducing IFN-γ production in T and NK cells, as well as enhancing cell-mediated cytotoxicity and comitogenic effects on resting T cells (Gately *et al.*, 1986; Stern *et al.*, 1990; Kobayashi *et al.*, 1989). It was subsequently purified to purity by two groups although at the time it was given different names. The first group purified IL-12 using its ability to stimulate NK cell mediated cytotoxicity and NK cell production (Kobayashi *et al.*, 1989) and named the molecule natural killer cell stimulatory factor (NKSF). Around the same time, Stern and colleagues (Stern *et al.*, 1990) purified a molecule capable of synergizing with IL-2 to induce T cell and lymphokine-activated killer (LAK) cell activation. The molecule was appropriately named cytotoxic lymphocyte maturation factor (CLMF). Subsequent characterization revealed both to be the same cytokine, now named IL-12.

The spontaneous activation by IL-12 of both human and murine NK cells has since been confirmed by a number of groups using recombinant IL-12, although less potent than IL-2, IFN-α and IL-15 (Kobayashi *et al.*, 1989; Robertson *et al.*, 1992; Chehimi *et al.*, 1992; Chehimi *et al.*, 1993, Carson *et al.*, 1994). In addition, an increased NK cell granularity and a facilitation of granule exocytosis has been reported upon IL-12 treatment (Chehimi *et al.*, 1993; Bonnema *et al.*, 1994), as well as an upregulation of expression of a number of different genes including the β2 integrin adhesion molecules (Robertson *et al.*, 1992; Rabinowich *et al.*, 1993). The other known effector functions of IL-12 include enhancement of T cell proliferation and induction of preactivated T and NK cells (Gately *et al.*, 1991; Perussia *et al.*, 1992), enhanced survival and proliferation of hematopoietic stem cells and an increase in numbers and size of colonies formed by progenitor cells in synergy with steel factor (SF) and IL-3

(Jacobsen *et al.*, 1993; Ploemacher *et al.*, 1993; Hirayama *et al.*, 1994; Bellone and Trinchieri, 1994) although conversely, an inhibition of colony formation is observed by IL-12 in the presence of NK cells (Bellone and Trinchieri, 1994). In addition, IL-12 induces T and NK cells to produce a multitude of cytokines, in particular IFN-γ, where it is seen to be more effective than IL-2 (Chan *et al.*, 1991). This implicates IL-12 as a promoter of Th1 cell generation and therefore cell-mediated immunity (Hsieh *et al.*, 1993; Schmitt *et al.*, 1994; Seder *et al.*, 1993; Wu *et al.*, 1993; Manetti *et al.*, 1993; Manetti *et al.*, 1994), and an antagonist of Th2 type responses (Sypek *et al.*, 1993; Heinzel *et al.*, 1993; Finkelman *et al.*, 1994). The induction of IFN-γ (as well as TNF-α and GM-CSF) and T and NK cell cytotoxicity also implicate IL-12 in innate and adaptive immunity to infectious diseases and inhibition of tumour growth.

(b) The IL-12 Heterodimer

The purification to homogeneity of IL-12 allowed the identification of its structure. The discovery that it is composed of a heterodimer makes it a unique cytokine. Kobayashi *et al* (Kobayashi *et al.*, 1989) and Stern *et al* (Stern *et al.*, 1990) observed that running IL-12 on SDS-PAGE under non-reducing conditions revealed a 75 kD band whilst under reducing conditions, two bands were clearly observed, one of 40 kD and one of 35 kD, suggesting that IL-12 was composed of a cysteine-linked dimer. N-terminal sequencing of the two bands revealed that both subunits, now known as IL-12 p40 and IL-12 p35, were in fact novel and different (Stern *et al.*, 1990). The cDNAs encoding the two subunits of human IL-12 were cloned in parallel by two groups (Wolf *et al.*, 1991; Gubler *et al.*, 1991), both using amino acid sequence from the two subunits to design degenerate oligonucleotides for PCR amplification. IL-12 biological activity was reconstituted only in the supernatants of COS cells transfected simultaneously with p40 and p35 encoding expression plasmids, suggesting that both subunits were essential for activity. The p35 subunit is a 219 aa residue polypeptide, whilst the p40 subunit cDNA encodes a 328 aa residue polypeptide.

The murine subunits were cloned using the human cDNAs as probes (Schoenhaut *et al.*, 1992). Due to a lack of cell lines known to express murine IL-12, murine genomic

DNA was initially cloned, and exons identified corresponding to the IL-12 subunits. The sequence was used to design PCR primers, and the murine cDNAs subsequently clones from activated splenocyte poly(A)$^+$ RNA. The p40 cDNA encoded a 325 aa residue protein with 70% aa sequence identity to human p40. The p35 cDNA encoded a 215 aa residue protein with 60% sequence identity to the human p35. It was then demonstrated by Schoenhaut et al (Schoenhaut *et al.*, 1992) that murine IL-12 was active on both murine and human cells, although the activity of human IL-12 appeared to be restricted to human cells. Interestingly, when interspecies heterodimers were created by transient transfection in COS cells, activity was detected on cells of both human and murine origin when murine p35 was present, but the presence of human p35 restricted activity to human cells. Therefore species specificity appears to be dictated by the p35 chain. The fact that the two p40 chains are more homologous between human and mouse than the p35 chain could explain the lack of importance of the origin of p40 for IL-12 function.

Sequence and structural analysis of the p35 and p40 subunits have revealed that the IL-12 heterodimer is an extremely interesting biological entity. The p35 subunit is predominantly helical, and shows a 'distant but significant relationship' with the cytokines IL-6 and G-CSF (Merberg *et al.*, 1992). More striking however is the homology of the p40 subunit to members of the cytokine type-I receptor family. Gearing and Cosman (Gearing and Cosman, 1991) noted that p40 showed extensive homology to the extracellular domain of IL-6Rα, including the N-terminal Ig-like domain and the cytokine receptor-like domain. In addition, it contains the highly conserved four cysteines and W-S-x-W-S motif characteristic of the cytokine type-I receptor family (in fact human p40 has W-S-E-W-A-S and murine p40 has C-S-K-W-A). This has raised the possibility that IL-12 is in fact an irreversibly complexed cysteine linked cytokine/soluble cytokine receptor complex.

It should be mentioned that Fan *et al* (Fan *et al.*, 1996) more recently reported the identification of membrane-bound IL-12 on the surface of a human monocyte and macrophage cell line by FACS analysis with an IL-12 heterodimer specific mAb and its upregulation in response to inflammatory stimulation. It is not clear how this molecule is anchored. The authors suggest that it could due to a transmembrane form

of p40 possibly generated by alternative splicing. This would not be too hard to imagine as membrane bound and soluble forms of cytokine type-I receptors have been well documented. A membrane-bound p35 cannot however be ruled out, as human p35 cDNA is known to contain amino-terminal sequence found in other membrane-associated proteins (Wolf *et al.*, 1991; von Heijne and Gavel, 1988). They do however fail to mention the possibility that soluble IL-12 associated with its receptor could be detected on the cell surface. The authors do however speculate that the significance of such a membrane-bound form of IL-12 could be to generate a more potent inducer of Th1 and other target cells through cell-cell contact.

(c) *Control of Expression of p35 and p40 Subunits*

As the simultaneous expression of the two IL-12 subunits in a given cell is required for a functional IL-12 heterodimer, the control of IL-12 expression is complex. Constitutive expression of mRNA encoding p35 is observed, albeit at low levels in most analyzed cell types. Secretion of the p35 subunit does however appear to be more tightly controlled (D'Andrea *et al.*, 1992). This, in addition to the fact that cells transfected with p35 cDNA only secrete very small amounts of the subunit, suggests that p35 can only be efficiently secreted as a heterodimer in the presence of p40. It therefore follows that expression of p40 is restricted to those cells capable of producing the functional IL-12 heterodimer. The expression of the p40 gene is also more highly regulated by activation of IL-12 expressing cells that that of p35 (D'Andrea *et al.*, 1993). In addition, the p40 subunit is overexpressed at a ratio of between 1:10 to 1:50 compared to p35 in cells producing functional IL-12. It is unclear as to why such non-correlation exists between the expression of the two genes. It could be hypothesized that the individual chains associate with other unidentified molecules. Indeed, it was recently demonstrated that p35 was able to interact with the novel soluble molecule EBI-3, expressed in Epstein-Barr virus (EBV) transformed B cells and also sharing homology with type-I cytokine receptors (Devergne *et al.*, 1996 and Devergne *et al.*, 1997; discussed later). One can also hypothesize that p35 and p40 have biological functions as individual proteins.

To demonstrate this last point, mouse p40, recombinantly expressed in COS cell supernatants, was shown to possess antagonistic properties in terms of IL-12 activity (Mattner *et al.*, 1993). They showed that p40 blocked the IL-12 induced homotypic aggregation of Th1 cells and interfered with the PMA + IL-12 induced proliferation of splenocytes in a concentration dependent manner. In addition, it inhibited in a dose dependent manner IFN-γ production of stimulated $CD4^+$ and $CD8^+$ splenocytes as well as Th1 cells. Two subsequent publications demonstrated that the antagonistic agent was actually a p40 homodimer (Gillessen *et al.*, 1995; Ling *et al.*, 1995). Gillessen and co-workers purified mouse p40 by immunoaffinity chromatography from the supernatant of insect cells infected with baculovirus encoding the p40 subunit. The purified protein was seen to contain both p40 monomer and cysteine linked p40 homodimer (as detected by SDS-PAGE under non-reducing and reducing conditions). Monomer and homodimer were separated by Mono Q anion chromatography and tested for their ability to inhibit the effects of IL-12. Its was clearly seen that the dimer was antagonistic for IL-12 activity in a variety of assays at concentrations only two fold higher that that of the IL-12 itself, whilst the monomer was much less potent. The p40 dimer was also able to block the binding of IL-12 to responsive cells in a dose dependent manner similar in potency to unlabeled IL-12. Monomer also inhibited binding, but again it was much less efficient, with 50% binding inhibition of IL-12 achieved at concentrations 25- to 50- fold higher that that of the dimer.

Similar results were obtained when human p40 homodimer and monomer were studied for their ability to bind to the IL-12 receptor and mediate biological activity. Monomer was separated from dimer in COS cell supernatants by affinity chromatography, gel filtration and hydrophobic interactive chromatography. The homodimer was subsequently shown to inhibit IL-12 binding to KIT225/K6 cells. Although the monomer inhibited IL-12 binding to the same cells, it was found to be at least 10- to 20-fold less efficient than the p40 homodimer. In addition, p40 homodimers inhibited IL-12 induced PHA-blast proliferation with a minimal inhibition seen by the monomer. The fact that only the IL-12 heterodimer and p40 homodimers but not monomers bind efficiently to the surface of IL-12 responsive cells suggests that p40 binding epitopes to the IL-12 receptor are conformational and

induced by association with p35 or a second p40 subunit. The antagonistic effects of two p40 subunits also suggests that p35 is the subunit responsible for signal transduction through the receptor.

So does the p40 homodimer play a physiological role in the inhibition of IL-12 mediated responses *in vivo*?. It has been extensively reported that in cells producing IL-12, the p40 subunit is expressed in substantial excess of the p35 subunit (Podlaski *et al.*, 1992; D'Andrea *et al.*, 1992; Zhang *et al.*, 1994). Although the biological effects of p40 administration have been observed *in vivo* (Kato *et al.*, 1996; Mattner *et al.*, 1997), whether it is actually produced during a biological response still needs to be clarified.

Figure 1.18 shows the consequences of the differential expression of the heterologous subunits of IL-12.

(d) The IL-12 Receptor

The first IL-12 receptor component was identified by Chua et al (Chua *et al.*, 1994) using an expression cloning strategy with an Ab raised against a cell surface IL-12/IL-12 binding protein complex. The Ab was used in a panning technique to identify CHO cells transfected with a human lymphoblast cDNA library expressing the counterstructure. The isolated cDNA encoded a protein of 662 aa residues, now known as IL-12Rβ1. The type I membrane glycoprotein has a extracellular domain of 516 aa residues, a cytoplasmic tail of 91 aa residue and a molecular weight of 110 kD. Predictably, IL-12Rβ1 contains motifs associated with the cytokine type-I receptor family, showing closest homology to gp130, G-CSFR and LIFR. The extracellular domain contains five fibronectin type III modules, the first and second from the N-terminus containing the unique cytokine receptor-like domain. Sequence similarities were also observed between IL-12Rβ1 and gp130/LIFR/G-CSFR within the cytoplasmic domain, with IL-12Rβ1 having box 1 and box 2 sequence motifs, thus implicating the receptor component in signal transduction. Indeed, α-IL-12Rβ1 Abs

were subsequently shown to inhibit IL-12 induced cellular responses, implicating this chain in IL-12 signalling.

Both human and murine IL-12 bound the human IL-12Rβ1 on the surface of transfected CHO cells with low affinity. It was known however that high affinity IL-12 sites exist on IL-12 responsive cells (Chizzonite *et al.*, 1992). It was also observed that IL-12Rβ1, although containing box 1 and box 2 motifs, contained no cytoplasmic tyrosine residues. It was therefore likely that a second, high affinity converting and signal transducing component of the IL-12 receptor existed. The second receptor component was identified by Presky et al (Presky *et al.*, 1996), again using an expression cloning strategy. The IL-12Rβ1 chain was co-expressed with a human lymphoblast cDNA library and cDNAs reconstituting high affinity IL-12 binding sites were isolated. The cDNA for IL-12Rβ2 encoded a protein of 862 aa residues with 596 and 216 aa residue extracellular and cytoplasmic domains respectively. The protein is also a member of the cytokine type-I receptor family, and like IL-12Rβ1, shows closest homology to gp130, G-CSFR and LIFR. The extracellular domain resembles that of gp130, with six fibronectin type III modules, the second and third from the N-terminus containing the unique cytokine receptor-like domain. The cytoplasmic domain also has box 1 and box 2 motifs as well as three tyrosine residues, suggesting as suspected a role for the receptor subunit in signal transduction.

Similar to IL-12Rβ1, IL-12Rβ2 binds IL-12 with low affinity. Co-expression of the two subunits however, resulted in high affinity binding. It has been observed that IL-12 p40 primarily interacts with the IL-12Rβ1 (Gillessen *et al.*, 1995), and that this interaction is antagonistic. This would therefore suggest that IL-12 p35 interacts with IL-12Rβ2, and that this interaction is important for receptor subunit association and signal transduction. Like the signalling through many of the receptors pertaining to the cytokine type-I receptor family, IL-12 signalling appears to be mediated through the Jak/STAT signalling pathway, specifically utilizing STAT 3 and STAT 4. (Jacobson *et al.*, 1995; Bacon *et al.*, 1995; Thierfelder *et al.*, 1996; Kaplan *et al.*, 1996).

The generation of chimeric receptors revealed that the IL-12Rβ2 cytoplasmic domain was an absolute requirement for signalling, and it could signal independently of the β1 chain (Zou *et al.*, 1997). Only the β2 chain cytoplasmic tail was seen to be phosphorylated upon ligand binding. Jak2, Tyk2 and STAT 3 were implicated in signalling in this study, the expression of STAT 4 in the particular cell line used being too low to be detected by immunoprecipitation. It appears that Jak 2 is phosphorylated in the presence of either the β1 or β2 chain cytoplasmic tail, whilst Tyk 2 is phosphorylated only in the presence of the β1 chain. These results would appear to suggest that the β1 chain acts purely to interact with the β2 chain and recruit Jak kinase family members, whilst the β2 chain not only recruits Jak kinases, but also becomes phosphorylated at tyrosine residues, thereby driving signal transduction. The box 1 motif in both receptors is presumably responsible for recruitment of Jak kinase family members. STAT 3 has been associated with the box 3 motif found within the subfamily of receptors showing close homology to gp130 (Discussed in Taga and Kishimoto, 1997). The absence of a box 3 motif in the β1 chain and the lack of β1 chain phosphorylation suggests that IL-12 dependent STAT 3 activation occurs through association with the phosphorylated β2 chain. Interestingly, the β2 chain does have a region containing a tyrosine residue which weakly resembles a box 3 motif. Obviously, STAT 3 and STAT 4 interactions with the IL-12 receptor will need to be elucidated further.

With the elucidation of the components of the IL-12 heterodimer and the functional IL-12 receptor, interesting comparisons can be drawn with the family of cytokines signalling through LIF and gp130. In particular, the IL-12/IL-12R signalling complex resembles that formed by CNTF, soluble CNTFRα, LIFR and gp130, the only difference being that the cytokine/soluble cytokine receptor complex (i.e. the p35 and p40 subunits) has become irreversibly bound by a disulphide bond. It is difficult to speculate as to why such a cytokine/cytokine receptor complex would evolve in such a way. Could this represent a step forward in evolution, whereby p35 has overcome the requirement of a ligand-binding chain (p40) on its target cell surface in order to exert its effects by simply associating with it in solution. The presence of the more ubiquitously expressed signal transducing subunits would therefore be sufficient for

signalling. This would however implicate a lack of control of specificity, which has quite possibly been overcome by expression of the antagonistic p40 homodimer. On the other hand, could this represent a survivor of earlier stage in the evolution of cytokine receptors, where ligands and so called ligand binding chains were actually irreversibly associated in solution, with evolution bringing a higher degree of control of cytokine signalling through the requirement that ligand binding chains were expressed on the target cell surface in order for the signal to be transduced. Although this is difficult to answer, it will be interesting to see whether any other such novel cytokine/cytokine receptor complexes are identified in the future.

Interactions involving IL-12 and its receptor are shown in Figure 1.19.

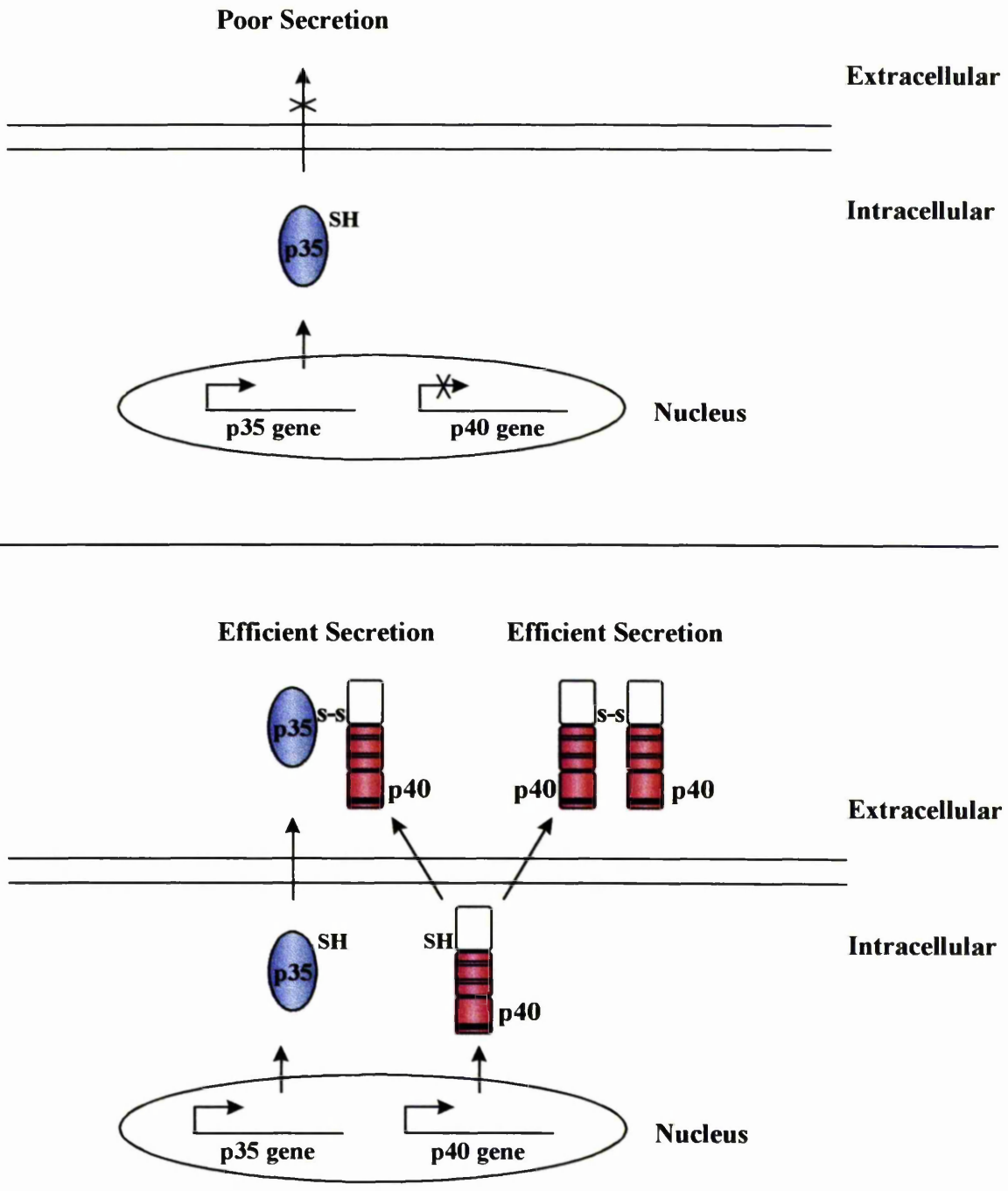


Figure 1.18. Expression and Secretion of the Heterologous Subunits of IL-12.

IL-12 p35 is represented by blue ovals, whilst the cytokine receptor-like domain of IL-12 p40 is shown in red.
(Above) Transcription of the IL-12 p35 subunit alone results in expression of the protein but poor secretion by the cell.
(Below) Transcription of both the IL-12 p35 and IL-12 p40 genes in parallel results in the efficient secretion of a disulphide linked heterodimer. Overexpression of the p40 subunit, in cells transfected with an IL-12 p40 expression construct for example, has also been shown to lead to the secretion of disulphide linked p40 homodimers.

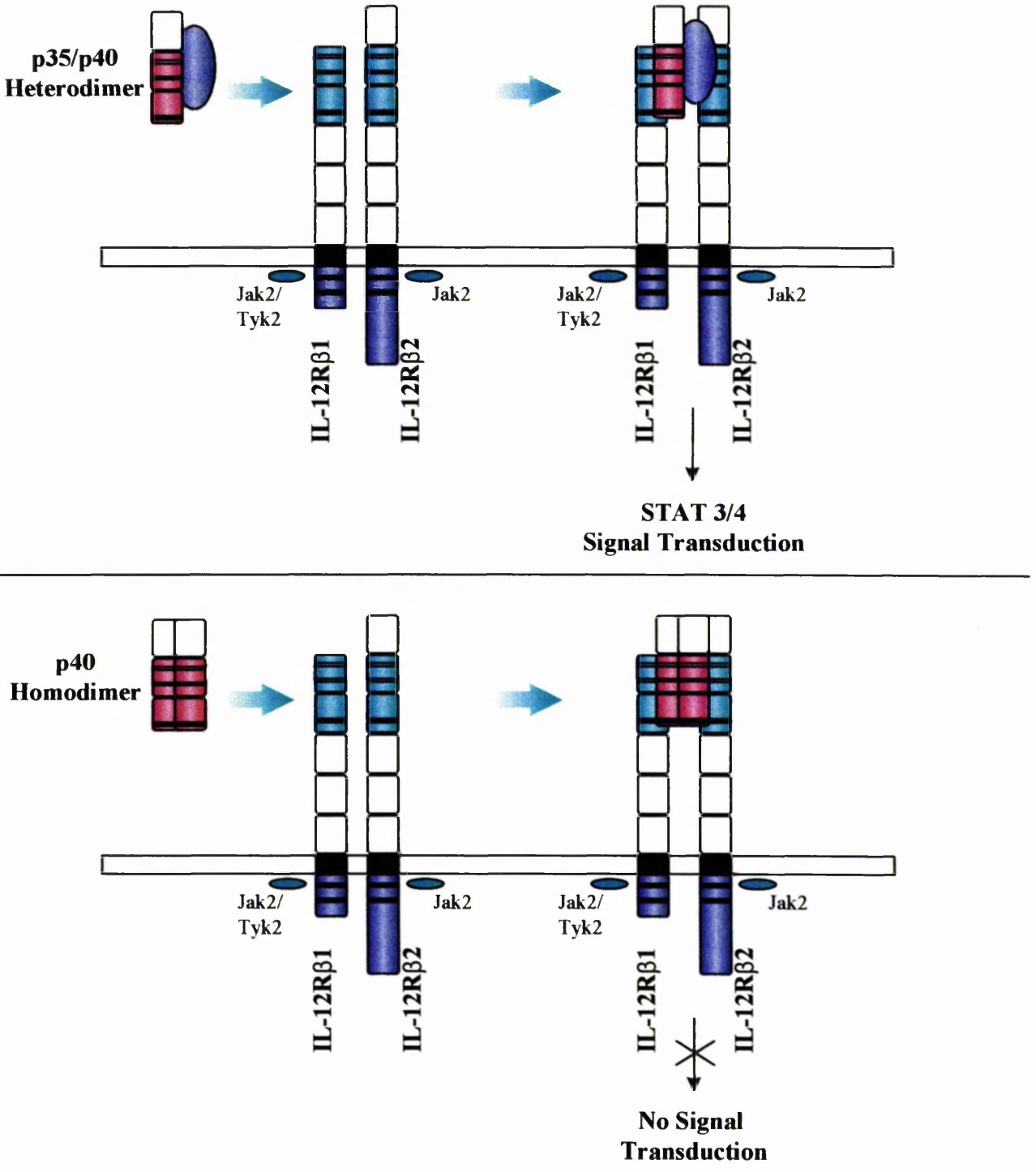


Figure 1.19. IL-12 Subunit Interactions with the IL-12 Receptor.

The cytokine receptor-like domain IL-12 p40 subunit is shown in red, whilst those of IL-12Rβ1 and β2 are shown in green. Intracellular domains of the receptors are shown in blue, whilst IL-12 p35 is represented as a blue oval and Jak family members as small green ovals.

(Above) The functional IL-12 molecule comprising the p35 and p40 subunits interacts with IL-12Rβ1 and β2 to form a high affinity binding and signal transducing complex involving Jak2, Tyk2 and STATs 3 and 4.

(Below) The p40 homodimer associates with IL-12Rβ1 and β2 but does not induce signal transduction.

(1.3.2) Epstein-Barr Induced Protein 3 (EBI-3)

This novel member of the cytokine type-I receptor family was identified by Devergne *et al* (Devergne *et al.*, 1996) as a factor whose expression was induced by Epstein-Barr virus (EBV) infection and shared homology with IL-12 p40 and CNTFRα. The cDNA was detected by subtractive hybridization and encoded a 229 aa residue, 33kD glycoprotein showing structural features characteristic of the cytokine type-I receptor family, including the 4 conserved cysteines and an L-S-x-W-S closely resembling the W-S-x-W-S motif. The protein shares 30% and 27% identity with CNTFRα and IL-12 p40 respectively. Similar to IL-12 p40, the protein lacks a transmembrane domain or GPI linkage consensus site (Englund, 1993). EBI-3 is expressed in spleen, placenta and pokeweed mitogen stimulated PBMC. The protein has four cysteines all implicated in intramolecular disulphide linkage, therefore it is unlikely that EBI-3 is secreted as a disulphide linked homo- or heterodimer similar to IL-12 p40.

Devergne *et al* also observed that much of the EBI-3 expressed by a given cell remained associated in the endoplasmic reticulum (ER) with the molecular chaperone calnexin. This led to the suggestion that EBI-3 had to associate with a second subunit to be efficiently secreted as a heterodimer. A second publication by the same group a year later (Devergne *et al.*, 1997) suggested that this second subunit was actually IL-12 p35. Cell were generated that overexpressed both subunits, the p35 subunit being expressed as a 'flag-tagged' protein. Immunoprecipitation with the α-flag mAb and western blotting with EBI-3 antisera revealed that between 10 and 25% of the expressed EBI-3 protein coprecipitated with the flag-tagged p35. As expected, the association between the two proteins was non-covalent. In addition, the two proteins were shown to be capable of associating in solution by cocultivating cells expressing either EBI-3 or IL-12 p35 and immunoprecipitating proteins from the supernatant. It also appeared that co-expression of the two proteins assisted their respective secretion from the cell. In parallel, it was demonstrated that the two proteins could be coprecipitated from extracts made from the syncitial trophoblast-enriched part of a human full term placenta, thus establishing that the association between the two proteins occurs *in vivo* and is not just an *in vitro* artifact.

The functional relevance of this unexpected association remains to be determined. Similar to IL-12 p40, EBI-3 expression is restricted. As p40 expression is the key regulatory event in IL-12 synthesis, it follows that EBI-3 expression would also regulate synthesis of this novel heterodimer. The poor secretion of EBI-3 when expressed on its own may however limit its role as an antagonist of the function of p35/EBI-3 as seen with p40 and IL-12. It is plausible that the p35/EBI-3 also utilizes the functional IL-12 receptor. This would imply that, considering these heterodimers as cytokine/cytokine receptor α chain complexes, p35 actually uses two ligand binding chains (EBI-3 and p40) to signal. As the p35/EBI-3 heterodimer is not covalently linked, this complex would be more similar to IL-6/sIL-6Rα or CNTF/CNTFRα for example. One could loosely draw a parallel with human OSM. Although not associating with an α chain, OSM can utilize LIFR or OSMR to signal through gp130. It cannot however be ruled out that p35/EBI-3 signals through an altogether independent receptor complex. Whatever the physiological role of p35/EBI-3 may turn out to be, it is likely that it is an antagonist of IL-12 function, if not by binding to the same functional receptor complex then by reducing the efficiency of IL-12 production by reducing the amount p35/p40 association in cells expressing all three subunits.

(1.4) Identification of Novel Cytokine Type-I Receptor Family Members

The work described in this report was carried out at the Geneva Biomedical Research Institute between August 1996 and April 1998. The aims of the research project were to identify novel members of the cytokine type-I receptor family, characterize their expression in fetal and adult tissues as well as in cells and cell lines, and to develop tools to allow the identification and characterization of native and recombinant protein.

An approach involving bioinformatics was chosen to allow the identification these novel proteins. The expressed sequence tag (EST) database (dbEST) has been compiled from two major sources. The first was constructed to identify novel genes and contains 174 000 sequences from randomly primed cDNA fragments from various tissues of different origins (Adams *et al.*, 1991; Adams *et al.*, 1995). The second source contains some 280 000 sequences constructed to obtain probes for gene mapping (Hillier *et al.*, 1996; Schuler *et al.*, 1996). The majority of cDNA libraries used to generate ESTs have been 'normalized' to reduce the number of frequently appearing clones (Soares *et al.*, 1994). This database represents an impressively deep gene pool for 'fishing' new members of gene families of homologous of genes from other species. In addition to this, 'the WorldWideWeb' (more familiarly known as 'the Web'), has become a very powerful a tool for searching the EST database, giving biologists almost instantaneous access not only to the database but also the software required to perform the searches (such as BLAST).

Our approach was therefore to take advantage of the presence of a highly conserved W-S-x-W-S sequence motif found within almost all of the sequences for the cytokine type-I receptors. The TBLASTN program translated the EST database in all six reading frames and compared amino acid sequences obtained with the 'query' sequence, which in our case was the region surrounding the W-S-x-W-S motif from one of the known cytokine receptors. Candidate 'hit' sequences were then screened for

the presence of probable open reading frames in which the homology was identified. In addition the each candidate EST was screened for the correct orientation of sequence alignment, as many of the ESTs have been directionally cloned.

The following report describes the identification through bioinformatics of the human, mouse and rat homologues of the cDNA for previously unidentified soluble protein CLF-1, whose amino acid sequence and secondary structure show significant homology to members of the cytokine type-I receptor family. Analysis of CLF-1 mRNA distribution and recombinant forms of the CLF-1 protein have provided the initial characterization of this novel molecule.

CHAPTER 2. MATERIALS AND METHODS

(2.1) Identification of Human and Mouse DNA for CLF-1

The amino acid sequence N K L C F D D N K L W S D W S E A Q S I G K E Q N from the murine IL-13 receptor (mIL-13R; Hilton *et al.* 1996) was used to search the GenBank database with expressed sequence tags (ESTs) using TBLASTN, in order to identify cDNAs encoding novel receptors with related sequence. ESTs with apparent homology were then screened for open reading frames (ORFs) within the region of homology, alignment with the query sequence in the correct orientation (as many of the ESTs are directionally cloned) and the presence of conserved sequence motifs present in the cytokine type-I receptor family (Bazan, 1990a; Bazan 1990c). ESTs of interest were translated, and amino acid sequences from regions of alignment with the initial mIL-13R amino acid query sequence were compared against the Swissprot protein database using BLASTP. BLASTN searches of the GenBank database with candidate ESTs allowed the identification of murine, human and rat ESTs with overlapping sequence homology.

(2.2) Cloning of murine CLF-1 cDNA

The mouse cDNA clone 479043 in the vector pT7T3 was purchased from Research Genetics Inc. (Birmingham, AL) and sequenced using vector specific and mCLF-1 specific synthetic oligonucleotides. The rapid amplification of 5' cDNA ends (5'-RACE) (Frohman *et al.*, 1988) on poly A+ RNA extracted from mouse lung allowed the cloning of murine cDNA upstream of the 5' end of the cDNA clone using the Marathon cDNA amplification kit (Clonetech, Palo Alto, CA). First-strand cDNA was synthesized from 1 μg of mouse lung poly A^+ RNA (Clonetech) using a dT_{30} synthetic oligonucleotide primer (containing two degenerate nucleotide positions at the 3' end) and Moloney murine leukemia virus (MMLV) reverse transcriptase. Second-strand cDNA synthesis was performed using an enzyme cocktail containing *E. coli* DNA polymerase I, *E. coli* DNA ligase and RNase H, with dNTPs added to final concentration of 10 mM each. T4 DNA polymerase was subsequently added to create blunt-ends on the double-stranded cDNA. Following purification by phenol/

chloroform extraction and ethanol precipitation, synthetic partially double stranded oligonucleotide adapters, phosphorylated at the 5' end, were ligated onto the double-stranded cDNA using T4 DNA ligase.

The adapter ligated cDNA was diluted 1/20 or 1/100 with H_20 and subjected to 40 cycles of PCR amplification using the following conditions: 94°C, 1'; 60°C, 1'; 72°C, 2'. The primer used in the PCR amplification (along with the adapter primer provided) was 5'-CGTACCACCTCAGCTTGTACTTG-3'. PCR products were cloned into the TA cloning vector pCRII (Invitrogen, Leek, The Netherlands) and colonies screened by hybridization with the ^{32}P-labeled synthetic oligonucleotide probe 5'-AAGGATCTCACGTGCCGCTGGACACCGGGT-3' (Sambrook *et al*, 1989). The hybridization was performed at 37°C in a buffer conatining 6 x SSPE, 0.1% SDS, 20% formamide (V/V) and 100 µg/ml yeast RNA. Filters were washed in 0.1 x SSPE, 0.1% SDS at room temperature. Inserts from plasmids derived from positive colonies were sequenced using vector specific synthetic oligonucleotides.

(2.3) Cloning of human CLF-1 cDNA

A portion of the hCLF-1 cDNA was amplified by PCR using cDNA derived from human lung poly A^+ RNA (Clonetech) with the primers 5'-ACCGCCGAGGGCCTC TACTG-3' and 5'TTGAGGGAGTAGTTGGTGTGGAGG-3'. 40 cycles of PCR amplification were performed using the following conditions: 94°C, 30''; 60°C, 30''; 72°C, 1'. The primers were designed from sequences obtained from hCLF-1 ESTs. The amplified product was cloned into the TA cloning vector pCRII and sequenced using vector specific synthetic oligonucleotides. The hCLF-1 cDNA fragment was subsequently used as a ^{32}P labeled probe to screen a human placental cDNA library in λgt10 (Sambrook *et al.*, 1989). The hybridization was performed at 42°C in a buffer containing 0.2% polyvinyl pyrolydone, 0.2% BSA, 50mM Tris-HCl (pH 7.5), 1M NaCl, 0.15 sodium pyrophosphate, 1% SDS and 100 µg/ml tRNA. Filters were

washed in 2 x SSC, 0.1% SDS at 55°C. The largest two cDNAs identified (1740 bp and 1517bp) were excised from λgt10 phage by digestion with *Eco* RI, recloned into pBluescript II SK- (Stratagene, La Jolla, CA), and sequenced using vector and hCLF-1 specific synthetic oligonucleotides.

(2.4) DNA and Protein Sequence Analysis

DNA sequencing was performed on an ABI Prism™ 377 DNA Sequencer, using fluorescent dye terminators (Perkin-Elmer International, Inc., Rotkreuz, CH). Sequences obtained from cDNA clones, as well as all relevant ESTs were imported into and analyzed by the sequence analysis software Sequencher (Genecodes Corporation, Ann Arbor, MI). The signalP server (htpp://www.cbs.dtu.dk/signalp/cbssignalp.html) was used to identify the predicted cleavage site of the signal peptide for CLF-1. The TFSearch server (htpp://www.pdap1.trc.rwcp.or.jp) was used to identify predicted transcription factor binding sites within the DNA upstream of the first hCLF-1 exon. DNA and amino acid sequence alignments as well as prediction of hydrophobic regions were analyzed with the Wisconsin package version 8.1 (Genetics Computer Group Inc., Madison, WI).

(2.5) In Vitro Transcription/Translation of hCLF-1 cDNA

pBluescript II SK- containing either clone 18 (full length) or clone 3 (5' truncated) hCLF-1 cDNA was linearized by restriction enzyme digestion with *Spe* I and subjected to in vitro transcription/translation using the TNT® T7 Coupled Reticulocyte Lysate System (Promega Corp., Zurich, CH). One-step transcription and translation was achieved by mixing 1 µg of the linearized hCLF-1 cDNA template with T7 RNA polymerase, rabbit reticulocyte lysate, a 1 mM amino acid mixture (without methionine), 5 µl ^{35}S-methionine at 10mCi/ml and 40 units of RNAguard®

ribonuclease inhibitor (Pharmacia LKB Biotechnology, Uppsala, Sweden). As a negative control, a reaction was performed without addition of DNA template. Reaction mixtures were subjected to SDS-PAGE using an 8-16 % acrylamide gel (Novex, San Diego, CA) which was dried following migration using a Model 583 Gel Dryer (BioRad Laboratories AG, Postfach, CH). ^{35}S labeled proteins were subsequently revealed by autoradiography by exposure of the dried gel to X-Omat AR film in the presence of an intensifying screen (Eastman Kodak Co., Rochester, NY) for 15 hours at room temperature.

(2.6) Source of Cells and Culture Conditions

(a) Cells/Cell Lines of Human Origin

The cell line HMC-1 was obtained from Dr. J. Butterfield (Mayo Clinic, Rochester, MN). The human cell lines EOL-3, HL-60, JY, RPMI-8866, RPMI-8226 and THP-1 were obtained from the American Type Culture Collection (ATCC; Rockville, MD). All the above cell types were maintained in RPMI 1640 medium (Life Technologies, Basel, CH) supplemented with 10% FCS (PAA laboratories GmbH, Linz, Austria). JY cells were maintained with or without 200 U/ml rIL-4 (Amersham International plc., Amersham, UK). THP-1 cells were maintained with or without 10 ng/ml phorbol myristate acetate (PMA; Sigma Chemical Co.). The Jurkat cell line was obtained from A. Bernard (University of Nice, France) and maintained with RPMI 1640 medium supplemented with 10% FCS with or without 10 ng/ml PMA and 1μM ionomycin (Calbiochem-Novabiochem, La Jolla, CA). The TT7 human T cell clone was maintained in Iscoves modified Dulbecco's medium (IMDM) supplemented with transferrin (20 μg/ml), insulin 5 μg/ml, rIL-2 (100 units/ml; Geneva Biomedical Research Institute) and 10% FCS. Human bronchial epithelial HBE-140 cells (Geneva Biomedical Research Institute) were maintained in Ham's F12 medium (Flow Laboratories, Irvine, Scotland) supplemented with 10% FCS. HBE-140 cells were stimulated with the cytokines TNF-α and IL-1β at a final concentration of 20 ng/ml per cytokine. Human umbilical vein endothelial cells (HUVEC) were isolated as previously described (Schnyder *et al.* 1996) and maintained in MCDB 131 medium

supplemented with 2% FCS, hydrocortisone (1 ng/ml), epidermal growth factor (10 ng/ml), and bovine brain extract containing heparin, gentamicin and amphotericin (Clonetics Corp., San Diego, CA). Tissue culture dishes were precoated with human fibronectin, (1 $\mu g/cm^2$; Boerhinger Mannheim, Mannheim, Germany). HUVEC were stimulated with the cytokines TNF-α and IL-1β at a final concentration of 20 ng/ml per cytokine.

Human peripheral blood leukocytes (PBL) were isolated from whole blood by centrifugation over a Ficoll-Hypaque gradient (Pharmacia LKB Biotechnology) and maintained in RPMI 1640 medium supplemented in 10% FCS. If stimulated, cells were incubated with 10ng/ml PMA and 1 μM ionomycin. Human T cells were purified from PBL by positive selection rosetting with sheep red blood cells and were maintained in IMDM supplemented with 10% FCS. Cells were incubated with or without 10 ng/ml PMA and 1 μM ionomycin. Human monocytes were purified from PBL by negative selection rosetting with sheep red blood cells and were maintained in RPMI 1640 medium supplemented with 10% FCS. Cells were incubated with or without LPS (1 μg/ml; Sigma Chemical Co.), IFN-γ (10 ng/ml; R&D Systems), IL-6 (10 ng/ml; Sigma Chemical Co.), TNF-α (10 ng/ml; Sigma Chemical Co.) or IL-1β (10 ng/ml; Sigma Chemical Co.). In order to isolate human tonsillar B cells, mononuclear cells were derived from tonsils, passed over a Ficoll-Hypaque gradient and B lymphocytes were isolated by negative selection rosetting with sheep red blood cells, and maintained in RPMI 1640 medium supplemented with 10% FCS. Cells were incubated with or without 200 U/ml rIL-4 and 1μg/ml αCD40 (Serotec, Oxford, UK).

The cell line HEK 293 was obtained from the ATCC. The EBNA-1 expressing HEK 293 (HEK 293-E) derivative was purchased from Invitrogen. Palmar fibromatosis lesion and mammary gland fibroblasts were a kind gift from Professor G. Gabbiani, (University Medical Center, Geneva, CH). All the above cell types were maintained in DMEM and Ham's F12 nutrient solution supplemented with 10% FCS. Tonsillar fibroblasts were derived from whole tonsils obtained from surgery as follows: surgically removed tonsils were rinsed in Dulbecco's modified Eagle's medium

(DMEM; Life Technologies) and cells teased from the tissue into warm DMEM using scissors and forceps. Following centrifugation, the cells were resuspended in DMEM and passed over a Ficoll-Hypaque gradient. Cells were then pelleted and resuspended in DMEM and Ham's F12 nutrient solution supplemented with 10% FCS. The cell suspension was plated onto sterile petri dishes containing the same medium and plated at 37°C to allow fibroblast growth. Adherent cells became visible after 2-3 days in culture. Fibroblasts were stimulated with the cytokines TNF-α, IL-1β, IL-6 (Sigma Chemical Co., St. Louis, MO) and IFN-γ (R&D Systems, Abingdon, UK) at a final concentration of 20 ng/ml per cytokine.

(b) Cells/Cell Lines of Murine Origin

The murine B cell lymphoma cell line A20 was obtained from the ATCC and maintained in RPMI 1640 medium supplemented with 10% FCS with or without 1 μg/ml lipopolysaccharide (LPS 026:26; Sigma Chemical Co.). Murine T cells were obtained from PBL, derived from murine blood by centrifugation over a Ficoll-Hypaque gradient, by positive depletion rosetting with sheep red blood cells. T cells were maintained in RPMI 1640 medium supplemented with 10% FCS. For T cell stimulation, NUNC 96 well immunoplates (439454; Life Technologies) were coated with α-CD3 and α-CD28 mAbs (Pharmingen, San Diego, CA) as follows: The mAbs were diluted to 10 μg/ml in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.4) and 100 μl added to each well. Plates were then incubated for 16 hours at 4°C and the wells washed once with PBS. T cells were subsequently added to the wells at a concentration of 1 x 10^6 cells/ml and incubated for 48 hours at 37°C. Murine macrophages and eosinophils were purified from mouse bronchial-alveolar lavage (BAL). Macrophages were isolated through there adherent properties whilst eosinophils were enriched by Ab panning. The murine myeloma Sp2 was obtained from the ATCC and maintained in IMDM supplemented with 10% FCS and 100 μM 2-mercaptoethanol (2-ME).

(d) Cells of Other Origin

The cell lines COS7 and CHO were obtained from the ATCC and maintained in DMEM and Ham's F12 nutrient solution supplemented with 10% FCS. The Sf9 and Sf21 cell lines were obtained from the ATCC and maintained in SF900II medium (Life Technologies) supplemented with 10% FCS.

(2.7) Detection of the CLF-1 Gene by Southern Blot Analysis

The source of the genomic DNA was as follows: the human cell line HEK 293, and African green monkey Cos-7 cell lines (ATCC), C57BL/6 mouse tails, rat liver, rabbit liver, cow liver and chicken embryo liver. Genomic DNA was isolated from cell lines or tissues with the DNAzol® reagent (Life Technologies). For cell lines: cells were washed once with PBS and resuspended in DNAzol® reagent at a concentration $1x10^7$ cells/ml. For tissues: 50 mg of tissue/ml of reagent was homogenized using a glass-Teflon® homogenizer. In both cases, the homogenate was centrifuged at full speed in a bench-top microcentrifuge to remove insoluble debris, the supernatant transferred to a clean tube and genomic DNA precipitated out of solution by the addition of 500 µl ethanol/ml DNAzol. The DNA was subsequently spooled onto a glass rod and transferred into a clean 1.5 ml eppendorf tube containing 1ml ethanol. The DNA was left to sediment to the bottom of the tube, the ethanol removed and replaced with 1ml of fresh ethanol. The ethanol was subsequently removed and the genomic DNA precipitates were air-dried and resuspended in 1x TE (10mM Tris-HCl (pH 8.0), 1mM EDTA).

Aliquots of genomic DNA (5 µg) were digested with either *SacI* or *BamHI* and the products migrated on a 0.8 % agarose TAE gel. The gel was treated sequentially with 0.125 M HCl (15'), 0.5 M NaOH (1 hr) and 0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl (30'). The DNA was subsequently transferred to a GeneScreen *Plus*® hybridization transfer membrane (NEN® Research Products, Boston, MA) by capillary transfer using 20 x SSC (Southern, 1975). DNA was fixed to the membrane by UV cross-linking using a UV Stratalinker™ 8400 (Stratagene, La Jolla, CA).

A 1.2 kb *Eco* RI/*Sac* II restriction enzyme digestion fragment of hCLF-1 cDNA was ^{32}P labeled using random hexamer priming for ^{32}P-dCTP incorporation (Feinberg and Vogelstein, 1983) with the Prime-a-Gene® labeling system (Promega Corp.). 50 ng of the cDNA fragment was denatured by heating to 95oC for 5' and rapidly cooling on ice. The denatured cDNA was incubated for 4 hrs at room temperature with dATP, dGTP, dTTP (33 μM each), random hexadeoxyribonucleotides (at 5 OD_{260} U/ml), BSA (20 μg/ml) 5 U klenow fragment of DNA polymerase I and ^{32}P-dCTP (1 mCi/ml final, 3000Ci/mmol; Amersham International plc). ^{32}P-labeled cDNA was separated from unincorperated ^{32}P-dCTP by Sephadex G-50 gel filtration using a Nick column (Pharmacia). Specific activity of the cDNA probe was determined using an LS 6000 TA scintillation counter (Beckman Instruments Inc., Palo Alto, CA) and the probe added to the hybridization solution at 1 x 10^6 counts per minute (cpm)/ml. Prehybridization and hybridization was performed in 5 x SSC, 5 x Denhardt's solution (0.1% w/v polyvinylpyrolidone, 0.1% w/v BSA, 0.1% w/v Ficoll 400), 5% SDS for 1 and 24 hours at 55°C respectively. Filters were washed 3 times for 30' with 0.1 x SSC, 1% SDS at 55°C. Hybridized ^{32}P-labeled probes were revealed by autoradiography, exposing the membrane to X-Omat AR film in the presence of an intensifying screen (Eastman Kodak Co., Rochester, NY) for 48 hours at -70°C.

(2.8) Mouse Immunization with KLH-Alum Conjugate

8 week old female BALB/c mice were immunized by subcutaneous injections in the neck (100 μl) and footpad (50 μl) with a keyhole limpet hemocyanin (KLH)-alum precipitate (1 mg/ml in PBS). Control mice were injected with PBS. The precipitate was formed as follows: 800 μl KLH (5 mg/ml; Calbiochem) was mixed with 800 μl potassium alum (9% in H_20; A7197; Sigma Chemical Co.). NaOH was added dropwise until a pH of 7.0 was attained. The precipitate was then washed 3 times in PBS and resuspended in 10 ml of PBS. 14 days post injection, mice were killed and the spleen, thymus, lymph node and bone marrow processed.

(2.9) RNA Purification from Tissues, Cells and Cell Lines

Total RNA was extracted from murine tissue using TRIzol® reagent (Life Technologies). Murine tissues were homogenized in TRIzol® reagent at a ratio of 50 mg tissue/ml TRIzol® using an Ultra-Turrax T8 (IKA Labortechnik, Bioblock, Frenkendorf, CH). The solution was extracted once with 0.2 volumes of chloroform and once with 1 volume of phenol/chloroform /isoamyl alcohol (25:24:1). Total RNA was then precipitated by the addition of 0.9 volumes of isopropanol, pelleted and washed with 80% ethanol. The RNA pellet was air-dried and resuspended in diethyl pyrocarbonate (DEPC)-treated H_20.

Total RNA was extracted from cells and cell lines using TRIzol reagent. Cells were washed once with PBS and resuspended in TRIzol. In order to shear genomic DNA, the solution was passed 10 times through a 0.8 mm gauge needle. The solution was extracted once with 0.2 volumes of chloroform and once with 1 volume of phenol/chloroform /isoamyl alcohol (25:24:1). Total RNA was then precipitated by the addition of 0.9 volumes of isopropanol, pelleted and washed with 80% ethanol. The RNA pellet was air-dried and resuspended in DEPC-treated H_20.

Poly A^+ RNA was obtain from total RNA using Oligotex™ latex particles coated with dT_{30} synthetic oligonucleotides. 100 µg total RNA was incubated with 20 µl of Oligotex™ suspension for 10 minutes at room temperature in order to anneal the poly A^+ sequence of the RNA to the dT_{30} synthetic oligonucleotides. Oligotex™ particles were then transferred to a spin column and washed twice. poly A^+ RNA was eluted from the Oligotex™ particles with 10mM Tris-HCl (pH 7.5) prewarmed to 70°C.

(2.10) Detection of CLF-1 mRNA by Northern Blot Analysis

(a) RNA Migration and Transfer

Aliquots (2 µg) of poly A^+ RNA were heated to 65°C for 2' and 37.5% (w/v) formaldehyde and 5 x MOPS buffer (Sigma Chemical Co.) added to each sample to give a final concentration of 6% formaldehyde and 1 x MOPS. Samples were loaded on a 1% agarose gel in 1 x MOPS, 6% formaldehyde and subjected to agarose gel

electrophoresis in 1 x MOPS running buffer. RNA was transferred from the gel to a GeneScreen *Plus*® hybridization transfer membrane (NEN® Research Products) by capillary transfer using 20 x SSC (Southern, 1975). RNA was fixed to the membrane by UV cross-linking using a UV Stratalinker™ 8400 (Stratagene). Membranes were subsequently stained with 0.1% methylene blue in order to localize and quantify RNA, and to assess the uniformity of transfer to the membrane.

(b) Probe Preparation, Hybridization and Detection

For detection of hCLF-1 mRNA transcripts, a 1.2 kb *Eco* RI/*Sac* II restriction enzyme digestion fragment of hCLF-1 cDNA was ^{32}P labeled using random hexamer priming for ^{32}P-dCTP incorporation as described in section 2.6. The cDNA probe was added to the hybridization solution at 1 x 10^6 cpm/ml.

Membranes were prehybridized and hybridized in ExpressHyb solution (Clonetech) for 30' and 2 hrs at 65°C respectively. Filters were washed three times 30' at 55°C with 0.1% SSC, 1% SDS. Hybridized ^{32}P-labeled probes were revealed by autoradiography, exposing the membranes to X-Omat AR film in the presence of an intensifying screen (Eastman Kodak Co.) for 24 hours at -70°C. In all cases, RNA integrity and equal loading was verified using a β-actin cDNA probe, ^{32}P-labeled by random hexamer priming for ^{32}P-dCTP incorporation as described in section 2.6.

For detection of mCLF-1 mRNA transcripts, ^{32}P-labeled cRNA probes were generated with mCLF-1 cDNA as a template for RNA transcription using the MAXIscript™ *In Vitro* Transcription Kit (Ambion Inc., Austin, TX). The vector pBluescript II KS- (Stratagene) containing a 700 bp fragment of the mCLF-1 cDNA was linearized by restriction enzyme digestion with *Bln* I. 1 μg linearized DNA was incubated for 1 hr at 37°C with ATP, CTP, and GTP (500 μM each), 40 U RNAguard (Pharmacia), 5 U T3 RNA polymerase and 10 μl ^{32}P-UTP (1mCi/ml, 3000 Ci/mmol; Amersham International Inc.). 10 U DNase I (Boerhinger Mannheim) were subsequently added to the reaction mixture in order to remove the DNA template. ^{32}P-labeled cRNA probes were separated from unincorperated ^{32}P-dCTP by Sephadex G-50 gel filtration using a Nick column (Pharmacia). Specific activity of the cRNA

probes were determined using an LS 6000 TA scintillation counter (Beckman Instruments Inc., Palo alto, CA) and the probe added to the hybridization solution at 1 x 10^6 cpm/ml.

Membranes were prehybridized and hybridized at 57.5°C for 2 hrs and 24 hrs respectively in a solution containing 0.75mM NaCl, 50 mM Na citrate (pH 7.5) 25 mM Na phosphate (pH 6.8), 1 mM EDTA, 50% formamide, 10% polyethylene glycol, 0.2% Ficoll 400, 0.2% polyvinylpyrolidone, 0.2% BSA, 1% SDS, 0.1% Na pyrophosphate, 200 µg/ml denatured sonicated salmon sperm DNA (Sigma Chemical Co.), 50 µg/ml yeast RNA (Boerhinger Mannheim) and 10 µg/ml poly(A) and poly(C). Membranes were rinsed twice in 0.1 x SSC and washed twice for 30' at 65°C in 0.1 x SSC, 1% SDS followed by two rinses in 0.1 x SSC. Membranes were then incubated for 30' at 37°C in Tris-HCl (pH 7), 300mM NaCl containing 2 mg/ml RNase A (Boerhinger Mannheim) and subsequently washed for 30' at 65°C in 0.1 x SSC, 1% SDS and rinsed in 0.1 x SSC. Hybridized ^{32}P-labeled probes were revealed by autoradiography, exposing the membranes to X-Omat AR film in the presence of an intensifying screen (Eastman Kodak Co.) for 48 hours at -70°C. In all cases, RNA integrity and equal loading was verified using a β-actin cDNA probe, ^{32}P-labeled by random hexamer priming for ^{32}P-dCTP incorporation as described in section 2.6.

(2.11) Detection of CLF-1 RNA by RT-PCR

In all cases, 5 µg total RNA was reverse transcribed using the first strand cDNA synthesis kit (Pharmacia LKB Biotechnology, Uppsala, Sweden). Total RNA was mixed with 0.2 µg of *Not* I-dT_{18} synthetic oligonucleotide, heated for 2' at 65°C and cooled rapidly on ice. DTT was added to a final concentration of 5 mM along with dNTPs (33 µM each) and 5 U MMLV reverse transcriptase, and the reaction mixture incubated at 37°C for 1 hr. 1/10 of the volume of the reaction mixture was used for subsequent PCR amplifications. hCLF-1 cDNA was amplified by 32 cycles of PCR using the primers 5'-GGATCAGGAGCCCACACAGC-3' and 5'-AGCGGCAGGTC AAGTCCTTC-3'. β-actin cDNA was amplified as a control for the integrity of the cDNA samples using the primers 5'-GGCGACGAGGCCCAGAGCAA G-3' and 5'-

CGATTTCCCGCTCGGCCGTG-3'. The conditions for each cycle of the PCR were as follows: 94°C, 30"; 60°C, 30"; 72°C, 1'. PCR amplified products were analyzed by agarose gel electrophoresis in 1 x TBE running buffer.

mCLF-1 cDNA was amplified by 30 cycles of PCR using the primers 5'-CTAGGCTCAGCAAGATCTGGATG-3' and 5'-GCTCCAGATTCCCGCCTTTTTC G -3'. HPRT cDNA was amplified as a control for the integrity of the cDNA samples using the primers 5'- GTTGGATACAGGCCAGACTTTGTTG-3' and 5'- GAGGGT AGGCTGGCCTATAGGCT-3'. Amplified mCLF-1 cDNA products were resolved by agarose gel electrophoresis on a 1.5% agarose gel in 1 x TBE gel runnung buffer. The gel was treated sequentially with 0.5 M NaOH (1 hr) and 0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl (30'). The DNA was subsequently transferred to a GeneScreen *Plus*® hybridization transfer membrane (NEN® Research Products, Boston, MA) by capillary transfer using 20 x SSC (Southern, 1975). DNA was fixed to the membrane by UV cross-linking using a UV Stratalinker™ 8400 (Stratagene, La Jolla, CA). mCLF-1 cDNA transcripts were detected by hybridization with the ^{32}P-labeled synthetic oligonucleotide probe 5'-CGCGTGGAGGACAGCGTGGACTGGAAGGTG -3' (Sambrook *et al.*, 1989). Amplified HPRT cDNA products were analyzed by agarose gel electrophoresis in 1 x TBE running buffer.

(2.12) Production of Recombinant Soluble hCLF-1 (rshCLF-1)

(a) Generation of Baculovirus Encoding rshCLF-1

hCLF-1 cDNA in pBluescript II SK- was digested with *Eco* RI and *Stu* I and cloned into the pFASTBAC-1 plasmid (Life Technologies) digested with the same enzymes. The synthetic oligonucleotides 5'CGCGTGCCTCGAACCCTACACCGCCTGCGAC CATCACCATCACCATCACTGA-3' and 5'-TCAGTGATGGTGATGGTGATGGT CGCAGGCGGTGTAGGGTTCGAGGCA-3' were annealed by heating to 94°C a 40 pmole solution of each oligonucleotide in the presence of 10 mM $MgCl_2$ and cooling gradually to room temperature. The annealed DNA was then ligated to the 3' end of the hCLF-1 cDNA in pFASTBAC-1 by digestion of the plasmid with the restriction enzymes *Mlu*I and *Stu*I. The annealed oligonucleotides encoded six histidines and the

CLEPYTACD epitope, an epitope recognized by mAb 179 (mAb 179 tag; Affymax, Palo Alto, CA).

Composite bacmid DNA was generated using the BAC-TO-BAC kit (Life Technologies; Luckow *et al.*, 1993). Competent DB10Bac *E. coli* cells, containing the baculovirus shuttle vector (bacmid), were transformed with the recombinant pFASTBAC-1 plasmid following a standard heat shock protocol (Sambrook *et al.*, 1989). Briefly, ligated DNA was mixed with 100 ml competent bacteria and incubated on ice for 30' followed by a 45'' incubation at 42°C. 900 µl Luria Broth was added to the solution which was subsequently incubated at 37°C for 1 hr. Bacteria containing pFASTBAC-1 transposed bacmids were selected on Luria Agar plates using antibiotics (50 µg/ml kanamycin sulphate, 10 µg/ml tetracycline, 7 µg/ml gentamicin) and histochemical substrates (300 µg/ml X-gal, 40 µg/ml IPTG; Sambrook *et al.*, 1989). Composite bacmid DNA was purified from *E. coli* using a standard protocol for plasmid DNA preparation from bacteria (Sambrook *et al.*, 1989). Briefly, bacteria were grown to confluence in 2 ml Luria Broth containing 50 µg/ml kanamycin sulphate, 10 µg/ml tetracycline, 7 µg/ml gentamicin, pelleted by centrifugation and resuspended in 300 µl 25mM Tris-HCl (pH 8.0), 10mM EDTA (pH 8.0), 50mM glucose. Bacteria were lysed by mixing with an equal volume of 0.2 N NaOH, 1% SDS followed by a 5' incubation at room temperature. 300 µl of 3M potassium acetate, 10% acetic acid was added and the solution gently mixed and incubated on ice for 10'. The precipitate was removed by centrifugation and the supernatant (900 µl) transferred to a clean tube. DNA was precipitated by the addition of 560 µl isopropanol and pelleted by centrifugation. The DNA pellet was washed with 70% ethanol, dried, and resuspended in 50 µl 1 x TE.

For the production of recombinant baculovirus, the cell line Sf21 was transfected with composite bacmid using CellFECTIN™ reagent. Sf21 cells during growth mid-log phase were plated at 9 x10^5/35mm cell culture well and incubated for 1 hr at 27°C for attachment. The transfection solution was prepared as follows: in one tube, 2 µg bacmid DNA was mixed with 100 µl Sf900 II medium without FCS. In a second tube,

6 µl CELLFECTIN reagent was mixed with 100 µl Sf900 II medium without FCS. The two solutions were mixed and incubated for 15' at room temperature. The mixture was diluted with 800 µl Sf900 II medium and overlaid on the adherent Sf21 cells. Cells were incubated sequentially with the transfection solution for 5 hrs at 27°C and 2 ml Sf900 II containing 10% FCS 72 hrs at 27°C. Virus was harvested in the cell supernatant and titered by serial dilution in Sf900 II medium with incubation of the dilutions on Sf21 cell monolayers for 2 hours at 27°C. Cell monolayers were subsequently covered with a 1:1 mixture of Sf900 II medium and low melting point agarose (Life Technologies) maintained at 37°C. The agarose was set, overlaid with Sf900 II medium supplemented with 10% FCS and incubated for 5 days at 27°C. Plaques were counted and the virus titer calculated in plaque forming units (pfu)/ml according to the dilutions.

(b) Expression and Purification of rshCLF-1

Sf9 cells in log growth phase were infected at 1.2 x 10^6 cells/ml with a multiplicity of infection (MOI) of 10. In order to achieve the desired MOI, the following formula was used to estimate the volume of viral innoculum required (ml): (desired MOI (pfu/ml) x total number of cells) / titer of viral innoculum (pfu/ml). The cell suspension was harvested at 72 hours post infection and the supernatant recovered from cells by centrifugation. The supernatant was subsequently passed through a 0.22 µm filter (Millipore Corp., Bedford, MA) and the proteins concentrated approximately 20-fold with Centriprep-10 concentrators (10 kD cut off; Amicon, Beverly, MA).

rshCLF-1 was purified from the concentrated Sf9 cell supernatant using a column packed with 50ml of Ni-NTA agarose resin (Qiagen AG, Basel, Switzerland). The concentrated supernatant was dialyzed against 100 mM Tris-HCl (pH 8.0) using Spectra/Por molecular porous membrane tubing with a 12-14 kD molecular weight cut-off (Spectrum Medical Industries Inc., Laguna Hills, CA) and applied to Ni-NTA agarose resin equilibrated in 100 mM Tris-HCl (pH 8.0). The resin was sequentially washed with 500 ml 100 mM Tris-HCl (pH 8.0), 0.1% w/v Tween-20 and 500 ml Tris-HCl (pH 8.0). Bound proteins were eluted with 100 mM Tris-HCl (pH 8.0), 500 mM imidazole and the eluate dialyzed against 100 mM Tris-HCl (pH 8.0) using Spectra/Por molecular porous membrane tubing with a 12-14 kD molecular weight

cut-off and concentrated with a Centriprep-10 concentrator (10 kD cut off; Amicon). The recovered fraction contained approximately 30% monomeric hCLF-1 as determined by SDS-PAGE under reducing conditions and staining with silver nitate (Silver Stain Plus; Bio-Rad Laboratories AG; Sambrook *et al.*, 1989).

(2.13) Expression of Recombinant Cell Membrane-Bound hCLF-1 in Sf9 Cells

(a) Generation of Baculovirus Encoding Recombinant Membrane-Bound hCLF-1

The human IL-13Rα1 (hIL-13Rα1; Gauchat *et al.*, 1997) transmembrane and cytoplasmic domains were amplified by PCR from pBluescript II SK- containing the hIL-13Rα1 cDNA using the synthetic oligonucleotides 5'- TCCCCGCGGTACATAA CCATGTTACTCATTGTT-3' and 5'-TCCCCGCGGGAATTCCATCACTGAGAGG CTTTCT -3'. The PCR product was digested with the restriction enzyme *Sac* II and ligated into *Sac* II digested pBluescript II SK- containing the full length hCLF-1 cDNA. DNA encoding the polyoma virus middle T antigen epitope EYMPME (EE tag; Grussenmeyer *et al.*, 1985) was inserted at the 5' end of the hCLF-1 cDNA by digesting the plasmid with *Xho* I and *Bam* HI and cloning into these restriction enzyme sites the two annealed oligonucleotides 5'TCGAACTAGTGAATACATGCC AATGGAAGCCCACACAGCTGTGATCAGCCCCAG-3' and 5'-GATCCTGGGG ACTGATCACAGCTGTGTGGGCTTCCATTGGCATGTATTCACTAGT-3'. Oligo-nucleotides were annealed as described above. The cDNA encoding the tagged fusion protein was excised from pBluescript II SK- by digestion with the restriction enzyme *Spe* I (whose site was subsequently blunt ended using the Klenow Fragment of DNA polymerase I) and *Eco* RI, and ligated into the pFASTBAC-1 plasmid (containing the mellitin signal peptide; Life Technologies) digested with *Bsp120* I (whose site was subsequently blunt ended using the Klenow Fragment of DNA polymerase I) and *Eco* RI. Recombinant baculovirus was generated using the protocol described above.

(b)Expression, Detection and Purification of Recombinant Membrane-Bound hCLF-1

In order to express recombinant membrane-bound hCLF-1, Sf9 cells were infected with recombinant baculovirus encoding the hCLF-1/hIL-13Rα1 fusion protein as described in section 2.12b. Expression of the recombinant protein at the surface cell

surface was analyzed at 24, 36 and 48 hours post-infection by flow cytometry. Cells were harvested by centrifugation, washed once in FACS buffer (1 x PBS, 1% w/v BSA, 0.01% w/v Na azide) and resuspended at 1 x 10^6 cells/ml in FACS buffer containing either10 μg/ml mouse IgG1 control Ab (Becton Dickinson Immunocytochemistry Systems, Erembodegem, Belgium) or 10 μg/ml α-EE tag mAb. Cells were incubated for 30' at 4°C, washed twice with FACS buffer and resuspended at 1 x 10^6 cells/ml in FACS buffer containing 10μg/ml flourescein conjugated sheep α-mouse Abs (Silenus Laboratories, Hawthorn, Australia) for 30' at 4°C. Following two washes in FACS buffer, cells were resuspended at 1 x 10^6 cells/ml in FACS buffer and analyzed for fluorescent staining using a FACSCalibur (Becton Dickinson Immunocytochemistry Systems).

To purify the membrane-bound fusion protein, cells at 48 hours post infection were harvested by centrifugation, resuspended at 0.5g/ml in cell lysis buffer (50 mM Tris pH 8.5, 0.5% Triton-X-100 (Sigma Chemical Co.), 0.5% Nonidet p40 (Boerhinger Mannheim), 0.1 M NaCl, 2 mM $CaCl_2$, 10 mM iodoacetamide (IAM; Sigma Chemical Co.), 1 mM phenylmethylsulphonyl fluoride (PMSF; Sigma Chemical Co.), 1 mM tosyl-lysine chloromethyl ketone (TLCK; Sigma Chemical Co.)), passed 3 times through a French press cell, sonicated and incubated for 1 hr at 4°C on a roller. The solution was centrifuged at 100 000 x g for 1 hr at 4°C and the supernatant passed through a 0.22μm filter (Millipore Corp.).

The filtered supernatant was then cycled for 16 hrs at 4°C through a column packed with BSA-coupled affigel 10 (equilibrated in 50 mM Tris-HCl (pH 8.5), 0.1 M NaCl, 1 % Triton-X-100; Bio-Rad Laboratories AG) to remove proteins with non-specific binding properties. The BSA was coupled to affigel 10 as follows: 2.5 mg/ml BSA (Sigma Chemical Co.) in coupling buffer (0.1 M MOPS pH 7.5, 0.3 M NaCl) was mixed with affigel 10 resin equilibrated in H_20 at a ratio of 5mg BSA/ml resin and incubated for 16 hrs at 4°C on a roller. The resin was subsequently pelleted by centrifugation, washed with coupling buffer and the remaining chemically active groups blocked by incubation for 1 hr at room temperature in 0.1 M ethanolamine pH

8.0 (Sigma Chemical Co.). The resin was washed with 1 x PBS, preluted with 3 M NH_4SCN (Sigma Chemical Co.) and rewashed with 1 x PBS.

The Sf9 cell lysate was then cycled for 16 hrs at 4°C through a column packed with α-EE tag mAb-coupled affigel 10 resin equilibrated in 50 mM Tris-HCl (pH 8.5), 1% Triton-X-100. The α-EE tag mAb was coupled to the affigel 10 resin following the procedure described above. The column was washed sequentially with 50 mM Tris-HCl (pH 8.5), 1% Triton-X-100 and 50 mM Tris-HCl (pH 8.5), 50 mM n-octyl-β-D-glucopyranoside (OGP; Alexis Corp., Läufelfingen, CH) and bound protein eluted with 50 mM Tris-HCl (pH 8.5), 50 mM OGP, 3 M NH_4SCN. The eluate was dialyzed extensively against 50 mM Tris-HCl (pH 8.5), 50 mM OGP using Spectra/Por molecular porous membrane tubing with a 12-14 kD molecular weight cut-off, and concentrated with a Centriprep-10 concentrator (10 kD cut off; Amicon). The recovered fraction contained approximately 90-95% monomeric hCLF-1/hIL-13Rα1 fusion protein as determined by SDS-PAGE under reducing conditions and staining with silver nitrate (Silver Stain Plus; Bio-Rad Laboratories AG; Sambrook *et al.*, 1989).

(2.14) Generation of Mouse α-hCLF-1 mAbs

(a) Immunization with hCLF-1 Protein

A Balb/c mouse was immunized on day 0, 7 and 28 subcutaneously in the limbs and behind the neck with 100 µg of purified rshCLF-1 in MPL+TDM emulsion (RIBI; Inotech, Dottikon, Switzerland) per injection. Three days after the final injection, the draining lymph nodes were obtained and placed in small tissue culture plates (3035; Costar, Cambridge, MA) with an enzyme cocktail containing 2.5 mg/ml collagenase (Worthington Biochemical Corp., Freehold, NJ) and 1%/ml DNase I (Sigma Chemical Co.) in IMDM. Lymph nodes were opened by passage through a 26 gauge needle and incubated for 30' at 37°C. The partially digested stroma was then gently pipetted and released cells in the supernatant placed in a tube containing IMDM supplemented with 5% FCS. A fresh aliquot of the enzyme cocktail was added to the remaining tissue and returned to a 37°C incubator. After 30' the tissue was re-pipetted and the supernatant

collected. All the supernatants were subsequently pooled, washed once in IMDM, resuspended at 1 x 10^8 cells/ml in IMDM, layered over pre-prepared continuous Percoll gradients (Pharmacia LKB Biotechnology), centrifuged at 400 x g for 30' and the 1.060 g/ml to 1.065 g/ml low density band removed. After 2 washes with IMDM, the cell suspension was resuspended in IMDM supplemented with 10% FCS and placed in a small tissue culture dish at 37°C for 1 hour to deplete adherent populations (macrophages and lymphoid dendritic cells).

The resulting cell suspension was resuspended at 10^6 cells/ml and fused with Sp2 myeloma cells. Equal volumes of culture medium containing Sp2 cells at 5 x 10^5 cells/ml and the lymph node cells at 5 x 10^5 cells/ml were mixed, washed with IMDM and pelleted. Cells were gently resuspended in a 1ml of 50% poylethylene glycol (PEG) 1500 in IMDM by stirring with a pipette tip during 1'. 10 ml IMDM was gradually added to the cells whilst continually stirring, the cells pelleted and resuspended in 200 ml prewarmed HAT selection medium (IMDM supplemented with 20% FCS, 0.015% w/v oxalacetate (Sigma Chemical Co.), 0.005% w/v sodium pyruvate (Sigma Chemical Co.) 20 IU bovine insulin (Sigma Chemical Co.), 100 μM hypoxanthine (Sigma Chemical Co.), 16 μM thymidine (Sigma Chemical Co.) and 0.4 μM aminopterin (Sigma Chemical Co.)). 100 ml of cells were dispensed into the wells of 96-well microtiter plates (Falcon 3912; Becton Dickinson Labware Europe, Meylan, France). 7-10 days after the fusion, the supernatants were harvested for screening.

(b) α-hCLF-1 mAb Screening

96 well NUNC immunoplates (439454; Life Technologies) were coated at 4°C with 10 μg/ml rshCLF-1 in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.4) by incubation for 16 hrs at 4°C. The wells were washed once with 1 x PBS, blocked with 1% BSA in 1 x PBS, incubated for 2 hrs at room temperature with 200 μl hybridoma supernatant and washed 4 times with 1 x PBS, 0.05% Tween-20 (Boerhinger Mannheim). Bound Ab was revealed with horseradish-peroxidase coupled goat α-mouse IgG (Southern Biotechnology Associates, Inc.). The conjugated Ab was diluted 1:1000 in 1 x PBS, 0.05% Tween-20, added to the wells and the plates incubated at

room temperature for 1 hr. Wells were washed 6 times with 1 x PBS, 0.05% Tween-20, and 100 µl of the enzyme substrate added to each well (0.5 mg/ml o-phenylenediamine dihydrochloride (OPD; Sigma Chemical Co.) in 0.2 M Na_2HPO_4, 0.1 M citric acid, 0.03% H_2O_2). The enzymatic reaction was terminated after 5' by the addition of 10% H_20_2 final and the OD of the solutions in each well at 490 and 570 nm wavelength determined using a Multiskan EX microplate photometer (Labsystems, Helsinki, Finland). Ab specificity was checked using an ELISA with recombinant soluble hIL-13Rα1 (rshIL-13Rα1; GBRI, Geneva, CH) having the same recognition tags as recombinant hCLF-1, following the procedure outlined above.

Specific positive supernatants were further screened by flow cytometry on Sf9 cells either mock infected or infected with baculovirus encoding the membrane bound hCLF-1/hIL13Rα1 fusion protein. Sf9 cells were infected with recombinant baculovirus encoding either the hCLF-1/hIL13Rα1 fusion protein or an irrelevant membrane-bound protein, p2X2 (Radford *et al.*, 1997) following the procedure outlined in section 2.12b). Cells were harvested 48 hours post-infection and cell surface expression of the recombinant fusion protein verified by flow cytometry using the α-EE tag mAb (as described in section 2.13b. To screen for α-hCLF-1 mAbs, Sf9 cells expressing the irrelevant cell surface protein or the hCLF-1 fusion protein were resuspended in hybridoma cell supernatant at 1 x 10^6 cells/ml for 30' at 4°C, washed twice with FACS buffer (1 x PBS, 1% w/v BSA, 0.01% w/v Na azide) and resuspended in FACS buffer containing 10 µg/ml flourescein conjugated sheep α-mouse Abs (Silenus Laboatories) for 30' at 4°C. Following two washes in FACS buffer, cells were resuspended at 1 x 10^6 cells/ml in FACS buffer and analyzed for fluorescent staining using a FACSCalibur (Becton Dickinson Immunocytochemistry Systems). Specificity was determined by comparing fluorescence from a given supernatant between Sf9 cells expressing the irrelevant protein and the hCLF-1 fusion protein.

(c) mAb Purification

Antibodies were purified by chromatography on protein A sepharose (Pharmacia LKB Biotechnology). Hybridoma supernatants were centrifuged and passed through a 0.22

μm filter (Millipore Corp.) to remove cells and cell debris, and cycled for 16 hours at 4°C through a column packed with protein A sepharose. The column was washed with 1 x PBS and bound protein eluted in 0.1 M Citrate pH 4.5. Eluates were then subjected to gel filtration on superdex-200 (Pharmacia LKB Biotechnology) equilibrated in 1 x PBS.

(d) Immunoglobulin Isotype Determination

Isotypes were determined using the MonoAb-ID EIA Kit (Zymed Laboratories, Inc., San Francisco, CA). 96 well NUNC immunoplates (439454; Life Technologies) were coated with hCLF-1 protein and blocked as described above. 200 μl hybridoma supernatant was added to each well, the plates incubated for 2' at room temperature and subsequently washed 4 times with 1 x PBS, 0.05% Tween-20. To each well, either horseradish-peroxidase coupled rabbit α-mouse IgG1, IgG2a, IgG2b or IgG3 was added (1:1000 dilution in 1 x PBS, 0.05% Tween-20) and incubated for 1' at room temperature. Wells were washed 6 times with 1 X PBS, 0.05% Tween-20, and 100 μl of the enzyme substrate added to each well (0.5 mg/ml OPD in 0.2 M Na_2HPO_4, 0.1 M citric acid, 0.03% H_2O_2). The enzymatic reaction was terminated after 5' by the addition of 10% H_2O_2 final and the OD of the solutions in each well at 490 and 570 nm wavelength determined using a Multiskan EX microplate photometer.

(2.15) Expression of Recombinant Cell Membrane-Bound hCLF-1 in CHO Cells

pBluescript II SK- containing the hCLF-1/hIL-13Rα1 cDNA without the EE tag (section 2.13a) was digested with *Bam* HI and *Eco* RI and ligated into the expression vector pCDNA3 (Invitrogen) digested with the same restriction enzymes. In order to insert the native signal peptide from hCLF-1 at the 5' end of the fusion construct, pBluescript II SK- containing the full length hCLF-1 was digested with *Kpn* I and *Bam* HI and the excised fragment (encoding the signal peptide) was inserted into pCDNA3 containing the hCLF-1/hIL13Rα1 cDNA digested with the same enzymes. The expression vector was linearized and introduced into CHO cells by electroporation. In parallel, linearized pCDNA3 encoding full length hIL-13Rα1 was introduced into CHO cells as a control. For cell transfections, CHO cells were grown

to 75% confluence, removed from culture by incubation with 1 x PBS, 1mM EDTA for 5' at 37°C, and washed sequentially in 1 x PBS and transfection buffer (20 mM HEPES (pH 7.4; Life Technologies), 150 mM NaCl). 25 μg linearized DNA for transfection resuspended in transfection buffer was added to the cells and the mixture placed in a Gene Pulser® cuvette with a 0.4 cm electrode gap (Bio-Rad Laboratories AG). Cells were electroporated in a Gene Pulser® (Bio-Rad Laboratories AG) using the following conditions: 260 V, 960 μF, ∞ Ω. Cells were placed in culture for 24 hrs after which time the growth medium was replaced with selection medium (growth medium supplemented with 500 μg/ml geneticin (Life Technologies) and 25 mM HEPES (pH 7.4)) for 2-3 weeks in order to select for transfected cells .

Expression of hCLF-1 on the surface of the resistant CHO cells was detected by flow cytometry with a monoclonal antibody recognizing hCLF-1. Resistant cells transfected with either pCDNA3 encoding the hCLF-1/hIL-13Rα1 fusion construct or hIL-13Rα1 alone were removed from culture by incubation with 1 x PBS, 1mM EDTA for 5' at 37°C and washed once with FACS buffer (1 x PBS, 1% w/v BSA, 0.01% w/v Na azide). The cells were resuspended at 1 x 10^6 cells/ml in FACS buffer containing 10 mg/ml α-hCLF-1 mAb and incubated for 30' at 4°C. Cells were washed twice with FACS buffer and resuspended in FACS buffer containing 10 μg/ml flourescein conjugated sheep α-mouse Abs (Silenus Laboatories) for 30' at 4°C. Following 2 washes with FACS buffer, cells were resuspended at 1 x 10^6 cells/ml in FACS buffer and analyzed for fluorescent staining using a FACSVantage (Becton Dickinson Immunocytochemistry Systems). Cells transfected with cDNA encoding the hCLF-1/hIL-13Rα1 fusion protein showing a significant level of fluorescence when compared to control cells were selected by the FACSVantage and replaced in culture. After three rounds of selection, a population of CHO cells expressing high levels of membrane-bound hCLF-1 were obtained.

(2.16) Expression of rshCLF-1 in HEK 293-E Cells

(a) Generation of HEK 293-E Cell Transfectants

cDNA encoding the six histidine/CLEPYTACD epitope tagged hCLF-1 derivative (section 2.12a) was excised from the pFASTBAC-1 plasmid by digestion with the restriction enzymes *Sal* I and *Xho* I and ligated into the EBV expression vector pEBS-PL (Boutron *et al*, 1997; a gift from V. Steimle, Max-Planck Institüt für Immunobiologie, Freiburg, Germany) digested with the same restriction enzymes. The purified plasmid was introduced into HEK 293-E cells by electroporation. As a control, HEK 293-E cells were transfected in parallel with pEBS-PL with no insert. Cell transfections were performed following the procedure described in section 2.15 except that transfected DNA was not linearized and cells were selected in selection medium containing 100 μg/ml hygromycin B (Calbiochem-Novabiochem). Following 2-3 weeks selection in selection medium, resistant cells were cloned at 1 cell/well in 96 well plates (Falcon 3912) using the FACSVantage and grown to confluence in selection medium.

Transfected cells expressing rshCLF-1 were screened by ELISA on cell supernatants. 96 well NUNC immunoplates (439454; Life Technologies) were coated at 4°C with 10 μg/ml mAb 179 in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.4) by incubation for 16 hrs at 4°C. The wells were washed once with PBS, blocked with 1% BSA in PBS, incubated for 2 hrs at room temperature with 200 μl transfected HEK 293-E cell supernatants and washed 4 times with 1 x PBS, 0.05% Tween-20 (Boerhinger Mannheim). Wells were subsequently incubated for 1 hour at room temperature with 1 x PBS, 0.05% Tween-20 containing 10 μg/ml biotin conjugated α-hCLF-1 mAb and washed 4 x with 1 x PBS, 0.05% Tween-20. For biotinylation, mAbs at 1 mg/ml in 1 x PBS were incubated for 2 hours at room temperature with D-biotinoyl-ε-aminocaproic acid-N-hydroxylsuccinimide ester (biotin-7-NHS; 30 μg/ml final). The remaining reactive biotin-7-NHS was blocked by the addition of Tris-HCl (pH 7.5; 0.2 M final) and dialyzed extensively against PBS using Spectra/Por molecular porous membrane tubing with a 12-14 kD molecular weight cut-off. The wells were then incubated with 1 x PBS, 0.05% Tween-20

containing horseradish peroxidase-conjugated streptavidin (1:1000 dilution; Amersham International plc) for 1 hour at room temperature, washed 6 times with 1 x PBS, 0.05% Tween-20 and 100 µl of the enzyme substrate added to each well (0.5 mg/ml OPD in 0.2 M Na_2HPO_4, 0.1 M citric acid, 0.03% H_2O_2). The enzymatic reaction was terminated after 5' by the addition of 20% H_2S0_4 final and the OD of the solutions in each well at 490 and 570 nm wavelength determined using a Multiskan EX microplate photometer.

Cell clones with supernatants giving positive results by ELISA were tested for recombinant hCLF-1 expression by flow cytometry using intracellular Ab staining with the Fix and Perm Cell Permeabilization Kit (Caltag Laboratories, Burlingame, CA). Cells were removed from culture by a 5' incubation with 1 x PBS, 1 mM EDTA washed with 1 x PBS and fixed by incubation at 5 x 10^6 cells/ml in fixation medium (containing formaldehyde) for 15' at room temperature. The cells were washed 2 times in FACS buffer (1 x PBS, 1% w/v BSA, 0.01% w/v Na azide), and incubated at 5 X 10^6 cells/ml in Permeabilization buffer containing either 10 µg/ml mAb 179 or α-hCLF-1 mAb for 15' at room temperature. The cells were washed twice in FACS buffer and incubated at 5 X 10^6 cells/ml in Permeabilization buffer containing 10 µg/ml flourescein conjugated sheep α-mouse Abs (Silenus Laboatories) for 15' at room temperature. Following 2 washes with FACS buffer, cells were resuspended at 1 x 10^6 cells/ml in FACS buffer and analyzed for fluorescent staining using a FACSVantage (Becton Dickinson Immunocytochemistry Systems).

(b) Purification of rshCLF-1 from Transfected HEK 293-E Cell Supernatants

Supernatants from HEK 293-E cells expressing rshCLF-1 were centrifuged and passed through a 0.22 µm filter (Millipore Corp.) in order to remove cell debris and cycled for 16 hrs at 4°C through a column packed with BSA-coupled affigel 10 equilibrated in 1x PBS as described above, to remove proteins with non-specific binding properties. The supernatant was then cycled for 16 hrs at 4°C through a column packed with mAb 179-coupled affigel 10. The mAb 179-coupled resin was prepared following the same procedure described above for the preparation of BSA-coupled affigel 10 resin. The column was washed sequentially with 1 x PBS, 1% Triton-X-

100, 2 M urea in 1 x PBS, and 1 x PBS alone. Bound proteins were eluted from the column by incubating the column in Tris-glycine-SDS sample buffer (Novex, San Diego, CA) for 5' at 94°C using a 1:1 of sample buffer to column volume. The recombinant protein was subsequently analyzed by Western blot analysis (see below).

(2.17) Characterization of Recombinant Forms of hCLF-1 by Western Blot Analysis

When purified, the recombinant hCLF-1 at a concentration of 20 µg/ml was diluted 1:1 with Tris-glycine-SDS sample buffer (Novex) and heated to 94°C for 5 minutes. In the case of recombinant hCLF-1 expressed in CHO cells, cells were removed from culture by incubation at 37°C for 5' in 1`x PBS, 1 mM EDTA, centrifuged and resuspended in 1 x PBS at 1 x 10^7 cells/ml. The cell suspension was diluted 1:1 in sample buffer, the solution passed extensively through a 0.8 gauge needle and heated at 94°C for 5'. In order to reduce proteins, 2–ME was added to the samples to a final concentration of 375mM prior to heating at 94°C.

The proteins were resolved by SDS-PAGE on 8% polyacrylamide gels (Novex) in Tris-glycine electrophoresis buffer (25 mM Tris, 250 mM glycine (pH 8.3), 0.1% SDS) using an X Cell II Mini-Cell (Novex). A constant current of 4 mA was applied to the gel, which was run until the bromophenol blue in the sample buffer had reached the bottom. The SeeBlue pre-stained standard (Novex) was migrated in parallel to allow the approximation of the molecular weight of the proteins. The proteins were transferred from the gel onto a nitrocellulose membrane (Novex) using an X Cell II Blot Module (Novex). The gel was placed in contact with the membrane and the two sandwiched between pieces of Whatman 3MM paper. The sandwich was placed between porous sponge pads which were in turn placed in the Blot Module. The above procedure was carried out immersed in transfer buffer (25mM Tris, 250 mM glycine (pH 8.3), 20% methanol). The transfer was performed in transfer buffer with the X Cell II Blot Module placed in ice. A constant current of 0.23 mA was applied for 2 hrs.

Following the transfer, membranes were blocked in 1 x PBS containing 5% dried milk and 0.15% Tween-20 for 1 h at RT and incubated for 1 h at RT with PBS containing 2.5% dried milk and 5 μg/ml of the indicated mAb. Membranes were washed 3 times for 15' at room temperature in 1 x PBS, 0.15% Tween-20, and incubated for 1 hr at room temperature with 1 x PBS, 0.15% Tween-20 containing horseradish peroxidase labeled goat α-mouse Ab (1:1000; Jackson ImmunoResearch Laboratories, Milan Analytica AG, La Roche, CH). Following 3 washes with 1 x PBS, 0.15% Tween-20, bound Ab was revealed by chemiluminescence. The membrane was incubated for 1' at room temperature in ECL™ western blot detection reagent (Amersham International plc) and chemiluminescence detected by exposure of the membrane to Hyperfilm™ ECL™ (Amersham International plc).

(2.18) Formation and Detection of Cell Surface IL-6 Type Cytokine Receptor Complexes

(a) Expression of gp130 on the Surface of HEK 293 Cells

Cells were removed from culture by incubation for 5'at 37°C with 1 x PBS, 1 mM EDTA, washed once with FACS buffer (1 x PBS, 1% w/v BSA, 0.01% w/v Na azide) and incubated for 1 hr at room temperature at 1 x 10^6 cells/ml with either biotin conjugated goat α-rat Ab (1:100; Jackson ImmunoResearch Laboratories) or 10 μg/ml biotin conjugated goat α-human gp130 Ab (R&D Systems) in FACS buffer. Cells were washed twice with FACS buffer, incubated for 30' at room temperature with 10 μg/ml fluorescin conjugated rabbit α-goat Ab in FACS buffer, washed a further 2 times with FACS buffer, resuspended at 1 x 10^6 cells/ml in FACS buffer and fluorescent staining analyzed by flow cytometry using a FACSCalibur.

(b) Receptor Complex Formation on the Surface of HEK 293 Cells

Cells were removed from culture by incubation for 5'at 37°C with 1 x PBS, 1 mM EDTA, washed once with FACS buffer and incubated for 1 hr at room temperature at 2 x 10^6 cells/ml with either 10 μg/ml recombinant soluble human LIF-R (rshLIF-R; R&D Systems) alone, 10 μg/ml rshLIF-R and 10 μg/ml human OSM (R&D Systems) or 10 μg/ml rshLIF-R and 10 μg/ml human CT-1 (Research Dianostics Inc., Flanders,

NJ) in FACS buffer. The cells were washed twice in FACS buffer and incubated for 30' at room temperature either biotin conjugated goat α-rat Ab (1:100; Jackson ImmunoResearch Laboratories) or 10 μg/ml biotin conjugated goat α-human LIF-R Ab (R&D Systems) in FACS buffer. Cells were washed twice in FACS buffer and incubated for 30' at room temperature with 10 μg/ml fluorescin conjugated rabbit α-goat Ab in FACS buffer, washed a further 2 times with FACS buffer, resuspended at 1 x 10^6 cells/ml in FACS buffer and fluorescent staining analyzed by flow cytometry using a FACSCalibur.

(c) Receptor Complex Formation on the Surface of CHO Cells Expressing Membrane-Bound hCLF-1

Cells were removed from culture by incubation for 5'at 37°C with 1 x PBS, 1 mM EDTA, washed once with FACS buffer and incubated for 1 hr at room temperature at 2 x 10^6 cells/ml with either 10 μg/ml rshLIF-R alone, 10 μg/ml recombinant soluble human gp130 (rshgp130; R&D Systems) alone, 10 μg/ml rshLIF-R and 10 μg/ml rshgp130 or 10 μg/ml rshLIF-R and rshgp130 alone or together with 10 μg/ml human IL-6 (Sigma Chemical Co.), 10 μg/ml mouse LIF (Life Technologies), 10 μg/ml human OSM (R&D Systems), 10 μg/ml human IL-11 (R&D Systems) or 10 μg/ml human CT-1 (Research Dianostics Inc., Flanders, NJ) in FACS buffer. The cells were washed twice in FACS buffer and incubated for 30' at room temperature either biotin conjugated goat α-rat Ab (1:100; Jackson ImmunoResearch Laboratories), 10 μg/ml biotin conjugated goat α-human LIF-R Ab or 10 μg/ml biotin conjugated goat α-human gp130 Ab in FACS buffer. Cells were washed twice in FACS buffer and incubated for 30' at room temperature with 10 μg/ml fluorescin conjugated rabbit α-goat Ab in FACS buffer, washed a further 2 times with FACS buffer, resuspended at 1 x 10^6 cells/ml in FACS buffer and fluorescent staining analyzed by flow cytometry using a FACSCalibur.

(2.19) Cell Surface Binding with rshCLF-1

The cell lines THP-1, HMC-1, HEK 293, Jurkat, JY and RPMI 8226 were removed from culture and washed twice in FACS buffer (1 x PBS, 1% w/v BSA, 0.01% w/v

Na azide). Freshly isolated monocytes derived from human PBL were incubated for 24 hours with the appropriate proinflammatory cytokine (see section 2.4), removed from culture and washed twice with FACS buffer.

Cells were incubated for 1 hr at 4°C at 2 x 10^6 cells/ml with either FACS buffer alone or FACS buffer containing 10 μg/ml recombinant soluble human IL-5R α chain (rshIL-5Rα), or 10 μg/ml rshCLF-1. Cells were washed twice with FACS buffer, incubated for 30' at 4°C with 10 μg/ml mAb 179 (as well as 10mg/ml α-hCLF-1 mAb for the cell lines) in FACS buffer, rewashed 2 times with FACS buffer and incubated for 30' at 4°C with 10 μg/ml fluorescin conjugated sheep α-mouse Abs (Silenus Laboatories) in FACS buffer. Following a further 2 washes with FACS buffer, cells were resuspended at 1 x 10^6 cells/ml in FACS buffer and fluorescent staining analyzed by flow cytometry using a FACSCalibur. In the case of RPMI 8226 cells, cells were resuspended at 1 x 10^6 cells/ml in the supernatant of either HEK 293-E cell transfectant clones 5 or 17 or the supernatant of non-transfected HEK-293-E cells and incubated for 30' at 4°C. Supernants were either crude or concentrated approximately 1:5 with a centriprep 10 concentrator with a 10 kD molecular weight cut off. (Amicon, Beverly, MA). Cells were washed and rshCLF-1 binding analyzed by FACS using the α-hCLF-1 mAb as described above.

CHAPTER 3. RESULTS

(3.1) Cytokine-Like Factor 1 (CLF-1) is a Novel Soluble Factor Sharing Homology with Cytokine Type-I Receptors

The GenBank database containing ESTs was searched using TBLASTN with a 20 amino acid sequence surrounding the W-S-x-W-S motif of mIL-13R (Hilton *et al.*, 1996). ESTs within the BLAST output showing homology to the query sequence within a significantly long open reading frame and within a region of high quality of sequence were selected manually. The cDNA sequences were translated, and the open reading frames (ORFs) which showed homology to the original mIL-13R query sequence were compared against the Swissprot database using BLASTP. The amino acid sequence from the murine EST W66776 shared a high level of homology with the prolactin receptor (PRLR), the family of α chain receptor subunits sharing gp130, such as IL-6Rα, IL-11Rα and CNTFRα, and members of the gp130 subfamily, such as gp130 itself, LIFR, OSMR, G-CSFR and the leptin receptor, OBR. Using the sequence of W66776 to search the GenBank database overlapping ESTs of murine, human and rat origin were identified (Table 3.1 below) and assembled.

The cDNA clone 479043, which gave rise to the mouse EST found furthest 5' in the sequence assembly was obtained from the IMAGE consortium. After sequencing, it was found to contain an insert of 1 Kb having a 3' poly A tail. The rapid amplification of 5' cDNA ends (5'-RACE; Frohman *et al.*, 1988) on murine lung cDNA allowed the cloning of a further 308 bp upstream. The 1397 bp murine cDNA encoded a protein of 383 amino acids. The cDNA was incomplete at the 5' end as the first amino acid of the translated sequence aligned to amino acid 39 of the human sequence, and no starting methionine or putative signal peptide could be identified (Figure 3.1).

To clone the human cDNA encoding CLF-1, a 310 bp PCR product was amplified from human lung cDNA using primers designed from the human ESTs. The PCR product was in turn used as a probe to screen a human placental cDNA library, resulting in the isolation of two clones, one of 1736 bp (clone 18; Figure 3.2a) and the other 1552 bp (clone 3), both containing a 3' poly A tail. The clone 18 cDNA encoded a precursor protein containing an ORF of 422 amino acids. The methionine starting the ORF directly preceded a putative signal peptide of 37 amino acids. The first

nucleotide of the clone 3 cDNA aligned to nucleotide 188 of the clone 18 cDNA, demonstrating that clone 3 was truncated at the 5' end. A difference between the derived amino acid sequences from the clone 18 and clone 3 cDNAs was also observed near the C terminus, with the sequences diverging at amino acid 404 (amino acid 382 of the clone 3 ORF). This difference was shown to be the result of an additional 5 nucleotides in the clone 3 cDNA sequence at a position corresponding to nucleotide 1327 of the clone 18 cDNA sequence, causing a frame shift in the amino acid sequence (Figure 3.2b). This difference in cDNA sequence was found to be due to an inexact splicing between exons 7 and 8 of the hCLF-1 gene (Figure 3.2c and section 3.2).

In vitro transcription/translation was performed on the clone 18 and clone 3 cDNA in order to demonstrate that the clone 18 cDNA was in fact the full length hCLF-1 cDNA, and that the methionine situated at the start of the putative signal peptide could indeed initiate translation. The clone 3 cDNA represented a negative control as it did not encode the putative initiating methionine. The predominantly translated product of the clone 18 cDNA migrated as a band approximating to 45 kD, a size close to that predicted from the amino acid sequence of the ORF (46.3 kD). A second band was detected approximating to 30 kD, which also corresponded to the predominantly translated product of the clone 3 cDNA (Figure 3.3). This was most probably the result of translation initiating from a methionine situated 147 amino acids downstream of the putative initiating methionine in the ORF. These findings suggested that the clone 18 cDNA was indeed the full length hCLF-1 cDNA.

Sequence analysis of the human clone 18 and murine cDNAs showed 85% nucleic acid identity and 96% amino acid identity. The human sequence showed 88% nucleic acid identity and 99% amino acid identity with the only identified rat EST encoding CLF-1 (Table 3.1), whilst the mouse and rat sequences showed 96% and 100% nucleic acid and amino acid identity respectively (Figure 3.4). Interestingly, the known amino acid sequence for rCLF-1 lies entirely within the cytokine receptor-like domain. Both mouse and human CLF-1 contained 11 cysteine residues and 6 potential N-linked glycosylation sites. The N-terminal region of both sequences appeared to represent an Ig-like domain, most closely resembling the C2-set sequence (Williams

and Barclay, 1988). This domain was followed by two fibronectin type III modules of approximately 100 amino acid residues each. Alignment of the human and mouse amino acid sequences with gp130 and PRLR showed regions of conserved homology within these two functionally important modules (known as the cytokine receptor-like domain), most notably at the highly conserved four cysteine residues and the W-S-x-W-S motif characteristic of this domain (Bazan, 1990c). We were unable to identify any transmembrane domains within the amino acid sequence of the mature proteins or hydrophobic region at the C terminus of the sequence, characteristic of glycosyl-phosphatidylinositol (GPI) anchored proteins such as the CNTFRα (Englund, 1993; Davis *et al.*, 1991). This suggests that the cloned human and mouse cDNAs encode soluble proteins.

Table 3.1. *ESTs corresponding to CLF-1 in GenBank*

Species	*EST Accession Number*	*Source of Origin*
Mouse	AA014965	Placenta
	AA039053	Embryo
	AA049278	Embryo
	AA049280	Embryo
	AA2700365	Embryo
	W17583	Embryo
	W66776	Embryo
Rat	AA866388	Adult Organ Mixture
Human	AA042914	Pregnant Uterus
	AA043001	Pregnant Uterus
	AA121532	Pregnant Uterus
	AA127694	Pregnant Uterus
	AA377893	Synovial Sarcoma
	AA406406	Melanocyte/Fetal Heart/Uterus
	H14009	Chromosome 19
	N78873	Fetal Lung
	R87407	Brain
	W37175	Fetal Lung
	W46603	Fibroblast
	W46604	Fibroblast

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CACAGCTGTAATCAGCCCCCAGGACCCCACCCTTCTCATCGGCTCCTCCCTGCAAGCTAC     60
 T  A  V  I  S  P  Q  D  P  T  L  L  I  G  S  S  L  Q  A  T
CTGCTCTATACATGGAGACACACCTGGGGCCACCGCTGAGGGGCTCTACTGGACCCTCAA    120
 C  S  I  H  G  D  T  P  G  A  T  A  E  G  L  Y  W  T  L  N
TGGTCGCCGCCTGCCCTCTGAGCTGTCCCGCCTCCTTAACACCTCCACCCTGGCCCTGGC    180
 G  R  R  L  P  S  E  L  S  R  L  L  N  T  S  T  L  A  L  A
CCTGGCTAACCTTAATGGGTCCAGGCAGCAGTCAGGAGACAATCTGGTGTGTCACGCCCG    240
 L  A  N  L  N  G  S  R  Q  Q  S  G  D  N  L  V  C  H  A  R
AGACGGCAGCATTCTGGCTGGCTCCTGCCTCTATGTTGGCTTGCCCCCTGAGAAGCCCTT    300
 D  G  S  I  L  A  G  S  C  L  Y  V  G  L  P  P  E  K  P  F
TAACATCAGCTGCTGGTCCCGGAACATGAAGGATCTCACGTGCCGCTGGACACCGGGTGC    360
 N  I  S  C  W  S  R  N  M  K  D  L  T  C  R  W  T  P  G  A
ACACGGGGAGACATTCTTACATACCAACTACTCCCTCAAGTACAAGCTGAGGTGGTACGG    420
 H  G  E  T  F  L  H  T  N  Y  S  L  K  Y  K  L  R  W  Y  G
TCAGGATAACACATGTGAGGAGTACCACACTGTGGGCCCTCACTCATGCCATATCCCCAA    480
 Q  D  N  T  C  E  E  Y  H  T  V  G  P  H  S  C  H  I  P  K
GGACCTGGCCCTCTTCACTCCCTATGAGATCTGGGTGGAAGCCACCAATCGCCTAGGCTC    540
 D  L  A  L  F  T  P  Y  E  I  W  V  E  A  T  N  R  L  G  S
AGCAAGATCTGATGTCCTCACACTGGATGTCCTGGACGTGGTGACCACGGACCCCCCACC    600
 A  R  S  D  V  L  T  L  D  V  L  D  V  V  T  T  D  P  P  P
CGACGTGCACGTGAGCCGCGTTGGGGGCCTGGAGGACCAGCTGAGTGTGCGCTGGGTCTC    660
 D  V  H  V  S  R  V  G  G  L  E  D  Q  L  S  V  R  W  V  S
ACCACCAGCTCTCAAGGATTTCCTCTTCCAAGCCAAGTACCAGATCCGCTACCGCGTGGA    720
 P  P  A  L  K  D  F  L  F  Q  A  K  Y  Q  I  R  Y  R  V  E
GGACAGCGTGGACTGGAAGGTGGTGGATGACGTCAGCAACCAGACCTCCTGCCGTCTCGC    780
 D  S  V  D  W  K  V  V  D  D  V  S  N  Q  T  S  C  R  L  A
GGGCCTGAAGCCCGGCACCGTTTACTTCGTCCAAGTGCGTTGTAACCCATTCGGGATCTA    840
 G  L  K  P  G  T  V  Y  F  V  Q  V  R  C  N  P  F  G  I  Y
TGGGTCGAAAAAGGCGGGAATCTGGAGCGAGTGGAGCCACCCCACCGCTGCCTCCACCCC    900
 G  S  K  K  A  G  I  W  S  E  W  S  H  P  T  A  A  S  T  P
TCGAAGTGAGCGCCCGGGCCCGGGCGGCGGGGTGTGCGAGCCGCGGGGCGGCGAGCCCAG    960
 R  S  E  R  P  G  P  G  G  G  V  C  E  P  R  G  G  E  P  S
CTCGGGCCCGGTGCGGCGCGAGCTCAAGCAGTTCCTCGGCTGGCTCAAGAAGCACGCATA   1020
 S  G  P  V  R  R  E  L  K  Q  F  L  G  W  L  K  K  H  A  Y
CTGCTCGAACCTTAGTTTCCGCCTGTACGACCAGTGGCGTGCTTGGATGCAGAAGTCACA   1080
 C  S  N  L  S  F  R  L  Y  D  Q  W  R  A  W  M  Q  K  S  H
CAAGACCCGAAACCAGGACGAGGGGATCCTGCCCTCGGGCAGACGGGGTGCGGCGAGAGG   1140
 K  T  R  N  Q  D  E  G  I  L  P  S  G  R  R  G  A  A  R  G
TCCTGCCGGCTAAACTCTAAGGATAGGCCATCCTCCTGCTGGGTCAGACCTGGAGGCTCA   1200
 P  A  G  *
CCTGAATTGGAGCCCCTCTGTACCATCTGGGCAACAAAGAAACCTACCAGAGGCTGGGGC   1260
ACAATGAGCTCCCACAACCACAGCTTTGGTCCACATGATGGTCACACTTGGATATACCCC   1320
AGTGTGGGTAGGGTTGGGGTATTGCAGGGCCTCCCAAGAGTCTCTTTAAATAAATAAAGG   1380
AGTTGTTCAGTCCCGAG                                              1397
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Figure 3.1 mCLF-1 cDNA and Predicted Amino Acid Sequence.

The 5' end of the cDNA is incomplete as no putative methionine start codon or signal peptide sequence has been identified. Amino acid residues in red represent the putative Ig-like domain whilst those in green represent the cytokine receptor-like domain. The blue residues are those found C-terminal of the cytokine receptor-like domain. Underlined residues indicate potential N-linked glycosylation sites. Conserved motifs of the cytokine type-I receptor family are shown as black residues.

```
CGCCCAGCGACGTGCGGGCGGCCTGGCCCGCGCCCTCCCGCGCCCGGCCTGCGTCCCGCG     60
CCCTGCGCCACCGCCGCCGAGCCGCAGCCCGCCGCGCGCCCCCGGCAGCGCCGGCCCCAT    120
                                                            M
GCCCGCCGGCCGCCGGGGCCCCGCCGCCCAATCCGCGCGGCGGCCGCCGCCGTTGCTGCC    180
 P  A  G  R  R  G  P  A  A  Q  S  A  R  R  P  P  P  L  L  P
CCTGCTGCTGCTGCTCTGCGTCCTCGGGGCGCCGCGAGCCGGATCAGGAGCCCACACAGC    240
 L  L  L  L  L  C  V  L  G  A  P  R  A  G  S  G  A  H  T  A
TGTGATCAGTCCCCAGGATCCCACGCTTCTCATCGGCTCCTCCCTGCTGGCCACCTGCTC    300
 V  I  S  P  Q  D  P  T  L  L  I  G  S  S  L  L  A  T  C  S
AGTGCACGGAGACCCACCAGGAGCCACCGCCGAGGGCCTCTACTGGACCCTCAACGGGCG    360
 V  H  G  D  P  P  G  A  T  A  E  G  L  Y  W  T  L  N  G  R
CCGCCTGCCCCCTGAGCTCTCCCGTGTACTCAACGCCTCCACCTTGGCTCTGGCCCTGGC    420
 R  L  P  P  E  L  S  R  V  L  N  A  S  T  L  A  L  A  L  A
CAACCTCAATGGGTCCAGGCAGCGGTCGGGGGACAACCTCGTGTGCCACGCCCGTGACGG    480
 N  L  N  G  S  R  Q  R  S  G  D  N  L  V  C  H  A  R  D  G
CAGCATCCTGGCTGGCTCCTGCCTCTATGTTGGCCTGCCCCCAGAGAAACCCGTCAACAT    540
 S  I  L  A  G  S  C  L  Y  V  G  L  P  P  E  K  P  V  N  I
CAGCTGCTGGTCCAAGAACATGAAGGACTTGACCTGCCGCTGGACGCCAGGGGCCCACGG    600
 S  C  W  S  K  N  M  K  D  L  T  C  R  W  T  P  G  A  H  G
GGAGACCTTCCTCCACACCAACTACTCCCTCAAGTACAAGCTTAGGTGGTATGGCCAGGA    660
 E  T  F  L  H  T  N  Y  S  L  K  Y  K  L  R  W  Y  G  Q  D
CAACACATGTGAGGAGTACCACACAGTGGGGCCCCACTCCTGCCACATCCCCAAGGACCT    720
 N  T  C  E  E  Y  H  T  V  G  P  H  S  C  H  I  P  K  D  L
GGCTCTCTTTACGCCCTATGAGATCTGGGTGGAGGCCACCAACCGCCTGGGCTCTGCCCG    780
 A  L  F  T  P  Y  E  I  W  V  E  A  T  N  R  L  G  S  A  R
CTCCGATGTACTCACGCTGGATATCCTGGATGTGGTGACCACGGACCCCCCGCCCGACGT    840
 S  D  V  L  T  L  D  I  L  D  V  V  T  T  D  P  P  P  D  V
GCACGTGAGCCGCGTCGGGGGCCTGGAGGACCAGCTGAGCGTGCGCTGGGTGTCGCCACC    900
 H  V  S  R  V  G  G  L  E  D  Q  L  S  V  R  W  V  S  P  P
CGCCCTCAAGGATTTCCTCTTTCAAGCCAAATACCAGATCCGCTACCGAGTGGAGGACAG    960
 A  L  K  D  F  L  F  Q  A  K  Y  Q  I  R  Y  R  V  E  D  S
TGTGGACTGGAAGGTGGTGGACGATGTGAGCAACCAGACCTCCTGCCGCCTGGCCGGCCT   1020
 V  D  W  K  V  V  D  D  V  S  N  Q  T  S  C  R  L  A  G  L
GAAACCCGGCACCGTGTACTTCGTGCAAGTGCGCTGCAACCCCTTTGGCATCTATGGCTC   1080
 K  P  G  T  V  Y  F  V  Q  V  R  C  N  P  F  G  I  Y  G  S
CAAGAAAGCCGGGATCTGGAGTGAGTGGAGCCACCCCACAGCCGCCTCCACTCCCCGCAG   1140
 K  K  A  G  I  W  S  E  W  S  H  P  T  A  A  S  T  P  R  S
TGAGCGCCCGGGCCCGGGCGGCGGGGCGTGCGAACCGCGGGGCGGAGAGCCGAGCTCGGG   1200
 E  R  P  G  P  G  G  G  A  C  E  P  R  G  G  E  P  S  S  G
GCCGGTGCGGCGCGAGCTCAAGCAGTTCCTGGGCTGGCTCAAGAAGCACGCGTACTGCTC   1260
 P  V  R  R  E  L  K  Q  F  L  G  W  L  K  K  H  A  Y  C  S
CAACCTCAGCTTCCGCCTCTACGACCAGTGGCGAGCCTGGATGCAGAAGTCGCACAAGAC   1320
 N  L  S  F  R  L  Y  D  Q  W  R  A  W  M  Q  K  S  H  K  T
CCGCAACCAGGACGAGGGGATCCTGCCCTCGGGCAGACGGGGCACGGCGAGAGGTCCTGC   1380
 R  N  Q  D  E  G  I  L  P  S  G  R  R  G  T  A  R  G  P  A
CAGATAAGCTGTAGGGGCTCAGGCCACCCTCCCTGCCACGTGGAGACGCAGAGGCCGAAC   1440
 R  *
CCAAACTGGGGCCACCTCTGTACCCTCACTTCAGGGCACCTGAGCCACCCTCAGCAGGAG   1520
CTGGGGTGGCCCCTGAGCTCCAACGGCCATAACAGCTCTGACTCCCACGTGAGGCCACCT   1580
TTGGGTGCACCCCAGTGGGTGTGTGTGTGTGTGAGGGTTGGTTGAGTTGCCTAGAACC    1640
CCTGCCAGGGCTGGGGGTGAGAAGGGGAGTCATTACTCCCCATTACCTAGGGCCCCTCCA   1700
AAAGAGTCCTTTTAAATAAATGAGCTATTTAGGTGC
```

Figure 3.2a hCLF-1 cDNA and Predicted Amino Acid Sequence.

The black, underlined amino acid residues represent the putative signal peptide. Those in red represent the putative Ig-like domain whilst those in green represent the cytokine receptor-like domain. The blue residues are those found C-terminal of the cytokine receptor-like domain. Underlined residues indicate potential N-linked glycosylation sites. Conserved motifs of the cytokine type-I receptor family are shown as black residues.

B

```
AGCTTCCGCCTCTACGACCAGTGGCGAGCCTGGATGCAGAAGTCGCACAAGACCCGCAAC
S  F  R  L  Y  D  Q  W  R  A  W  M  Q  K  S  H  K  T  R  N

CAGGACGAGGGGATCCTGCCCTCGGGCAGACGGGGCACGGCGAGAGGTCCTGCCAGATAA
Q  D  E  G  I  L  P  S  G  R  R  G  T  A  R  G  P  A  R  *



AGCTTCCGCCTCTACGACCAGTGGCGAGCCTGGATGCAGAAGTCGCACAAGACCCGCAAC
S  F  R  L  Y  D  Q  W  R  A  W  M  Q  K  S  H  K  T  R  N

CAGCACAGGACGAGGGGATCCTGCCCTCGGGCAGACGGGGCACGGCGAGAGGTCCTGCCA
Q  H  R  T  R  G  S  C  P  R  A  D  G  A  R  R  E  V  L  P

GATAAGCTGTAG
D  K  L  *
```

C

	Splice Donor	Splice Acceptor
Genomic DNA	CCAGGTAG	.....CCAAGCACAGGACG
cDNA	CCAGGACG	
Amino Acid	Q D E	

	Splice Donor	Splice Acceptor
Genomic DNA	CCAGGTAG	.....CCAAGCACAGGACG
cDNA	CCAGCACAGGACG	
Amino Acid	Q H R T	

Figure 3.2b,c. Alternative Sequences of hCLF-1 Encoding cDNAs.

(B) *Upper Part.* Sequence of the 3' end of hCLF-1 cDNA clone 18. Amino acid residues in red represent those specific for clone 18.
Lower Part. Sequence of the 3' end of the hCLF-1 cDNA clone 3. Amino acid residues in blue represent those specific for clone 3. The nucleic acids in bold type are those specific for clone 3 and are not present in clone 18.
(C) *Upper Part.* The splice junction generating clone 18 specific sequence.
Lower Part. The splice junction generating clone 3 specific cDNA. Additional bases in the clone 3 sequence not present in the clone 18 sequence are underlined.
Nucleic acids in red are exon and cDNA specific whilst those in blue are intron specific.

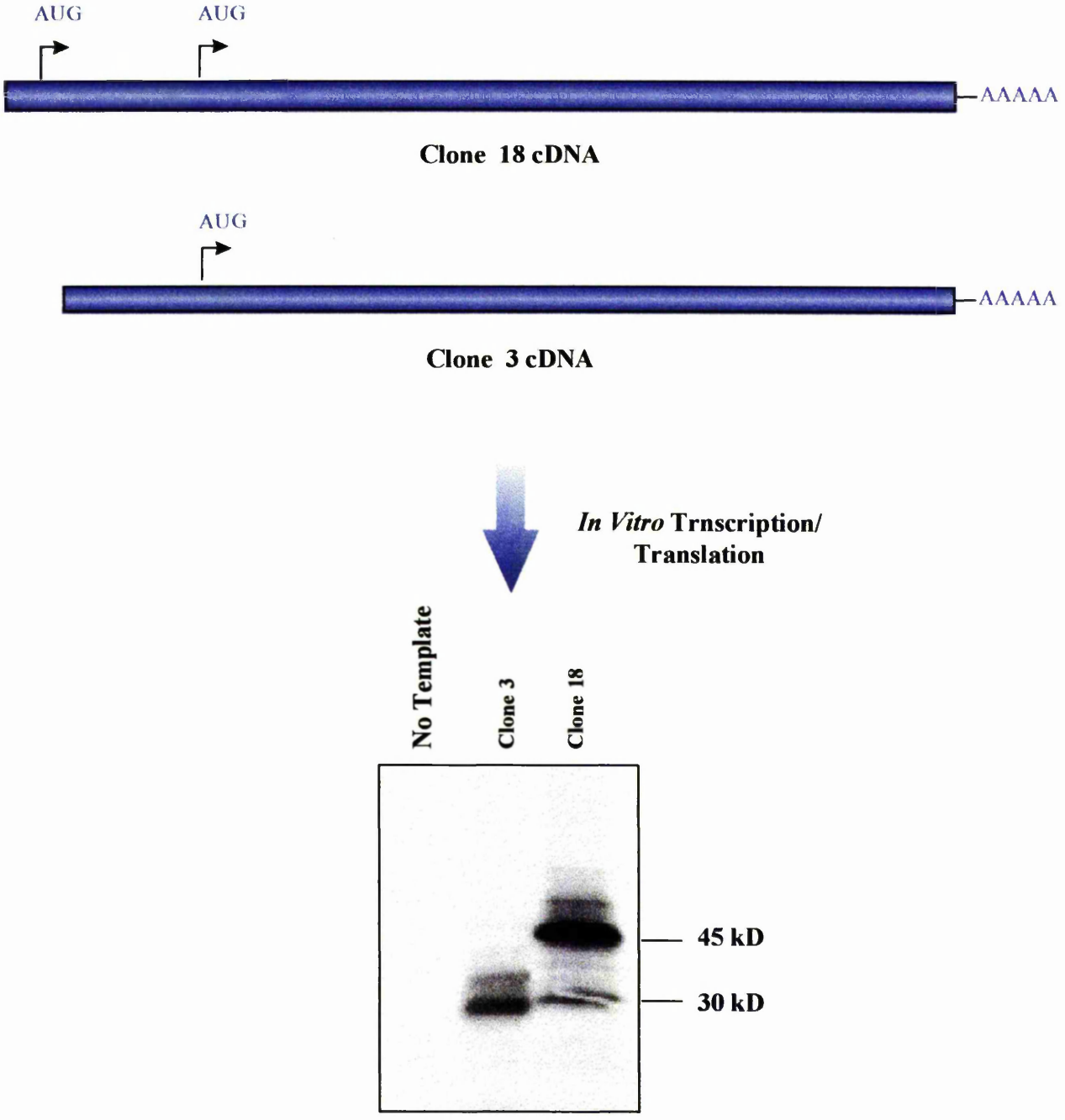


Figure 3.3. *In Vitro* Transcription/Translation of hCLF-1 cDNA.

Plasmids containing clone 18 and clone 3 cDNA (solid blue bars) were linearized and subjected to one step *in vitro* transcription and translation. As a control, a reaction was performed without addition of a cDNA template. ^{32}P-labeled protein products were detected by SDS-PAGE followed by auto-radiography.

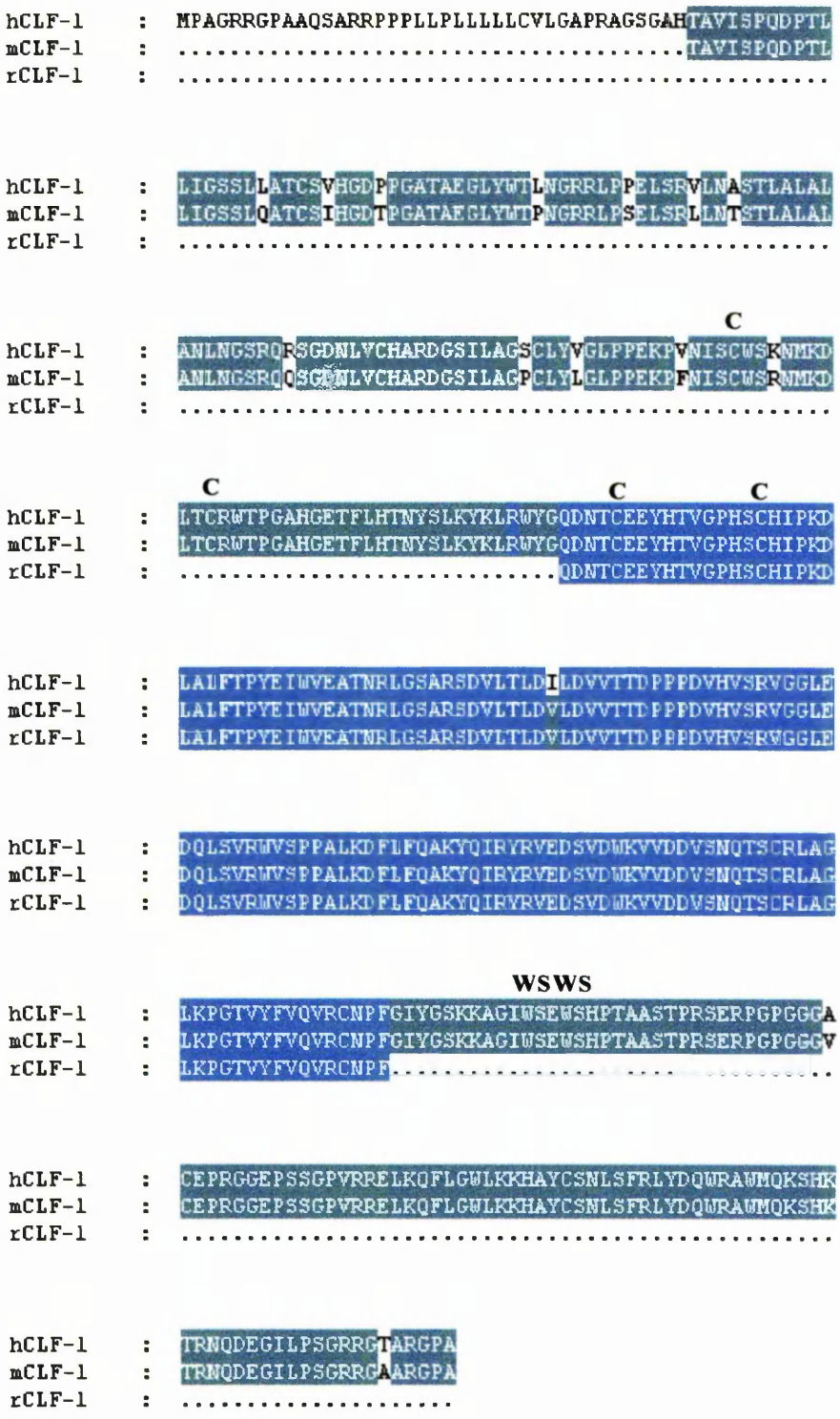


Figure 3.4. Amino Acid Sequence Alignment of CLF-1 from Human, Mouse and Rat.

Residues sharing identity between all three sequences are boxed in blue. Residues sharing identity between the human and mouse sequences outside of the region where the partial rCLF-1 sequence aligns are shown in green, as are residues sharing sequence identity between two of the three sequences within the region to which the partial rCLF-1 sequence aligns. Regions where no alignment is obtained due to incomplete sequence information, such as the that for the N terminal mCLF-1 sequence, are dotted. The four cysteine and W-S-x-W-S motifs are indicated above the sequence.

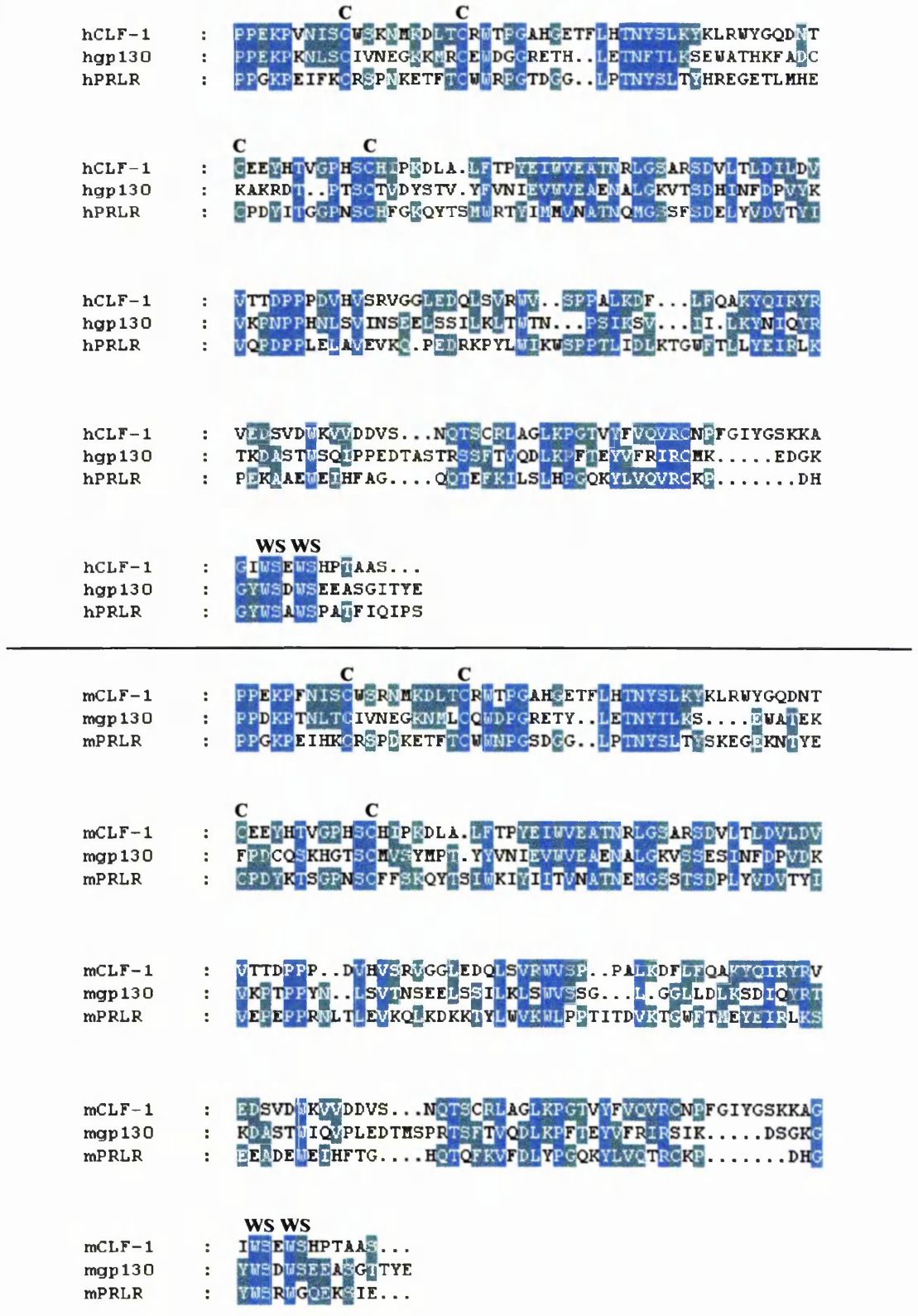


Figure 3.5. Alignment of the Cytokine Receptor-Like Domains of CLF-1, gp130 and PRLR

Amino acid residues showing similarity throughout the three sequences are boxed in blue. Residues showing conserved similarity between two of the three sequences are boxed in green. The four cysteine and W-S-x-W-S motifs are indicated above the alignment. (A) Human sequences; (B)Mouse sequences.

(3.2) The hCLF-1 Gene is Localized on Chromosome 19 and Structurally Related to Other Type-I Receptor Genes

The hCLF-1 gene sequence, under the accession number AC003112, was identified in the GenBank database using the hCLF-1 cDNA as the search sequence. The gene was found to be within a 14 kb region of the chromosome 19 specific cosmid R30292 mapping to chromosome 19p12. This confirmed the location predicted by the EST with accession number H14009, that had been generated by a PCR based exon amplification technique (Buckler *et al.*, 1991) using DNA from chromosome 19p12-19p13.1). Three other members of the cytokine type-I receptor family, the erythropoietin receptor (Winkelmann *et al.*, 1990), sharing homology with CLF-1, the Epstein-Barr virus induced factor, EBI-3 (Devergne *et al.*, 1996) and IL-12Rβ1 (Yamamoto *et al.*, 1997) are known to be localized on this part of chromosome 19.

The intron/exon organization of the gene for hCLF-1 was in agreement with the predicted structure of the domains within the hCLF-1 protein, the gene being constructed of 9 exons, arranged in a similar fashion to other type-I receptor genes Figure 3.6a). The first exon encoded the putative signal peptide, exon 2 encoded the Ig like domain, exons 3 and 4 encoded the N-terminal cytokine receptor-like domain with one of the two pairs of cysteine residues found in each exon. Exons 5 and 6 encoded the C-terminal cytokine receptor-like domain, with exon 6 containing the W-S-x-W-S motif. Exons 7 and 8 showed no homology to any other type I receptor family member whilst exon 9 encoded the stop codon close to its 5' end and the polyadenylation signal close to its 3' end. Each intron had typical splice donor and acceptor motifs (Breathnach and Chambon, 1981), beginning with a GT and ending with an AG. The intron phases (Sharp, 1981) were also in agreement with those found for other type-I receptor genes. These findings demonstrated that the gene for hCLF-1 followed the rules proposed for the cytokine receptor family (Nakagawa *et al.*, 1994; Figure 1.7). We were unable to identify anything resembling an exon encoding a putative transmembrane domain within the 17.4 Kb of DNA sequence contained within the contig downstream of the exon containing the W-S-x-W-S motif (exon 6), which is often the last extracellular region encoding exon within the type-I receptor family.

The 5 kb of genomic DNA situated upstream of the methionine start codon was analyzed for sequences typifying eukaryotic promoters using the TFSearch server, which identified potential transcription factor binding sites with a 85 % minimum homology to the consensus. An 8 bp consensus for cap signal for transcription initiation (TCAGACTT) was identified some 20 bp upstream of the DNA representing the start of the clone 18 hCLF-1 cDNA. This suggested that the DNA representing the first bp of the clone 18 cDNA could be a point of transcription initiation. Although no TATA box was identified, 40-100 bp upstream of the cap signal, a GC box containing a number of putative Sp-1 (stimulating protein-1) binding sites were found, along with a putative MZF-1 (myeloid zinc finger protein) binding site. A further 70-180 bp upstream, two putative GATA binding factor sites were found. Within the 4.8 kb upstream of this region, many other potential binding sites were identified, most notably several other MZF-1 and GATA family binding sites as well as C/EBP family, STAT family, NF-kB, AP-1 and upstream stimulating factor (USF) binding sites (Figure 3.6b).

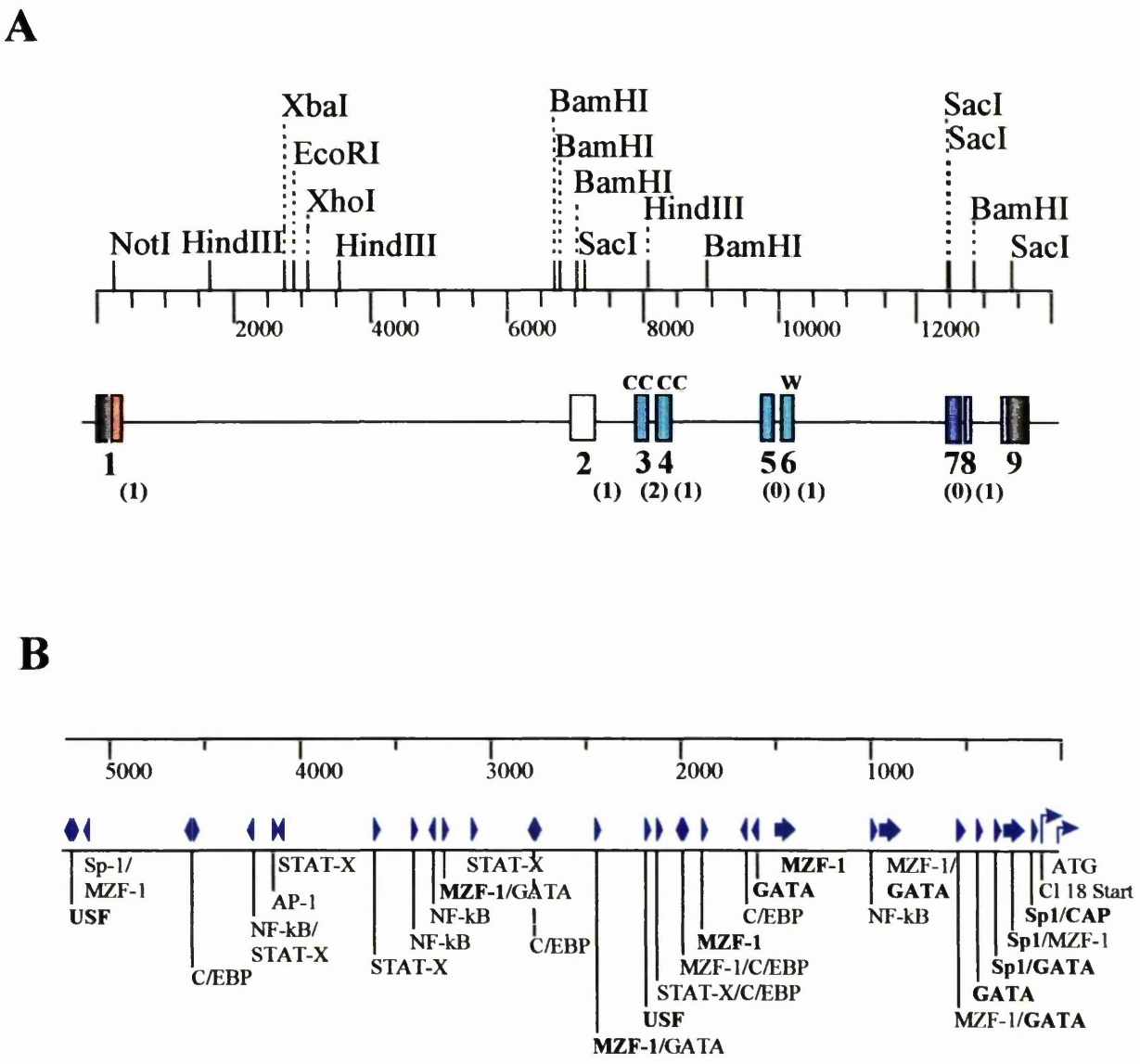


Figure 3.6. Structure of the hCLF-1 Gene and its Promotor.

(A) Exon organization of the hCLF-1 gene. Each box represents an individual exon. Red represents the putative signal peptide, white the Ig-like domain, green the cytokine receptor-like domain and blue the C-terminal region. Black represents the 5' and 3' UTRs. The exons are numbered below. The numbers in brackets represent the intron phases. A partial restriction map and the distance in bp is shown above.

(B) Putative transcription factor binding sites in the hCLF-1 gene promotor. The blue arrows represent the localization and orientation of the putative binding sites. Transcription factors in bold are those whose putative binding site in the hCLF-1 promotor has 95% or more sequence homology with the consensus. Those in normal type have between 85 and 95 % sequence homology with the consensus. The line above shows distance in bp from the translation initiating ATG codon.

(3.3) The hCLF-1 Gene is Highly Conserved

The very high nucleic acid and amino acid homology between the human, mouse and rat protein sequences for CLF-1 suggested that the gene had been highly conserved during recent evolution. As the cDNA encoding hCLF-1 shared 85% and 88% homology with mouse and rat CLF-1, respectively, we estimated that Southern blot analysis using the hCLF-1 cDNA as a probe would allow the detection of the CLF-1 gene within the genomic DNA of a number of different animal species. We examined the conservation of the gene between human, monkey, mouse, rat, rabbit, cow and chicken (Figure 3.7). The analysis was performed with stringent hybridization and washing conditions, thus reducing the possibility of obtaining signals resulting from non-specific probe hybridization. As expected, human and monkey genomic DNA gave the strongest signals. Distinct bands were also obtained with rat and rabbit DNA, with feint but detectable bands also seen with cow and chicken genomic DNA. These results suggest that the CLF-1 gene is indeed highly conserved between the animal species tested.

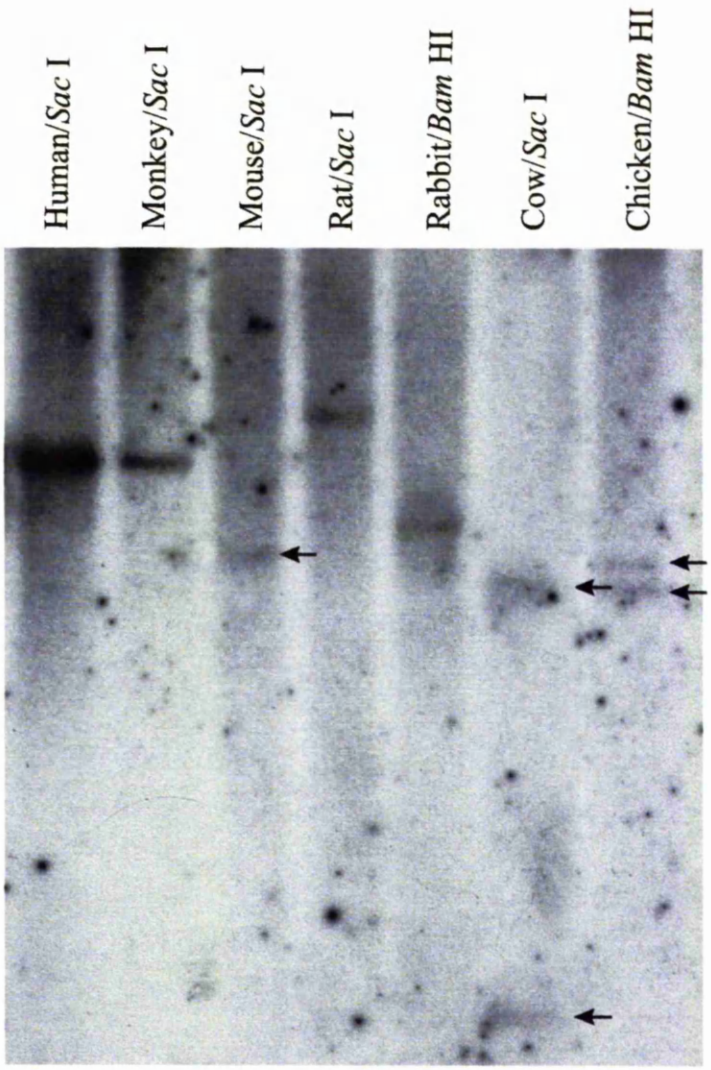


Figure 3.7. CLF-1 Interspecies Genomic Southern Blot.

5 μg of genomic DNA was digested with either *Bam* HI or *Sac* I as indicated, the restriction fragments resolved on an 0.8% agarose gel, transferred to a nylon filter and hybridized with a radiolabeled hCLF-1 cDNA probe. Autoradiography exposure time was 24 hrs at -70°C. Feint bands are indicated with an arrow.

(3.4) Tissue and Cellular Distribution of mCLF-1 mRNA Expression

The level of hCLF-1 mRNA expression was examined with two Northern blots (Clonetech) using a ^{32}P labeled cRNA probe (Figure 3.8a (i and iii)). The first blot contained poly A^+ RNA derived from adult mouse testis, kidney, skeletal muscle, liver, lung, spleen, heart and brain and the second contained poly A^+ RNA derived from fetal tissue at 9, 11, 15 and 17 days post-coitum (d.p.c.). Amongst the adult tissues examined, expression was detected most predominantly in lung, with detectable levels of expression in skeletal muscle, heart and brain. In the fetus, no expression was detected at 9 d.p.c. with a constant level of expression clearly detected from 11 through to 17 d.p.c. Fetal expression of mCLF-1 was confirmed by the source of origin of mouse ESTs (Table 3.1), with 7 of the 8 ESTs being derived from embryonic tissue.

We also examined mCLF-1 mRNA expression by Northern blot analysis in the lymphoid tissues of naive mice, and mice immunized with KLH-alum (Figure 3.8a (ii)). There was a detectable level of expression in lymph nodes before immunization with a modest up-regulation of expression in the lymph node afterward. The thymus also expressed mCLF-1 mRNA with no difference in steady state levels versus immunized mice. Expression in bone marrow and both spleen samples was at the limit of sensitivity for the Northern blot.

RT-PCR was used to examine mCLF-1 mRNA levels in primary immunocompetent cells and cell lines of murine origin (Figure 3.8b). As a positive control for mCLF-1 expression, mouse lung RNA was included in the RT-PCR. Expression was seen in the B cell lymphoma line A20 with no change in levels upon LPS stimulation, and in peripheral blood T cells with again no change upon stimulation by cross-linking CD3 and CD28 with mAbs. Freshly isolated BAL macrophages and eosinophils also expressed a detectable level of mCLF-1 mRNA, as well as six microvascular endothelial cell lines (RNA kindly provided by Dr M. Pepper, University Medical Center, Geneva, CH).

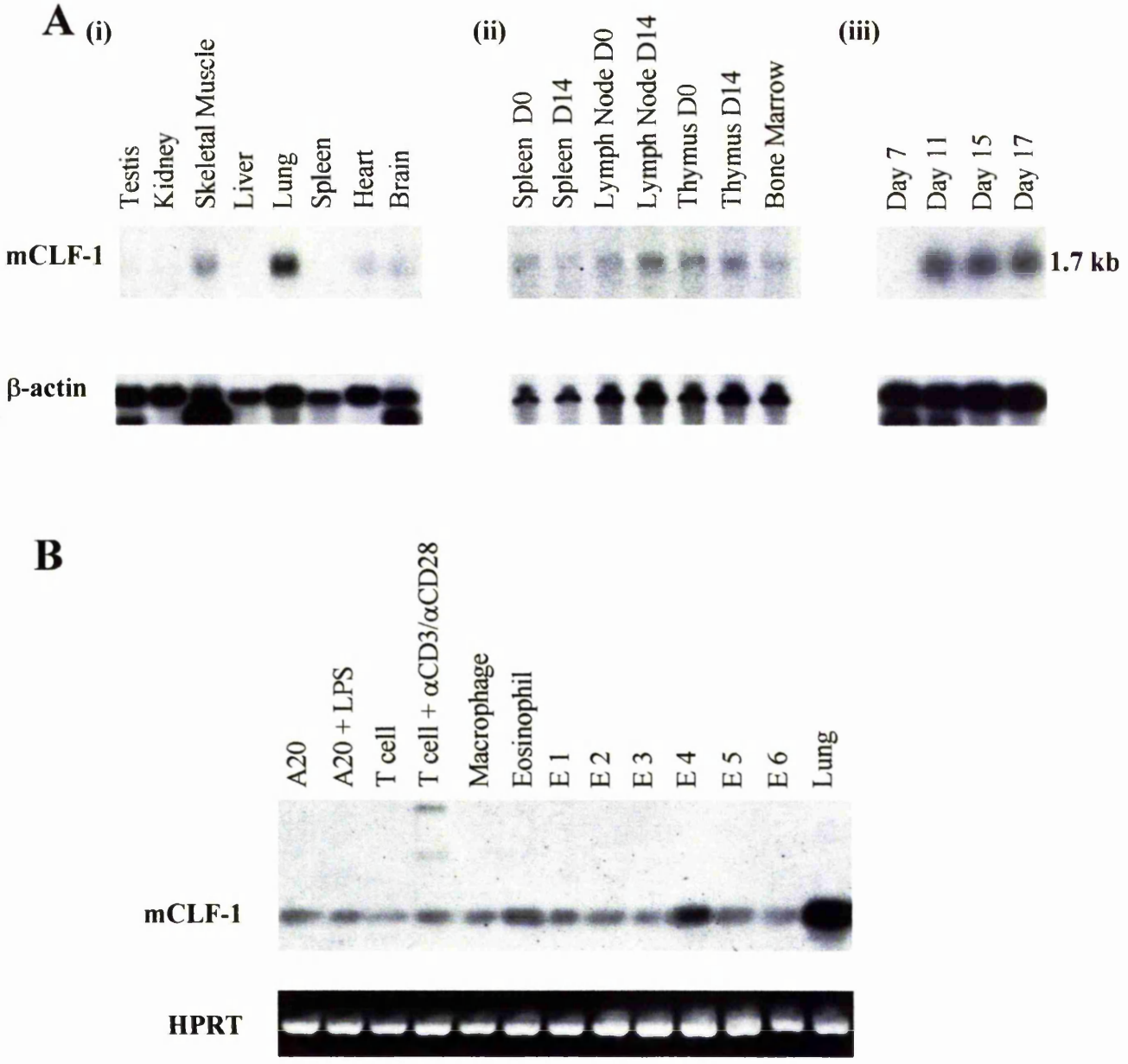


Figure 3.8. Distribution of Expression of mCLF-1 mRNA

(A) Detection of mCLF-1 mRNA transcripts by Northern blot analysis using a cRNA probe derived from mCLF-1 cDNA. (i) Murine adult tissues, (ii) murine adult immune tissues before (D0) and after (D14) immunization and (iii) murine embryonic tissue at 7, 11, 15 and 17 d.p.c. Autoradiography exposure times at -70°C: (i) and (iii) 24 hrs; (ii) 72 hrs.

(B) Detection of mCLF-1 mRNA transcripts in murine cells and cell lines by RT-PCR and Southern blot analysis using an mCLF-1 specific oligonucleotide probe. E1 to E6 represents six microvascular endothelial cell lines (see text).

(3.5) Tissue and Cellular Distribution of hCLF-1 mRNA Expression

hCLF-1 mRNA levels were examined in different human tissues of both fetal and adult origin using poly A^+ Northern blots (Clonetech) with a ^{32}P-labeled hCLF-1 cDNA probe (Figure 3.9a). Amongst the tissues tested, highest levels of expression were seen in a variety of immune tissues, including lymph node, spleen, thymus, appendix and bone marrow, as well as stomach, placenta, heart, skeletal muscle, ovary and, interestingly, fetal lung. A detectable level of expression of hCLF-1 mRNA was found in prostate, testis, small intestine, PBL, thyroid and adrenal cortex. The source of origin of human ESTs (Table 3.1) confirmed the expression seen in fetal lung, with two of the ESTs being derived from that tissue. We also observed that hCLF-1 mRNA was expressed in pregnant female uterus, with 4 ESTs being derived from that tissue. These results point to a possible role for hCLF-1 in immune regulation and fetal development.

Northern blotting was again used to study the expression of hCLF-1 mRNA in a variety of primary immunocompetent cells and cell lines of human origin (Figure 3.9b). Of the cells tested, strongest expression was seen in the human fetal kidney fibroblastic cell line, HEK 293. Detectable levels of expression were also observed, however, in the immature mast cell line, HMC-1, the lymphoblastoid cell line JY (with or without stimulation with IL-4), the monocyte cell line, THP-1, following PMA stimulation and HBE-140 bronchial epithelial cells following stimulation with TNF-α and IL-1β.

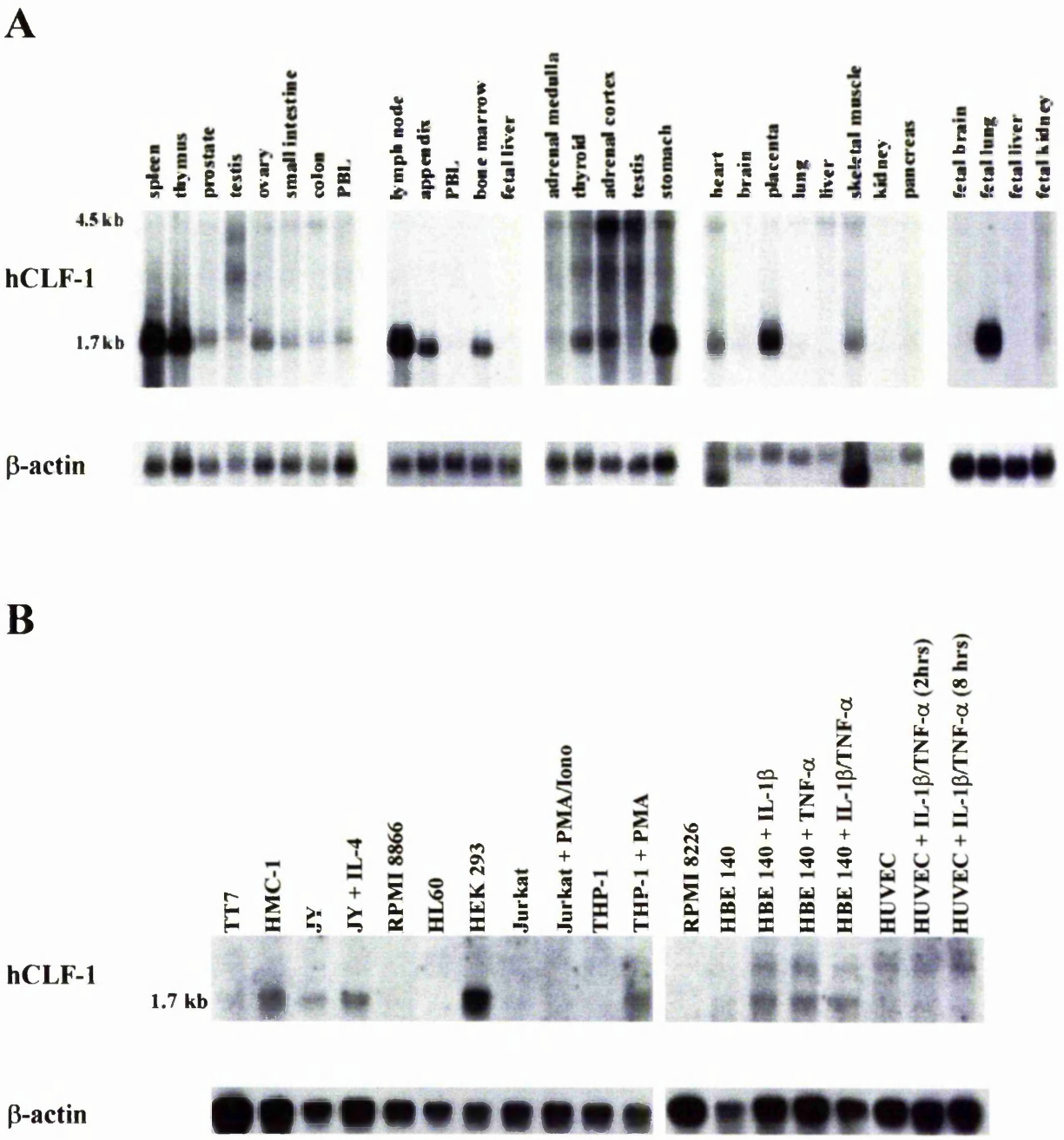


Figure 3.9. Distribution of Expression of hCLF-1 mRNA

hCLF-1 mRNA transcripts were detected by Northern blot analysis using a ^{32}P-labeled hCLF-1 cDNA probe.

(A) Detection of hCLF-1 mRNA expression in adult and fetal human tissues. Autoradiography exposure time was 24 hrs at -70°C.

(B) Detection of hCLF-1 mRNA expression in human cells and cell lines with or without cytokine treatment as indicated. Autoradiography exposure time was 72 hrs at -70°C.

(3.6) Proinflammatory Cytokines Upregulate hCLF-1 mRNA Levels in Fibroblasts

RT-PCR was used to investigate the effect of the proinflammatory cytokines IL-1β, TNF-α, IL-6 and IFN-γ on the expression of hCLF-1 mRNA in HEK-293 cells and fibroblasts derived from tonsil, mammary gland and a palmar fibromatosis lesion. mRNA levels were examined by 32 cycles of PCR on cDNA derived from the fibroblasts after a 48 hour incubation without cytokines, with the cytokines added separately or with all four cytokines added together, as indicated (Figure 3.10).

Although the cytokines appeared to have no effect on the steady state mRNA level found in HEK 293 cells (Figure 3.10a), clear differences were observed when the cytokines were added to the primary fibroblast cell cultures. In the palmar lesion fibroblasts, hCLF-1 mRNA expression was clearly up-regulated with TNF-α and IFN-γ and the four cytokines together. IL-6 slightly up-regulated the mRNA expression, whilst IL-1β appeared to down-regulate the message (Figure 3.10b). In mammary gland fibroblasts, hCLF-1 mRNA was clearly up-regulated by TNF-α, IFN-γ and the four cytokines together. (Figure 3.10c). In the tonsillar-derived fibroblasts, IL-6, IFN-γ and the four cytokines together had an upregulatory effect on hCLF-1 mRNA production, with TNF-α having no effect. IL-1β was again seen to have a down-regulatory effect on mRNA expression (Figure 3.10d). The finding that hCLF-1 mRNA could be upregulated by the proinflammatory cytokines TNF-α, IL-6 and IFN-γ (alone or in combination) again suggests a role for the protein in immune regulation.

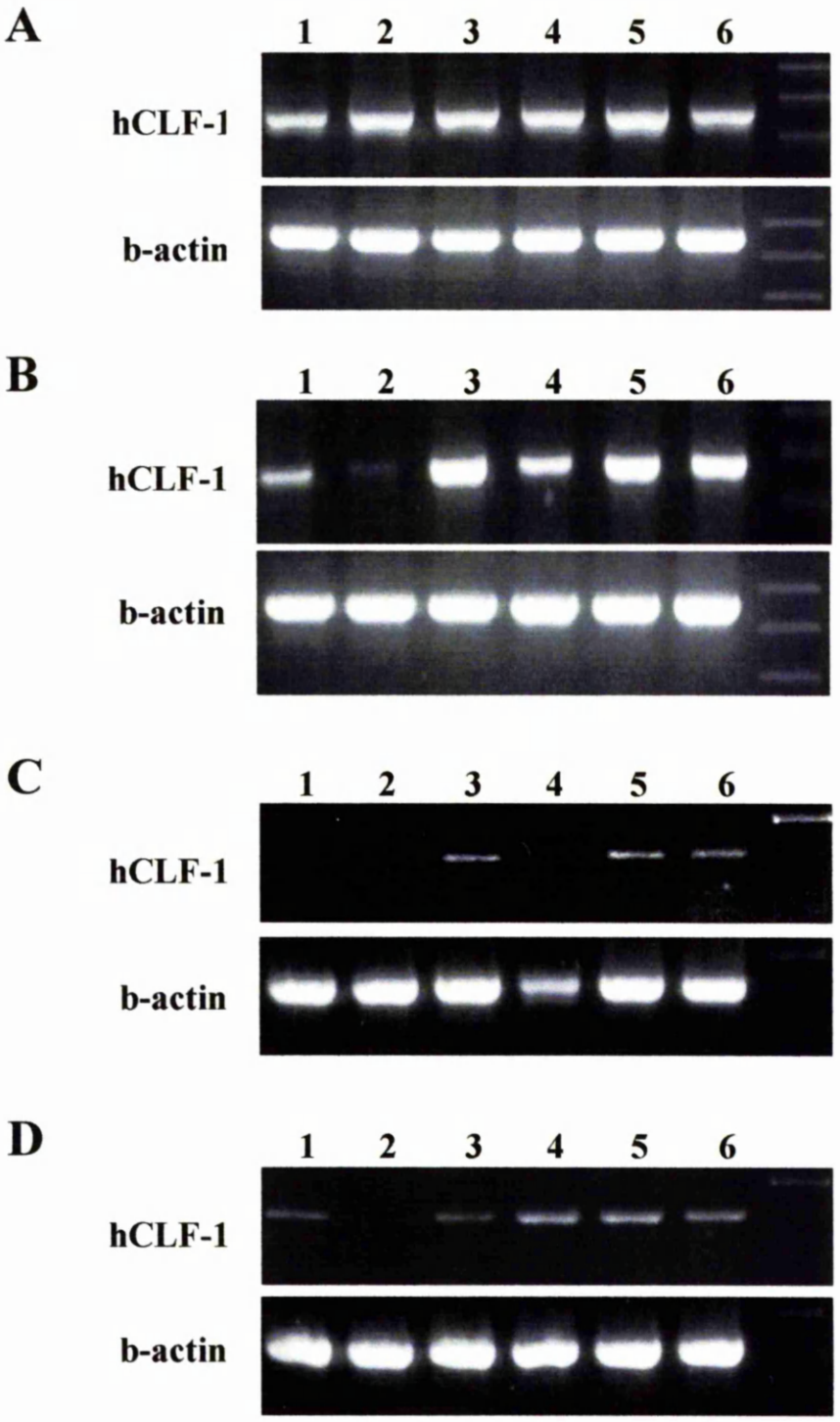


Figure 3.10. Expression of hCLF-1 mRNA in Human Fibroblast Cells

hCLF-1 transcripts were detected by RT-PCR using oligonucleotide primers specific for the hCLF-1 cDNA sequence. As a positive control for the reaction, PCR was performed with primers specific for the β-actin cDNA sequence. Cells were cultured for 48 hrs in the presence of (1) Medium alone, (2) IL-1β, (3) TNF-α, (4) IL-6, (5) IFN-γ, or (6) a mixture of all four cytokines. (A) HEK 293 cells, (B) Palmar fibromatosis lesion fibroblasts, (C) Mammary gland fibroblasts and (D) Tonsillar fibroblasts.

(3.7) The Mouse mAb $92A_{10}$ Recognizes Recombinant Forms of hCLF-1

In order to generate a mAb against hCLF-1, mice were immunized with a recombinant form of the protein (rshCLF-1) containing the 6 histidine and mAb 179 tags generated in Sf9 insect cells using the baculovirus expression system. Hybridomas resulting from the fusion between plasma cells derived from the lymph nodes of the immunized mouse and a mouse myeloma cell line were tested by ELISA. The supernatants were tested against both the rshCLF-1 and as a control, a recombinant soluble form of the hIL-13Rα1 (rshIL-13Rα1) that contained the same Ab recognition tags. This facilitated the selection of wells specifically recognizing only hCLF-1, i.e. not the tags or any structurally conserved motifs within the type-I receptor family.

Supernatants specifically recognizing rshCLF-1 were then tested for their recognition of cell surface bound hCLF-1 by flow cytometry. Sf9 cells infected with baculovirus encoding either an irrelevant cell surface protein (p2X2) or the hCLF-1/hIL-13Rα1 membrane bound fusion protein were used. Expression of the fusion protein on the cell surface was verified using the α-EE mAb. Using an IgG1 isotype control, cells infected with the hCLF-1 fusion protein had a mean fluorescence intensity (MFI) of 4.15, whereas the α-EE mAb bound to 64% of the cells with an MFI of 60 (Figure 3.11a). When using supernatants on the p2X2 or hCLF-1 expressing Sf9 cells, one single hybridoma clone, identified as $92A_{10}$, was found to express mAbs against hCLF-1. When incubated with the supernatant of $92A_{10}$, P2X2 expressing Sf9 cells showed an MFI of 8.32 whereas 62% of the hCLF-1 expressing Sf9 cells showed fluorescence with an MFI of 56 (Figure 3.11b). As both fluorescent profiles were comparable, it appeared that the mAb produced by hybridoma $92A_{10}$ specifically recognized hCLF-1. After subcloning, the hybridoma $92A_{10}$ was determined to have the IgG2a isotype.

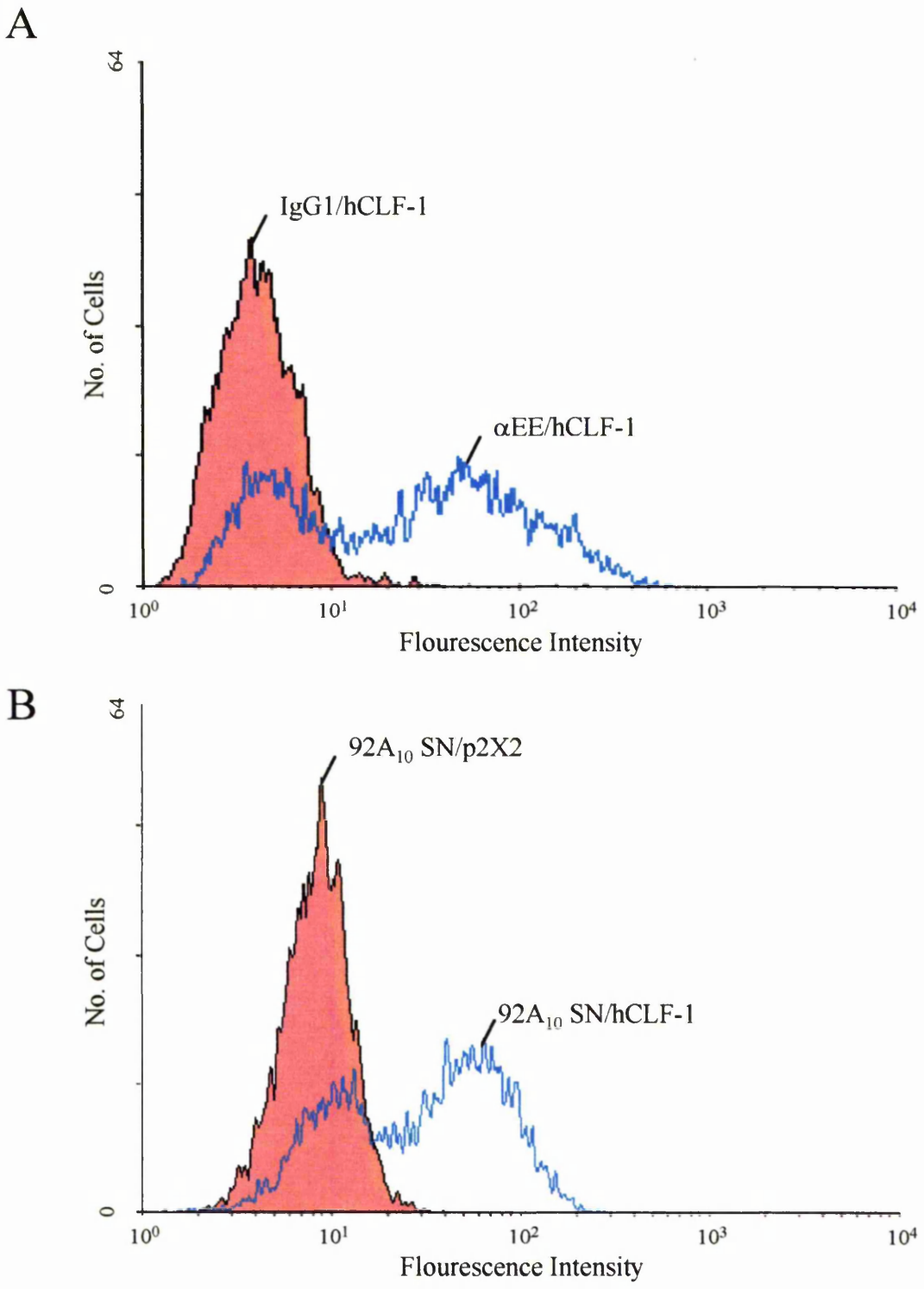


Figure 3.11. Detection of Membrane-Bound hCLF-1 by FACS analysis with the $92A_{10}$ Hybridoma Supernatant.

(A) Expression of the EE-tagged hCLF-1 fusion protein on the surface of Sf9 cells was analyzed 48 hrs post-baculovirus inoculation with the αEE mAb (αEE/hCLF1) or with an IgG1 isotype matched control (IgG1/hCLF-1).

(B) Cellular fluorescence 48 hours post baculovirus innoculation following incubation with the $92A_{10}$ hybridoma supernatant. Cells were expressing either the EE-tagged hCLF-1 fusion protein (as above; $92A_{10}$ SN/hCLF-1) or the EE-tagged p2X2 cell surface ($92A_{10}$ SN/p2X2) as a control.

(3.8) Recombinant Forms of hCLF-1 are Expressed as Covalently-Linked Homomers

The anti-tag mAbs and the α-hCLF-1 mAb (mAb $92A_{10}$) were used in parallel to analyze recombinant hCLF-1 by Western blotting under non-reducing and reducing conditions. rshCLF-1 purified from Sf9 and HEK 293-E cell supernatants were analyzed along with the hCLF-1/hIL-13Rα1 fusion protein purified from Sf9 cells or contained within CHO cell lysates (Figure 3.12).

Using mAb 179, rshCLF-1 expressed in Sf9 cells was seen to migrate under reducing conditions as a single band (corresponding to the monomeric protein) with an approximate molecular weight of 45-50 kD, somewhat bigger than the theoretical molecular weight of 39.3 kD. This difference in molecular weight is most likely to be due to glycosylation of the protein. Under non-reducing conditions, although the band corresponding to monomeric protein was seen to account for a large proportion of the total recombinant protein. Bands with a significantly larger molecular weight were also detectable. The most intense of these bands had an approximate molecular weight of 200 kD. The same experiment done with the α-hCLF-1 mAb under non-reducing and reducing conditions revealed the band corresponding to monomeric protein only after overexposure of the membrane (30''), whereas the 200 kD band was revealed after a time of exposure equivalent to that used with mAb 179 (10'') (Figure 3.12a).

A similar pattern of expression was observed with the recombinant hCLF-1 fusion protein expressed on the surface of Sf9 and CHO cells. Using the α-EE mAb to detect the Sf9 expressed hCLF-1 fusion protein under reducing conditions, a single band was revealed with an approximate molecular weight of 55-60 kD, glycosylation again being probably responsible for the size difference when considering the theoretical molecular weight of 45.3 kD. Under non-reducing conditions, a similar length of exposure (10'') revealed a band with a molecular weight of approximately 110-120 kD, with a band of weaker intensity detected at approximately 200-220 kD. A band corresponding to monomeric protein was only detected after a longer exposure (20-30''). The α-hCLF-1 mAb revealed monomeric protein under reducing conditions only after a long exposure

of the membrane (30''), whilst under non-reducing conditions, a 10'' exposure revealed the same bands (110-120 kD and 200-220 kD) seen with the α-EE mAb under non-reducing conditions (Figure 3.12b).

The hCLF-1 fusion protein expressed on CHO cells did not contain the EE tag, so the protein was analyzed under reducing and non-reducing conditions with the α-hCLF-1 mAb alone. Following a 10'' exposure, no bands were visible under reducing conditions, with a band corresponding to molecular weight of 120-150 kD being observed under non-reducing conditions. A band representing monomeric protein was seen under non-reducing and reducing conditions only after a relatively long exposure of the membrane (30''), and corresponded to a molecular weight of approximately 60-65 kD (Figure 3.12c). The slight difference in molecular weight seen between the Sf9 and CHO expressed hCLF-1 fusion protein is probably the result of a greater degree of glycosylation of the protein expressed in the mammalian (CHO) cells compared to that expressed in the insect (Sf9) cells.

rshCLF-1 expressed by HEK 293-E cells (see materials and methods) was analyzed with mAb 179 and the α-hCLF-1 mAb. When comparing the bands revealed with mAb 179 under reducing and non-reducing conditions, it was clear that the majority of the protein was expressed as a monomer, with a strong band detected under both conditions following a 10'' exposure. The band corresponded to a protein with a molecular weight of 55-60 kD. The difference in molecular weight observed between the HEK 293-E and Sf9 expressed rshCLF-1 is again probably due to the difference in glycosylation levels between mammalian and insect cells. More diffuse bands of a weaker intensity corresponding to covalently linked multimers with a higher molecular weight (from 110-250 kD) were also detected under non-reducing conditions, the most intense of which had a molecular weight of approximately 120 kD. With the α-hCLF-1 mAb, the bands representing monomeric protein were far less intense than those seen with the mAb 179 following a similar length exposure. A band with an approximate molecular weight of 200 kD was however readily visible, the equivalent band being much weaker at the same time point with the mAb 179 western blot (Figure 3.12d).

The comparison of recombinant hCLF-1 under reducing and non-reducing conditions by Western blotting indicated that recombinant hCLF-1 has a tendency to form covalently-linked homodimers. The result also suggested that the α-hCLF-1 mAb has a higher affinity for homodimeric and multimeric hCLF-1 than monomeric hCLF-1.

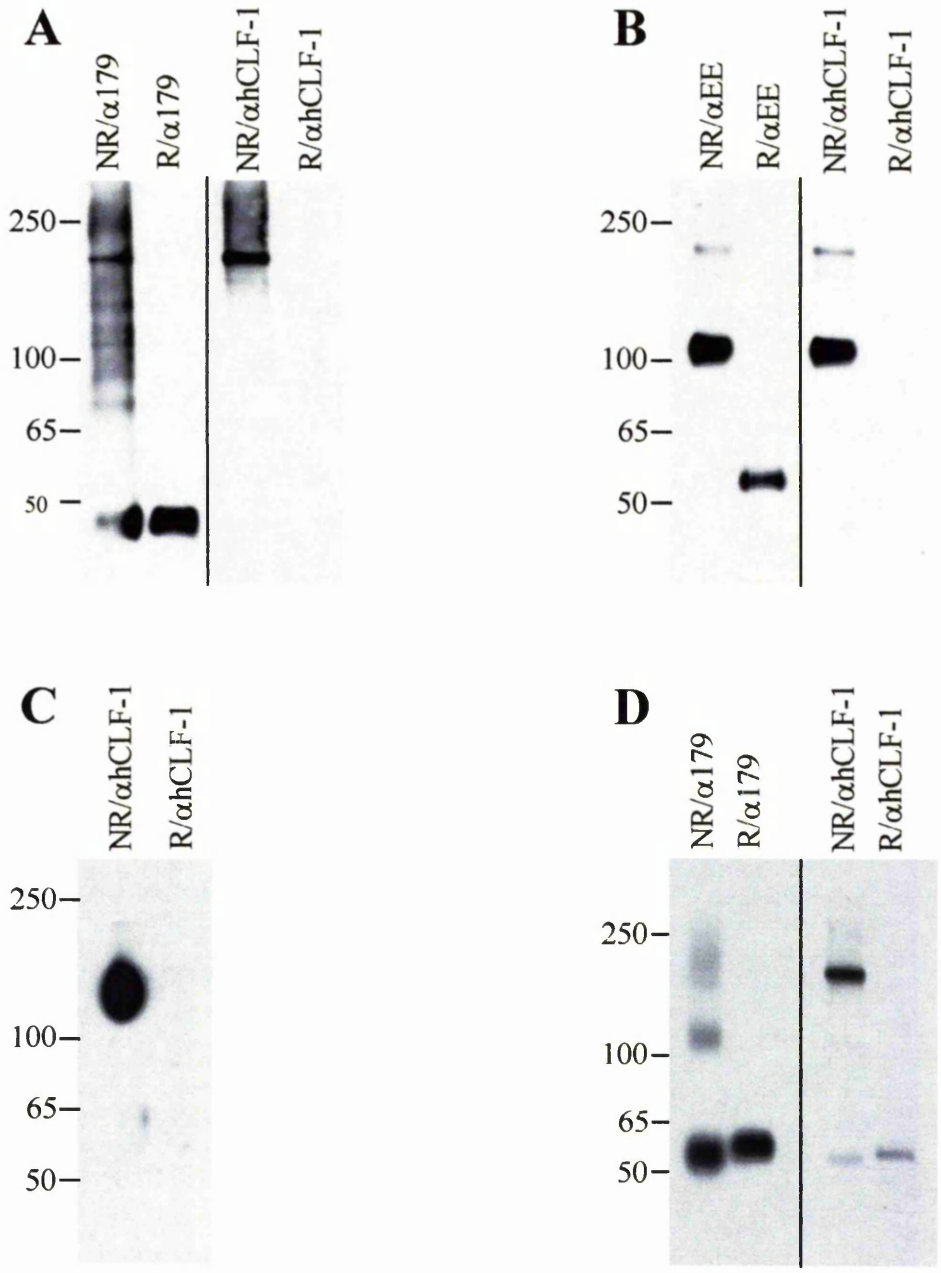


Figure 3.12. Detection of hCLF-1 by Western Blot Analysis.

Proteins were resolved under either non-reducing (NR) or reducing (R) conditions by SDS-PAGE on 8% acrylamide gels and electortransferred to nitrocellulose filters.

(A) rshCLF-1 expressed by Sf9 cells, detected with either mAb 179 (α179) or the α-hCLF-1 mAb (αhCLF-1). (B) Membrane-bound hCLF-1/hIL-13Rα1 fusion protein expressed on Sf9 cells detected with either the α-EE mAb (aEE) or the α-hCLF-1 mAb. (C) Membrane-bound hCLF-1/hIL-13Rα1 fusion protein expressed on CHO cells detected with the α-hCLF-1 mAb. (D) rshCLF-1 expressed by HEK 293-E cells detected with either mAb 179 or the α-hCLF-1 mAb.

(3.9) The Involvement of hCLF-1 in IL-6 Type Cytokine Receptor Complex Formation

Flow cytometry was used in an attempt to discover whether hCLF-1 was involved in a receptor complex with the known IL-6 type cytokines that use gp130 and LIF-R as signalling subunits. The study was performed with membrane-anchored hCLF-1 expressed on CHO cells, recombinant IL-6 type cytokines and recombinant soluble forms of the two signalling subunits.

In order to demonstrate the viability of the experimental approach, a number of human cell lines were screened for expression of gp130 by flow cytometry. HEK 293 cells were found to express the membrane bound protein, with a clear shift in cell fluorescence seen between cells incubated with control Ab (MFI=4.3) and cells incubated with α-gp130 Ab (MFI=15.3; Figure 3.13a). These cells were then used to form IL-6 type cytokine receptor complexes. As OSM and CT-1 form receptor complexes with LIF-R and gp130 (Gearing and Bruce, 1992a; Pennica *et al.*, 1995b), HEK 293 cells were incubated alone, or with sLIF-R, sLIF-R and OSM or sLIF-R and CT-1. Receptor complex formation was determined by the presence of sLIF-R on the cells surface, as measured by flow cytometry using an α-LIF-R mAb. HEK cells alone showed a MFI of 7.4 for the α-LIF-R Ab, whilst HEK 293 cells incubated with sLIF-R showed an MFI of 8.9, indicating a low level binding to the HEK 293 cells. When incubated with LIF-R and OSM in concert however, the HEK 293 cell MFI increased to 20, and likewise with sLIF-R and CT-1, 15.4 (Figure 3.13b,c). The MFI of the HEK cells obtained with the control Ab remained unchanged with all four conditions above, indicating that the change in cell fluorescence seen with the LIF-R Ab was specific. This confirmed that the formation of cell surface IL-6 type cytokine receptor complexes could be detected by flow cytometry.

The same experimental approach was then employed to investigate the involvement of hCLF-1 in receptor complex formation with the known IL-6 type cytokines, using CHO cells expressing either the membrane-bound hCLF-1/hIL-13Rα1 fusion protein or control CHO cells expressing hIL-13Rα1. hCLF-1 expression on the cell surface

was verified by flow cytometry with the α-hCLF-1 mAb. Control IL-13Rα1 cells showed an MFI of 3.53 with the α-hCLF-1 mAb, whilst with the same mAb on hCLF-1 expressing CHO cells the MFI increased to 76.1. Compared to the IL-13Rα1 expressing cells, 87% of the hCLF-1 cells were positive for fluorescence, with the positive cells showing an MFI of 107.7 (Figure 3.14a).

Cells were incubated without cytokine, or with IL-6, LIF, CNTF, IL-11, OSM and CT-1 in combination with either sLIF-R, sgp130 or both soluble receptors together. Receptor complex formation was determined by the amount of sLIF-R or sgp130 as measured by flow cytometry using an α-LIF-R or α-gp130 mAb. No difference was observed in cellular fluorescence between the control and hCLF-1 expressing CHO cells when incubated with any of the combinations of cytokines and soluble receptors (Figure 3.14b-g). This suggested either that hCLF-1 was not involved in receptor complex formation with gp130 or LIF-R and any of the known IL-6 type cytokines or that the conformation of the membrane-bound hCLF-1 did not permit the formation of such a complex.

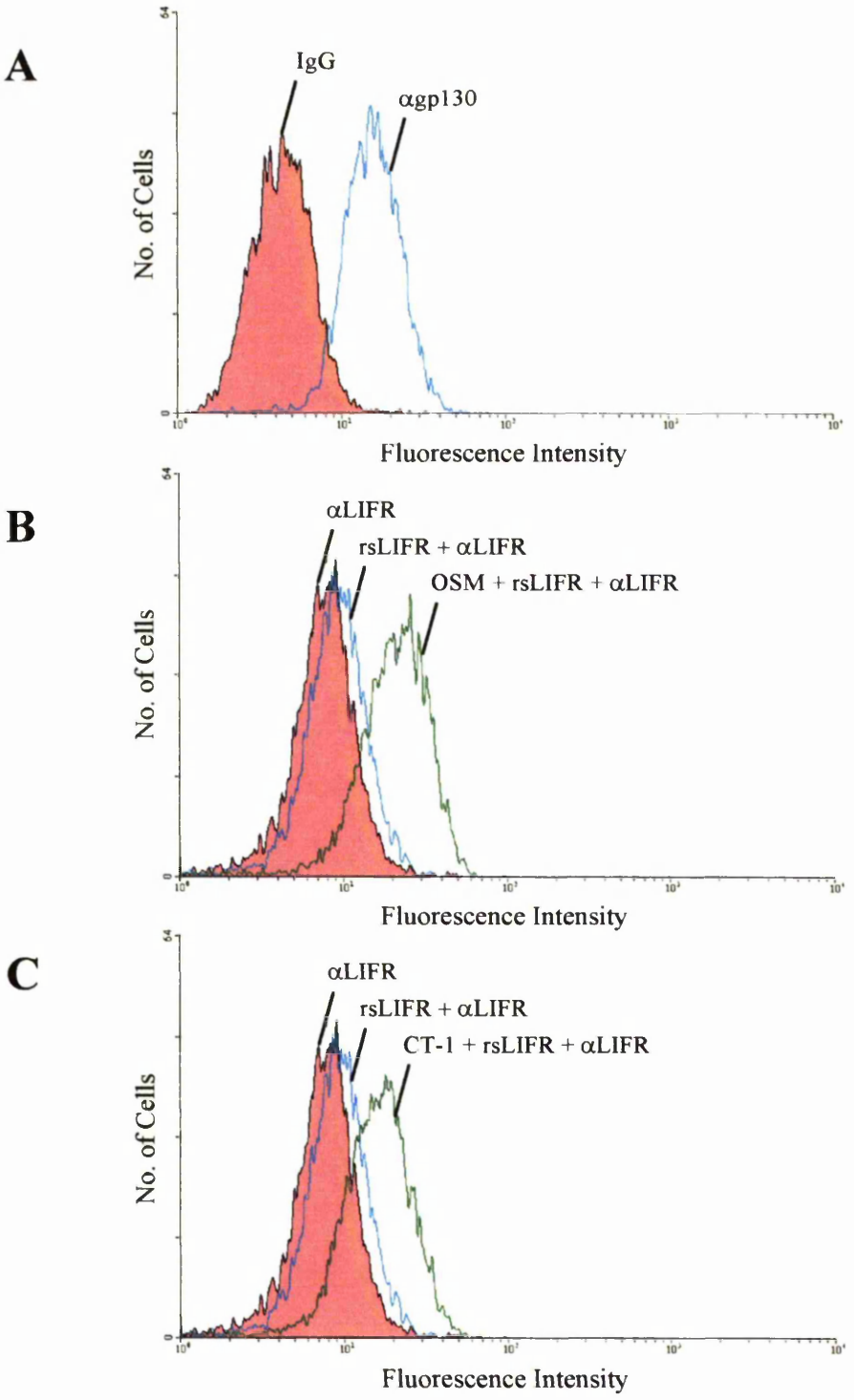


Figure 3.13. OSM and CT-1 Receptor Complex Formation on HEK 293 Cells.

(A) Expression of endogenous gp130 was detected by FACS analysis on the surface of HEK 293 cells with polyclonal goat α-gp130 Abs (αgp130). Non-specific goat IgG was used as a control.

(B) Detection of rsLIFR bound to the surface of HEK 293 cells by FACS analysis with polyclonal goat α-LIFR Abs. Cells were incubated alone (αLIFR), with rsLIFR (rsLIFR+αLIFR) or with OSM and rsLIFR (OSM+rsLIFR+αLIFR).

(C) As (B) but CT-1 was used instead of OSM.

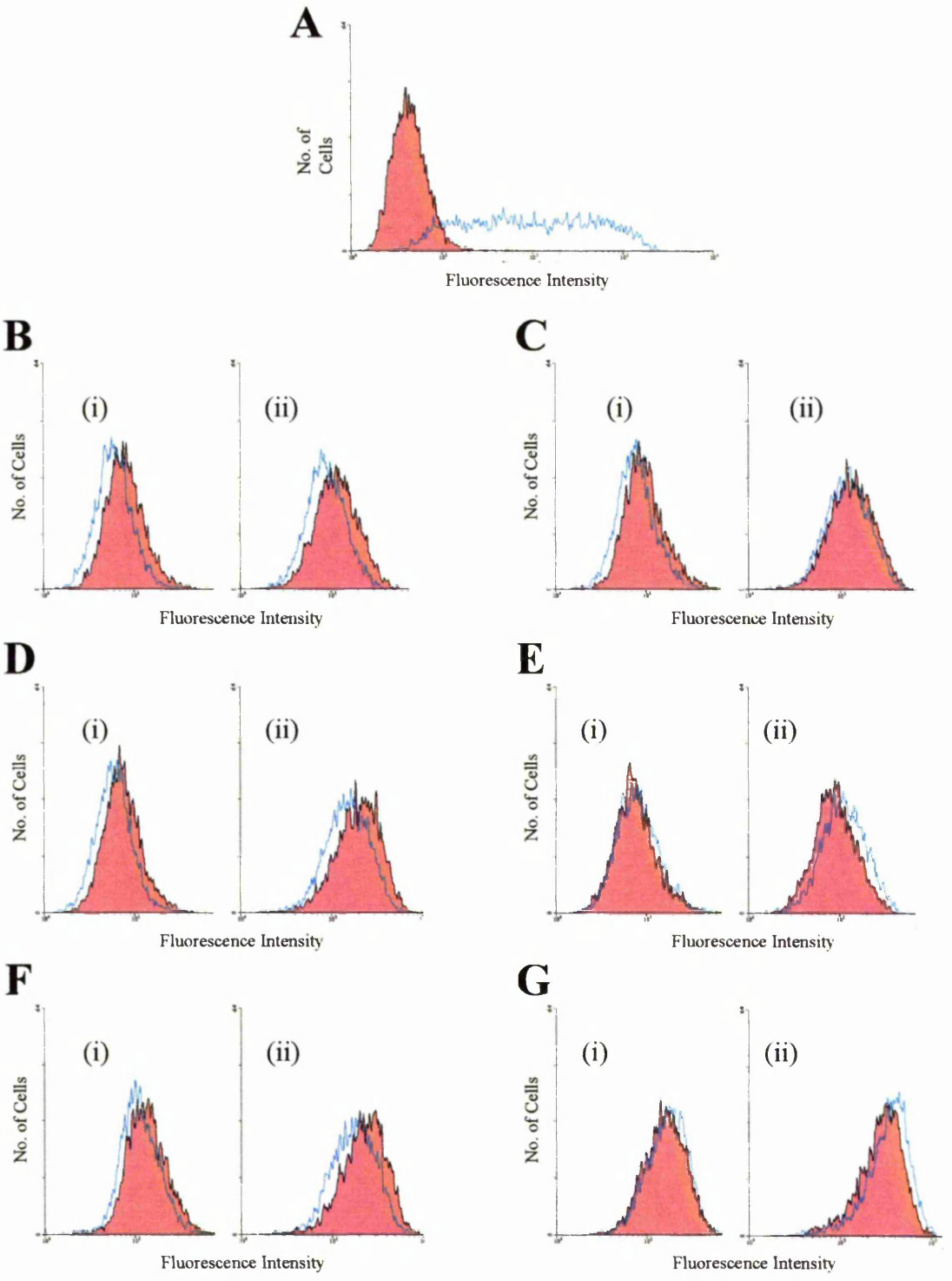


Figure 3.14. Analysis of rsgp130 and rsLIFR Binding to hCLF-1-Transfected CHO Cells.

(A) Expression of hCLF-1 on the surface of CHO transfected cells as detected with the α-hCLF-1 mAb (blue line profile). hIL-13Rα1 transfected CHO cells served as a control (red filled profile).
(B-G) hCLF- 1 (blue line profile) or hIL-13Rα1 (red filled profile) transfected CHO cells were incubated with rsgp130 + rsLIFR and either (B) IL-6, (C) LIF, (D) CNTF, (E) IL-11, (F) OSM or (G) CT-1. (i) gp130 and (ii) LIFR binding to the cell surface was detected using the appropriate polyclonal Abs. Similar results were obtained when cells were incubated with either rsgp130 alone or rsILFR alone and the above cytokines (data not shown).

(3.10) An hCLF-1 Counterstructure on Human Monocytes and Two Cell Lines

rshCLF-1 purified from Sf9 cell supernatants was used to identify cells expressing an hCLF-1 counterstructure. rhsIL-5Rα served as a control since it has a natural soluble form, shares 41% amino acid sequence similarity with hCLF-1 and like hCLF-1, has the mAb 179 and 6 histidine tags at its C terminus.

Flow cytometry with mAb 179 or the α-hCLF-1 mAb was used to detect binding on cell lines. Whereas rshIL5Rα showed no binding to any of the cell lines tested, hCLF-1 showed a significant amount of binding to the fibroblast cell line, HEK 293, and the B cell line, RPMI 8226. Using mAb 179, the MFI of the HEK cells increased from 5.76 with rshIL-5Rα to 38.9 with rshCLF-1 (Figure 3.15a(i)) and with the α-hCLF-1 mAb, the shift increased from 3.92 to 69.8 (Figure 3.15a(ii)). The RPMI 8226 cells showed a shift from 3.3 to 10.0 with mAb 179, and from 4.54 to 45.3 with the α-hCLF-1 mAb (Figure 3.15b(i and ii)). These results indicate that a counterstructure for hCLF-1 is expressed on the human cell lines HEK 293 and RPMI 8226.

In order to confirm the observation that HEK 293 cells express a counterstructure for CLF-1, HEK 293 stable transfectants expressing hCLF-1 with the mAb 179 epitope were generated. Supernatants from transfected cell clones resistant to G418 and hygromycin B were screened by ELISA using mAb 179 to capture hCLF-1 and a biotinylated form of the α-hCLF-1 mAb to reveal supernatants containing rshCLF-1. Two clones, 5 and 17, were identified as expressing rshCLF-1 (Figure 3.16a). The presence of rshCLF-1 was subsequently detected on the surface of the HEK 293 cells using the α-hCLF-1 mAb, suggesting that it was not only being secreted by the transfectants, but it was also recognizing a counterstructure on the cell surface (Figure 3.16b).

The supernatants of the HEK 293 transfectants containing rshCLF-1 were then used either directly or concentrated 1:5 to investigate binding of mammalian-cell derived rshCLF-1 to the surface of the RPMI 8226 cells by FACS analysis with the α-hCLF-1 mAb. A small but significant binding was detected using both clone 5 and clone 17

supernatants when compared to the supernatant of non-transfected HEK-293-E cells, which was increased when concentrated supernatants were used (Figure 3.17a,b).

Human PBL, either resting or stimulated with PMA and ionomycin, were also tested for binding of rshCLF-1 by flow cytometry using mAb 179. No binding was observed on cells having the morphology and size of T cells either resting or stimulated. A small amount of binding was, however, detected on cells with the size and morphology of monocytes following PBL stimulation with PMA and ionomycin (Figure 3.18a), with an MFI of 5.7 and 5.6 with mAb 179 alone and rshIL-5Rα + mAb 179, respectively, increasing to 8.9 with rshCLF-1.

Purified human monocytes, either resting or stimulated with LPS or pro-inflammatory cytokines, were therefore tested for rshIL-5Rα and rshCLF-1 binding by flow cytometry using mAb 179. rshIL-5Rα showed a small amount of binding to monocytes with or without monocyte stimulation, with an MFI for cells incubated with mAb 179 alone and cells incubated with rhsIL-5Rα + mAb 179 increasing from 6.4 to 7.8 without treatment, 5.2 to 7.1 with LPS, 3.5 to 4.6 with IFN-γ, 9.4 to 12.3 with IL-6, 4.5 to 5.9 with TNF-α and 6.4 to 6.8 with IL-1β. rshCLF-1, however, showed a greater affinity for the monocytes with the MFI's between mAb 179 alone and rshCLF-1 + mAb 179 shifting from 6.4 to 10.4 without treatment, 5.2 to 11.4 with LPS, 3.5 to 17.4 with IFN-γ, 9.4 to 18.4 with IL-6, 4.5 to 8.4 with TNF-α and 6.4 to 11.4 with IL-1β. The affinity was clearly seen to be greater than that shown by the rshIL-5Rα with or without monocyte stimulation (Figure 13.18b(i-vi)). When comparing the difference in MFI's between cells incubated with mAb 179 alone and cells treated with rshCLF-1 + mAb 179 following treatment with the different stimuli (4.0 with no treatment, 6.2 with LPS, 13.9 with IFN-γ, 9.0 with IL-6, 3.9 with TNF-α and 5.0 with IL-1β), it appears that a counterstructure for hCLF-1 is upregulated on monocytes by the different stimuli tested, with IFN-γ having the greatest effect.

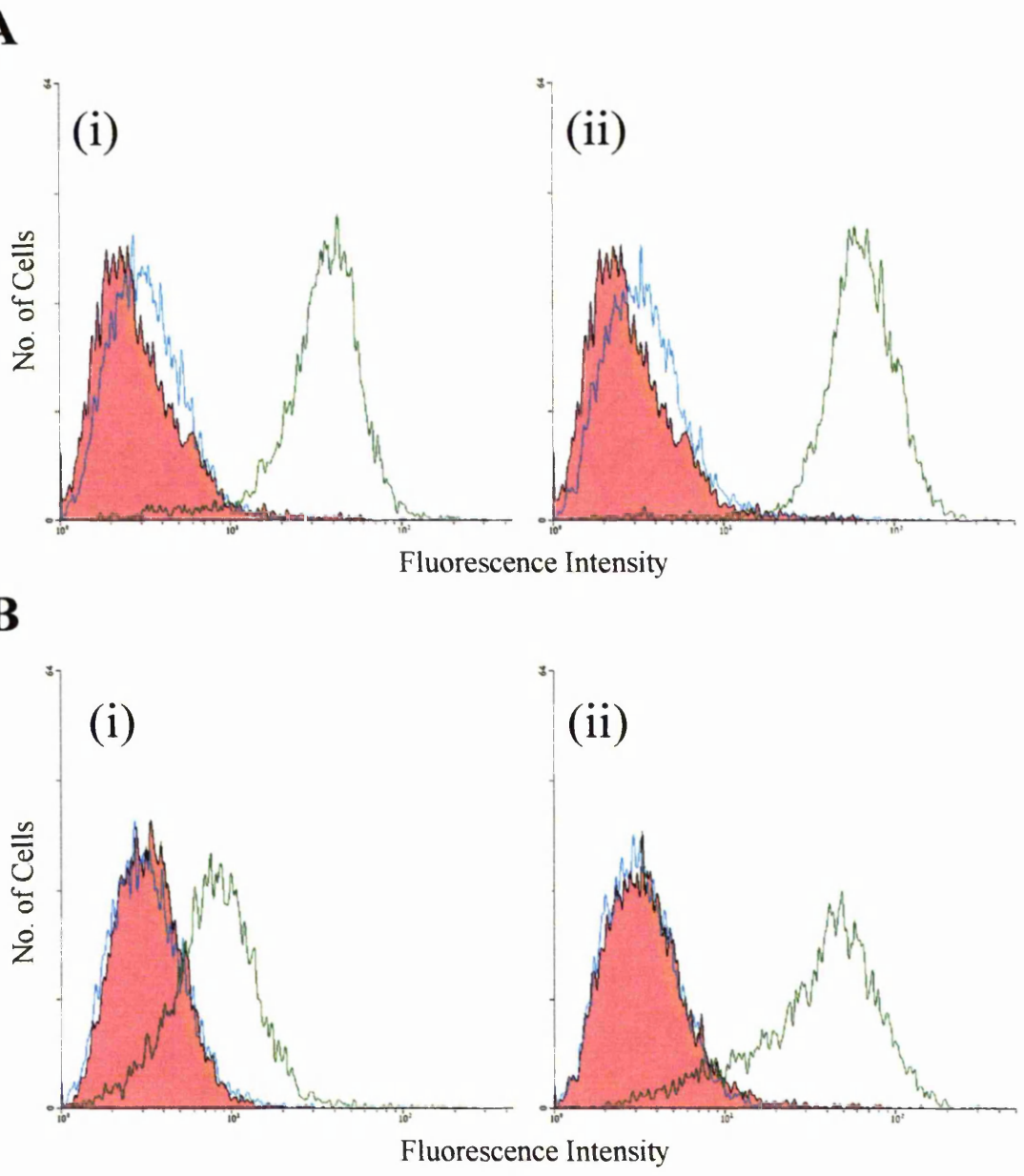


Figure 3.15. hCLF-1 Binding to to HEK 293 and RPMI 8226 Cells.

(A) HEK 293 cells were incubated with rshCLF-1 purified from Sf9 cells at 10μg/ml (blue line profile) rshIL-5Rα at 10μg/ml (green line profile) or medium alone (red filled profile) and cells surface binding detected by FACS analysis either with (i) mAb 179 or (ii) the α-hCLF-1 mAb.

(B) The same experiment repeated with RPMI 8226 cells.

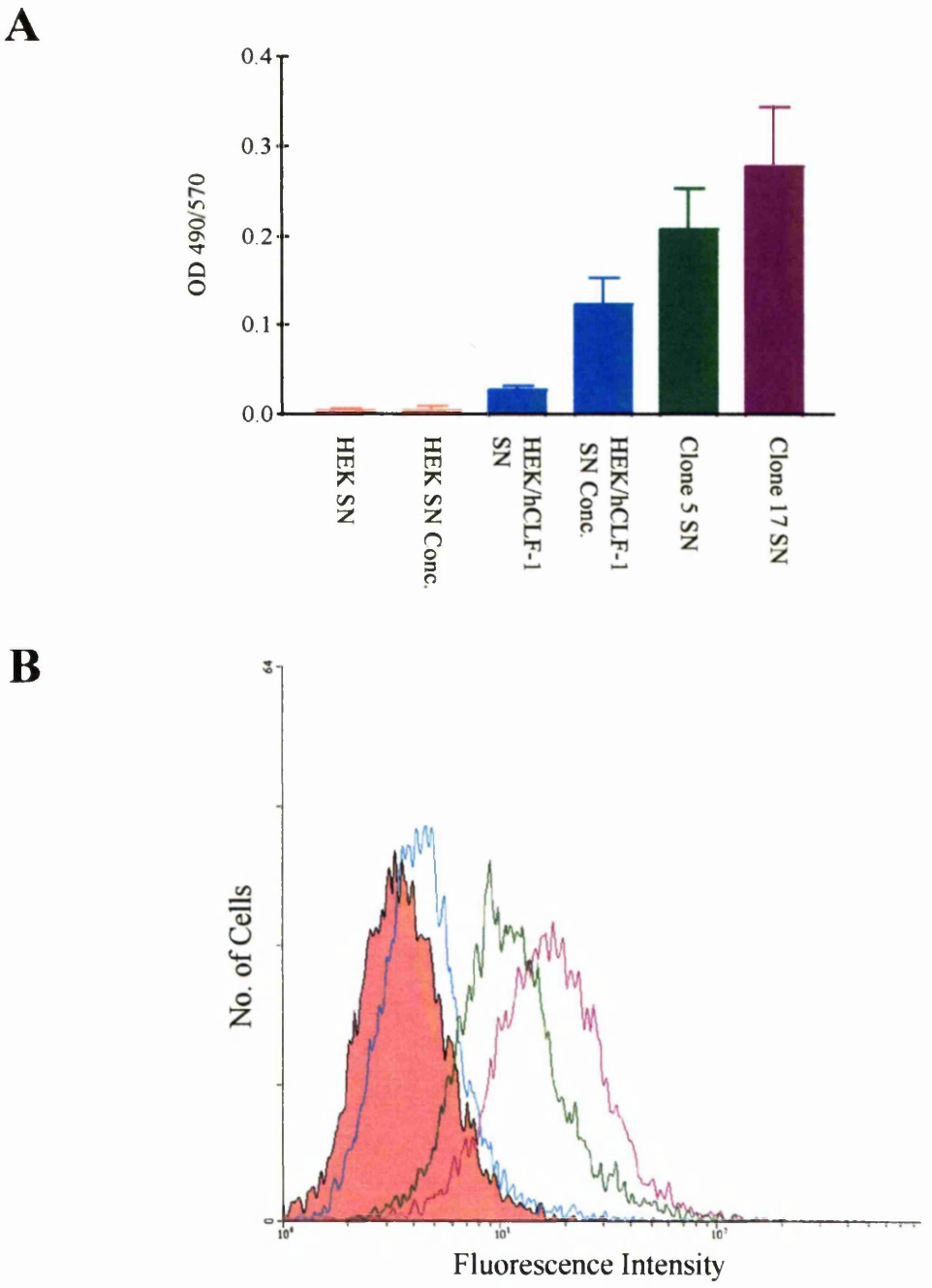


Figure 3.16. rshCLF-1 Expressed in Transfected HEK 293-E Cells and its Detection on the Cell Surface.

(A) Expression of rshCLF-1 in HEK 293-E cell supernatants. The supernatants of non-transfected HEK 293-E and transfected but non-cloned HEK 293-E cells were tested along with the supernatants of transfected clones by ELISA with mAb 179 and the α-hCLF-1 mAb. Where indicated, supernatant proteins were concentrated approximately 5-fold (SN Conc.).

(B) rshCLF-1 binding to the surface of transfected HEK 293-E cells, as detected by FACS analysis with the α-hCLF-1 mAb. Non-transfected HEK-293-E cells are represented by the red filled profile, transfected non-cloned cells are represented by the blue line profile, whilst clone 5 and clone 17 are represented by the green and violet line profiles respectively.

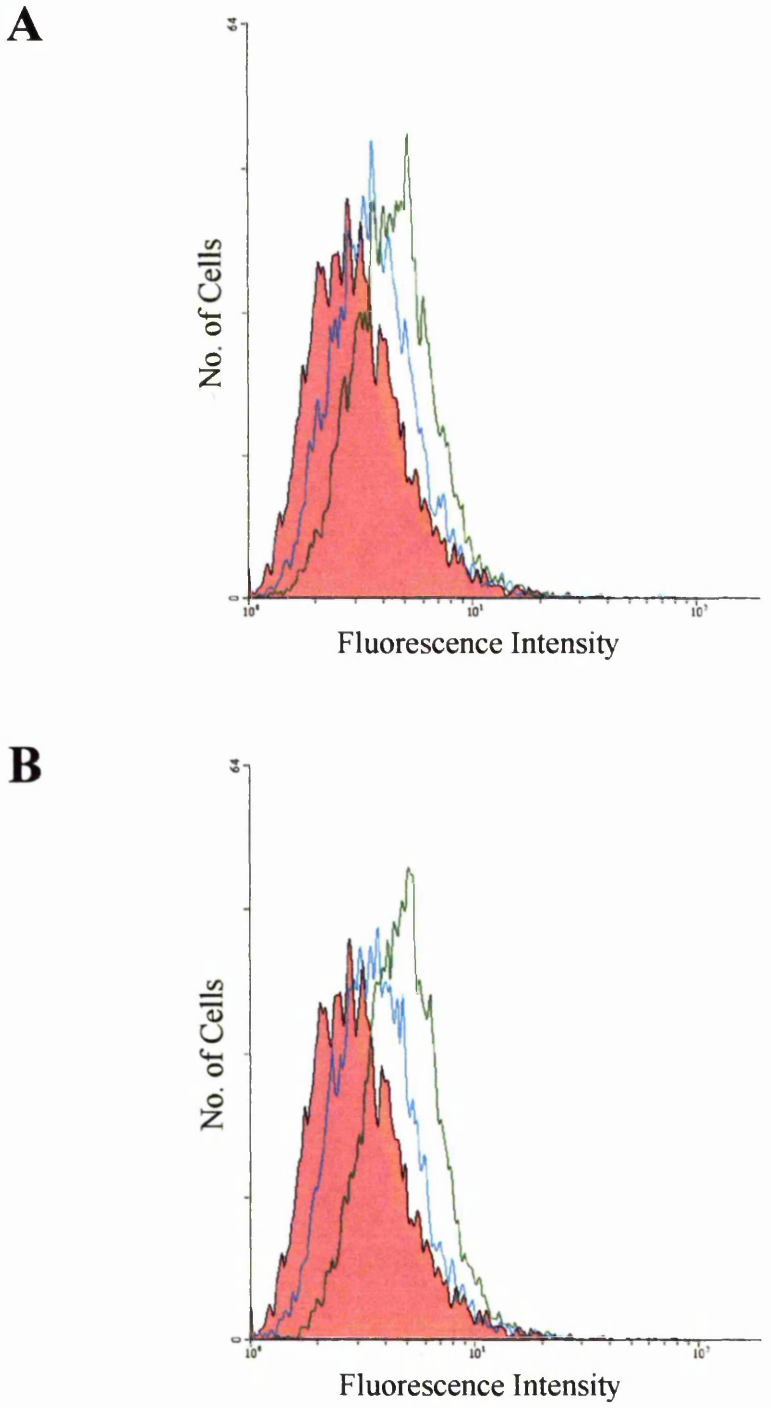
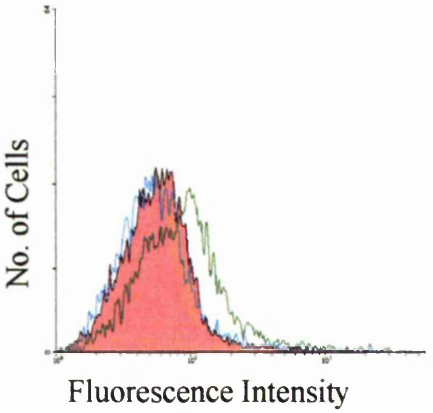


Figure 3.17. Binding of Mammalian Cell-Derived rshCLF-1 to the Surface of RPMI 8226 Cells

(A) RPMI 8226 cells were incubated with either the supernatant of HEK 293-E cells (red filled profile), the supernatant of HEK 293-E clone 5 cell transfectants (blue line profile) or the same supernatant concentrated approximately 5-fold (green line profile). hCLF-1 binding to the cell surface was detected by FACS analysis with the α-hCLF-1 mAb.

(B) The above experiment was repeated with the supernatant of HEK 293-E clone 17 cell transfectants.

A



B

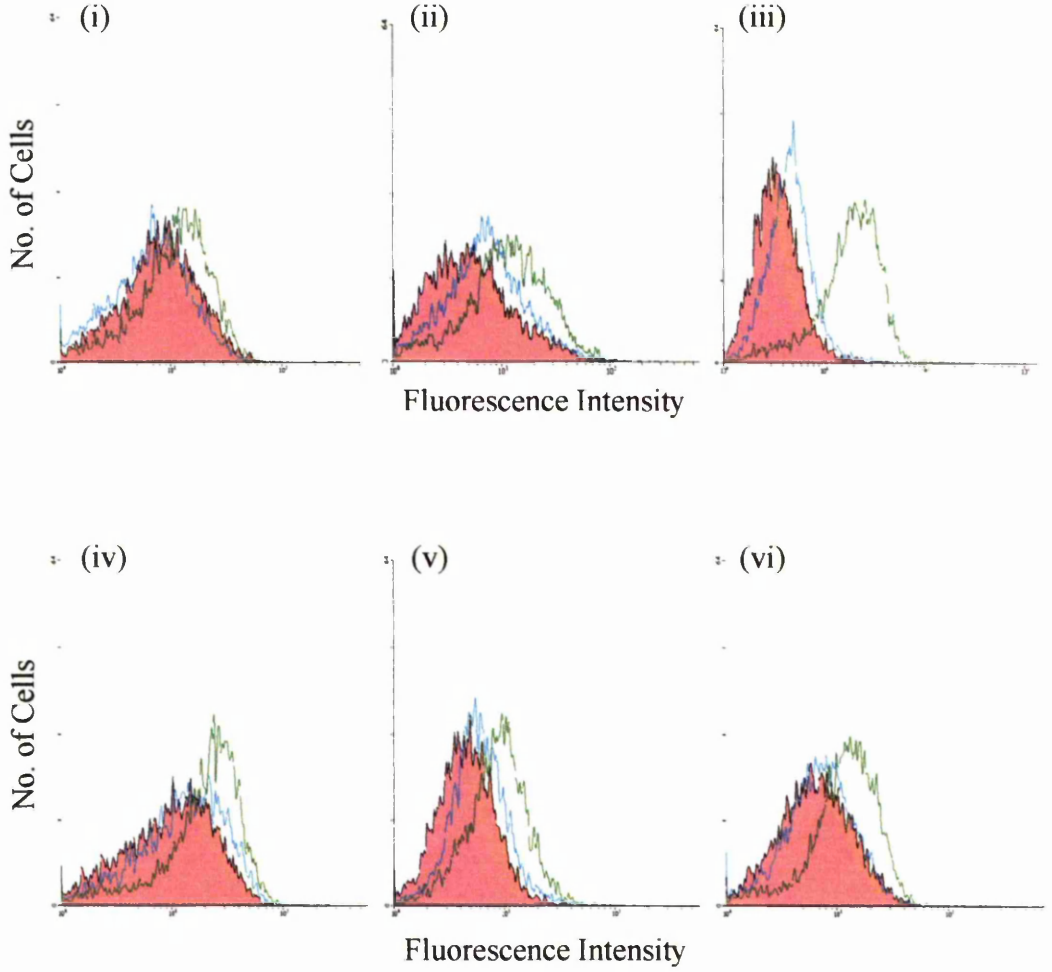


Figure 3.18. hCLF-1 Binding to Stimulated Monocytes

(A) rshCLF-1 (10 μg/ml; green line profile), rshIL-5Rα (10 μg/ml; blue line profile) or buffer alone (red filled profile) were incubated with PMA + ionomycin stimulated PBL and cells with the size morphology of monocytes were analyzed by FACS with mAb 179 for cell surface binding.

(B) Purified blood monocytes were treated with either (i) medium alone, (ii) LPS (iii) IFN-γ, (iv) IL-6, (v) TNF-α or (vi) IL-1β and subsequently analyzed for their capacity to bind hCLF-1 as described in (A) above.

Chapter 4. DISCUSSION

(4.1) Bioinformatics and EST Databases

The identification and cloning of the novel soluble CLF-1 using bioinformatics has demonstrated the validity and utility of an EST database screening approach for the rapid identification of unidentified proteins pertaining to families defined by sequence, sequence motif and secondary structure homology. This approach clearly has advantages over more traditional library screening, expression cloning, subtractive hybridization and differential display approaches in terms of speed in which sequence information is obtained and a reduction in laboratory bench work. The drawback of the approach however, especially when compared to expression cloning is the lack of functional data. For example, expression cloning has been used to identify a variety of different cytokine receptors. The identification typically involves a specific interaction, such as ligand binding, or high affinity receptor complex formation. This information gives an invaluable starting point for the functional characterization of such novel molecules. Similarly, subtractive hybridization and differential display give information about the expression pattern of a given protein.

Identification of a protein sequence from the EST database gives no additional information, except in the best case a variety of organs, tissues and cells which may be associated with expression. No information about the level of expression can be ascertained, although several ESTs derived from the same source could point to an elevated expression in that particular tissue. In the case of CLF-1, we had identified a novel protein of human, mouse and rat origin from dbEST showing homology to the type I family of cytokine receptors. The source of the ESTs for CLF-1 pointed predominantly to an expression in fetal tissue in mice and humans as well as the uterus in humans (Table 3.1). One additional piece of information was the chromosomal localization of the human gene (19p12-13.1) ascertained from one entry in the database derived by PCR from exon amplification (Table 3.1). As a consequence of this lack in functional data and the wide variety of functions shown by cytokines signalling through cytokine type-I receptors, we could not easily focus our initial characterization of CLF-1 to any one specific area.

(4.2) CLF-1 as a Soluble Cytokine Type-I Receptor

In terms of primary amino acid sequence, CLF-1 appears to be a member of the cytokine type-I receptor family, containing a region of approximately 200 aa residues resembling the functionally important cytokine receptor-like domain. This is best highlighted by the presence of the characteristic two pairs of cysteines and the highly conserved W-S-x-W-S motif (Bazan, 1990c; Figures 3.1 and 3.2a). Human CLF-1 has highest homology with human gp130 and the human prolactin receptor (PRLR) with 25% and 32% sequence identity, respectively. The homology is, however, quite restricted to the N-terminal extracellular region of both receptors. In fact, the homology shown between hCLF-1 and hPRLR falls principally in the cytokine receptor-like domain. CLF-1 also shares significant homology with members of the gp130 subfamily and the receptor subunits sharing the signalling subunits LIFR and gp130 (Figure 3.5). In structural terms, CLF-1 is more similar to the extracellular regions of IL-6Rα, IL-11Rα and CNTFRα in that it has an Ig-like domain followed by a cytokine-receptor-like domain. The difference is that the cytokine receptor-like domain in CLF-1 is not followed by a GPI-linkage motif or a transmembrane domain. In this respect, CLF-1 very much resembles a soluble cytokine type-I receptor.

Numerous examples of soluble forms of cytokine type-I receptors have been cited in the literature including those for the aforementioned IL-6Rα and CNTFRα. The function of soluble receptors may be to modify ligand concentration, downregulate membrane-receptor number by shedding or specifically inhibit ligand-receptor association, as is demonstrated by the soluble forms of gp130 (Narazaki *et al.*, 1993; Montero-Julien *et al.*, 1997) and IL-5Rα (Tavernier *et al.*, 1991). Soluble receptors can also act as agonists for cytokine signalling, as well as confer to cells the ability to respond to cytokines for which they do not normally express the cognate receptor (reviewed in Heaney and Golde, 1996).

As CLF-1 appears to exist predominantly, if not uniquely, as a soluble protein, one must assume that if it is indeed a cytokine receptor, it does not play a part in intracellular cytokine signalling directly. In this respect, it resembles the three receptor

subunits with which it shares significant sequence and structural homology, IL-6Rα, IL-11Rα and CNTFRα. These three receptor subunits, acting as ligand binding subunits and all sharing gp130 as a signal transducing receptor subunit, have very short (and in the case of CNTFRα, non-existent) cytoplasmic tails which do not play a part in signalling. They also exist naturally in soluble form (although this is not confirmed for IL-11Rα), and act as agonists for cytokine signalling (discussed in Chapter I). It is also interesting to note that the heterodimeric cytokine IL-12 is composed of the two subunits called p35 and p40. The p40 subunit resembles a soluble cytokine type-I receptor and shares homology with IL-6Rα and CNTFRα (Gearing and Cosman, 1991), whilst p35 shares structural homology with the family of cytokines signalling through LIFR/gp130 (Merberg, 1992). In this case, however, the soluble cytokine receptor subunit is covalently linked by a disulphide bridge to the heterologous subunit p35. The p35/p40 complex, therefore, resembles an irreversibly bound cytokine/soluble cytokine receptor complex.

The cDNA for hCLF-1 was obtained screening a placental cDNA library. An interesting feature of the two clones identified was the fact that they had different C-terminal amino acid sequence. Clone 18 encoded the full length hCLF-1 protein whereas clone 3 was incomplete at the 5' end. We had difficulty in sequencing the 5' region of the clone 18 cDNA, and this was attributed to a 118 bp 5' UTR upstream of the initiating ATG codon (determined by *in vitro* translation; figure 3.3) that was extremely GC-rich (89%). This could also explain our inability to clone the full length murine cDNA by 5'-RACE, as the denaturing conditions used in the PCR reaction were probably insufficient to separate the two DNA strands. Such GC-rich regions are not uncommon within the cytokine receptor family, and are probably implicated in transcription factor binding for transcription initiation (discussed later).

Another difference in the two cDNA sequences was the presence of an additional 5 bp of DNA sequence in clone 3 near the 3' end of the cDNA attributed to an inexact splicing between exons 7 and 8 of the hCLF-1 gene (Figure 3.2b,c). This difference results in a frameshift which causes a divergence in amino acid sequence. It is not clear whether this divergence in sequence is a rare event or fairly common. If the

latter is true, it could suggest that the C-terminal region of CLF-1 is of reduced importance in terms of CLF-1 function. Indeed, C-terminal variation has also been observed between the sequence encoded by different cDNA clones identified for murine CLF-1 (D. Hilton, personal communication).

(4.3) The High Conservation of the CLF-1 Gene

One of the striking features of CLF-1 was the observed homology between human, mouse and rat homologues. At the nucleic acid level, hCLF-1 clone 18 shares 85 and 88% sequence homology with mCLF-1 and rCLF-1, respectively, converting to 96 and 99% amino acid identity, respectively. Between mCLF-1 and rCLF-1, the homology is even more staggering, especially at the nucleic acid level with 96% and 100% nucleic and amino acid identity, respectively (Figure 3.4). It was also observed that by interspecies genomic southern blot analysis (commonly referred to as zooblot analysis), performed with high stringency hybridization and washing, the hCLF-1 cDNA probe gave a hybridization signal with not only human, mouse and rat genomic DNA but also monkey, rabbit, cow and chicken (Figure 3.7). This indicates even further that the CLF-1 gene is highly conserved.

Why would such an interspecies conservation have arisen for CLF-1 and not other members of the cytokine type-I receptor family? One could hypothesize that CLF-1 is an essential protein for the functioning of a given species and a significant degree of selective pressure is therefore exerted on the gene sequence. Indeed, in mice where the gene for CLF-1 has been inactivated by gene targeting, newborn pups were seen to be incapable of initiating the feeding process and died shortly after birth (D. Hilton, personal communication), indicating an essential function for CLF-1 in fetal development. However, other members of this cytokine receptor family are also lethal when their gene is inactivated, such as gp130 or LIFR, but the sequence identity between human and mouse gp130 and LIFR are a long way short of that found for CLF-1 (77 and 76% amino acid sequence identity, respectively).

If one hypothesizes that CLF-1 is indeed a cytokine receptor, it should interact with a cytokine like molecule, and this interaction, being absolutely essential for CLF-1

function, has led to a very high conservation of the cytokine receptor. Indeed, if one assumes that the cytokine receptor-like domain is directly responsible for cytokine binding, as has been shown with other cytokine receptors (Figure 1.6b; de Vos *et al.*, 1992), it is interesting that between the human and mouse amino acid sequences only one amino acid residue difference is observed. In the cytokine receptor-like domain, a valine is changed to a phenylalanine. This is compared to seven differences in the Ig like domain and three differences in the region C-terminal of the cytokine receptor-like domain. From these observations, it would follow that any CLF-1 binding cytokine-like molecule would itself be highly conserved between species. Having said this however, such a uniform homology in regions of CLF-1 outside of the cytokine receptor-like domain is more difficult to clarify.

As mentioned earlier, variations exist between the C-terminal sequences from different CLF-1 proteins of the same species (Figure 3.2b,c). This leads to speculation that the C-terminal region of the protein is not important for function. The very high homology between the predicted C-terminal region derived from the hCLF-1 clone 18 cDNA and the cloned mCLF-1 cDNA would appear to contradict this suggestion (Figure 3.4). The identification of proteins interacting with CLF-1 and mutational analysis will permit the determination of exactly how important this conserved sequence is for the functioning of the protein, as well as the determination of the precise regions of importance.

(4.4) CLF-1 mRNA Distribution

Expression of murine mRNA transcripts for CLF-1 using an mCLF-1 cDNA probe were detected predominantly in the adult lung, with detectable expression seen in skeletal muscle, heart, brain, lymph node, thymus and bone marrow (Figure 3.8a(i and ii)). A modest upregulation of expression was seen in the lymph node of immunized mice when compared to control mice. Due to the low levels of expression detected, this finding awaits confirmation, possibly using a more suitable and sensitive detection method such as ribonuclease protection assay or RT-PCR. The expression levels in adult tissues not giving us many clues as to the function of CLF-1 in the mouse, we looked at cells and cell lines expressing mCLF-1 RNA to point us towards

a function. As the lung was the tissue expressing highest levels of mCLF-1 amongst those tested, we focused on cell types which could be derived from lung tissue (Figure 3.8b). Although expression was detected in all cell types, no cell line was found to overexpress the mRNA transcript, or even express the mRNA at a level equivalent to that found in whole lung tissue. In order to elucidate which cells in vivo express significant amounts of mRNA encoding mCLF-1, an alternative method of mRNA detection could be more appropriate, such as *in situ* hybridization on murine lung sections.

Interestingly, it appeared that peripheral blood T cells stimulated with CD3 and CD28 Abs expressed specific hCLF-1 transcripts of a higher molecular weight (Figure 3.8b). The PCR primers used in this experiment were designed to amplify a region of mCLF-1 cDNA stiuated within the cytokine receptor-like domain. An alternatively spliced form of the mCLF-1 mRNA with a larger molecular weight than the cloned mCLF-1, as seen in the RT-PCR experiment, would implicate exon insertion in this functionally important region. This would represent a novel splice variant within the cytokine type-I receptor family. It should be pointed out that mRNA splice variants do exist within this family of proteins. However, modifications almost invariably involve the transmembrane encoding exon or exons encoding the cytoplasmic tail (Nakamura *et al.*, 1992; Mignotte *et al.*, 1994; Fukunaga *et al.*, 1990; Edery *et al.*, 1989; Lust *et al.*, 1992; Horiuchi *et al.*, 1994; Diamant *et al.*, 1997). The cloning and characterization of these stimulated T cell transcripts will determine whether they do indeed express alternatively spliced forms of the mCLF-1 mRNA transcript.

A contrasting distribution of expression is seen between mRNA encoding human and murine CLF-1 (Figure 3.8a and 3.9a). The most striking feature was the strong expression detected in immune and hematopoietic tissues such as the adult spleen, lymph node, thymus, appendix and bone marrow, pointing towards a possible role for hCLF-1 in hematopoietic/immune regulation. Indeed this finding was somewhat supported by the observation that the mRNA for hCLF-1 was upregulated in primary fibroblast cultures treated with the proinflammatory cytokines TNF-α, IL-6 and IFN-γ. The role of these cytokines in CLF-1 gene regulation will be discussed later. Strong

expression of hCLF-1 mRNA transcripts was also detected in adult stomach, placenta, heart, skeletal muscle and ovary. This fairly ubiquitous expression of hCLF-1 mRNA could point to a rather pleiotrophic function for the protein. As previously discussed, CLF-1 shares significant homology with the receptors for cytokines signalling through gp130. It is interesting to note that these cytokines exhibit widespread pleiotrophy.

One puzzling feature of the expression pattern of CLF-1 in humans and mice was a clear difference in their expression pattern in certain tissues (Figures 3.8a and 3.9a). For example, strongest expression of mCLF-1 was seen in adult lung, whereas in adult human lung no expression was detected, even though the quality and quantity of the lung-derived polyA$^+$ RNA on the Northern blot was verified using a β-actin probe. Similarly, CLF-1 was clearly expressed in human spleen but barely detectable in mouse spleen and the expression seen in mouse brain was not reproducible in humans. The easiest explanation involves the poor choice of tissue area used to extract the RNA. With murine RNA extraction, for example, the whole organ or tissue is removed and homogenized. This is impossible for human tissues due to size restraints. Therefore, in practical terms, only a small region of each tissue is used. It is plausible that CLF-1 is expressed only in a particular region of a given tissue, for example, and if this region was not taken for extraction when preparing human RNA, a negative result would be obtained. In contrast, with the mouse organ a positive signal can be obtained. Having said this, it is equally possible that such a differential expression of CLF-1 does exist between human and mouse. Whether this means that the functional activities of CLF-1 differ between humans and mice will need to be clarified.

Due to its expression in human immune tissues, we studied hCLF-1 mRNA levels in a number of available cells and cell lines, with a bias towards immunocompetent cells (Figure 3.9b). Although expression was found to be weak in most cell lines, we found a reasonable signal in the embryonic kidney fibroblast cell line, HEK 293. Fibroblasts are well known for their role in matrix production, but they also play an important role as antigen-independent effector cells in inflammation through production of various mediators and cytokines (Gauldie *et al.*, 1992). This prompted us to investigate the expression of hCLF-1 mRNA in primary fibroblast cell cultures and the effect of the proinflammatory cytokines IL-1β, TNF-α, IL-6 and IFN-γ on hCLF-1 mRNA

expression levels (Figure 3.10). The finding that TNF-α, IL-6 and IFN-γ were capable of upregulating the constitutive hCLF-1 mRNA levels found in primary fibroblast cell cultures again points to a role for this soluble factor in immune regulation. In accordance with the observation that hCLF-1 mRNA was expressed in fibroblasts, murine CLF-1 mRNA has been detected in stromal tissue by *in situ* hybridization (D. Hilton, personal communication), which is known to be rich in that particular cell type.

It was suprising to find that IL-1β had no effect on the level of hCLF-1 mRNA production in the cultured fibroblasts, especially as TNF-α upregulates hCLF-1 mRNA and IL-1 and TNF-α are capable of utilizing similar signalling pathways terminating in activation of the transcription factor NF-kB (May and Ghosh, 1998). In fact, a downregulation was observed when comparing transcript levels between IL-1β treated and non-treated cells, especially with fibroblasts derived from palmar lesions and tonsils. IL-1 is a cytokine whose signalling can be downregulated by the expression of two molecules. IL-1 has two receptors, IL-1RI and IL-1RII. IL-1RII has no signal transducing capacity and therefore acts as a decoy for IL-1Rβ. In addition, IL-1 receptor antagonist (IL-1Ra) is a naturally occurring protein that efficiently binds both IL-1 receptor types but transduces no signal with IL-1RI (reviewed in Dinarello, 1996). One can therefore speculate that after 48 hours of IL-1β treatment, the fibroblasts have upregulated expression of either IL-1RII or IL-1Ra or both to block IL-1 signalling and hence the upregulation of hCLF-1 mRNA. To really resolve this issue, a time course experiment would be the most appropriate approach, looking at the effect of hCLF-1 mRNA expression levels after IL-1β treatment at different time-points. In parallel, one could determine the levels of expression of IL-1RII and IL-1Ra mRNA.

CLF-1 was also found to be expressed in murine embryonic and human fetal tissue (Figures 3.8a and 3.9a). In addition to this, several ESTs (especially murine) encoding CLF-1 were derived from fetal sources. This, coupled to the phenotype of CLF-1 deficient mice suggests a role for CLF-1 in fetal development. The localization of expression of hCLF-1 to fetal lung indicates that the protein may function to regulate

development in this tissue. Cytokine and cytokine receptor expression in fetal lung has been documented to play a role in lung development, with the mesenchyme and extracellular matrix shown to be an important region for their production (reviewed in Hilfer, 1996). Furthermore, fetal lung fibroblasts have been shown to upregulate mRNA for soluble differentiation factors involved in lung development in response to certain stimuli such as glucocorticoids (Wang *et al.*, 1995). It will therefore be interesting to see whether mouse fetal lung expresses CLF-1. In situ hybridization on mouse embryos would be the logical approach to resolve this issue. If CLF-1 is indeed found to be expressed in mouse fetal lung, as it is in the adult, an analysis of the morphology of the lungs of newborn mice deficient in the CLF-1 gene could give some indications as to the role of the protein in fetal lung development.

(4.5) CLF-1 Gene Structure and Chromosomal Localization

As mentioned earlier, the identification of an EST encoding CLF-1 derived by exon amplification from human chromosome 19p12-13.1 DNA allowed the chromosomal localization of the hCLF-1 gene (Table 3.1). A more precise localization was attained by the identification of a cosmid derived from chromosome 19p12 containing the complete CLF-1 gene and promoter region, the sequence of which had been deposited in the GenBank database. There are three genes encoding members of the cytokine type-I receptor family located on the same arm of chromosome 19, the EPO receptor (EPOR; Winkelmann *et al.*, 1990; p13.2), EBI-3 (Devergne *et al.*, 1996; p13.2/3) and IL-12Rβ1 (Yamamoto *et al.*, 1997; p13.1). This suggests that the region is rich in genes for cytokine type-I receptors and could therefore represent a gene cluster such as that found within human chromosome 5p12-14 containing LIFR (p12-13), GHR (p13.1-12), PRLR (p12-14) and IL-7Rα (p13). If so, this specific vicinity of chromosome 19 represents a potentially excellent source for the identification and cloning of new members of this receptor family.

The localization to a specific region of a chromosome can also give information about the role of a gene in diseases such as inherited disorders. Evidence for a direct association between a given gene locus and a genetic disorder leads to the

identification of candidate genes. For example, the severe combined immuno-deficiency disorder is linked to the X chromosome (SCIDX1) and is characterized by a deficiency in T and NK cell development. The γc signalling subunit for the IL-2, IL-4, IL-7, IL-9 and IL-15 receptors, localized to chromosome Xq13.1 became a candidate gene responsible for SCIDX1. Indeed, characterization of the γc gene in SCIDX1 patients has revealed a number of defects including frameshift mutations and mutations in the WSXWS and four cysteine motifs of the cytokine receptor-like domain (Noguchi *et al.*, 1993; Puck *et al.*, 1993; DiSanto *et al.*, 1994). Other cytokine receptors have also been associated with human genetic disorders such as GHR in Laron dwarfism (Amselem *et al.*, 1989). The p arm of Chromosome 19 has been well mapped in terms of genetic markers, genes and associated genetic disorders (Ashworth *et al.*, 1995), with cosmids covering almost the full length of the arm. Several disorders have been linked to this arm of chromosome 19, including acute lymphoblastic leukemia and lymphoid leukemia. These are disorders that could conceivably implicate modifications in cytokine receptors situated in the proposed chromosome 19 gene cluster. To our knowledge, there are at present no genetic disorders linked to the precise region of chromosome 19 where the hCLF-1 gene is situated. The identification of any genetic disorder linked to this region could however give an important insight into the functional role of hCLF-1.

The exon structure of the gene for hCLF-1 is in accordance with the structure of genes for other cytokine type-I receptor extracellular regions, with the signal peptide and Ig-like domain being encoded by a single exon and the two halves of the cytokine receptor-like domain being encoded by two exons each (Figures 3.6a and 1.7). The intron phases of the gene are also in accordance with the model for the evolution of type-I and type-II cytokine receptors proposed by Nakagawa *et al* (Nakagawa *et al.*, 1994; discussed in Chapter I) whereby phase 1 introns define the boundaries of the two pairs of exons encoding the N- and C-terminal regions of functionally important cytokine receptor-like domain. Each pair of exons therefore encodes a fibronectin type III domain which can essentially be duplicated or inserted without causing a frameshift. This model states that the cytokine receptor-like domain evolved from a common ancestral gene generated from a prototype exon encoding a primordial

Ig/fibronectin type-III like-module by internal duplication and intron insertion. Figure 1.7b describes schematically the putative model for receptor evolution.

The sequence of the gene was also used to investigate the potential to generate an alternatively spliced mRNA encoding a membrane-bound form of hCLF-1. It is quite feasible that a membrane-bound form indeed exists, as many of the soluble cytokine type-I receptors also have alternatively spliced membrane-bound forms. The detection of a larger mRNA transcript of approximately 4.5 kb in certain adult human tissues by Northern blot analysis in addition to the predominant 1.7 kb transcript (Figure 3.9a), raises the possibility of the existence of alternatively spliced transcripts encoding hCLF-1. Assuming that a transmembrane domain would be situated C-terminal of the cytokine receptor-like domain, we looked for a transmembrane domain-encoding exon situated in the genomic DNA lying downstream of the exon encoding the W-S-x-W-S motif (exon 6). Although we found no clear evidence of a putative transmembrane encoding domain situated between exons 6 and 7, 7 and 8 or 8 and 9, we cannot rule out that a splice alternative skips exon 9 (containing the putative stop codon of the soluble protein) or even exons 7, 8 and 9 to an exon or exons encoding a transmembrane domain further downstream.

It should be mentioned that when studying the expression of mCLF-1 mRNA in murine tissue by Northern blot analysis, we saw no evidence for the existence of mCLF-1 mRNA transcripts of higher molecular weights. It is possible that the larger mRNA transcript observed by Northern blot in certain human tissues such as adrenal cortex and testis is in fact an artifact resulting from the partial processing of hCLF-1 mRNA or even background with heterologous mRNA species, although due to the employment of high stringency hybridization and washing conditions, the latter seems less probable. The cloning of cDNA representing the larger mRNA transcripts will firstly ascertain whether they encode hCLF-1, and if so, whether they encode a soluble or membrane bound form of the protein. By studying the results of the Northern blot (Figure 3.9a), it is clear that certain tissues contain greater quantities of the larger transcript than others. In fact, in adrenal cortex and testis, the amount of the larger transcript appears to exceed the amount of transcript encoding soluble hCLF-1. Using such tissues as a source of mRNA for cDNA synthesis, the appropriate cloning

strategy such as library screening or 3'-RACE should allow the preferential identification of cDNA derived from the larger transcripts as opposed to the 1.7 kb hCLF-1 mRNA transcript.

Analysis of the genomic DNA sequence upstream of the first hCLF-1 encoding exon using the TF search program, which identifies DNA sequences that have a >85% homology with consensus binding motifs for transcription factors, identified a putative promoter region with a number of potential transcription factor binding sites (Figure 3.6b). The 300 bp region upstream of the ATG start codon was found to be extremely GC-rich with the presence of an 8 bp consensus cap signal for transcription initiation. The cap signal was found to be 20 bp upstream of the first bp of the clone 18 cDNA sequence suggesting that this was the point of transcription initiation. As a consequence of being GC-rich, the region of DNA immediately upstream of the cap signal contained putative binding sites for the transcription factor Sp-1 as well as putative binding sites for the GATA family of transcription factors. No obvious TATA or CAAT box was identified. GC-rich TATA-less regions have already been described for some of the promoter regions of cytokine type-I receptor genes, four examples of which include human and murine c-mpl (Mignotte *et al.*, 1994) and murine IL-3Rα (Miyajima *et al.*, 1995). Both of these contain putative Sp-1 and GATA binding sites, as well as murine LIFR (Chambers *et al.*, 1997).

In addition to putative Sp-1 and GATA binding sites, the 1 kb of DNA upstream of the cap signal contained several putative MZF-1 binding sites. Significantly, both the GATA family and MZF-1 are transcription factors implicated in the control of gene expression in hematopoietic cells. MZF-1 is a zinc finger protein playing an important role in myelopoiesis, and, more specifically, granulopoiesis. Its expression is upregulated in premyelocytic HL-60 cells stimulated with GM-CSF or retanoic acid (Hui *et al.*, 1995). As hCLF-1 expression in these cells is undetectable (Figure 3.9b), it would be interesting to see whether GM-CSF upregulates expression of its mRNA, thus confirming a role for MZF-1 in the control of hCLF-1 gene transcription. The GATA-binding family of proteins are transcription factors also playing a role in the control of a number of genes involved in hematopoiesis (reviewed in Orkin, 1992). The tissue distribution of hCLF-1 mRNA expression shows that it is expressed in

tissues with hematopoietic potential such as the bone marrow and thymus, indirectly pointing to the control of expression of hCLF-1 mRNA by the aforementioned transcription factors. Interestingly, GATA-binding proteins have also been shown to control gene expression in non-hematopoietic cells such as endothelial cells (Orkin, 1992). Indeed, they have more recently been shown to control gene expression in gastric parietal cells, responsible for acid secretion into the stomach (Maeda *et al.*, 1996). It is therefore interesting to note that hCLF-1 mRNA was strongly expressed in tissue derived from human stomach (Figure 3.9a).

In accordance with the observation that hCLF-1 mRNA was upregulated by TNF-α, IL-6 and IFN-γ in primary fibroblast cell cultures, the hCLF-1 gene promoter region was also found to contain several putative binding sites for the STAT family of transcription factors as well as NF-κB. The putative binding sites, six for the STAT family and four for NF-κB, were located throughout a 4.3 kb region upstream of the cap signal. TNF-α signalling has been shown to activate NF-κB to induce signal transduction (reviewed in Baeuerle and Henkel, 1994). STAT 1 and 2 activation by IFN-γ, and STAT 3 activation by IL-6 and have also been well documented (reviewed in Ihle *et al.*, 1995). Several putative binding sites for the C/EBP family of transcription factors (Wedel and Ziegler-Heitbrock, 1995) were also detected within the 4.5 kb of genomic DNA upstream of the cap signal. Interestingly, NF-IL-6 and NF-IL-6β (Isshiki *et al.*, 1990; Kinoshita *et al.*, 1992) are two members of this family whose expression is upregulated by the cytokines TNF-α and IL-6.

It should be stated that the identified binding sites are only putative and identified using computational analysis whereby motifs are identified showing a minimum homology of 85% with the consensus binding site for a given transcription factor. In order to elucidate the promoter regions important for transcription of hCLF-1 and the transcription factors involved, experiments will have to be designed targeting specific regions of the promoter for their importance. For example, the minimal promoter region required for transcriptional activation can be identified by the construction of different length deletion mutants for the promoter region controlling the expression of a reporter gene transfected into a cell line expressing hCLF-1 such as HEK 293. A

CAT activity assay would be appropriate to measure transcriptional activity. In a similar way, point mutations of critical bases involved in transcription factor binding within putative binding sites would allow the identification of those transcription factors playing a role in hCLF-1 expression. In the case of identifying transcription factors upregulating hCLF-1 message as a consequence of TNF-α, IL-6 and IFN-γ signalling, experiments involving promoter region mutagenesis would require the identification of a cell line in which the expression of hCLF-1 mRNA is upregulated by the aforementioned proinflammatory cytokines. Experiments localizing promoter regions essential for the activity of the same inflammatory mediating transcription factors by mutational analysis have been well documented. An example is the characterization of the promoter region of the IL-8 gene in terms of transcriptional activation by NF-κB and NF-IL-6 (Kunsch *et al.*, 1994).

(4.6) CLF-1 as a Cytokine Receptor Subunit

In order to facilitate the elucidation of a function for CLF-1, we generated both recombinant protein and α-hCLF-1 mAbs. Two different forms of recombinant hCLF-1 were expressed. Soluble hCLF-1 (rshCLF-1) with the mAb 179 recognition epitope and a six histidine tail was expressed in Sf9 insect cells using the baculovirus expression system, allowing the expression of large quantities of insect derived recombinant protein. Recombinant soluble protein with the same Ab recognition tag was also expressed in the human cell line HEK 293-E. This cell line has been stably transfected with a plasmid constitutively expressing the EBNA-1 protein, thus allowing the episomal replication of plasmids containing the Epstein-Barr virus (EBV) origin of replication. We therefore used the mammalian expression vector pEBS-PL (Bontron *et al.*, 1997) to express mammalian derived recombinant soluble hCLF-1. Recombinant membrane-bound hCLF-1 was expressed by generating a fusion protein cDNA construct containing hCLF-1 cDNA encoding the N-terminal region of the protein up to the end of the cytokine receptor-like domain (close to the W-S-x-W-S motif) followed by human IL-13Rα1 cDNA encoding the transmembrane and cytoplasmic tail of the receptor. The EE epitope tag, recognized by the α-EE mAb

(Grussenmeyer *et al.*, 1985) was inserted at the N-terminus of the protein. The recombinant fusion protein was expressed in insect cells using the baculovirus, and expression was verified by FACS analysis. A similar construct was also expressed in stably transfected CHO cells, the only difference being that the protein did not contain the EE epitope tag.

The expression of recombinant hCLF-1 on the surface of CHO cells allowed us to investigate whether hCLF-1 was involved as a ligand binding α-chain in the formation of a receptor complex for one of the cytokines signalling through LIFR and/or gp130. We chose this family of cytokines for two reasons. Firstly, CLF-1 shows close homology with the receptor subunits utilized by these cytokines and secondly, the α-chains complexing with gp130/LIFR have been shown to exist and function in soluble form (Muller-Newen *et al.*, 1996; Davis *et al.*, 1993a). In addition to this, there are thought to be additional as yet unidentified receptor subunits implicated in receptor complex formation for certain cytokines within this family, in particular IL-11 (Ward *et al.*, 1994; Paonessa *et al.*, 1995), LIF (Heymann *et al.*, 1996) and CT-1 (Robledo *et al.*, 1997).

The strategy employed was to incubate the hCLF-1 expressing or control CHO cells with recombinant soluble gp130 (rsgp130) and/or recombinant soluble LIFR (rsLIFR), with or without the appropriate cytokine in an attempt to reconstitute a cytokine/cytokine receptor complex involving hCLF-1. Complex formation was detected by FACS analysis with α-LIFR or α-gp130 polyclonal Abs. As a control to demonstrate the viability of the approach, we reconstituted the OSM and CT-1 cytokine/cytokine receptor complexes on the surface of HEK 293 cells using the appropriate cytokine and rsLIFR, exploiting the fact the gp130 is constitutively expressed on the surface of HEK 293 cells. α-LIFR Abs detected the binding of the rsLIFR to the cell surface in the presence of either OSM or CT-1.

We did not however detect any significant binding of LIFR or gp130 to CHO cells expressing hCLF-1 with any of the cytokines used when compared to control CHO transfectants, indicating that hCLF-1 played no part in the formation of receptor

complexes involving LIFR and/or gp130 for the cytokines IL-6, IL-11, LIF, CNTF, OSM or CT-1. These results must be interpreted with caution, however, as the artificial hCLF-1/hIL-13Rα1 fusion protein expressed on the CHO cell surface may be incapable of interacting with cytokines and/or receptor subunits due to structural constraints. A logical reciprocal experiment to confirm the above data would be to find a cell line expressing significant quantities of both gp130 and LIFR and look for rshCLF-1 binding in the presence of the appropriate cytokine. If binding was indeed observed with a particular cytokine, one could subsequently assess the contribution of gp130 and LIFR within the receptor complex by preincubating the cells with α-LIFR or α-gp130 polyclonal Abs, thus blocking the interaction between hCLF-1 and the appropriate signalling subunit.

(4.7) CLF-1 as a Covalently-Linked Molecule

Purified rshCLF-1 expressed in insect cells was used to immunize mice in order to generate hybridoma clones for α-hCLF-1 mAb production. A double screen by ELISA with rshCLF-1 followed by FACS analysis with insect cell expressed membrane-bound hCLF-1 fusion protein allowed the identification of one single hybridoma expressing a mAb recognizing hCLF-1 out of approximately 3000 screened. This very low efficiency is most probably explained by the extremely high homology existing between the human and mouse proteins. There are only 10 amino acid differences between human and mouse CLF-1 throughout the length of the protein, and in no position are there two or more consecutive amino acid differences (Figure 3.4). There is therefore a very limited potential for the mouse to raise an immune response against the human protein. Indeed, we failed in our attempted to raise antisera against hCLF-1 by injection of the purified protein in rabbits, suggesting that the high homology found between the two species results in the failure of the rabbit to mount an immune response (data not shown). The mAb raised against insect cell derived hCLF-1 was also capable of recognizing recombinant mammalian forms of hCLF-1 as demonstrated by ELISA with the soluble protein expressed in HEK 293-E cell

supernatants and FACS analysis with the membrane-bound form of the hCLF-1 expressed on stably transfected CHO cells (Figures 3.16a and 3.14a, respectively).

The α-hCLF-1 mAb and α-epitope tag mAbs were subsequently used to characterize recombinant forms of the hCLF-1 protein under non-reducing and reducing conditions. Purified soluble forms of hCLF-1 expressed in both insect and mammalian cells appeared to have a tendency to form covalently linked homomer. This comes form the observed molecular weight of the recombinant protein under non-reducing and reducing conditions (Figure 2.12). It therefore seems likely that the homomeric forms of the protein arise from the formation of disulphide bridges. Amino acid sequence analysis reveals that the mature protein contains nine cysteine residues. Four of these lie in the cytokine-receptor like domain, form intramolecular bonds and are functionally important. Two cysteines are present in the sequence C-terminal of the cytokine receptor-like domain, that have the potential to also create intramolecular bonds. The Ig-like domain has three cysteines. If we are to assume that two of these form an intramolecular disulphide bond, as is seen with other Ig-like domains (Williams and Barclay, 1988), this leaves a free cysteine with the capacity to form an intermolecular disulphide bond, either with another hCLF-1 molecule to form a homodimer, or a heterologous protein producing a heterodimer. The fact that covalently-linked forms of hCLF-1 of a molecular weight greater than that predicted for the homodimer were also visible under non-reducing conditions implies an aggregration of the protein, or a covalently bound complex of more than two hCLF-1 subunits. This in turn suggests that one or more of the other cysteine residues is also implicated in the formation of intermolecular disulphide bonds.

The majority of the membrane-bound hCLF-1 appears to form homodimeric protein. This is certainly the case for the insect cell expressed protein, where the α-EE mAb revealed a major band under non-reducing conditions at a size corresponding to the molecular weight of homodimeric hCLF-1. Although hCLF-1 homodimers are also expressed in mammalian cells, it is difficult to ascertain the ratio of monomeric to dimeric protein due to the absence of an Ab epitope tag and the uncertainty concerning the ability of α-hCLF-1 mAb to recognize monomeric protein (discussed

below). As the recombinant protein is actually a fusion protein between hCLF-1 and the transmembrane/cytoplasmic region of hIL-13Rα1, one could argue that disulphide bridges form within the region of the protein pertaining to hIL-13Rα1. This possibility can be ruled out as the region of hIL-13Rα1 present in the fusion protein contains no cysteine residues.

It should also be clarified that although the recombinant protein appears to form homomeric structures, we cannot be certain of this. There is a possibility that hCLF-1, when expressed in these cell types, interacts with a endogenously expressed heterologous protein to form a covalently linked heteromer, and it is indeed heteromeric protein and not homomeric protein that is detected by Western blot analysis under non-reducing conditions. To resolved this question therefore, one would need to separate monomeric hCLF-1 from higher-molecular weight covalently-linked hCLF-1 complexes within the purified hCLF-1 fraction using a suitable technique such as gel filtration. The non-monomeric fractions could then be analyzed for homogeneity following reduction using techniques which allow the separation of proteins not just by molecular weight but also by other physical properties, such as their overall charge or overall hydrophobicity. 2-dimensional gel electrophoresis is one such technique, separating proteins in the first dimension by isoelectric focusing according to the overall charge of the protein, and in a second dimension according to protein molecular weight.

The band pattern revealed by Western blot analysis of recombinant hCLF-1 with the α-hCLF-1 mAb demonstrated that the mAb had a tendency to preferentially recognize homomeric forms of hCLF-1 over the monomeric form. This was seen when directly comparing the intensity of bands revealed after the same length of exposure by either α-epitope tag or α-hCLF-1 mAbs on the same protein samples. Figure 3.12 shows that whereas the di- and multimeric forms of hCLF-1 were recognized with approximately the same intensity by the two mAbs, the α-epitope tag mAbs were remarkably more efficient at detecting monomeric hCLF-1 than was the α-hCLF-1 mAb. The most plausible explanation for this is that the α-hCLF-1 mAb recognizes a structural

epitope formed by two or more hCLF-1 proteins complexed together as opposed to a sequence epitope.

With this in mind, our failure to detect hCLF-1 in the supernatant of HEK 293 (by Western blot analysis or immunoprecipitation followed by Western blot analysis) or on the surface of HEK 293 cells by FACS analysis (data not shown) suggests one of two possibilities. Firstly, the hCLF-1 mRNA expression detected constitutively in HEK 293 cells is subject to a tight translational control, resulting in a block in the production of native hCLF-1 protein, or the production of such small amounts that they are undetectable using the α-hCLF-1 mAb. Secondly, and perhaps more plausible, is that the native hCLF-1 is not expressed in homomeric form. The protein could be secreted as monomer and therefore be less sensitive to detection by the α-hCLF-1 mAb. We have shown however that the protein has the ability to form covalently-linked dimers, and therefore, it is equally likely that in HEK 293 cells, hCLF-1 is expressed as a covalently-linked heteromeric molecule with another, heterologous subunit. As we suspect that the mAb recognizes a structural epitope formed by a complex of two or more hCLF-1 proteins, a heteromeric complex would also be inefficiently recognized by the α-hCLF-1 mAb. Furthermore, if naturally occuring CLF-1 does only exist as a heterodimer, it seems plausible that mAbs could be generated against an artificial hCLF-1 homodimer, such as is suspected for the α-hCLF-1 mAb.

Having characterized the recombinant protein expressed in both insect and mammalian cells, it is tempting to draw parallels for hCLF-1 with the IL-12 p40 subunit. P40 shares structural and sequence homology with members of the cytokine type-I receptor family, especially IL-6Rα and CNTFRα. It is composed of three domains, an Ig-like domain and two fibronectin type III modules resembling a cytokine receptor-like domain (Gearing and Cosman, 1991). When p40 is expressed in the same cells as the second IL-12 subunit, the cytokine-like molecule p35, a covalently-linked heterodimeric structure is formed, which is active in terms of IL-12 function (Wolf *et al.*, 1991; Gubler *et al.*, 1991). When expressed alone in transfected cells, the p40 subunit has been shown to form covalently-linked homodimers that

antagonize IL-12 signalling (Gillessen *et al.*, 1995; Ling *et al.*, 1995). It would appear then that hCLF-1 and IL-12 p40 have very similar structural characteristics. Both are members of the cytokine type-I receptor family, and both are capable of forming covalently-linked homodimers. The fact that p40 interacts with p35 to form functional IL-12 raises the possibility that hCLF-1 also interacts by covalent association with a second subunit to form a functionally active molecule. As mentioned above, our inability to detect hCLF-1 in the supernatant or on the surface of HEK 293 cells (which constitutively express the mRNA for hCLF-1) with an hCLF-1 mAb which preferentially recognizes the hCLF-1 homomer, suggests that hCLF-1 could indeed interact with a second, p35-like subunit also expressed in HEK 293 cells.

The HEK 293-E cell transfectants constitutively expressing Ab epitope tagged rshCLF-1 could therefore be an ideal tool for the identification of such an hCLF-1 associating protein. The transfected cell supernatants have been shown by Western blot analysis with mAb 179 to contain a majority of monomeric hCLF-1 detected at approximately 55-60 kD (Figure 3.12d). There is a band detected at approximately 110-120 kD which probably represents rshCLF-1 homodimer and/or a heterodimer between rshCLF-1 a covalently-associated heterologous protein. One approach would be to separate the monomeric and dimeric proteins according to the difference in their molecular weight by gel filtration, followed by purification of covalently-linked hCLF-1 complexes from fractions enriched in proteins of the appropriate molecular weight by immunoprecipitation or affinity chromatography with mAb 179. Following reduction, 2D gel electrophoresis or high performance liquid chromatography (HPLC) could be used to distinguish hCLF-1 from heterologous proteins representing candidate subunits of an hCLF-1 heterodimer, the candidate proteins then being subjected to sensitive methods of analysis such as mass spectrometry (Figure 4.1).

The IL-12 p35 subunit can also interact in a non-covalent manner with another member of the cytokine type-I receptor family, EBI-3 (Devergne *et al.*, 1997). This protein appears to exist uniquely in soluble form and like IL-12 p40, is most closely related to IL-6Rα and CNTFRα. As hCLF-1 is also structurally related to these receptor subunits, it could be worth investigating a potential covalent or non-covalent interaction with IL-12 p35.

(4.8) A Counterstructure for CLF-1

The IL-12 p40 homodimer binds to and blocks signalling through the IL-12 receptor (Gillessen *et al.*, 1995; Ling *et al.*, 1995). Bearing in mind the hypothesis that hCLF-1 behaves in a similar way to IL-12 p40, we investigated the ability of rshCLF-1 purified from insect cells to bind to the surface of a variety of cells and cell lines. Using either mAb 179 or the α-hCLF-1 mAb to detect rshCLF-1 on the cell surface by FACS analysis, we found a significant amount of binding to the B cell line RPMI 8226 and HEK 293-E cells (Figure 3.15). Indeed, in HEK 293-E cell transfectants expressing rshCLF-1, the soluble protein was not only found in the supernatant by ELISA, but also detected on the cell surface, suggesting that the rshCLF-1 was being secreted into the supernatant and subsequently binding to the HEK 293 cell surface in an autocrine-like fashion (Figure 3.16). To confirm that mammalian cell-derived rshCLF-1 was indeed recognizing the same counterstructure as the insect cell-derived protein, the HEK 293 cell transfectant supernatant was used to demonstrate binding of rshCLF-1 on the surface of 8226 cells (Figure 3.17). It appeared that a counterstructure for hCLF-1 is expressed on the surface of the two cell lines, RPMI 8226 and HEK 293-E.

The availability of cells lines expressing the counterstructure greatly facilitates its identification. An experimental approach involving chemical cross-linking of rshCLF-1 to the counterstructure and the subsequent purification of the complex by immunoprecipitation with mAb 179 or the α-hCLF-1 mAb should allow the initial characterization of the protein (Figure 4.2).

If one follows the hypothesis that hCLF-1 is an IL-12 p40 like molecule, it is logical to estimate that the counterstructure will consist of members of the cytokine type-I receptor family, similar to IL-12Rβ1 and β2 (to which IL-12 p40 homodimers bind), forming part of a receptor complex for a functional hCLF-1 dimer. It would therefore be interesting to separate homodimeric from monomeric hCLF-1 and see which of these two fractions has the capacity to bind to the hCLF-1 counterstructure,

remembering that only p40 homodimer and not monomer binds to IL-12R (Gillessen *et al*, 1995; Ling *et al*, 1995).

The counterstructure for hCLF-1 was subsequently found to be upregulated on PBL-derived monocytes following a 24 hour stimulation with either LPS or one of the proinflammatory cytokines IL-1β, TNF-α, IL-6, or IFN-γ (Figure 3.18). The most striking upregulation of expression was seen following treatment with IFN-γ. This pattern of expression correlates well with the expression pattern of hCLF-1 mRNA seen in primary fibroblasts cultures. Here it was also upregulated by the same proinflammatory cytokines, with the exception of IL-1β, which failed to upregulate mRNA for hCLF-1 in fibroblasts but did upregulated expression of its counterstructure in monocytes. It should however by pointed out that the monocytes were treated with IL-1β for 24 hours whereas the fibroblasts were treated for 48 hours. This difference in time of exposure to the cytokine could account for such differences, with prolonged exposure to IL-1β upregulating certain negative regulators of IL-1 signalling, as discussed earlier.

The upregulation of both hCLF-1 and its counterstructure with such proinflammatory cytokines is a very significant observation in terms of immune regulation. In certain types of immune response, such as those raised against viral and bacterial infection, activated T and NK cells produce IFN-γ as well as TNF-α. These proinflammatory cytokines stimulate monocyte-derived macrophages at the site of inflammation to produce, amongst other molecules, the proinflammatory cytokines IL-1, IL-6 and TNF-α. Therefore T cell derived IFN-γ and TNF-α would have the effect of upregulating the hCLF-1 counterstructure on monocyte-derived macrophages. At the same time, one could imagine that the proinflammatory cytokines are capable of stimulating fibroblasts situated in the surrounding tissue to produce hCLF-1. The local production of hCLF-1 and the upregulation of expression of its counterstructure suggests that the functional hCLF-1 molecule, whether it be heterodimeric, homodimeric or in another form, plays a role in immune regulation by signalling through its functional receptor on monocyte-derived macrophages during an immune response. To support this hypothesis, fibroblasts are known to be important immune-

effector cells producing a variety of soluble mediators of immune function (reviewed in Gauldie *et al.*, 1992).

The fact that hCLF-1 mRNA was seen to be expressed in secondary lymphoid tissues such as spleen and lymph node, as well as the thymus further implicates a role for hCLF-1 in immune regulation. Although the expression of a counterstructure for hCLF-1 was not detected on PBL-derived T cells following treatment with the non-physiological stimuli PMA and ionomycin, it could be worthwhile investigating the upregulation of the hCLF-1 counterstructure on T cells in response to more physiological stimuli such as IL-2, IL-4, IL-12 or stimulation through CD3 and/or CD28. In a similar fashion, expression on purified B cells from a source such as human tonsils should be investigated in response to stimuli such as IL-4, CD40 ligand, IL-6, TNF-α and surface IgM cross-linking amongst others.

In conclusion, this report has described the identification, cloning and initial characterization of CLF-1, a novel soluble protein pertaining to the cytokine type-I receptor family. From the data presented, it would appear that CLF-1 has a role to play in fetal development and immune regulation. Due to its similarity to the p40 subunit of IL-12, it is possible that hCLF-1 interacts in solution with other heterologous proteins to give a functionally active molecule signalling through a counterstructure expressed on activated monocytes. The elucidation of hCLF-1 in its functionally active form and the identification and characterization of the hCLF-1 counterstructure will help to determine the functions of the molecule.

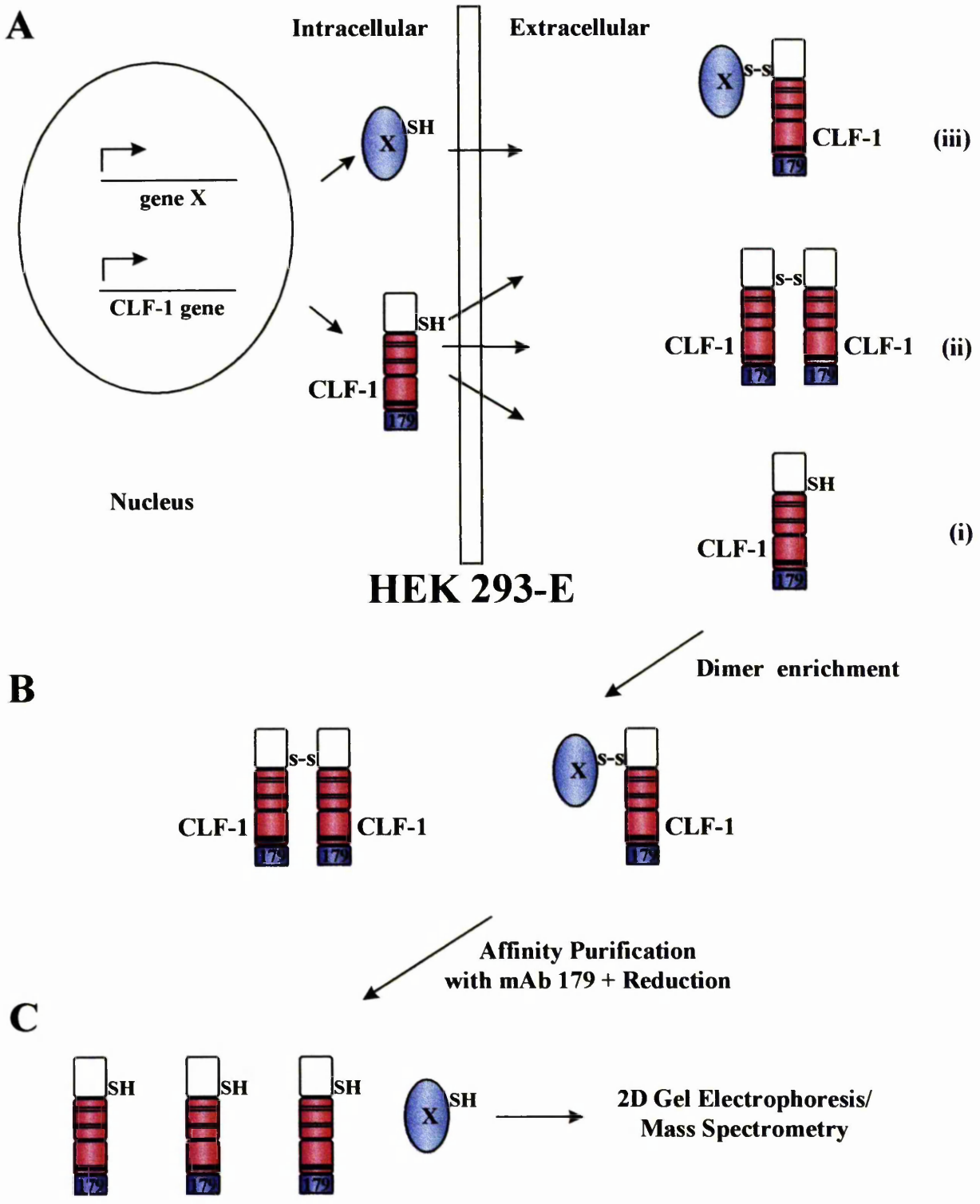


Figure 4.1. Characterization of Covalently-Linked hCLF-1 Heterodimers.

(A) Expression of rshCLF-1 in HEK 293-E cells results in the secretion of (i) monomer (ii) homodimer and (iii) possibly heterodimer.

(B) Enrichment of dimeric forms of hCLF-1 using techniques such as gel filtration.

(C) Affinity purification of dimeric hCLF-1 complexes using mAb 179 followed by reduction allows the separation and identification of heterologous proteins which can by analyzed by 2D gel electrophoresis and mass spectrometry.

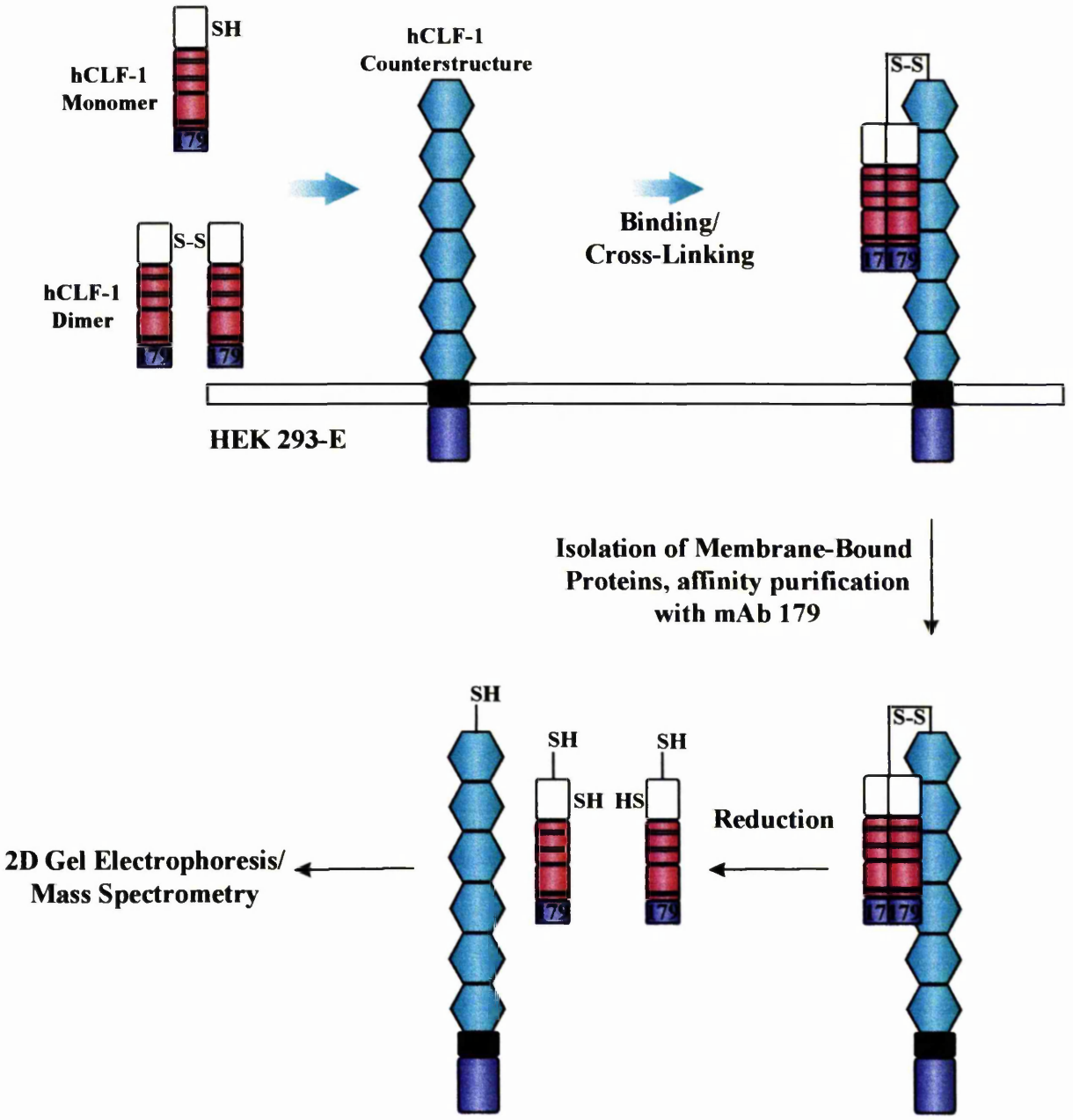


Figure 4.2. Characterization of a Counterstructure for hCLF-1

rshCLF-1 is chemically bound to the surface of HEK 293 cells using a suitable reducible cross-linking reagent. Membrane-bound proteins are then isolated and rshCLF-1 complexes affinity purified using mAb 179. Reduction allows the association of hCLF-1 from its counterstructure, with separation of the proteins by 2D gel electrophoresis and subsequent analysis by mass spectrometry.

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APPENDIX

1. CLF-1, A NOVEL SOLUBLE PROTEIN SHARES HOMOLOGY WITH MEMBERS OF THE CYTOKINE TYPE I RECEPTOR FAMILY.
   ELSON, G.C.A., *ET AL.*, *J. IMMUNOL.*, IN PRESS

2. A NOVEL 4-KB INTERLEUKIN-13 RECEPTOR α MRNA EXPRESSED IN HUMAN B, T AND ENDOTHELIAL CELLS ENCODING AN ALTERNATE TYPE-II INTERLEUKIN-4/INTERLEUKIN-13 RECEPTOR.
   GAUCHAT, J-F., *ET AL.*, EUR. J. IMMUNOL., (1997). **27**;971-978

3. TISSUE LOCALISATION OF IGE CLASS SWITCHING DURING AN ALLERGIC RESPONSE TO AN AERO-ALLERGEN

4. BUFFERS AND SOLUTIONS

Appendix 1

Cytokine-Like Factor-1, a Novel Soluble Protein, Shares Homology with Members of the Cytokine Type I Receptor Family[1]

Greg C. A. Elson,[2] Pierre Graber,[3] Christophe Losberger,[3] Suzanne Herren,[3] Denise Gretener,[3] Laurent N. Menoud,[3] Timothy N. C. Wells,[3] Marie H. Kosco-Vilbois,[3,4] and Jean-François Gauchat[2]

In this report we describe the identification, cloning, and expression pattern of human cytokine-like factor 1 (hCLF-1) and the identification and cloning of its murine homologue. They were identified from expressed sequence tags using amino acid sequences from conserved regions of the cytokine type I receptor family. Human CLF-1 and murine CLF-1 shared 96% amino acid identity and significant homology with many cytokine type I receptors. CLF-1 is a secreted protein, suggesting that it is either a soluble subunit within a cytokine receptor complex, like the soluble form of the IL-6R α-chain, or a subunit of a multimeric cytokine, e.g., IL-12 p40. The highest levels of hCLF-1 mRNA were observed in lymph node, spleen, thymus, appendix, placenta, stomach, bone marrow, and fetal lung, with constitutive expression of CLF-1 mRNA detected in a human kidney fibroblastic cell line. In fibroblast primary cell cultures, CLF-1 mRNA was up-regulated by TNF-α, IL-6, and IFN-γ. Western blot analysis of recombinant forms of hCLF-1 showed that the protein has the tendency to form covalently linked di- and tetramers. These results suggest that CLF-1 is a novel soluble cytokine receptor subunit or part of a novel cytokine complex, possibly playing a regulatory role in the immune system and during fetal development. *The Journal of Immunology,* **1998, 161: 1371–1379.**

Cytokines and growth hormones are secreted molecules controlling important cell functions, such as proliferation, differentiation, and survival (1–3). They exert their effects via specific receptors located on the target cell surface. These receptors are grouped into families according to both structural and amino acid sequence similarities (4–6). The cytokine receptor superfamily comprises the receptors for many families, including IFN, TNF, and hemopoietic growth factors. The cytokine type I receptors make up the largest subclass of this superfamily and are characterized by the presence of a conserved extracellular region of approximately 200 amino acids containing two fibronectin type III modules (7). This region, known as the cytokine receptor-like domain, has been shown to play an essential role in receptor function in terms of ligand binding (8) and receptor complex formation (9). It is characterized by four conserved cysteine residues in the first module and a W-S-x-W-S motif in the second (5).

The receptors for the IL-6-type cytokines (10), including IL-6, ciliary neurotrophic factor (CNTF),[5] IL-11, leukemia inhibitory factor (LIF), oncostatin M, and cardiotropin-1 are typical of the cytokine type I receptor family, forming multicomponent complexes comprising ligand binding and shared signaling subunits (11). Pleiotrophy and redundancy within this family can be accounted for by the fact that the receptors for many of these cytokines use the signaling subunits gp130 (12) or the LIF receptor (13), both with long intracytoplasmic tails and involved in the induction of the JAK-STAT signaling pathway (14, 15). Receptor specificity is provided by the ligand binding subunit. In the case of the IL-6, IL-11, and CNTF receptors (IL-6R, IL-11R, and CNTFR), a soluble form of this subunit is capable of rendering cells sensitive to the appropriate ligand provided the correct signaling subunit (gp130 or LIF receptor) is present on the cell surface (16–19). In the case of the receptors for LIF and cardiotropin-1, LIF receptor serves as both a ligand binding and a signaling subunit (13, 20, 21), as is the case for gp130 within the oncostatin M receptor complex (22, 23).

Another interesting configuration is that demonstrated by the organization of IL-12 and its receptor. IL-12 has been shown to be composed of two covalently linked polypeptide chains known as p35 and p40. Certain regions of p35 share homology with IL-6 and granulocyte CSF, while p40 shares homology with the extracellular region of IL-6R and CNTFR (24). p40 also displays the hallmarks of the cytokine type I receptor family, containing the four conserved cysteines residues and a WSEWAS sequence, resembling the W-S-x-W-S motif (5). This has led to the suggestion that IL-12 may have originated from a cytokine/soluble cytokine receptor complex, a suggestion supported by the homology between the IL-12R and gp130 (25).

Department of Immunology, Geneva Biomedical Research Institute, Glaxo Wellcome Research and Development, Plan-les-Ouates, Switzerland

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[1] The human CLF-1 mRNA sequence has been deposited in GenBank (accession no. AF059293).

[2] Current address: Centre d'Immunologie Pierre Fabre, 74164 Saint-Julien-en-Genevois, France.

[3] Current address: Serono Pharmaceutical Research Institute, Ares Serono Research and Development, 1228 Plan-les-Ouates, Switzerland.

[4] Address correspondence and reprint requests to Dr. M. H. Kosco-Vilbois, Serono Pharmaceutical Research Institute, 14 Chemin des Aulx, 1228 Plan-les-Ouates, Switzerland.

[5] Abbreviations used in this paper: CNTF, ciliary neurotrophic factor; LIF, leukemia inhibitory factor; CNTFR, ciliary neurotrophic factor receptor; CLF-1, cytokine-like factor-1; EST, expressed sequence tag; hCLF-1, human cytokine-like factor-1; mCLF-1, murine cytokine-like factor-1; hIL-13Rα1, human IL-13 receptor α1-chain.

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In this report, bioinformatics was used to identify human and murine homologues of a novel cytokine-like factor, CLF-1, which was subsequently cloned and analyzed. Human and murine CLF-1 share 96% amino acid identity, suggesting an important function for the protein. CLF-1 shares significant homology with many members of the cytokine type I receptor family. Analysis of CLF-1 mRNA distribution and recombinant forms of the CLF-1 protein provide the initial characterization for this novel member of the cytokine type I receptor family.

Materials and Methods

Cloning of human and mouse cDNA for CLF-1

The amino acid sequence N K L C F D D N K L W S D W S E A Q S I G K E Q N from the murine IL-13R (26) was used to search the GenBank database with expressed sequence tags (ESTs) using TBLASTN to identify cDNAs encoding receptors with related sequence. BLASTN searches of the GenBank database with the identified EST allowed the identification of ESTs with overlapping sequence homology.

The mouse cDNA clone 479043 was purchased from Research Genetics (Birmingham, AL). 5′-RACE on poly(A)$^+$ RNA extracted from mouse lung allowed the cloning of 310 bp of the murine cDNA upstream of the 5′ end of the cDNA clone using the Marathon cDNA amplification kit (Clontech, Palo Alto, CA) following the manufacturer's guidelines. The primer used in the PCR amplification (along with the adaptor protein-1 primer provided) was 5′-CGTACCACCTCAGCTTGTACTTG-3′. PCR products were cloned into the vector pCRII (Invitrogen, Leek, The Netherlands), and colonies were screened by hybridization with the oligonucleotide probe 5′-AAGGATCTCACGTGCCGCTGGACACCGGGT-3′.

A portion of the hCLF-1 cDNA was amplified by PCR using cDNA derived from human lung poly(A)$^+$ RNA with the primers 5′-ACCGC CGAGGGCCTCTACTG-3′ and 5′TTGAGGGAGTAGTTGGTGTG GAGG-3′. The amplified product was cloned into the vector pCRII and subsequently used as a ^{32}P-labeled probe to screen a human placental cDNA library in gt10. The largest cDNA identified (1740 bp) was recloned in pBluescript II SK$^-$ (Stratagene, La Jolla, CA).

DNA and protein sequence analysis

Sequences obtained from cDNA clones as well as all relevant ESTs were imported into and analyzed by the sequence analysis software Sequencher (Genecodes, Ann Arbor, MI). The signalP server (htpp://www.cbs.dtu.dk/signalp/cbssignalp.html) was used to identify the predicted cleavage site of the signal peptide for CLF-1. DNA and amino acid sequence alignments as well as prediction of hydrophobic regions were analyzed with the Wisconsin package version 8.1 (Genetics Computer Group, Madison, WI).

Source of cells and culture conditions

The cell lines HEK-293 and CHO were obtained from the American Type Culture Collection (Manassas, VA). Palmar fibromatosis lesion and mammary gland fibroblasts were gifts from Prof. G. Gabbiani (University Medical Center, Geneva, Switzerland). Tonsillar fibroblasts were derived from whole tonsils obtained from surgery following a previously described protocol (27). All the above cell types were maintained in DMEM and Ham's F-12 nutrient solution supplemented with 10% FCS. Fibroblastic cells and cell lines were stimulated with the appropriate cytokines at a final concentration of 10 ng/ml/cytokine.

Detection of the CLF-1 gene by Southern blot analysis

Genomic DNA was isolated from cell lines or tissues with the DNAzol reagent (Life Technologies, Grand Island, NY) following the manufacturer's guidelines. The source of the genomic DNA was as follows: HEK-293 human fibroblastic cells, African green monkey COS-7 cells (American Type Culture Collection), C57BL/6 mouse tails, rat liver, rabbit liver, cow liver, and chicken embryo liver. Aliquots of genomic DNA (5 μg) were digested with either *Sac*I or *Bam*HI and subjected to Southern blot analysis using a ^{32}P-labeled 1.2-kb fragment of hCLF-1 cDNA as a probe. Hybridization was performed in 5× SSC, 5× Denhardt's solution, and 5% SDS at 55°C. Filters were washed with 0.1× SSC and 1% SDS at 55°C and exposed at −70°C.

Detection of hCLF-1 mRNA by Northern blot analysis

The same ^{32}P-labeled 310-bp hCLF-1 cDNA fragment that was used to screen the human placental cDNA library (see above) was used to identify hCLF-1 mRNA transcripts in the Human, Human II, Human Immune System, Human Endocrine, and Human Fetal Multiple Tissue Northern Blots (Clontech). Hybridization was performed in ExpressHyb solution (Clontech). Hybridization and washing were performed according to the manufacturer's guidelines.

Detection of hCLF-1 mRNA by RT-PCR

Total RNA was purified from the appropriate source using TRIzol reagent solution (Life Technologies) following the manufacturer's guidelines. Five micrograms of total RNA was reverse transcribed using the first strand cDNA synthesis kit (Pharmacia LKB Biotechnology, Uppsala, Sweden) following the manufacturer's guidelines, and 1/10th of the cDNA was amplified by 32 cycles of PCR using the primers 5′-GGATCAGGAGCCCA CACAGC-3′ and 5′-AGCGGCAGGTCAAGTCCTTC-3′ for hCLF-1 cDNA and 5′-GGCGACGAGGCCCAGAGCAAG-3′ and 5′-CGATTTC CCGCTCGGCCGTG-3′ for β-actin cDNA. PCR products were analyzed by agarose gel electrophoresis.

Production of recombinant soluble hCLF-1

To generate recombinant soluble protein, hCLF-1 cDNA in pBluescript II SK$^-$ was digested with *Eco*RI and *Stu*I and cloned into pFASTBAC-1 (Life Technologies) digested with the same enzymes. The oligonucleotides 5′-CGCGTGCCTCGAACCCTACACCGCCTGCGACCATCACCATCA CCATCACTGA-3′ and 5′-TCAGTGATGGTGATGGTGATGGTCGCA GGCGGTGTAGGGTTCGAGGCA-3′ were annealed and cloned into pFASTBAC-1 containing the hCLF-1 cDNA digested with *Mlu*I and *Stu*I. The annealed oligonucleotides encoded six histidines and the CLEPYTACD tag, an epitope recognized by mAb 179 (Affymax, Palo Alto, CA), at the 3′ end of the hCLF-1 cDNA.

Recombinant baculovirus was generated using the BAC-TO-BAC kit (Life Technologies) and was used to infect Sf9 cells (American Type Culture Collection) expanded in SF900II medium (Life Technologies). Recombinant protein was purified from the Sf9 culture medium using a column packed with 50 ml of Ni-NTA (Qiagen, Basel, Switzerland) following the manufacturer's guidelines. The recovered fraction contained approximately 30% monomeric hCLF-1 as determined by SDS-PAGE under reducing conditions.

Expression of cell membrane-bound hCLF-1 in Sf9 cells

To generate membrane-bound hCLF-1, pBluescript II SK$^-$ containing hCLF-1 cDNA was digested with *Sac*II. The human IL-13Rα1 (hIL-13Rα1) (28) transmembrane and cytoplasmic domains were amplified by PCR from pBluescript II SK$^-$ containing the hIL-13Rα1 cDNA using the oligonucleotides 5′-TCCCCGCGGTACATAACCATGTTACTCAT TGTT-3′ and 5′-TCCCCGCGGGAA TTCCATCACTGAGAGGCTTTC TT-3′. The PCR product was digested by *Sac*II and ligated into the *Sac*II-digested pBluescript II SK$^-$ containing hCLF-1 cDNA. DNA encoding the polyoma virus middle T Ag epitope EYMPME (EE tag) (29) was inserted at the 5′ end of the hCLF-1 cDNA by digesting the plasmid with *Xho*I and *Bam*HI and cloning into these restriction enzyme sites the two annealed oligonucleotides (5′-TCGAACTAGTGAATACATGCCAATGGAAGCC CACACAGCTGTGATCAGTCCCCAG-3′ and 5′-GATCCTGGGGACT GATCACAGCTGTGTGGGCTTCCATTGGCATGTATTCACTAGT-3′). The cDNA encoding the tagged fusion protein was excised from pBluescript II SK$^-$ by digestion with *Spe*I (whose site was subsequently blunt ended using the Klenow fragment of DNA polymerase I) and *Eco*RI, and ligated into pFASTBAC-1 (containing the mellitin signal peptide) digested with *Bsp*120I (whose site was subsequently blunt ended using the Klenow fragment of DNA polymerase I) and *Eco*RI.

Recombinant baculovirus was generated using the BAC-TO-BAC kit and used to infect Sf9 cells. Expression of the fusion protein on the surface of the Sf9 cell line was confirmed at 24 and 48 h by flow cytometry using a mAb recognizing the EE tag. Briefly, cells were washed with FACS buffer (1% BSA and 0.01% sodium azide in PBS) and successively incubated for 30 min with the anti-EE mAb at 10 μg/ml in FACS buffer and FITC-labeled sheep anti-mouse F(ab′)$_2$ fragments (Silenius Laboratories, Hawthorn, Australia) diluted 1/100 in FACS buffer. Fluorescence was measured using a FACSCalibur (Becton Dickinson, Erembodeggen, Belgium).

Expression of cell membrane-bound hCLF-1 in CHO cells

To generate membrane-bound hCLF-1 expressed in mammalian cells, pBluescript II SK$^-$ containing the hCLF-1/hIL-13Rα1 cDNA without the EE tag was digested with *Bam*HI and *Eco*RI and inserted into the expression vector pCDNA3 (Invitrogen) at the same restriction enzyme sites. To insert the native signal peptide from hCLF-1 at the 5′ end of the fusion construct, pBluescript II SK$^-$ containing the full-length hCLF-1 was digested with *Kpn*I and *Bam*HI, and the excised fragment (encoding the

signal peptide) was inserted into pCDNA3 containing the hCLF-1/hIL13Rα1 cDNA digested with the same enzymes. The expression vector was linearized and introduced into CHO cells by electroporation using previously described conditions (28).

Transfected cells were selected using 500 μg/ml geneticin (Life Technologies). Control cells were transfected with pCDNA3 containing the full-length hIL-13Rα1 cDNA. Expression of hCLF-1 on the surface of the CHO cells was detected by flow cytometry with a mAb recognizing hCLF-1 (see below) using the conditions described above. Positive cells were selected and sorted by flow cytometry and replaced in culture. After three rounds of sorting, a population of CHO cells expressing high levels of membrane-bound hCLF-1 was obtained.

Generation of mouse anti-hCLF-1 mAbs

A BALB/c mouse was immunized on days 0, 7, and 28 s.c. in the limbs and behind the neck with 100 μg of purified recombinant hCLF-1 in MPL+TDM emulsion (RIBI, Inotech, Dottikon, Switzerland) per injection. Three days after the final injection, the draining lymph nodes were obtained, and the tissue was digested using a DNase and collagenase mixture as reported previously (30). The resulting cell suspension was resuspended at 10^6 cells/ml and fused with Sp2 myeloma cells using a standard protocol (31). The hybridomas were selected in hypoxanthine-aminopterin-thymidine medium, and 7 to 10 days after fusion, the supernatants were harvested for screening.

Screening hybridoma supernatants for anti-hCLF mAbs

Ninety-six-well plates (Falcon 3912, Becton Dickinson Labware Europe, Meylan, France) were coated at 4°C with 1 μg/ml recombinant hCLF-1 in carbonate buffer, pH 9.6. Plates were washed, blocked with 1% BSA in PBS, incubated for 2 h with 200 μl of hybridoma supernatant, and washed again. Bound Ab was revealed with horseradish peroxidase-coupled goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL). Specificity was checked using an ELISA with recombinant soluble hIL-13Rα1[6] that had the same recognition tags as recombinant hCLF-1 at 1 μg/ml. Specific positive supernatants were further screened by flow cytometry on Sf9 cells either mock infected or infected with baculovirus encoding the membrane-bound hCLF-1/hIL13Rα1 fusion protein or on transfected CHO cells expressing hIL-13Rα1 or the hCLF-1/hIL-13Rα1 fusion protein.

Purification of anti-hCLF-1 mAbs

Abs were purified by chromatography on protein A-Sepharose Fast Flow in PBS and eluted in 0.1 M citrate, pH 4.5. Eluates were then subjected to gel filtration on Superdex-200 (Pharmacia) equilibrated in PBS.

Detection of hCLF-1 by Western blot analysis

The supernatants or cell lysates containing the recombinant protein were diluted 1/1 with Tris-glycine-SDS sample buffer (Novex, San Diego, CA) and heated to 95°C for 5 min. To reduce proteins, β-ME was added to the samples to a final concentration of 375 mM. The proteins were resolved on an 8% polyacrylamide gel (Novex) and electrotransferred onto a nitrocellulose membrane (32). The membrane was blocked in PBS containing 5% dried milk and 0.15% Tween-20 for 1 h at room temperature and incubated for 1 h at room temperature with PBS containing 2.5% dried milk and 5 μg/ml of the appropriate mAb. Bound mAb was detected with horseradish peroxidase-labeled sheep anti-mouse Ab and ECL (Amersham Life Sciences, Aylesbury, U.K.) following the manufacturer's guidelines.

Results

Identification and characterization of a new CLF

The GenBank database with ESTs was searched using TBLASTN with a 20-amino acid sequence surrounding the W-S-x-W-S motif of murine IL-13R (26). ESTs showing significant homology were then translated, and the open reading frames were used to search the Swissprot database using BLASTP for homologous proteins. The amino acid sequence from the murine EST W66776 shared a high level of homology with the prolactin receptor (33) and the receptors for the IL-6-type cytokines (10). Using the sequence of W66776 to search the GenBank database allowed the identification of overlapping homologous sequences (of both murine and human origin), which, in turn, were run against the GenBank database to identify more overlapping ESTs (Table I). This allowed the assembly of overlapping sequences encoding the human and mouse cDNAs. The cDNA clone 479043, which gave rise to the mouse EST found furthest 5′ in the sequence assembly was obtained from the IMAGE consortium. After sequencing, it was found to contain an insert of 1 kb, including a 3′ poly(A) tail. The rapid amplification of 5′ cDNA ends on murine lung cDNA allowed the cloning of a further 308 bp upstream.

Table I. *ESTs corresponding to human and mouse CLF-1 in GenBank*

Receptor	EST Accession Number	Source of Origin
Mouse	AA014965	Placenta
	AA039053	Embryo
	AA049278	Embryo
	AA049280	Embryo
	AA2700365	Embryo
	W17583	Embryo
	W66776	Embryo
Human	AA042914	Pregnant uterus
	AA043001	Pregnant uterus
	AA121532	Pregnant uterus
	AA127694	Pregnant uterus
	AA377893	Synovial sarcoma
	AA406406	Melanocyte/fetal heart/uterus
	H14009	Chromosome 19
	N78873	Fetal lung
	R87407	Brain
	W37175	Fetal lung
	W46603	Fibroblast
	W46604	Fibroblast

To clone the human cDNA encoding CLF-1, a 310-bp PCR product was amplified from human lung cDNA using primers designed from the human ESTs. The PCR product was, in turn, used as a probe to screen a human placental cDNA library, resulting in the isolation of a full-length clone of 1740 bp, which included a 3′ poly(A) tail. The human cDNA encoded a precursor protein of 422 amino acids with a putative signal peptide of 37 amino acids. In vitro translation revealed that the AUG codon coding the methionine at the start of the putative signal peptide was indeed used to initiate translation (data not shown).

The murine cDNA encoded a protein of 383 amino acids. The cDNA was incomplete at the 5′ end as the first amino acid of the translated sequence aligned to amino acid 39 of the human sequence, and no starting methionine or putative signal peptide could be identified. Both mouse and human CLF-1 contained 11 cysteine residues and 6 potential *N*-linked glycosylation sites. Sequence analysis of the human and murine cDNAs showed 85% nucleic acid identity and 96% amino acid identity (Fig. 1*A*). Human and murine CLF-1 showed close homology to the prolactin receptor and receptors of the IL-6-type cytokines (10) (Table II). The N-terminal region of both sequences appeared to represent an Ig-like domain, most closely resembling the C2-set sequence (34). This domain was followed by two fibronectin type III modules of approximately 100 amino acid residues each. Alignment of the human and mouse amino acid sequences to members of the IL-6-type cytokine receptor family showed regions of conserved homology within these two functionally important modules known as the cytokine receptor-like domain, and both sequences contained the highly conserved four cysteine residues and the W-S-x-W-S motif characteristic of this domain (5) (Fig. 1*B*). We were unable to identify any transmembrane domains within the amino acid sequence of the mature proteins or hydrophobic region at the C-terminus of the sequence, characteristic of glycosyl phosphatidylinositol-anchored proteins such as the CNTFR α-chain (CNTFRα)

[6] P. Graber et al. The distribution of IL-13Rα1 expression by B cells, T cells and monocytes and its regulation by IL-13 and IL-4. *Submitted for publication.*

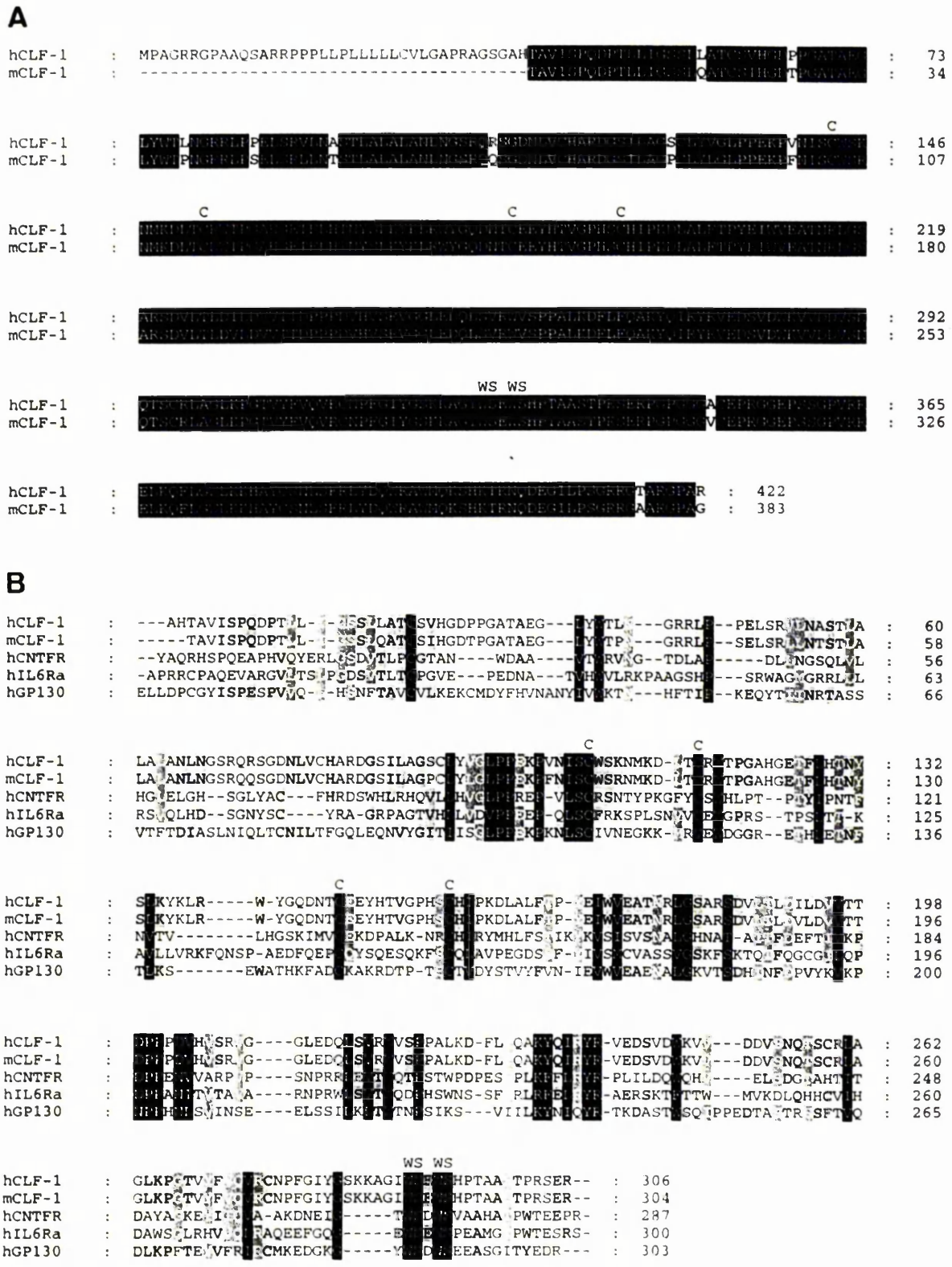

FIGURE 1. *A*, Alignment of the human and mouse CLF-1 amino acid sequences. Residues sharing identity between the two proteins are boxed in black. Unknown residues at the N-terminus of mCLF-1 due to the incomplete cloning of the 5′ end of the cDNA are indicated by a dash. The four conserved cysteines and the W-S-x-W-S motif are indicated above the alignment. *B*, Alignment of the Ig-like and cytokine receptor-like domains of human and mouse CLF-1 with related cytokine type I receptors. Regions of amino acid identity are boxed in black. The four conserved cysteines and the W-S-x-W-S motif are indicated above the alignment. hgp130, human gp130.

Table II. *Homology between CLF-1 and IL-6-type cytokine receptors*[a]

Cloned Receptor	Alignment with	Identical Residues	Similar Residues
Human	hGP130	87/306	120/306
	hCNTFR	79/306	102/306
	hGCSFR	85/306	102/306
	hIL-6Rα	71/306	104/306
Mouse	mGP130	89/304	125/304
	mGCSFR	81/304	115/304
	mIL-6Rα	71/304	107/304

[a] Homology was ascertained using Bestfit in the Wisconsin package, version 8.1.

(35). This suggested that the cloned human and mouse cDNAs encode soluble proteins.

Characterization of the hCLF-1 gene

Using the hCLF-1 cDNA sequence to search the GenBank database we identified the hCLF-1 gene sequence, under the accession number AC003112. The gene was encoded by nine exons contained within a 14-kb region of the chromosome 19-specific cosmid R30292 mapping to 19p12 (confirming the location predicted by the EST with accession no. H14009). The erythropoietin receptor (36), sharing homology with CLF-1, and the soluble protein EBI3 (37) are the only known members of the cytokine type I receptor family shown to be localized on this arm of chromosome 19. The intron/exon organization of the gene for hCLF-1 is in agreement with the predicted structure of the domains within the hCLF-1 protein, following the rule proposed for the cytokine receptor-like domain (Fig. 2) (38). The first exon encodes the signal peptide, exon 2 encodes the Ig-like domain, exons 3 and 4 encode the N-terminal cytokine receptor-like domain, and exons 5 and 6 encode the C-terminal cytokine receptor-like domain. Exons 7 and 8 showed no homology with any other family members, while exon 9 had the stop codon close to its 5′ end and the polyadenylation signal close to its 3′ end. We were unable to identify anything resembling an exon encoding a putative transmembrane domain within the 17.4 kb of DNA sequence contained within the contig downstream of the exon containing the W-S-x-W-S motif (exon 6).

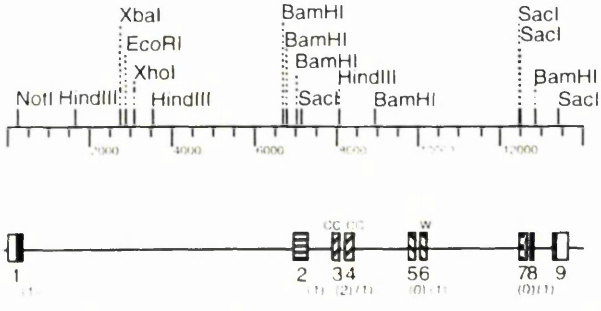

FIGURE 2. Genomic organization of the hCLF-1 gene. Each box represents one exon, and the exons are numbered. Numbers in brackets represent the phases of the introns (38). Open boxes indicate noncoding regions, the solid box represents the signal peptide. The horizontally hatched box represents the Ig-like domain. Shaded boxes represent the N-terminal and C-terminal cytokine receptor-like domains. The conserved cysteine doublets (CC) and the W-S-x-W-S motif (W) are also indicated. Checkered boxes represent the coding region downstream of the C-terminal cytokine receptor-like domain. A partial restriction map covering the 14-kb DNA region is shown above.

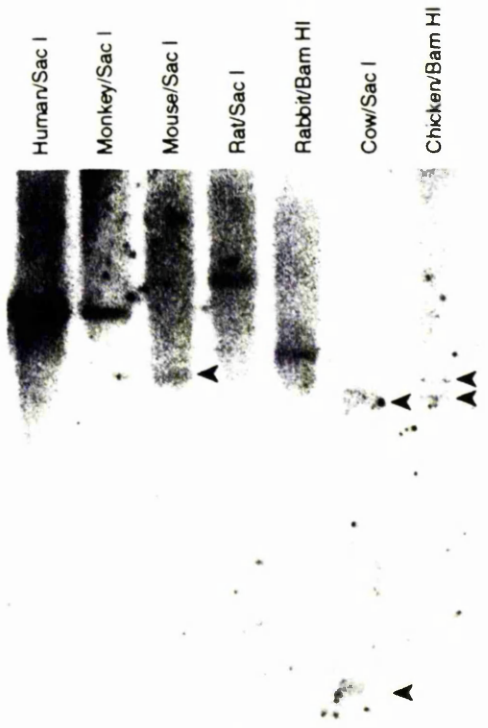

FIGURE 3. Genomic Southern blot analysis of CLF-1 in five animal species. Five micrograms of genomic DNA was digested with either *Sac*I or *Bam*HI as indicated, migrated on a 0.8% agarose gel, transferred to a nylon filter, and hybridized with the radiolabeled human cDNA probe. The autoradiography exposure time was 48 h at −70°C.

Conservation of the CLF-1 gene

The very high amino acid identity between the human and mouse protein sequences for CLF-1 suggested that the gene had been highly conserved during recent evolution. As the cDNAs encoding human and mouse CLF-1 shared 85% homology, we examined the conservation of the gene in a number of animal species by Southern blot analysis of genomic DNA using an hCLF-1 cDNA probe (Fig. 3). As expected, human and monkey genomic DNA gave the strongest signals, but signals could be detected in all six mammalian species studied, with faint bands also seen with chicken genomic DNA, indicating that the gene is highly conserved within these species.

Tissue and cellular distribution of hCLF-1 mRNA

hCLF-1 mRNA expression was studied in human tissues by Northern blot analysis. The mRNA transcript migrated as a 1.7-kb species, a size close to that predicted from the clone obtained by library screening. Expression of the 1.7-kb transcript could be detected in several tissues (Fig. 4). The strongest expression of the hCLF-1 mRNA was detected in the spleen, thymus, lymph node, appendix, bone marrow, stomach, placenta, heart, thyroid, and ovary. Interestingly, a strong level of expression was also detected in fetal lung. We studied the level of mRNA expression by Northern blot analysis in several cell lines, including the fibroblastic cell line HEK 293, the monocyte cell line THP-1, JY lymphoblastoid cells, RPMI 8226 myeloma cells, the mast cell line HMC-1, HBE-140 bronchial epithelial cells, and HUVEC. A detectable level of expression could be seen in JY cells, HMC-1 cells, and PMA-stimulated THP-1 cells. The strongest expression, however, was detected in HEK-293 cells (data not shown).

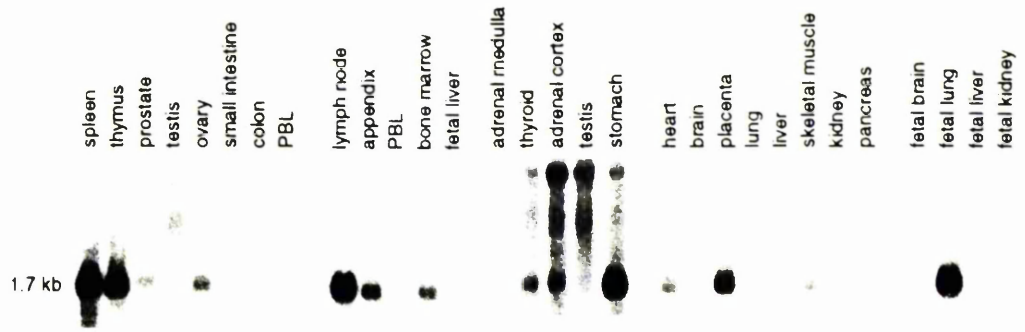

FIGURE 4. Northern blot analysis of hCLF-1 mRNA in human organs and tissues. Poly(A)$^+$ RNA blots were hybridized with a radiolabeled hCLF-1 cDNA probe. The autoradiography exposure time was 24 h at −70°C.

The expression of hCLF-1 mRNA in HEK 293 cells and fibroblast primary cell cultures was studied by RT-PCR following stimulation with proinflammatory cytokines. Whereas no effect was seen on the constitutive mRNA level found in HEK 293 cells (Fig. 5*A*), an up-regulation of hCLF-1 mRNA was observed in all three fibroblast primary cell cultures under stimulation with IFN-γ. TNF-α up-regulated hCLF-1 mRNA in palmar fibromatosis lesion and mammary gland fibroblasts, while IL-6 up-regulated hCLF-1 mRNA in palmar fibromatosis lesion and tonsillar fibroblasts. Interestingly, IL-1β had a down-regulatory effect on hCLF-1 mRNA production in two of the three fibroblast cultures (Fig. 5, *B–D*).

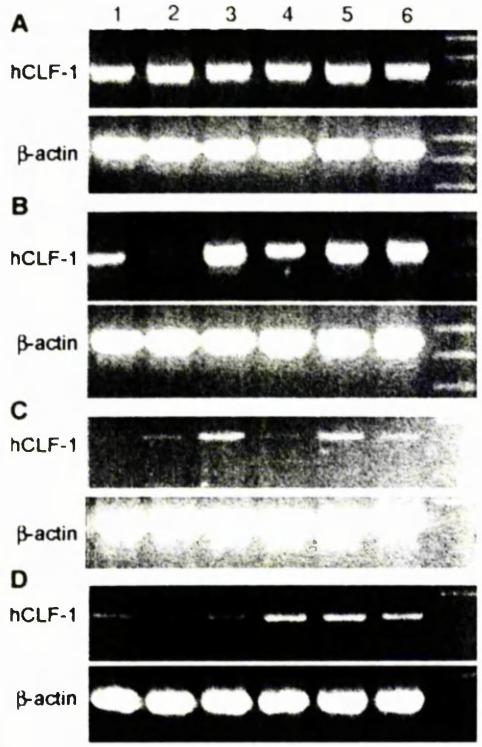

FIGURE 5. Detection of expression of hCLF-1 mRNA in human fibroblast cells and cell lines by RT-PCR. Total RNA was extracted from the cells and subjected to RT-PCR using primers specific for the hCLF-1 cDNA sequence. As a positive control for RNA preparation and RT-PCR was performed with primers specific for the β actin cDNA sequence. Products were analyzed on a 1.5% agarose gel. Cells were cultured in the presence of 1) medium alone, 2) IL-1β, 3) TNF-α, 4) IL-6, 5) IFN-γ, or 6) a mixture of TNF-α, IL-1β, and IL-6 for 48 h. *A*, HEK 293 cells. *B*, palmar fibromatosis lesion fibroblasts. *C*, mammary gland fibroblasts. *D*, tonsillar fibroblasts.

Generation of mAbs recognizing hCLF-1

A BALB/c mouse was immunized with recombinant soluble hCLF-1 generated with the baculovirus expression system. Supernatants from the resulting hybridomas were tested by ELISA using the same recombinant protein. An ELISA using a recombinant soluble form of hIL-13Rα1 with the same recognition tags as the recombinant hCLF-1 was performed on the positive hybridomas to eliminate Abs raised against the tags or conserved cytokine receptor epitopes. Specific positive mAbs were then tested by flow cytometry using Sf9 cells infected with baculovirus encoding the hCLF-1/hIL-13Rα1 membrane-bound fusion protein or CHO cells stably transfected with an expression vector encoding the same fusion construct. A strong signal was seen with one hybridoma, $92A_{10}$, with no signal detected on mock-transfected Sf9 cells or hIL-13Rα1-transfected CHO cells (Fig. 6). The mAb expressed by this hybridoma was determined to be an IgG2a.

Characterization of recombinant forms of hCLF-1

The recombinant forms of hCLF-1 expressed in Sf9 and CHO cells were examined by Western blot analysis under nonreducing and reducing conditions using either the appropriate anti-tag mAb or the anti-hCLF-1 mAb. The mAb 179 revealed that in the supernatant of Sf9 cells infected with baculovirus encoding recombinant soluble hCLF-1, the protein formed covalently linked tetramers. This was demonstrated by the presence of a high molecular mass band (~200 kDa) detected under nonreducing conditions. Under reducing conditions, only the band corresponding to monomeric protein could be detected (~48–49 kDa; Fig. 7*A*). A similar pattern was detected using the anti-FE mAb on the cell lysate of baculovirus-infected Sf9 cells expressing the hCLF-1/hIL-13Rα1 fusion protein. Western blotting revealed a predominant band corresponding to covalently linked homodimeric protein (~120 kDa) when performed under nonreducing conditions. A larger band with a molecular mass of ~200kDa was also detected, probably corresponding to covalently linked tetrameric protein. Under reducing conditions, the only band detected was that which corresponded to the monomeric form of the protein (~60 kDa; Fig. 7*B*). The hCLF-1/hIL-13Rα1 fusion protein was detected in the cell lysate of stably transfected CHO cells using the anti-hCLF-1 mAb. Again, under nonreducing conditions the protein formed covalently linked homodimers of 130 to 150 kDa (Fig. 7*C*).

In contrast to the di- and tetrameric forms of hCLF-1, the monomeric form of the protein was not recognized by the anti-hCLF-1 mAb by Western blotting (Fig. 7, *A–C*).

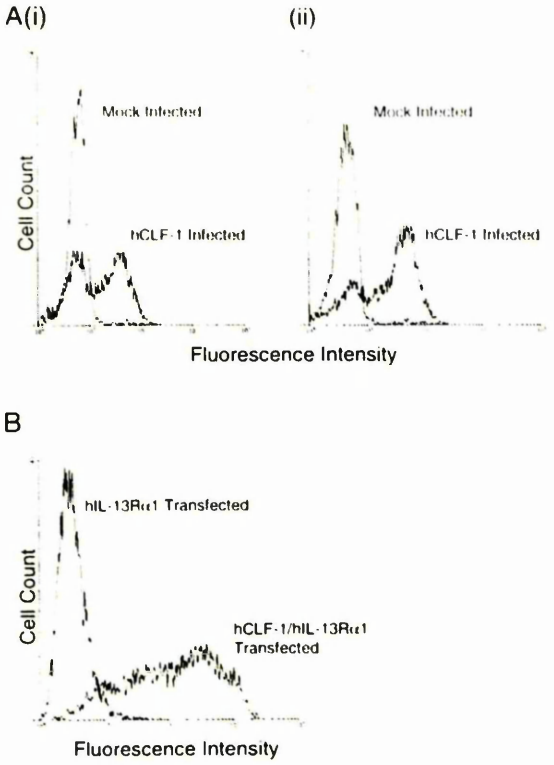

FIGURE 6. Expression of an hCLF-1/hIL-13Rα1 membrane-bound fusion protein on the surface of Sf9 cells and CHO cells and its detection using an anti-hCLF-1 mAb. *A*, Sf9 cells were either mock infected or infected with recombinant baculovirus encoding the fusion protein. *i*) Cells were analyzed at 24 h postinfection by flow cytometry using the anti-EE mAb and an FITC-conjugated sheep anti-mouse secondary Ab. *ii*) Cells were analyzed at 24 h postinfection by flow cytometry using the anti-hCLF-1 mAb and an FITC conjugated sheep anti-mouse secondary Ab. *B*, CHO were stably transfected with an expression construct expressing either hIL-13Rα1 as a negative control or the hCLF-1/hIL-13Rα1 fusion construct. Expression was detected by flow cytometry using the anti-hCLF-1 mAb, and an FITC-conjugated sheep anti-mouse secondary Ab.

Discussion

In this report we describe the identification and cloning of the human and mouse homologues of a novel gene, CLF-1. The human cDNA encodes a 422-amino acid precursor protein with a 37-amino acid putative signal peptide. Based on their deduced amino acid sequences, human and murine CLF-1 would appear to constitute new members of the cytokine type I receptor family. The expression pattern of hCLF-1 suggests a role for the protein in the immune system and in fetal development.

Human and murine CLF-1 were seen to share 96% amino acid identity (Fig. 1*A*) and also show highest homology with the prolactin receptor (33) and gp130 (12). It is interesting to note that amino acid identity between human and mouse gp130 is 77%, and that between the human and mouse prolactin receptors is 69%. Southern blot analysis, using hCLF-1 cDNA as a probe, indicated that the high conservation of the CLF-1 gene sequence between human and mouse can be extended to monkey, rat, rabbit, cow, and chicken (Fig. 3). The hybridization and washing conditions were relatively stringent, thus reducing the risk of nonspecific signals. This extremely high level of conservation during recent evolution suggests a functionally important role for CLF-1.

Information concerning the homology between the N-terminal region of human and mouse CLF-1 and the extracellular region of members of the cytokine type I receptor family is provided in Figure 1*B* and Table II. The four conserved cysteines and the W-S-x-W-S motif are shown above the alignment in Figure 1*B*. These sequence motifs characterize members of the type I cytokine receptor family (5) and are located within two fibronectin type III modules (7) that have been shown to play an important role in receptor function (8, 9). This homology suggests that CLF-1 constitutes a new member of the cytokine type I receptor family. The CLF-1 gene structure is also typical of those found within this family of receptors (Fig. 2) (38).

Although CLF-1 appears to exist uniquely as a soluble protein, there are numerous examples of soluble forms of receptor subunits in the cytokine type I receptor family. These soluble subunits can exhibit either antagonistic effects in terms of ligand signaling, such as those shown by soluble gp130 (39, 40) and the soluble IL-5Rα chain (IL-5Rα) (41), or agonistic effects, such as those shown by the IL-6Rα chain (IL-6Rα) (16, 17), the IL-11Rα chain (IL-11Rα) (18), and CNTFRα (19). Receptor chains within the cytokine type I receptor family generally function as receptor signaling or ligand binding subunits (11). If we are to assume that CLF-1 exists solely in soluble form and is indeed a soluble receptor chain, it is likely that it serves as a ligand binding subunit. Further analysis must be

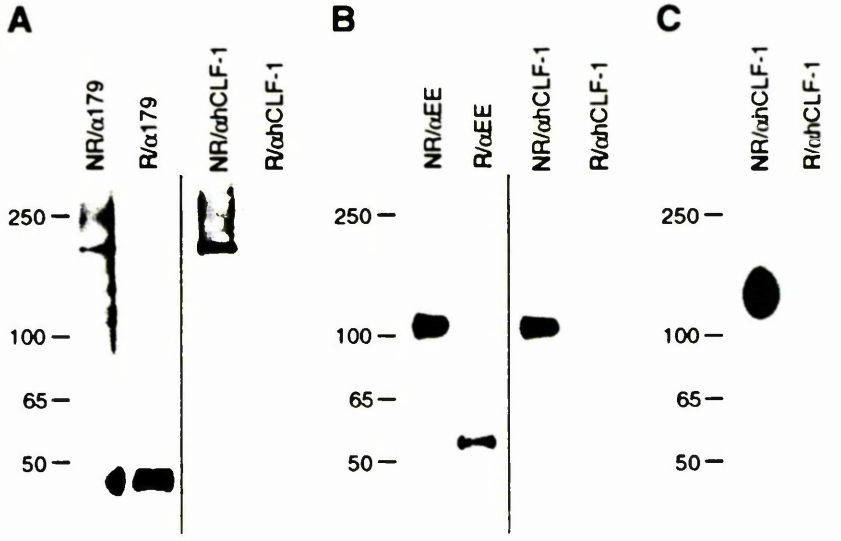

FIGURE 7. Detection of recombinant hCLF-1 by Western blot analysis. Proteins were resolved under either nonreducing (NR) or reducing (R) conditions on 8% polyacrylamide gels and electrotransferred to nitrocellulose filters. *A*, Recombinant soluble hCLF-1 expressed in Sf9 cell supernatants following baculovirus infection, detected with either the mAb 179 (α179) or the anti hCLF-1 mAb (αhCLF-1). *B*, Recombinant membrane bound hCLF-1/hIL-13Rα1 fusion protein expressed on Sf9 cells following baculovirus infection, detected with either the anti-EE mAb (αEE) or the anti hCLF-1 mAb (αhCLF-1). *C*, Recombinant membrane bound hCLF-1/hIL-13Rα1 fusion protein expressed on stably transfected CHO cells detected with the anti hCLF-1 mAb (αhCLF-1).

undertaken to determine whether any of the known ligands is able to bind CLF-1.

Human CLF-1 mRNA is predominantly expressed in adult spleen, thymus, lymph node, appendix, bone marrow, stomach, placenta, heart, thyroid, and ovary (Fig. 4). We can also predict that the transcript is well expressed in pregnant female uterus when looking at the source of origin of hCLF-1 ESTs (Table I). Expression of hCLF-1 mRNA was also studied by Northern blot analysis in a number of different primary and transformed cells. Strongest expression was observed in the fibroblastic cell line HEK 293 with detectable levels in the B cell line JY, the immature mast cell line HMC-1, and the monocyte cell line THP-1 following stimulation (data not shown). The expression seen in HEK 293 cells prompted us to study the modulation of hCLF-1 mRNA levels in HEK 293 cells and fibroblast primary cell cultures in response to the proinflammatory cytokines IL-1β, TNF-α, IL-6, and IFN-γ. Although these cytokines had no real effect on the constitutive level of hCLF-1 mRNA expression in HEK 293 cells, a significant up-regulation of mRNA was observed in the primary fibroblast cultures in response to IFN-γ with an up-regulation in two of the three cultures in response to TNF-α and IL-6, as detected by RT-PCR (Fig. 5, *A–D*). The tissue distribution of hCLF-1 mRNA and its up-regulation by proinflammatory cytokines in fibroblast cultures suggest that hCLF-1 may be involved in regulation of the immune system during an inflammatory response.

A strong expression of hCLF-1 mRNA was also observed in fetal lung (Fig. 4). In addition, mCLF-1 mRNA transcripts were detected by Northern blot analysis in total mouse embryos on days 11, 15, and 17 days postcoitum, with no expression seen on day 9 postcoitum (data not shown). Furthermore, many ESTs encoding human and mouse CLF-1 were derived from fetal tissues (Table I). This points to an additional role for CLF-1 in mediating regulatory signals during fetal development.

Western blot analysis of the recombinant forms of hCLF-1 baculovirus-infected Sf9 cell culture supernatants, membrane-bound hCLF-1 fusion protein from baculovirus-infected Sf9 cell lysates and membrane-bound hCLF-1 fusion protein from stably transfected CHO cell lysates under nonreducing and reducing conditions, provided two significant observations (Fig. 7, *A–C*). Firstly, the recombinant protein had a tendency to exist in covalently linked homomeric forms when comparing the difference in molecular mass under nonreducing or reducing conditions. Secondly, it appeared that the anti-hCLF-1 mAb recognized the nonreduced form of the protein, while reduced (i.e., monomeric) forms of the protein were poorly recognized. These findings suggested that the mAb bound to a structural epitope, formed when two or more hCLF-1 chains are covalently linked together, as opposed to a sequence epitope on the protein. Furthermore, HEK 293 cells (which constitutively expressed hCLF-1 mRNA) were used in an attempt to detect hCLF-1 protein in its native form using the mAb raised against the recombinant protein. No evidence was obtained of hCLF-1 being expressed on the cell surface by flow cytometry or in the cell supernatant by Western blot or immunoprecipitation, suggesting one of three possibilities. Firstly, a tight translational control could result in a level of protein production below the threshold of detection of the Ab. Secondly, hCLF-1 could be secreted from the cell in monomeric form, rendering itself undetectable to the mAb. Thirdly, hCLF-1 could form a covalently linked heterodimeric structure with a different subunit, again rendering itself invisible to the mAb.

The cytokine IL-12 is composed of two disulfide-linked *N*-glycosylated polypeptides of approximately 40 kDa (p40) and 35 kDa (p35). Certain regions of the p35 amino acid sequence show homology to IL-6 and granulocyte CSF, suggesting that p35 is derived from a cytokine-like molecule. The p40 amino acid sequence, however, shows homology with the extracellular domain of members of the cytokine type I receptor family, most notably with IL-6Rα and CNTFRα. Interestingly, recombinant p40 can form a covalently linked homodimer, a form that in the mouse acts as a physiologic antagonist of IL-12, probably competing for IL-12 binding to its receptor (24, 42, 43).

From these observations, it is tempting to compare CLF-1 with IL-12 p40. Firstly, both proteins share homology with the extracellular regions of IL-6Rα and CNTFR. Secondly, when recombinant protein is expressed in cells transfected with the appropriate cDNA, both CLF-1 and IL-12 p40 can form covalently linked homodimers. If, indeed, as with the IL-12 p40 subunit, CLF-1 exists in its native functional form as a covalently linked heterodimer, this could well account for our failure to detect the protein using the anti-hCLF-1 mAb in cells expressing hCLF-1 mRNA as discussed earlier.

The generation of Abs recognizing the native form of CLF-1 could allow the purification and thus structural characterization of the native protein, leading to the identification of covalently linked heterologous subunits. The identification of a cell membrane-bound counterstructure for CLF-1 is ongoing and will also further the characterization of this novel protein.

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Appendix 2

Jean-François Gauchat[1], Edith Schlagenhauf[2], Ning-Ping Feng[2], René Moser[2], Mat Yamage[2], Pascale Jeannin[1], Sami Alouani[1], Greg Elson[1], Luigi D. Notarangelo[3], Timothy Wells[1], Hans-Pietro Eugster[2] and Jean-Yves Bonnefoy[1]

[1] Geneva Biomedical Research Institute, Glaxo Wellcome Research and Development, Plan-les-Ouates, Switzerland
[2] Institute of Toxicology, Swiss Federal Institute of Technology, Schwerzenbach, Switzerland
[3] Dipartimento Materno-Infantile, Università di Brescia, Brescia, Italy

A novel 4-kb interleukin-13 receptor α mRNA expressed in human B, T, and endothelial cells encoding an alternate type-II interleukin-4/ interleukin-13 receptor

A 4 kb human interleukin-13 receptor (IL-13R) chain cDNA was cloned from a B cell cDNA library using expressed sequence tags homologous to mouse IL-13R as probes. The deduced protein sequence shows a significant level of sequence identity with the IL-5R and the human IL-13R identified recently by expression cloning. The cytoplasmic region is very highly conserved between human and mouse homologs and contains a consensus binding motif for a signal transducer and activator of transcription. The cDNA encodes a protein binding IL-13 when expressed alone which participates in a receptor complex for both IL-4 and IL-13 when expressed in conjunction with the IL-4Rα chain. Transcripts for this IL-13R chain could be detected in most tissues and organs studied and in T, B, endothelial cells, basophilic, immature mast cell, and monocytic cell lines. The pattern of expression is different from the other recently cloned IL-13R molecule, and correlates with sites where IL-4 and IL-13 signaling is known to occur. This novel receptor is therefore likely to be implicated in reactions involved in IgE responses, T helper 2 differentiation, adhesion of leukocytes to endothelium, and therefore in pathological phenomena such as allergy, atopy, and asthma.

1 Introduction

IL-4 and IL-13 are related cytokines that share numerous biological activities [1, 2]. Both have been shown to be important in the induction of IgE and IgG4 synthesis in human B cells [3–6] and the differentiation of Th2 cells [7, 8]. Among the events leading to IgE synthesis, induction of germ-line ε RNA transcription, which precedes the class switching to the corresponding H chain C region, has been shown to be triggered by IL-4 and IL-13 [6, 9, 10].

Th cells can be subdivided into two major subtypes according to their polarized cytokine production capacities [7]: Th1 cells can secrete IFN-γ, whereas Th2 cells are able to produce IL-4 and IL-5 [7]. Th2 cells are thought to be implicated in the development of atopy, allergy, and some forms of asthma [11, 12]. Th2 differentiation can be induced by IL-4 [7]. IL-13 was first considered to be inactive on T cells [2]; however, it has been shown recently to induce Th2 differentiation in mice [8].

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J.-F. Gauchat and E. Schlagenhauf contributed equally to this work.

Correspondence: Jean-François Gauchat, Immunology Department Geneva Biomedical Research Institute, Glaxo Wellcome Research and Development S.A., 14 chemin des Aulx, CH-1228 Plan-les-Ouates, Switzerland
Fax: + 41-22 794 69 65; e-mail: jfmg28928@ggr.co.uk

Abbreviations: γ_c: Common γ chain **HUVEC:** Human umbilical cord endothelial cell **EST:** Expressed sequence tag

Key words: Interleukin-13 / Interleukin 4 / Interleukin-13 receptor / Interleukin-4 receptor

In addition to their effects on lymphocytes, IL-4 and IL-13 share the ability to inhibit the production of inflammatory cytokines by macrophages [1, 2] and to up-regulate the expression of the vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells [13–15], leading to adhesion and transendothelial migration of very late antigen 4 (VLA-4)-expressing leukocytes [16]. This provides a basis for selective extravasation of eosinophils, the hallmark of the pathological inflammation seen in allergy and asthma [17–19]. These two cytokines activate common cytokine receptor signaling pathways involving 4PS/IRS-1 [20–24] and the signal transducer and activator of transcription 6 (STAT-6 [25–27]). Inactivation of STAT-6 has been demonstrated to affect both IL-4 and IL-13 signaling and to block IL-4 and IL-13-induced IgE synthesis or Th2 differentiation [28–30].

Studies have been conducted to examine whether these two cytokines share a receptor or receptor subunits [2, 15, 31–33]. The IL-4R is composed of two chains, the IL-4Rα chain and the common γ chain (γ_c). The γ_c is a subunit of the receptors of many of the other four-helix bundle cytokines, such as IL-2, IL-4, IL-7, IL-9, and IL-15 [34, 35]. The IL-4Rα chain alone forms a tight complex with its ligand, whereas the γ_c was thought to be mainly responsible for signal transduction. However, IL-4- and IL-13-induced responses could be observed in cells which naturally do not express γ_c or in lymphocytes obtained from severe combined immunodeficiency (SCID) patients who are deficient for γ_c [15, 33, 36–38]. It has therefore been proposed that a second form of an IL-4R exists which would be activated by both IL-4 and IL-13 (IL-4R type II/ IL-13R [39]). Recently, cDNA encoding proteins sharing high levels of sequence identity with known cytokine receptors and capable of binding IL-13 have been cloned [40, 41]. The first, which we refer to as IL-13Rα1, was cloned in the mouse [40]. The second was identified by expression cloning using cDNA derived from a human cell

line [41]. This receptor has only 26% sequence identity with mouse IL-13Rα1. We refer to this receptor as IL-13Rα2. The highest protein sequence identity of mouse IL-13Rα1 and human IL-13Rα2 is with the IL-5Rα chain (26–27%). Similar to the IL-5Rα, both appear to consist of three fibronectin type III domains [42]. The sequence identity with the IL-4Rα chain (14%) is not higher than that of the other hematopoietic cytokine receptors.

We have cloned the cDNA for a human homolog of mouse IL-13Rα1. Transcript analysis of human IL-13R chain showed a receptor specific pattern of expression in different organs, primary cells, and cell lines. Transient expression of the cloned cDNA in COS7 cells confers low-affinity binding of human IL-13. Co-expression with IL-4Rα generates majority of low and a limited number of high-affinity receptor complexes for IL-4 and IL-13.

2 Materials and methods

2.1 Cytokines and reagents

The CHO-derived human IL-4 was a gift of Dr. W. Sebald (Theodor-Boveri-Institute, Würzburg, Germany). The human IL-4Rα chain-specific mAb S697 was kindly provided by Dr. J. Banchereau (Schering Plough, Dardilly, France). CHO-derived human IL-13 was generously provided by Dr. G. Zurawski (DNAX Research Institute, Palo Alto, CA).

2.2 Cells and tissue culture conditions

The mast cell line HMC1 was from Dr. J. Butterfield (Mayo Clinic, Rochester, MN) and was maintained in Dulbecco's modified Eagle's medium and Ham's F-12 nutrient solution supplemented with 10% FCS. The source and culture conditions of the cell lines EOL-3, HFB-1, JY, and the human T cell clones JF7 and MAB.PHA.3.6 have been described [43–45]. The EBV-transformed B cell lines from the Janus kinase 3 (JAK-3)-deficient SCID patient CM [46] and γ_c-deficient X-SCID patient VA (L. D. Notarangelo, unpublished data) were maintained in RPMI 1640 supplemented with 10% fetal calf serum. The cell lines COS7, HEK-293, RPMI-8226, Jijoye, Daudi, and IM-9 were obtained from the American Type Culture Collection (Rockville, MD) and cultured according to their specifications. B cells were purified from tonsillar mononuclear cells following depletion of T cells by rosetting with SRBC (>98% $CD20^+$). Peripheral blood T cells were purified by rosetting (>95% $CD3^+$). T cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated AB^+ human serum (CTS, Annemasse, France) and stimulated with 10 μg/ml immobilized anti-CD3 (Immunotech, Marseille, France) alone or in combination with 10 μg/ml immobilized anti-CD28 (Immunotech) or PMA (10 nM). Human umbilical vein endothelial cells (HUVEC) were prepared and cultured as described [15].

2.3 Cloning of human IL-13Rα1 chain cDNA

The Gene Bank expressed sequence tag (EST) database was searched using the mouse IL-13Rα extracellular domain protein sequence as the query. Two EST with open reading frames with high degrees of amino acid identity were identified (EST H57074 and H89334). The EST sequence was used to design the PCR primers CTGAGCTACATGAAGTGTTCTTGGCTCCCT and CAGAGTTTGTCATCCTCATAGCATAACTTA and the probe AATACCACTCCCGACACTAACTATACTCTC.

Poly(A)$^+$ RNA was isolated [47] from tonsillar B cells incubated for 5 days in the presence of 200 U/ml rIL-4 and 1 μg/ml anti-CD40 mAb. cDNA was synthesized using SuperScript II reverse transcriptase (Life Technologies AG, Basel, Switzerland) and used as the template for PCR. The amplification product which hybridized with the radiolabeled probe was cloned into the vector pCRII (Invitrogen, Leek, The Netherlands) and analyzed by DNA sequencing. Double-stranded cDNA was synthesized, ligated to Not I-Eco RI adapters (Pharmacia LKB Biotechnology, Uppsala, Sweden), size-selected by Sephacryl S 400 gel filtration and cloned in the Eco RI site of λgt10. The amplified cDNA library was screened using the human IL-13Rα1 chain cDNA fragment as a probe. The largest cDNA (named 3.1) was recloned into pBluescript II SK (Stratagene, Zürich, Switzerland) and fully sequenced.

2.4 Subcloning of the human IL-13Rα1 and IL-4Rα cDNA in the expression vector pCDLSRα296

A segment of cDNA 3.1 containing the open reading frame was isolated from the vector pBluescript II SK with the restriction enzymes Not I and Xba I. The cDNA fragment was made blunt-ended using T4 DNA polymerase and recloned in the expression vector pCDLSRα296 [48]. The IL-4Rα cDNA was isolated from pBSKS-hIL-4R ([49], a kind gift of Dr. J.-P. Galizzi, Schering-Plough, Dardilly, France) using the restriction enzymes Eco RV and Nco I. The insert was recloned in pCDLSRα296 as described for the IL-13Rα1 cDNA.

2.5 Detection of the IL-13Rα1 and IL-13Rα2 chain mRNA by Northern blot assays

We used either cDNA or cRNA probes for the detection of the IL-13Rα1 and IL-13Rα2 chain mRNA. IL-13Rα1 chain ^{32}P-labeled cDNA probes were obtained by labeling the Xmn I-Sca I restriction fragment of the cDNA by random hexamer priming [50]. To produce a cRNA probe specific for IL-13Rα1 chain, the same fragment was recloned in the Eco RV site of pBluescript II SK and used for the transcription of a ^{32}P-labeled probe [51]. To produce probes specific for the IL-13Rα2 chain, the cDNA, amplified by PCR from the tonsillar B cell cDNA using the primers GGAGAAATGGCTTTCGTTTGCTTGGCTATC and TACCATGTCTCTTGATATGGAAAGTCTTCA, was cloned in the pCRII vector. cDNA probes were labeled with ^{32}P by random hexamer priming [50]. cRNA probes labeled with ^{32}P were generated by transcribing the region of the cDNA insert in 3′ of the Eco RV restriction site [51]. Total RNA was isolated either by the guanidium thiocyanate-CsCl procedure [47] or using TRIzol (Life Technologies). Poly(A)$^+$ RNA was isolated by one cycle of oligo(dT)-cellulose chromatography.

The Human Immune System Multiple Tissue Northern blot and the Human Multiple Tissue Northern blot (Clontech, Palo Alto, CA) were hybridized with the IL-13Rα1 or IL-13Rα2 and with actin cDNA [10] probes in ExpressHyb Hybridization Solution (Clontech). The other Northern blot assays were performed as described previously [52]. Membranes were stained with methylene blue to localize and quantify rRNA before hybridization.

2.6 Transfection with the IL-13Rα1 and IL-4Rα cDNA

For the transfection with the pCDLSRα296 constructs containing the cDNA or empty vector, COS7 cells were electroporated with 25-μg aliquots of DNA in 20 mM Hepes pH 7.4, 150 mM NaCl. Electroporation conditions were: 260 V, 960 μF and ∞ resistance. Binding studies were performed 48 h after transfection.

2.7 Affinity cross-linking of ^{125}I-IL-4 and ^{125}I-IL-13 to their cognate receptors

Iodination of recombinant IL-4 and IL-13 was performed as described [15]. Aliquots of 200 μl containing 2×10^6 cells were incubated on ice in αMEM medium containing 1% BSA and 0.5 nM ^{125}I-IL-4 or 3 nM ^{125}I-IL-13. The specific activities of ^{125}I-IL-4 and ^{125}I-IL-13 were 60–70 μCi/mg and 90–100 μCi/mg, respectively. For competition, a 1000-fold excess of unlabeled ligand was added 20 min prior to the iodinated cytokines. The cross-linking procedure was performed as described [15] and samples were analyzed by SDS-PAGE (3–10%, w/v) under reducing conditions.

2.8 Binding studies with radioiodinated IL-4 and IL-13

The binding experiments were performed as described [15]. Nonspecific binding was determined by incubating the same number of cells with a 1000-fold excess of unlabeled IL-4 or IL-13 at 4 °C for 30 min. Binding data were analyzed with the computerized weighted least-squares curve fitting software described by Munson and Rodbard [53].

3 Results

3.1 Cloning of human IL-13Rα1 chain

To clone the human cDNA homolog of the mouse IL-13Rα1 chain, the Gene Bank EST data base was searched using the sequence of the extracellular protein domain of the mouse IL-13Rα1 chain. The sequence of two EST (H57074 and H89334) were used to design PCR primers. A segment of the human IL-13Rα1 cDNA was amplified from tonsillar B cell cDNA and used to screen a B cell cDNA library. Sequencing of the largest cDNA insert obtained from the screening revealed a 4-kb mRNA sequence with an open reading frame encoding a 427-amino-acid polypeptide (EMBL Nucleotide Sequence Database acc. no. Y 10659 and Fig. 1). The deduced polypeptide includes two hydrophobic regions likely to represent a signal peptide and a transmembrane domain. The transmembrane domain is followed by a 59-amino-acid cytoplasmic region that is highly conserved between human and mouse. Interestingly, this region contains a YXXQ sequence motif which has been identified as a consensus sequence for STAT binding [54]. The extracellular domain includes the four cysteine residues and WSXWS motif conserved among the type-I cytokine receptor superfamily members [42] (Fig. 1A).

As expected, the deduced polypeptide sequence of human IL-13Rα1 is highly homologous to its murine counterpart ([40], 75% identity) (Fig. 1B). The level of identity is particularly high in the cytoplasmic region, suggesting that this chain plays a role in receptor signal transduction. It is also related to the IL-5Rα chain (26% identity) and to the other human IL-13R (27% identity) cloned recently [41] which we refer to as IL-13Rα2 due to its lower level of sequence identity with mouse IL-13Rα (Fig. 1A).

3.2 IL-13Rα1 chain mRNA is expressed in different tissues

The steady state level of IL-13Rα1 chain mRNA in human organs was examined by Northern blot assay using commercially available poly(A)$^+$ RNA blots (Fig. 2). The results are similar to those described for the mouse homolog [40]. Two major classes of mRNA hybridized with the IL-13Rα1 cDNA probe: a band of 4.2 kb, representing the predominant species, and a doublet around 2 kb. The apparent molecular weight of the largest RNA species is close to the size of the cloned cDNA (Fig. 2). The IL-13Rα1 mRNA levels in nonlymphoid organs were highest in adult liver, intermediate in skeletal muscle, heart, placenta, lung, and pancreas, and almost undetectable in kidney and brain (Fig. 2). In lymphoid tissues, hybridization signals were most prominent in lymph nodes, fetal liver, PBL, appendix, and spleen, and lowest in bone marrow and thymus. These data reveal a correlation between organs having a large population of mature T and B cell and a high level of IL-13Rα1 mRNA.

Expression of IL-13Rα2 transcripts was studied by using a cDNA probe corresponding to the entire open reading frame described by Caput et al. [41]. The signal in the Northern blot was much lower than that for IL-13Rα1. However, the expression of a 1.7-kb IL-13Rα2 mRNA could be detected in the poly(A)$^+$ RNA from all organs tested. Its highest steady state levels were detected in placenta and liver. Among the lymphoid organs and PBL, the IL-13Rα2 mRNA level was highest in bone marrow and fetal liver. As a control, the same blots were rehybridized with a human actin probe (Fig. 2). In summary, the data for IL-13Rα1 and for IL-13Rα2 reveal a different expression pattern in the organs studied, most notably in thymus, bone marrow, brain, and kidney.

3.3 The expression of IL-13Rα1 mRNA can be detected in a variety of primary cells and cell lines

The levels of the IL-13Rα1 transcripts in total RNA isolated from a panel of cells and cell lines were compared (Fig. 3A). Interestingly, IL-13Rα1 mRNA was detectable in HUVEC cells, the monocytic cell line THP-1, the immunoglobulin-secreting lymphoblast cell line IM-9, the

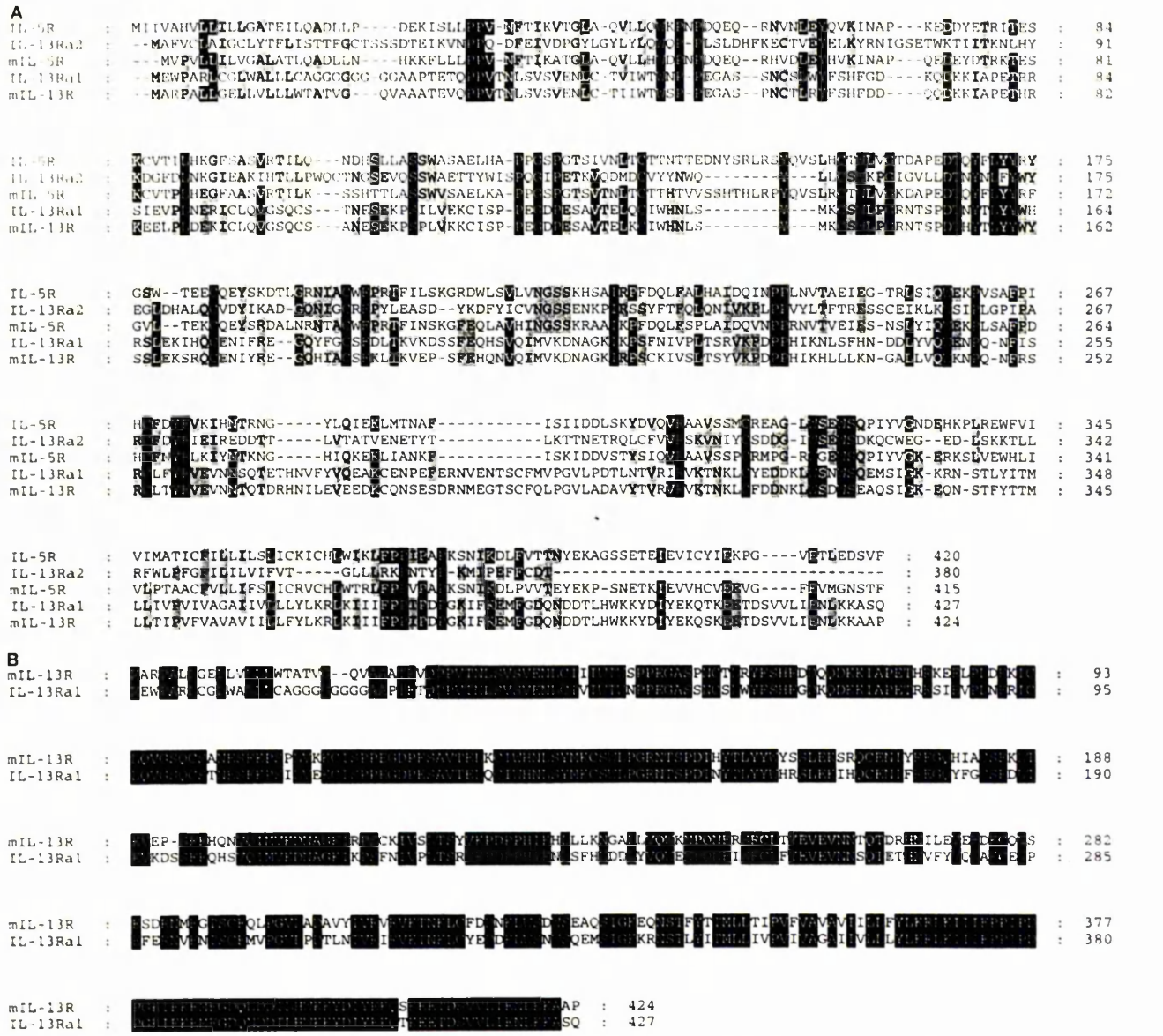

Figure 1. The deduced amino acid sequence of the cDNA clone 3.1 is compared with related cytokine family members (A) and with mouse IL-13R (B). Regions of amino acid identities are boxed in black.

immature mast cell line HMC-1, and the eosinophilic cell line Eol-3 (Fig. 3A). No signal could be detected by Northern blot analysis in total RNA isolated from the adenovirus-transformed human embryonic kidney cell line 293, the Burkitt lymphoma cell lines Jijoye and Daudi, and the B cell line HFB-1. When identical parallel Northern blots where hybridized with a IL-13Rα2 cRNA probe, a signal was detected in RNA isolated from the immature mast cell line HMC-1.

3.4 IL-13Rα1 chain is expressed by B cells

The IL-13Rα1 cDNA was cloned from a tonsillar B cell library. When B cell total RNA was subjected to a Northern blot assay with a cRNA probe for IL-13Rα1, a hybridization signal was detected (Fig. 3B).

Germ-line ε transcripts are induced by IL-4 or IL-13 in the B cell line JY (Fig. 3B). We therefore examined whether this capacity to respond to both IL-4 and IL-13 was paralleled by the expression of the IL-13Rα1 mRNA. As shown in Fig. 3B, IL-13Rα1 mRNA was indeed detectable in a Northern blot of this cell line (Fig. 3B). It was apparently not affected by the stimulation conditions tested (Fig. 3B).

We and others have previously used EBV-transformed B cell lines from SCID patients deficient for γ_c or JAK-3 to study the role of the IL-4R type I chain and its associated kinases for the induction of germ-line ε responses to IL-4 and IL-13 ([40], Gauchat et al., manuscript submitted for publication). Results demonstrated that a JAK-3- and γ_c-independent response to IL-4 occurred, suggesting that these two proteins were unlikely to be involved in the

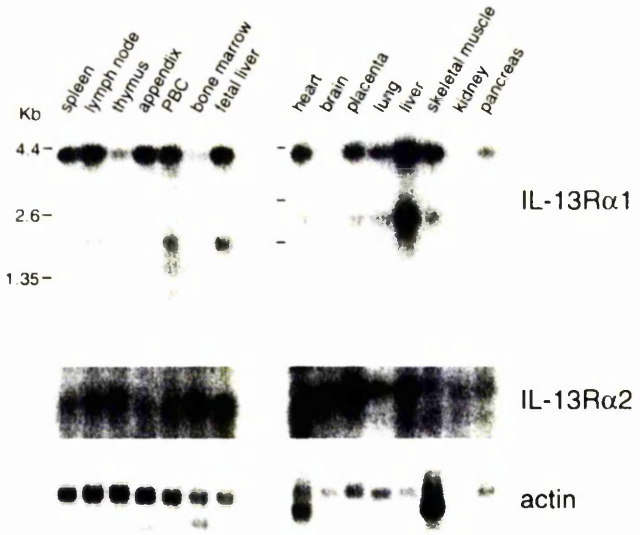

Figure 2. Northern blot analysis of IL-13Rα1 and IL-13Rα2 mRNA in human organ and tissues. Poly(A)$^+$ RNA blots were hybridized with cDNA probes for IL-13Rα1, IL-13Rα2 or actin mRNA. The autoradiography exposure times: IL-13Rα1, 18 h; IL-13Rα2, 7 days; actin, 3 h.

induction of a germ-line ε transcript response by IL-13. A simple interpretation is that the response observed in the SCID patient cell lines involves an IL-4R type II/IL-13R which would not comprise γ_c nor signal through JAK-3. Northern blot assays were therefore used to study whether IL-13Rα1 chain expression could be detected in the SCID patient B cell lines. As shown in Fig. 3B, IL-13Rα1 chain mRNA was detectable in the EBV-transformed cells from both SCID patients tested (VA, γ_c deficient; CM, JAK-3 deficient).

The IL-13Rα2 cDNA probe was obtained from tonsillar B cell cDNA, indicating that this mRNA is expressed in these cells. The signal observed was however at the limit of detection of the Northern blot assay (Fig. 3B). No signal could be detected in the JY B cell line or in the cell lines from the SCID patients VA or CM (Fig. 3B) or B cell lines from normal donors (data not shown).

3.5 IL-13Rα1 chain mRNA is expressed in T cells

Recent studies in mice have shown that IL-13 can induce Th2 T cell differentiation [8]. IL-13Rα1 and IL-13Rα2 mRNA expression in T cells was therefore evaluated (Fig. 3C). The IL-13Rα1 mRNA was clearly detected in the CD4$^+$ T cell clones JF7 and MAB.PHA.3.6. This observation was not restricted to T cell clones, but could be extended to peripheral blood T cells (Fig. 3C). In peripheral T cells, IL-13Rα1 mRNA levels were reduced in response to mitogenic stimulation by immobilized anti-CD3 alone or used in conjunction with phorbol esters or

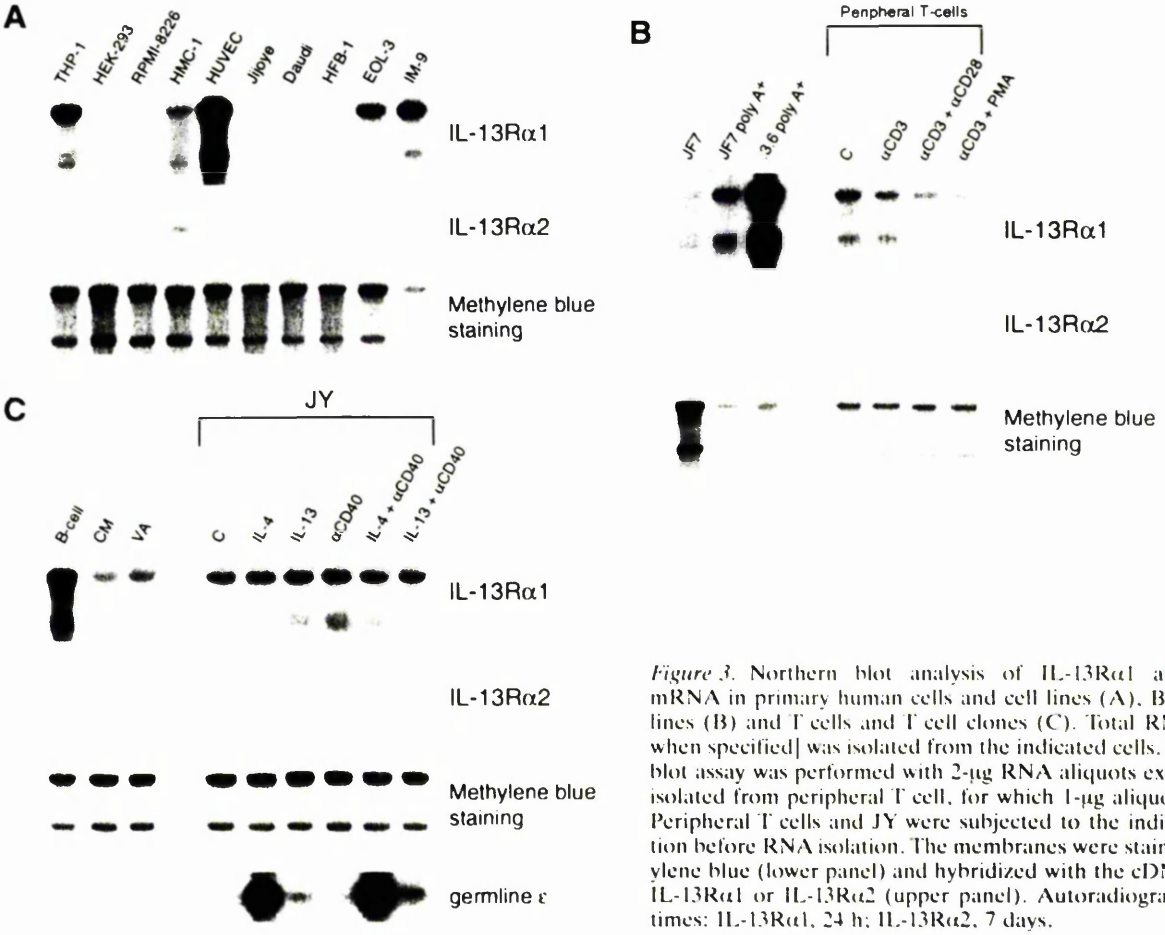

Figure 3. Northern blot analysis of IL-13Rα1 and IL-13Rα2 mRNA in primary human cells and cell lines (A), B cells and cell lines (B) and T cells and T cell clones (C). Total RNA [poly(A)$^+$ when specified] was isolated from the indicated cells. The Northern blot assay was performed with 2-μg RNA aliquots except for RNA isolated from peripheral T cell, for which 1-μg aliquots were used. Peripheral T cells and JY were subjected to the indicated stimulation before RNA isolation. The membranes were stained with methylene blue (lower panel) and hybridized with the cDNA probes for IL-13Rα1 or IL-13Rα2 (upper panel). Autoradiography exposure times: IL-13Rα1, 24 h; IL-13Rα2, 7 days.

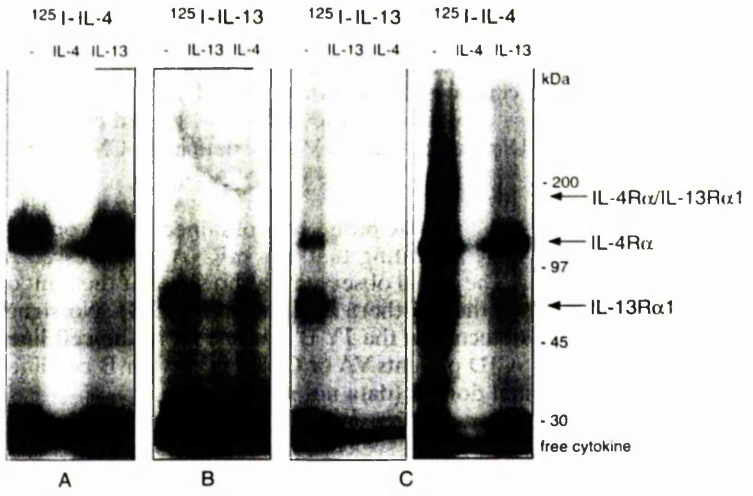

Figure 4. Radioligand affinity cross-linking of IL-4 and IL-13 to IL-4Rα and IL-13Rα1 transfectants. COS7 cells transfected either with the cDNA of the human IL-4Rα (A), the human IL-13Rα1 (B) or both (C) were labeled with 0.5 nmol/l ^{125}I-IL-4 or 3 nmol/l ^{125}I-IL-13 as indicated. Displacement of the radioiodinated ligand was performed with a 1000-fold excess of unlabeled cytokine or with buffer (−) before cross-linking was performed using 2.5 nmol/l disuccinimidyl suberate. The lysates were analyzed under reducing conditions by gradient SDS-PAGE (3–10% polyacrylamide), and exposed to X-ray films for 1–3 days. Net molecular masses of the receptors (R) were calculated by subtracting 19 kDa for bound IL-4 or 15 kDa for bound IL-13.

anti-CD28 for 4 h (data not shown) or 16 h (Fig. 3C), and by ionomycin and phorbol esters or the lectin concanavalin A (data not shown). When identical parallel blots were subjected to hybridization with the IL-13Rα2 cRNA probe, no signal could be detected, even with poly(A)$^+$ RNA isolated from the T cell clones (Fig. 3C and data not shown).

3.6 Cross-competition between IL-4 and IL-13 for the heterologous IL-4/IL-13R complex

The cDNA of human IL-13Rα1 and IL-4Rα were transiently transfected in COS7 cells either individually or together to characterize the cloned human IL-13Rα1 alone or in the context of a co-expressed IL-4Rα chain. Chemically cross-linking iodinated IL-13 to COS7 cells co-expressing both receptors showed predominant binding to the 65–75-kDa IL-13Rα1, and to a lesser extent to the IL-4Rα migrating at 130 kDa. In addition, a diffuse band was recognized at 200 kDa. Binding was completely abolished in the presence of cold IL-4 and IL-13 (Fig. 4).

Iodinated IL-4 predominantly cross-linked to the 130 kDa protein, with a small amount binding to the IL-13Rα1 at 65–75 kDa. Again, a trace band was observed at 200 kDa and cold IL-4 totally blocked ^{125}I-IL-4 binding. In contrast, cold IL-13 completely abolished binding of ^{125}I-IL-13 to the 65–75-kDa IL-13Rα1, but only decreased the radioactivity at the 130-kDa level. This partial cross-competition might reflect a stochiometric imbalance of the expressed receptor or spatial differences between bound IL-4 and IL-13. The 200-kDa band, which probably represents a small population of heterodimeric receptors recruited by both cytokines, was cross-competed by either cold IL-4 or IL-13.

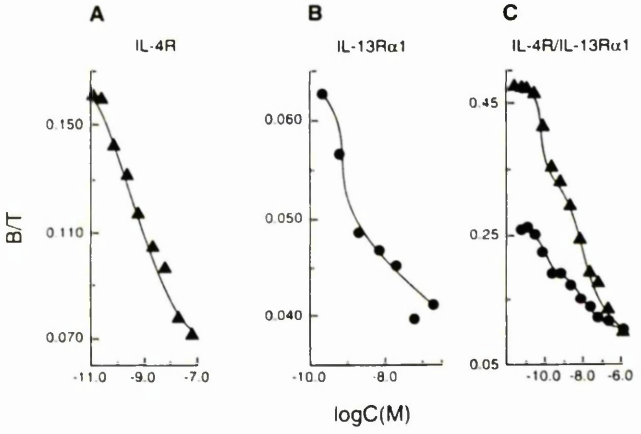

Figure 5. Binding of radiolabeled IL-4 and IL-13 to IL-4Rα and IL-13Rα1 transfectants. Receptor binding analysis of radiolabeled IL-4 (▲) and IL-13 (●) was performed with COS7 cells transfected with (A) the cDNA of either the human IL-4Rα (full line), the IL-13Rα1 (dashed line) or (B) co-transfected with both cDNA. Data were analyzed with the computerized weighted least-squares curve fitting software described by [53].

3.7 IL-13 binding studies

To address the issue of whether the IL-13Rα1 confers binding properties similar to those reported for its mouse homolog, binding of iodinated IL-4 and IL-13 was studied using COS7 cells expressing IL-13Rα1, IL-4Rα, or both. Using single-receptor transfectants, IL-4 and IL-13 bound with low affinity to its cognate receptors (IL-4: K_D = 0.9 ± 0.4 nM; IL-13: K_D = 1.4 ± 0.4 nM; Fig. 5A and B). Analysis of the data fit only with the one-binding site model (Table 1). In co-transfection experiments, binding of both cytokines fit with high statistical significance (p = 0.001) to the two-binding-site model, indicating a limited number of high-affinity receptors for IL-4 (K_{D1} = 32 ± 14 pM; 300 ± 100 sites/cell) and IL-13 (K_{D1} = 50 ± 20 pM; 500 ± 200 sites/cell). A second population of low-affinity receptors was the predominant binding site for IL-4 (K_{D2} = 2.5 ± 0.6 nM; 15000 ± 3000 sites/cell) and IL-13 (K_{D2} = 6.2 ± 3.0 nM; 45000 ± 15000 sites/cell; Fig. 5C).

4 Discussion

A mouse cDNA encoding an IL-13 binding protein was recently cloned [40]. We used human EST homologous to the mouse receptor IL-13Rα to generate a cDNA probe to clone a 4-kb cDNA encoding a human homolog with 75%

Table 1. Binding parameters of IL-4 and IL-13 to transfected COS7 cells

Transfection	Ligand	K_D (pM)	B max	Receptor/cell
IL-13Rα1	IL-13	1430 ± 360	$5.8\ 10^{-11}$	3620 ± 1010
IL-4Rα1	IL-4	870 ± 430	$9.4\ 10^{-11}$	6100 ± 4700
IL-13Rα1	IL-4	2460 ± 640	$2.6\ 10^{-10}$	15180 ± 2530
+ IL-4Rα1		31.5 ± 14.2	$3.9\ 10^{-12}$	240 ± 108
IL-13Rα1	IL-13	6290 ± 3020	$7.3\ 10^{-10}$	45450 ± 15450
+ IL-4Rα1		45.9 ± 22.5	$7.4\ 10^{-12}$	460 ± 220

amino acid identity. The sequence of the deduced polypeptide is related to IL-5Rα (26% identity). The human IL-13Rα1 contains three fibronectin type III domains also present in the IL-5Rα. IL-13Rα1 is also related to the human IL-13R identified by expression cloning (27% identity [41]).

The signal peptide area of IL-13Rα1 contains a long polyglycine stretch which is not present in the mouse homolog or in the IL-13Rα2. The mRNA segment encoding this polyglycine stretch forms GC-rich repeats. We observed deletions in this area when the cDNA was subjected to multiple cycles of polymerization (PCR). Preliminary observations suggest that the GC repeats might lead to allelic variations. It will be of interest to examine whether variations in this region of the protein result in changes of receptor surface expression, function, or both, related to allergy or atopy.

Transient expression of IL-13Rα1 in COS7 cells showed IL-13 binding with a unique affinity constant. When co-expressed with IL-4Rα chain, low- and high-affinity binding sites for both IL-4 and IL-13 were found, suggesting formation of a heterodimeric receptor complex. In related radioligand cross-linking experiments, the high-affinity heterodimeric receptors are likely to be represented by the trace band migrating at 200 kDa. The fact that disuccinimidylsuberate cross-linked this trimolecular cytokine/receptor complex, and cold IL-4 or IL-13 cross-competed with the opposite iodinated cytokine, indicates the spatial proximity of the two receptor chains. These data are in line with the properties of type II IL-4 receptors [39]. In view of the homology between IL-13Rα1 and IL-5Rα, we tested whether IL-13Rα could bind IL-5. We observed no IL-5 binding to IL-13Rα1 when it was expressed alone or in conjunction with IL-5Rβ in COS7 cell transfectants (data not shown).

We compared the expression of the mRNA encoding IL-13Rα1 and IL-13Rα2 in RNA purified from organs, leukocytes, and cell lines using Northern blot assays. Expression of the IL-13Rα1 and IL-13Rα2 were both ubiquitous, indicating that they are expressed by cells present in all organs tested. The variation in the steady-state levels of the two mRNA were not parallel, suggesting that the two receptors' mRNA steady state levels are not co-regulated and that the two receptors might have different functions. Among the organs of the immune system tested, the expression of the IL-13Rα1 was high in the organs with large proportions of mature lymphocytes and lower where selection and differentiation of immature lymphocytes predominantly occurs. It remains to be determined which cells express the IL-13Rα1 mRNA and the corresponding receptor protein in these organs.

When the study was extended to primary and transformed cells, the IL-13Rα1 mRNA was detected in B, T, transformed endothelial cells, as well as monocytic, eosinophilic, and immature mast cells lines. The pattern of expression observed is therefore compatible with a putative role of IL-13Rα1 as a receptor mediating the effects of IL-13 which have been described on B, T, and endothelial cells [6, 13–15, 28]. The mRNA encoding IL-13Rα2 chain was detected only in purified tonsillar B cells and the immature mast cell line HMC-1, indicating a more restricted pattern of expression or a much lower steady-state level which would preclude detection by Northern blotting.

The mRNA encoding IL-13Rα1 was also detected in B cell lines from γ_c-deficient SCID patients in which a type I IL-4R-independent IL-4 and IL-13 response can be detected. No signal for IL-13Rα2 mRNA was observed. The IL-13Rα1 therefore represents a candidate for a receptor subunit involved in a type II IL-4/IL-13R transducing IL-4 and IL-13 signaling in the absence of γ_c in B cells and HUVEC [39].

The results reported here correspond to those recently published by Aman and colleagues [55]. In contrast to our 4-kb cDNA, they cloned the cDNA for the 2-kb IL-13Rα1 mRNA. Analysis of transcript distribution in organs in the two studies are similar and we provide additional evidence of mRNA expression in individual immune cell types [55].

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Appendix 3 :

Tissue Localization of IgE Class Switching During an Allergic Response to an Aero-Allergen

The work described in this section concerns the original project undertaken at the start of time as a PhD student. The work was performed between the period of January 1995 to August 1996. The project involved the construction of a genetically modified mouse to localize the tissues in which B cells were undergoing the class switch to IgE following allergen challenge using the LacZ reporter gene. The project was eventually terminated due to our failure to pass the genetic modification across the germline. Fears over the time remaining for the PhD and the possibility of generating results more quickly with the project described in the main body of the thesis were the main reasons for this decision, as well as a change in direction of the research interests of the department at this time.

ABSTRACT

B cell switching to IgE is induced by the conjugation of signals given by interleukin-4 (IL-4) and CD40 ligand (CD40L). These signals are provided by T cells, presumably in secondary lymphoid organ germinal centres. The demonstration that mast cells and basophils in an induced state express the CD40L and can induce B cell IgE production *in vitro* leads to the possibility of IgE class switching in peripheral organs such as lung and skin, where the above cell types are localised.

Production of IgE in B cells is always preceded by transcription of the sterile ε transcript, driven by the IL-4 inducible Iε promoter. The transcript commences at the Iε exon and passes through the switch (S) region and constant exons of the ε region of the Ig heavy chain locus. The transcript's function is largely unknown, but it is a prerequisite of class switching to IgE and through its induction by IL-4, appears to direct switching to the ε constant region.

As identification of the sterile ε transcript in B cells is a good indication that they are undergoing class switching to IgE, the aim of the project is to localise tissues in which B cell class switching to IgE is occurring following the induction of an allergic response by studying induction of expression of the sterile ε transcript. We have constructed a gene targeting vector which will place a LacZ reporter gene under the control of the Iε promoter in a 'knock-in' mouse. Following an airway ovalbumin challenge, tissues from the mice will be examined for β-galactosidase activity in order to identify B cells with an active Iε promoter. We have demonstrated the viability of the chosen approach *in vitro* using both stable and transient transfection of mammalian cells culture systems. We have

Also investigated the expression of the sterile ε transcript in vivo using reverse transcription PCR (RT-PCR).

Two embryonic stem cell clones were subsequently obtained which had integrated the gene targeting construct by homologous recombination. Injection of these clones into blastocysts resulted in the generation of chimeric mice. We were unable however to achieve germline transmission of the genetic alteration.

1. INTRODUCTION

Type I hypersensitivity, or anaphylactic hypersensitivity is caused by an excessive secondary reaction to an antigen following immunological priming of an individual with the same antigen. Such hypersensitivity gives rise to the symptoms associated with atopic allergy, and the antigens provoking these symptoms called allergens. Atopic allergy affects almost 10% of the population, with some of the most common allergens include grass pollen, house dust mite faeces and the contents of a wasp sting. The synthesis of allergen specific IgE plays a major role in the induction of type I hypersensitivity. Binding of IgE to the high affinity IgE receptor FCεRI on the surface of mast cells and its subsequent cross linking by allergen results in degranulation of the mast cell, causing the release of an array of mediators such as histamine, proteoglycans, heparin and various neutral proteases. These mediators in turn act on endothelial cells, smooth muscle, mucous glands, inflammatory cells and connective tissue to produce the symptoms associated with an allergic response. An example of such an allergic response is the induction of asthma through exposure of the lung to relatively large amounts of allergen following sensitisation of an allergic individual to the allergen. The induction of asthma resulting from an allergic response can be studied in established murine asthma models. If mice are injected once with an allergen such as ovalbumin, no obvious symptoms are observed. If, however, these sensitised mice are subjected to an airway challenge of ovalbumin several weeks after the initial injection, they develop the symptoms associated with asthma such as bronchial restriction. This is largely due to contact of the allergen with cell-bound IgE in the bronchial tree of the lung and subsequent mast cell degranulation and mediator release.

IgE is produced and secreted by B cells in an activated state. Its production is induced by a physical interaction between B cells and T cells, involving signalling through surface adhesion molecules such as CD40 ligand (CD40L) (1,2) as well as soluble factors such as IL-4 and IL-13 (3,4). It has since been shown that mast and basophil cell lines can be induced to express CD40L and, in the presence of IL-4, provide the cell contact signals required for

the induction of IgE synthesis *in vitro* in the absence of T cells. This finding was also extended to purified lung mast cells and blood basophils, and in the case of blood basophils, IgE production could be induced in the absence of exogenous IL-4 (5). These data suggest that both mast cells and basophils can may play a direct role in regulating IgE synthesis *in vivo* independently of T cells. It also leads to the possibility that immunoglobulin class switching to IgE may be occurring in peripheral organs such as the lung and skin, where these cells are localised, and not simply restricted to germinal centres in secondary lymphoid organs. This, if shown to be the case, could have important clinical relevance in terms of local treatment of allergic disorders, such as asthma in the lung.

Manipulation of the Ig class switch mechanism in B cells would allow the investigation of class switching to IgE in B cells localised in the peripheral organs. 5 different immunoglobulin isotypes (or classes) are known to exist, IgM, IgD, IgG, IgE and IgA. B cells are known to express surface IgM and IgD, but given the appropriate signals, may 'switch' to the production of either IgG, IgE or IgA. In general, early immune responses are dominated by IgM expression, and class switching takes place after a committed B lymphocyte is stimulated by antigen. A B lymphocyte producing IgM expresses the μ constant region of the heavy chain locus but upon class switching may express the γ, ε or α heavy chain constant region. The switching is accompanied a large DNA deletion in the genome between the rearranged VDJ region and the selected constant region. Since the new heavy chain retains the original upstream variable region, antigen specificity is retained, whilst the function of the antibody changes due to the acquisition of a different constant region and therefore a different effector function (reviewed in 6 and 7).

The DNA rearrangement of the constant regions occurs at defined switch (S) regions, each constant region segment on the heavy chain locus being preceded by a S region of a few kilobases containing repetitive sequences (8). Following class switching, breakpoints for the switch rearrangements are found within the S regions. A class switch to IgE, for example, would involve the DNA rearrangement between the Sμ and the Sε regions, involving a looping out of the DNA followed by deletion due to S region recombination. Strong

evidence for such a model of DNA rearrangement exists through the identification and cloning of DNA loops excised as a result of the recombination (9,10,11,12). Evidence also exists for the occurrence of sequential class switching, an example of which being the sequential switch in mice from IgM to IgG1 and then IgE (11,12,13,14). The B lymphocyte switches to IgG1 by recombination between the Sμ and Sγ1 regions, (creating a hybrid SμSγ 1 region), and then switches to IgE by recombination between the SμSγ1 region and the Sε region (creating a SμSγ1Sε region hybrid). Evidence for a sequential switch from IgM to IgG4 to IgE has also been found in humans (15).

The mechanism of S region recombination is largely unknown, but production of a 'sterile transcript' has been shown to be an essential prerequisite for the switch to a given Ig isotype (16). For class switching to any given isotype, transcription of the sterile transcript commences upstream of the switch region, passes through the switch region itself and terminates downstream of the constant region exons. In terms of class switching to IgE, the sterile transcript expressed in the ε constant region of the Ig heavy chain locus (the sterile ε transcript) has been characterised for both humans (17) and mice (18,19), and found to be a 1.7 Kb RNA transcript encoding the I exon for the ε constant region (Iε exon) spliced to the ε constant region (Cε) exons. RNA encoding the Sε region is lost as a result of the splice. As production of the transcript implies transcription through the S region, it is believed that this transcription causes a change in the chromatin structure of the S region, making the region more accessible for the 'recombinase system' causing the DNA rearrangement (20). Figure 1 summarises the genomic events occurring during the IgE class switch. Recent work by Bottaro *et al* (21) has shown however that transcription in the absence of the I exon through this region is not sufficient to induce class switching, and work by Lorenz *et al* (22) has demonstrated that the splice junction between the I exon and the first C exon must be intact. This suggests that the RNA excised from the sterile transcript as a result of the splicing, and representing the S region, may have a functional role in the DNA rearrangement leading to class switching.

The promoter driving transcription of the sterile ε transcript, known as the Iε promoter, has been well characterised in both humans and mice and a number of IL-4 responsive elements mapped, in accordance with the fact that the sterile ε transcript is induced by IL-4 (23,24,25,26,27). As mentioned earlier, class switching to IgE relies upon the conjugation of two signals, one given by IL-4/IL-13 and the other by CD40L. The current model for this process is that IL-4 or IL-13 induces the IgE specific induction of sterile ε transcripts, whereas ligation of CD40 molecule via the CD40L renders B cells competent for class switching in general. This model is supported by the effects of disrupting the genes encoding IL-4, CD40 and CD40L in mice. Deletion of the IL-4 gene has little effect beside abolishing the synthesis of IgE (28), whereas disruption of the genes encoding CD40 and CD40L have numerous consequences on the immune system, including a blockage of class switching (29,30). The detection of induction of sterile ε transcript expression, (providing the specificity for the ε class switch) therefore appears to provide a tool for the localisation of B lymphocytes undergoing the class switch to IgE *in vivo*.

In order to detect the induction of the sterile ε transcript *in vivo*, a reporter gene system has been employed whereby the gene encoding the bacterial protein β-galactosidase, LacZ, will be inserted downstream of, and subsequently under the control of Iε promoter by gene targeting of the mouse genome. Following the induction of an allergic response in these mice by an ovalbumin airway challenge, a proportion of B lymphocytes will be undergoing the switch to IgE and therefore have an 'active' Iε promoter. This will result in the expression of β-galactosidase in the cells, leading to their tissue localisation using standard histochemical or fluorescent staining techniques.

Before placing the LacZ gene under the control of the Iε promoter, four important considerations must be taken into account. Firstly, insertion of a nuclear localisation signal (NLS) (31) directly upstream of the LacZ gene allows the enhancement of the signal given by β-galactosidase and therefore facilitates the detection of expression. Secondly, The transcript expressed from the Iε promoter is sterile and therefore does not produce functional protein. This is due to the presence of both ATG start codons and downstream stop codons

present in the small Iε exon. Translation could only therefore produce very short, non-functional peptides, and would also make the in-frame insertion of the LacZ gene very difficult. This can be overcome by the insertion of a viral internal ribosome entry sequence (IRES) (32,33,34) directly upstream of the NLS/LacZ ATG translation start site. The presence of an IRES allows ribosomes to bind to the first AUG after the sequence, regardless of any preceding AUG's in the mRNA, thus allowing β-galactosidase expression when downstream of the Iε exon. The third consideration is that of an Iε exon/Cε1 exon splice in the sterile transcript. Insertion of a LacZ into the DNA between the Iε exon and the Sε region would result in its loss from any mRNA produced by the Iε promoter as the region in which the LacZ gene will be inserted is considered as intronic DNA. The solution is therefore to insert the LacZ gene as an exon, with an upstream splice acceptor, and a downstream splice donor. Replacement of the polyA signal associated with the Lac Z gene in the gene targeting vector with a splice donor would also allow the switch to IgE to remain physiological in these mice, as it would also allow the transcription through the switch region and constant region exons to remain, as well as the 'splicing-out' of the switch region, proven to be an important feature of the class switch mechanism. Figure 2 outlines transcription for the Iε promoter before and after the gene targeting. The fourth consideration is that of constitutive transcription of the neomycin gene close to the Sε region following gene targeting. In order to target the ε constant region, the gene targeting construct must be transfected into murine embryonic stem (ES) cells and stable DNA integration selected for by resistance to G418. For selection of G418 resistant cells, the gene targeting vector must contain a constitutively expressed neomycin resistance (neo^R) gene downstream of the LacZ gene. As expression through the Sε region is known to play a role in class switching, constitutive transcription close to this region in mice resulting from successful gene targeting may lead to unwanted switching to IgE in the absence of induction of the Iε promoter. The Neo^R gene will therefore by flanked by *LoxP* recombination sites in the gene targeting vector, in order to remove it from ES cell clones arising as a result of homologous recombination. This will be achieved by transient transfection in such clones of a *Cre* recombinase expression construct, catalysing recombination at the *LoxP* sites (35,36,37). Recombination will be selected for using negative selection by inserting the thymidine

kinase (TK) gene within the LoxP sites alongside the neo^R gene in the gene targeting construct. Cells which have undergone the recombination event, and therefore not containing the TK gene can be selected for using the thymidine analogue FIAU (38).

The following report describes the construction of a gene targeting vector to be used in order to localise and monitor the induction of activity of the Iε promoter, an essential prerequisite to the IgE class switch. It outlines techniques used to clone the genomic DNA required in the gene targeting process, modification of the LacZ gene in order to make it a viable reporter gene in this system, and the selection and screening steps necessary in order to obtain the required genomic alterations resulting from the gene targeting.

In addition, the *in vitro* experiments performed in mammalian expression systems in order to examine the viability of the modified LacZ gene alone and the modified LacZ gene under the control of the Iε promoter are described. Finally, experiments performed in order to localise expression of the Iε sterile transcript *in vivo* by reverse transcription and PCR amplification (RT-PCR) are outlined.

2. MATERIALS AND METHODS

2.1 Library Screening and Cloning of Genomic Arms for Gene Targeting

The probe hybridising to the Iε promoter/exon region was amplified by PCR from mouse genomic DNA using the primers 5PIE and 3PIE whose sequences were 5'-TAGAGAGCCTTGACCAGAGTGTG-3' and 5'-GGTCCTCTGATAAGTGAGTGGCT-3' respectively. The probe hybridising to the Cε third exon region was amplified from mouse genomic DNA using the primers 5PCE and 3PCE, whose sequences were 5'-GATCATGAGCCACGGGGTGTGAT and 5'-CTTGGTGATGGAACGCACAATTG-3' respectively. PCR conditions were as follows; 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 1 minute, and elongation at 72°C for 2 minutes. Products of the PCR reaction were then cloned into the vector pCRII (Invitrogen, San Diego, CA) and the inserts verified by sequence analysis. To obtain probe DNA for the library screening, vector DNA was cut with the restriction enzyme *EcoRI,* the products of the digestion run on an agarose gel and the appropriate bands purified using the Sephaglas Bandprep kit (Pharmacia, Uppsala, Sweden), following manufacturer's guidelines.

A genomic DNA library of the mouse strain 129/OLA (Harlan, Indianapolis, ID) in the phage λ Fix II (Stratagene, La Jolla, CA) was screened as follows: the *E.coli* strain LE392 was infected with the phage, colonies transferred onto nitro-cellulose filters (Schleicher and Schuell, Dassel, Germany) and denatured with 0.5M NaOH, 1.5M NaCl. Following neutralisation with 0.5M Tris (pH 7.4), 1.5M NaCl, filters were washed with 2xSSC and DNA fixed by baking at 80°C for 2 hours. Filters were subsequently treated with 50 μg/ml proteinase K for 1 hour at 37°C and pre hybridised at 42°C for 2 hours with PSB

buffer (0.2 % polyvinyl pyrolydone, 0.2 % Ficoll, 0.2 % BSA, 0.05M Tris-HCl (pH 7.5), 1M NaCl, 0.1 % sodium pyrophosphate, 1% SDS, 100 µg/ml tRNA).

Probes were labelled with $^{\alpha 32}$P dCTP (3000 Ci/mmole, Amersham Life Sciences, Amersham, Bucks, UK) by random primer labelling using the oligolabelling kit (Pharmacia) following manufacturers guidelines. Radio labelled probe DNA was purified from unincorperated radioactivity using Nick columns (Pharmacia) following manufacturers guidelines. Filters were then placed in hybridisation buffer (50% PSB buffer, 50% formamide) and probe DNA added at a concentration of $1x10^6$ cpm/ml. Hybridisation was at 42°C for 4-16 hours. Following hybridisation, filters were washed 3 times 45 minutes with 2x SSC, 0.1% SDS at 55°C. Filters were then dried briefly at room temperature and subjected to autoradiography for 48 hours at -70°C. Plaques giving a positive signal were purified by 3 cycles of limiting dilutions followed by screening with ^{32}P labelled probes.

2.2 Subcloning of Genomic DNA containing the Iε and Sε Regions

Following preparation of DNA from the positive λ clone 3.2, the genomic insert was excised from the λ DNA arms by digestion with the restriction enzyme *NotI*, and the insert was separated from the λ arms by agarose gel electrophoresis. The 14 Kb insert was recovered by electroelution (Schleicher and Schuell). Genomic DNA was then digested with the restriction enzyme *PstI* and all the resulting fragments ligated into the vector pBluescript (Stratagene). The bacterial strain DH5α was transformed with the ligation products, and resistant colonies containing the required inserts identified by colony hybridisation using two probes in parallel. In order to identify colonies containing the Iε promoter/exon region genomic fragment, a probe was amplified by PCR from the λ

clone 3.2 using the primers 5PIE and 3PIE (see above). In order to identify colonies containing the Sε region genomic fragment, a probe was amplified using the primers HY5S and 3PSE, whose sequences are 5'-GGAATCGGCCAGCAAGCCCAT-3' and 5'-CTAGCCTAGTCTGATTCACC-3' respectively. The conditions used in the PCR amplification and colony hybridisation were the same as those described above. Plasmid DNA from the colonies positive for the hybridisation were sequenced in order to confirm the presence of the required inserts.

2.3 Insertion of a Nuclear Localisation Signal onto β-Galactosidase

The vector pGT1.8IRESβgeo (39) contains the gene encoding β-galactosidase preceded by a splice acceptor from the murine En-2 gene (En-2 SA) (40) and an IRES from the encephalomyocarditis virus (EMCV). Due to the absence of any convenient restriction sites, bacterial homologous recombination was chosen for the insertion of the NLS directly upstream of the β-galactosidase gene.

Two separate DNA fragments were amplified from the vector above. The first fragment, fragment 1, contained the En-2 SA the IRES and the 5' region of the NLS which was introduced downstream of the IRES sequence by adding a 22 base 5' extension to the downstream primer. The sequence of the two primers, En2-5P and IRES-3P is 5'-CGCG GATCCCCTAGTTTGTGATA-3' and 5'-ATTCCTGAAACTTTATCCATGGTTGTGG CAAGCTTATCATCG-3' respectively. The second fragment, fragment 2, contained the first 840 bases of the LacZ gene, starting at base 15. The upstream primer was designed so as to include a 74 base 5' extension containing the complete NLS and 20 bases of the 3' end of the IRES. The downstream primer region of hybridisation encompassed the unique *ClaI* site in the LacZ gene for use in future reclonings. The sequence of the two

primers, LacZ-5P and LacZ-3P is 5'-ATGATAAGCTTCCACAACCATGGATAAAGTT TTCAGGAATTCCGCCAAAAAGAAGAGAAAGGTAGAAGACCCCTCACTGGCC GTCGTTTTACAACGTCGT-3' and 5'-TAACCACCACGCTCATCGAT-3' respectively. The two fragments have a total region of homology of 41 bases, covering the last 20 bases of the IRES and the first 21 bases of the NLS. PCR conditions were as follows; 20 cycles of 30 seconds at 94°C and 3 minutes at 68°C. A mix between Taq polymerase and the thermostable proof-reading polymerase Pwo (Boerhinger Mannheim GmbH, Mannheim, Germany) was used in order to reduce the risk of unwanted mutations. For the same reason the number of amplification cycles during the PCR was reduced to 20. Both fragments were separately recloned into pCRII. pCRII containing fragment 1 was then digested with the restriction enzymes *NotI* and *SphI* and the DNA fragment of 3.63 Kb purified from an agarose gel. pCRII containing fragment 2 was digested with the restriction enzymes *SpeI* and *XmnI* and the DNA fragment of 2.95 Kb purified from an agarose gel. These two new DNA fragments have two separate regions of homology at either end, one of 466 bp containing regions of both the ampicillin and kanamycin resistance genes, and the other of 41 bp containing the 3' sequence of the IRES and the 5' sequence of the NLS. 100 ng of each fragment was mixed and the $CaCl_2$ competent *E.coli* DH5α was transformed with the mixture. DNA from colonies resistant to both kanamycin and ampicillin were characterised by both restriction enzyme and sequence analysis to detect plasmid formation by homologous recombination.

2.4 Mutagenesis to Revert the NLS back to Wild Type

PCR mutagenesis was performed on the mutant DNA template using primers LACMUT-5P (containing the reversion mutation) and LACMUT-3P whose sequences are 5'-CCATGGATAAAGTTTTCAGGAATTCCGCCAAAAAGAAGAGAAAGGTAGAAG

ACC-3' and 5'-GTTAACGCCATCAAAAATAATTCGC-3' respectively. PCR conditions were 20 cycles of 30 seconds at 94°C and 3 minutes at 68°C. Amplified DNA was digested with the restriction enzymes *NcoI* and *HpaI* and cloned into pBluescript containing the mutant mLacZ at the same restriction enzymes sites, thereby replacing the mutation with the wild type sequence.

2.5 Transient Transfection of Cos 7 Cells and Detection of β-Galactosidase

For electroporation, $1x10^7$ cells were transfected with 20 μg of supercoiled plasmid DNA. The DNA was re suspended in a volume of 50 μl 150 mM NaCl in the presence of 100 μg tRNA. Cos 7 cells were grown to 80% confluence, detached from the flask wall by the addition of PBS/1 mM EDTA and washed twice with PBS. After a further wash with 20 mM HEPES/150 mM NaCl, cells were re suspended in 500 μl of the same buffer, mixed with the DNA and placed in a gene pulsar cuvette with a 0.4 cm gap (BioRad, Richmond, CA). Cells were then subject to electroporation using a Gene Pulsar (BioRad) with the following conditions; 960 μF, ∞ Ω, and 260 V. β-galactosidase activity in the cells was measured at 72 hours post-transfection.

For lipofection, DNA was transfected into the Cos 7 cells using the LipofectAMINE reagent (GibcoBRL, Gaithersburg, MD) following manufacturers guidelines. Briefly, 5 μ g of DNA was re suspended in 100 μl of serum free Delbrucco's modified Eagles medium (GibcoBRL). In a separate solution, 10 μl LipofectAMINE was re suspended in 100 μl of the above serum free medium, and the two solutions mixed together and incubated at room temperature for 30 minutes and 0.8 ml of serum-free medium subsequently added. Cells were grown to 80% confluence in a 6 well plate rinsed once with serum free medium, overlaid with the DNA/liposome complex solution and incubated for 5 hours at

37°C in a 5 % CO_2 atmosphere. After 5 hours, 1 ml of medium containing 20 % serum was added to the cells. After a further 24 hours incubation, the medium was replaced with fresh medium containing 10% serum. Cells were assayed at 72 hours post-transfection.

Cells were assayed for β-galactosidase activity either by blue staining or fluorescent antibody binding. For the blue staining, cells were rinsed once with PBS, fixed for 5 minutes in freshly prepared 2 % formaldehyde/0.2 % gluteraldehyde in PBS, and washed a further two times. The cells were overlaid with 2 ml of histochemical reaction mixture containing 1 mg/ml X-Gal (Sigma, St. Louis, MO), 5mM potassium ferricyanide, 5mM potassium ferrocyanide, and 2 mM $MgCl_2$ in PBS. The cells were incubated for 16 hours at 37°C, washed in PBS and the blue staining cells counted under a light microscope.

For fluorescent antibody binding, transfected cells (grown on cover slips) were washed twice in sulphate buffer (90 mM Na_2SO_4, 30 mM K_2SO_4, 5.8 mM $MgCl_2$, 0.25 mM $CaCl_2$ and 1 mM NaH_2PO_4), fixed with 4 % formaldehyde in sulphate buffer and washed three times in MTBS (66 mM NaCl, 100 mM Trizma base). The anti-β-galactosidase antibody was added to the cells at a concentration of 0.4 µg/ml in antibody diluent (2 % BSA, 10 % rat serum, 0.3 % Triton X-100 in MTBS), in a volume of 250 µl per coverslip and the cells incubated 16 hours at 4°C. Cells were then washed three times in MTBS and incubated for 30 minutes at room temperature in the dark with FITC-conjugated anti-mouse antibody (Vector Laboratories Inc., Burlingame, CA) at a 1/75 dilution in antibody diluent (250 µl per coverslip). Cells were washed three times in MTBS, the coverslips placed onto microscope slides and sealed with nail varnish and fluorescent cells viewed using a confocal microscope (Zeiss, Zurich, Switzerland).

2.6 Stable Transfection of the A20 B Cell Line and Detection of β-Galactosidase

Detection of expression of the ε sterile transcript in the A20 cells by reverse transcription and PCR (RT-PCR) preceded stable transfection, and was performed as follows: Cells at a concentration of $1x10^6$ were incubated either with or without IL-4 (200 U/ml, Pharmigen, San Diego, CA) and LPS (10 μg/ml, Sigma) for 4 days at 37°C in a 5 % CO_2 atmosphere. Total RNA was then prepared from the cells using Triazol solution (GibcoBRL) following manufacturers guidelines. 5 μg of each RNA sample was then used to produce first-strand cDNA by reverse transcription, using the First strand cDNA Synthesis Kit (Pharmacia) following manufacturers guidelines. In the subsequent PCR reactions, first strand cDNA representing 1/4, 1/40 and 1/400 of the total reverse transcription reaction was used for both samples. Amplification of the HPRT housekeeping gene using the primers HPRT-5P (5'-GTTGGATACAGGCCAGACTTTG TTG-3') and HPRT-3P (5'-GAGGGTAGGCTGGCCTATAGGCT-3'). The sterile transcript was amplified using the primers 5P-CEI (5'-CCTCCATCATTCAACGAAAGT AGG-3') AND 3PCE (see earlier). Conditions for the PCR were 35 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes.

For the transfection of the A20 lymphoma B cell line, 30 μg of the appropriate plasmid DNA was linearised with the restriction enzyme *NotI*, precipitated with ethanol and re suspended in the presence of 100 μg tRNA. For each transfection, $1x10^7$ A20 cells were washed once with PBS and re suspended in 750 μl RPMI medium without serum. The cells were then mixed with the DNA and transferred to a Gene Pulsar cuvette with a 0.4 cm gap. Cells were electroporated with a Gene Pulsar using 960 μF, ∞ Ω and 260 V. Cells were then diluted in RPMI medium containing 10 % serum and rested for 24 hours. The selection medium, containing G418 (GibcoBRL) at a concentration 400 μg/ml and HEPES buffer (GibcoBRL) at a concentration of 25 mM was added to the cells, and the

cells were then incubated without dilution (crude) or diluted 1:3, 1:10 and 1:20 with selection medium. After approximately three weeks, small clumps of dividing cells began to appear in the crude and 1:3 diluted populations. Cells were then passed through Ficoll, and the boyant (living) cells reseeded for both populations (crude and 1:3). Genomic PCR of the two populations was performed using the primers 5PIE (see earlier) and OHR-2 (5'-AGTCACGACGTTGTAAAACG-3'). Genomic DNA was prepared by harvesting $5x10^5$ cells from the appropriate population and re suspended in 50 µl 1xPBS, incubating for 10 minutes at 95°C followed by 1 hour at 50°C in the presence of 40 mg proteinase K and a further 10 minutes at 95°C to inactivate the enzyme. 5 µl from the 50 µl total was used per PCR amplification. PCR conditions were 35 cycles of 1 minute at 95°C, 1 minute at 65°C and 2 minutes at 72°C.

Cells, at a concentration of $1x10^6$/ml, were incubated with or without murine IL-4 (1:250 dilution of an in-house stock) and LPS (10 µg/ml) for 4 days, and β-galactosidase activity was detected either by FACS analysis or blue staining of the cells. For the FACS analysis, cells expressing β-galactosidase were fluorescently stained using the FLUOROREPORTER LacZ Flow Cytometry Kit (Molecular Probes Europe BV, Leiden, The Netherlands), following manufacturers guidelines. Briefly, cells were washed once in staining medium (4 % (v/v) foetal calf serum and 10 mM HEPES pH 7.2 in PBS) and re suspended in the same medium at a concentration of 10^7 cells/ml. 100 µl of Cells were prewarmed at 37°C for ten minutes and 100 µl of the prewarmed fluorescence substrate, 2 mM FDG incubated with the cells for 1 minute. FDG uptake into the cells was stopped by the addition of 1.8 ml ice-cold staining buffer containing 1 µg/ml propidium iodide. After 15 minutes incubation on ice, β-galactosidase activity was inhibited by the addition of 40 µl 1 mM PETG. Cellular fluorescence was then detected on a FACScan flow cytometer (Becton Dickinson, Erembodegem, Belgium).

For blue staining, cells were washed once in PBS and re suspended at a concentration of $5x10^5$ cells/ml. Microscope slides were then treated with 100 µl of a 1 % BSA solution in PBS (freshly made) by centrifugation for 1 minute at 500 rpm in a 'Cytospin 2' (Shandon Inc., Pittsburgh, PA) and 100 µl of the cells loaded onto the slides by centrifugation for 5 minutes at 500 rpm. Cells were then air-dried for 5 minutes and fixed in 2 % formaldehyde, 0.2 % gluteraldehyde for 5 minutes. Following two washes in PBS, the cells were overlaid with 50 µl histochemical reaction mixture (see 2.5, Materials and Methods). Following 24 hours incubation at 37°C, cells were mounted with a coverslip and blue staining cells counted under a light microscope.

2.7 Construction of the Gene Targeting Vector

The *AscI* restriction site was introduced into the pGN vector by oligonucleotide annealing and ligation using the primers pGNASC-5P (5'-CTAGAGGCCGGCCTCTAGAGCGGC CGCGGTAC-3') and pGNASC-3P (5'-TCCGCGCGGAGATCTCGCCGGCGC-5'). 10 pmoles of each oligonucleotide was mixed in a volume of 100 µl 10 mM Tris HCl pH 8.0 10 mM $MgCl_2$. The mixture was heated to 95°C and slowly cooled to room temperature. The annealed oligonucleotides were then ligated into the vector at the *SpeI/KpnI* restriction sites. The upstream *LoxP* sequence was inserted at the *ApaI/AscI* restriction sites by oligonucleotide annealing and ligation (as above) using the oligonucleotides LoxAA-5P (5'-CATAACTTCGTATAATGTATGCTATACGAAGTTATGG-3') and LoxAA-3P (5'-CGCGCCATAACTTCGTATAGCATACATTATACGAAGTTATGGGC -3'). The downstream *LoxP* sequence with a 5' unique *PmlI* restriction site was inserted into the vector at the *Bpu1102 I/XhoI* sites by oligonucleotide annealing and ligation (as above) using six different oligonucleotide primers. The three upper-strand primers were ANLOX-51 (5'-TGACGCCCGGTCGCTACCATTACCAGTTGGTCT-3'), -52 (5'-GGT

GTCAAAAATAATAATAATAACACGTGATAA-3') and -53 (5'-CTTCGTATAATGT ATGCTATACGAAGTTATC-3'). The three lower strand primers were ANLOX-31 (5'-CACCAGACCAACTGGTAATGGTAGCGACCGGCGC-3'), -32 (5'-GAAGTTATCAC GTGTTATTATTATTATTTTTGA-3') and -33 (TCGAGATAACTTCGTATAGCATAC ATTATAC).

2.8 *Cre* Recombination of the Gene Targeting Vector in *E.Coli*

The E.coli strain Am-1 (Clonetech, Palo Alto, CA) containing a plasmid housing the *Cre* recombinase under the control of the Lac promotor was transformed by electroporation with 5 ng of either the final gene targeting construct (containing directly two repeated *LoxP* sequences) or the vector pGN (containing no *Lox P* sequences), as a negative control. Following electroporation, cells were grown at 30°C in order to recover, and expression of the *Cre* recombinase induced by the addition of IPTG to a final concentration of 0.5 mM. Bacteria were then plated on L-Broth/agar plates containing 20 µg/ml kanamycin. Resistant colonies were then screened by PCR, using the primers Lac3-5P (5'-CCGGTCGCTACCATTACCAG-3') AND LAC3-3P (5'-GTGTAGATAAC TACGATACGG-3'). PCR conditions were 30 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute. Using pGN as a template in the PCR gives an expected amplified product of 550 bp, the unrecombined gene targeting construct gives an amplified product of 350 bp, and the recombined gene targeting vector should not give any amplification products. All subsequent bacterial transformations (i.e. recloning of the genomic arms) were performed using the E.coli strain STBL2 (GibcoBRL) in order to avoid any unwanted recombination of the plasmid resulting from the presence of the directly repeated *LoxP* sites.

2.9 ES Cell Transfection Positive Control

Linearised plasmid DNA was re suspended in 100µl TE buffer containing 100mM NaCl. The embryonic stem cell (ES cell) line D3 was grown to 80% confluence, washed once with PBS and $2x10^7$ cells re suspended in the same buffer. The DNA was then mixed with the cells, and transferred to a Gene Pulsar cuvette with a 0.4 cm electrode gap. Cells were then electroporated using a Gene Pulsar with the following conditions: 500 µF, ∞ Ω and 240 V. Cells were subsequently rested on ice for 10 minutes before diluting in ES cell medium, containing murine leukaemia inhibiting factor (mLIF) at a concentration of 1 U/ml, and plated on 10 cm petri dishes. Following 24 hours incubation at 37°C in an atmosphere of 5 %, cells stably integrating the DNA were selected by addition of G418 to the medium at a concentration of 0.3 µg/ml. Clones resulting from the transfection were then grown up in 24 well culture dishes.

In order to obtain genomic DNA for the PCR reaction, the transfected cells were re suspended in 50 ml of 0.2xPBS, boiled for 10 minutes at 95°C, and 4 µl 10 mg/ml proteinase K added. Following incubation for 3 hours at 55°C, cells were then treated for a further 10 minutes at 95°C to inactivate the proteinase K. 5 µl of genomic DNA was used per reaction. In order to optimise the PCR, three upstream primers called ESEN-1 (5'-ACTTGAGGCTGGACATGTGC-3'), ESEN-2 (5'-TGTGCCCACTGACCAGAAGG -3') and ESEN-3 (5'-GATGGATTGGCAGATGTAGC-3'), were tested in conjunction with three upstream primers, called ESNCO-1 (5'-CCCAGAAGATGACCTTGCTC-3'), ESNCO-2 (5'-AACCCAGAAGATGACCTTGC-3') and ESNCO-3 (5'-GGCCTACAGA GACATCAGAG-3'). All three primer combinations were tested with PCR buffer (10X: 0.67M Tris (pH 8.5), 0.17M $(NH_4)_2SO_4$, 100mM β-Mecapthoethanol, 0.1% gelatin) containing either 0, 10, 20, 30, 40 or 50 mM $MgCl_2$. PCR conditions were 40 cycles of 1 minute at 94°C, 1 minute at 60°C and 7 minutes at 72°C.

2.10 Gene Targeting of the ES Cells

The gene targeting construct was linearized with *Not*I and resuspended in 100 µl TE containing 100 mM NaCl. The ES cell line HM-1 was grown to 80% confluence, detached by digestion with trypsin, washed once, and 2 x 10^7 cell resuspended in 750 µl 1 x PBS. The DNA was mixed with the cells, and transferred to a Gene Pulsar cuvette with a 0.4 cm electrode gap. Transfection of the cells and selection of cells integrating the DNA was performed as described above.

PCR screening of the resistant clones was performed using the primers ESNCO-3 and ESEN-3 (see above). The PCR buffer contained 10 mM $MgCl^2$ and the PCR conditions were the same as those used above.

The two clones positive for the homologous recombination were expanded, and transfected (using the same conditions used above) with 20 µg of the *Cre* recombinase expressing construct pGK-Cre pA, in order to induce the recombination of the LoxP sites and therefore the subsequent deletion of the Neo^R and TK genes. Cells were selected with FIAU (0.2 µM) for the absence of the TK gene and resistant clones screened by PCR using the primers Lac3-5P (see earlier) and ESSE-2 (5'-ACATCTCAGCCAATCCAGC-3').

2.11 Identification of the ε Sterile Transcript in Tissue Samples from Ova-Sensitised Mice

Mice were first sensitised by intra-peritoneal injection of either NaCl (control) of 10 μg of ovalbumin 6 times every two days for a period of two weeks. 26 days later, mice were subject to 3 airway challenges of either NaCl (control) or 20 μg of ovalbumin by deposition in the trachea. One challenge was given every three days. Liver, thymus, spleen, lymph node or lung tissue was taken from the contol or ova-challenged mice 2, 5 and 8 days after the final airway challenge of NaCl or ovalbumin.

Total RNA was prepared from these tissues using the Triazol solution (GibcoBRL), following manufacturer's guidelines, followed by treatment with DNase in order to remove any contaminating genomic DNA. First-strand cDNA was prepared from 10 μg total RNA in each case using the First-strand cDNA Synthesis Kit (Pharmacia) following manufacturer's guidelines.

PCR amplification on the first-strand cDNA was performed using either HPRT-5P and HPRT-3P (see earlier) for amplification of the HPRT housekeeping gene, or with RTIE-2 (5'-AGCCACTCACTTATCAGAGGACC-3') and 3PCE (see earlier) for amplification of the sterile ε transcript. PCR conditions of 40 cycles of 94°C for 1 minute, 55°C (HPRT) or 65°C (sterile ε transcript) for 1 minute and 72°C for 1 minute 30 seconds were used.

3. RESULTS

3.1 Library Screening and Cloning of Genomic Arms for Gene Targeting

In order to construct the gene targeting vector, two genomic DNA arms of approximately 1 Kb and of 4-6 Kb were required to target the LacZ gene to the ε constant region (41). The smaller 1 Kb arm is needed in order to facilitate PCR screening of ES cell clones, as this gives a suitable size for amplification (see later). In order to clone ε constant region genomic DNA, a mouse genomic library was screened using two probes, one hybridising to the Iε promotor/exon region, and the other hybridising to the third exon of the ε constant region of the IgH gene locus.

After the first round of screening, 14 phage clones were found to be positive for the two probes, 10 of which were selected for the subsequent rounds of screening. Following three rounds of screening, Lambda DNA from two of the ten clones, named 3.2 and 3.4, was purified. The genomic DNA contained in clone 3.2 was subsequently digested with the restriction enzyme *PstI*, products from the digestion recloned into pBluescript and colonies containing the 1.1 Kb fragment (housing the Iε promotor/exon) and the 4 Kb fragment (housing the Sε region) screened for by colony hybridisation (Figure 3).

3.2 Insertion of a Nuclear Localisation Signal onto β-Galactosidase

The vector pGT1.8IRESβGeo contains a β-Galactosidase gene preceded by a splice acceptor from the mouse En-2 gene and an IRES from the encephomyocarditis virus (EMCV), both of which are required in the gene targeting construct. In addition to these,

DNA encoding a NLS is required directly upstream of the β-galactosidase gene. Due to the lack of any convenient restriction enzyme sites the NLS was inserted using bacterial homologous recombination (see materials and methods and figure 4).

Transformation of *E.coli* strain DH5α with the appropriate DNA fragments resulted in the growth of 11 colonies which were resistant to both ampicillin and kanamycin. Of these 11 colonies, 2 were shown to contain DNA plasmids resulting from homologous recombination of the two overlapping DNA fragments in the bacteria, as demonstrated by restriction enzyme and sequence analysis. The other 9 colonies were found to be the result of trace amounts of undigested vector in the DNA preparations.

3.3 Mutagenesis to Revert the NLS back to Wild Type

Following sequencing, the NLS was found to contain an unwanted single base mutation, whereby at base 37, an A had been changed to a T, changing an AGA argenine codon to a TGA stop codon. This was most likely due to an error in the oligonucleotide primer (the mistake is in the primer binding site) or a mismatch error during the polymerisation step of the PCR reaction.

In order to correct the mutation, the DNA fragment containing the En-2 splice acceptor, IRES, NLS and partial β-galactosidase gene (modified LacZ or mLacZ) was recloned into pBluescript with the restriction enzymes *BamHI* and *ClaI*. PCR amplification was now performed using the mutant DNA as template using a long upstream primer containing the reversion mutation as well as a 5' *NcoI* restriction site. This *NcoI* restriction site is situated at the junction of the IRES and the NLS and is unique in the vector. The downstream primer was designed so as to contain the *HpaI* restriction site in the β-

galactosidase gene and also a unique restriction site in the vector. Amplification resulted in the production of a 500 bp DNA fragment which was subsequently recloned into the mutant vector using the *NcoI* and *HpaI* unique restriction sites (Figure 5). Reversion to wild type was confirmed by sequence analysis.

3.4 Expression of mLacZ in Cos 7 Cells

The functioning of the modified LacZ construct was demonstrated by transient expression in the Cos 7 cell line. Cos 7 cells constitutively express the SV40 large T antigen and therefore expression vectors containing the SV40 origin of replication (ori) can replicate episomally resulting in large numbers of plasmid per cell and therefore high amounts of protein expressed by the plasmid.

In order to obtain the full length LacZ, the mLacZ was recloned from pBluescript into pGN (a vector containing full length LacZ) using the restriction enzymes *NotI* and *ClaI*. Full length mLacZ could now be obtained by digestion with the restriction enzyme *BamHI* alone and was subsequently cloned into the expression vector pCDNA3 under the control of the CMV promotor. As a way of trying to demonstrate that the En-2 slice acceptor was functional, full length mLacZ was cloned using the *BamHI* restriction sites into a second vector, pCDNA3/SRα where the CMV promotor has been replaced by the SRα promotor (42) and an SV40 splice donor. Therefore, mRNA produced by the CMV promotor construct will have an exposed splice acceptor, whereas mRNA produced by the SRα promotor construct in theory has a functional splice junction. Both expression constructs contain the SV40 ori, allowing for episomal replication.

DNA was transfected into the Cos 7 cells using either electroporation or lipofection, β-galactosidase expression detected either by blue staining with X-gal or using fluorescent antibodies recognising the protein. Positive controls with and without a nuclear localisation signal were transfected in parallel. Blue staining demonstrated that the mLacZ was indeed functional, and the level of expression was strong (as strong as the positive controls when detecting time of appearance of blue cells after addition of the reaction mixture)(Not shown). The efficiency of the transfection was however lower than the controls, with also a marked difference between the efficiency of the pCDNA3 construct and the pCDNA3/SRα construct. The SRα construct had a ten-fold higher efficiency of transfection, suggesting that the splice junction could be playing a role in stabilising the mRNA produced by the expression vector in these cells (Figure 6). Nuclear localisation could clearly be seen for both constructs with the blue staining, as well as for the appropriate positive control. This finding was further supported by fluorescent antibody staining showing the β-galactosidase tightly localised in the nucleus where appropriate, and evenly distributed throughout the cytoplasm for the positive control without nuclear localisation (Figure 7).

3.5 Stable Transfection of the A20 B Cell Line with mLacZ under the control of the Iε Promotor

The murine lymphoma B cell line A20 was chosen in order to demonstrate that expression of β-galactosidase under the control of the Iε promotor could be detected in murine B cells.

RT-PCR was used in order to demonstrate that the sterile ε transcript is expressed in the A20 cell line upon stimulation with IL-4 and bacterial lipopolysaccharide (LPS). A very

weak band representing low level constitutive expression can be detected in the absence of stimuli, which is clearly and strongly unregulated in the presence of IL-4 and LPS. Amplification of the HPRT housekeeping gene was used as a positive control for the reverse transcription (Figure 8).

In order to prepare the vector for stable transfection, the mLacZ DNA fragment was excised from pBluescript using the restriction enzymes *BamHI* and *ClaI* and recloned into pBluescript containing the 1.1 Kb Iε promotor/exon region using the restriction enzymes *BglII* and *ClaI*. The mLacZ was now located downstream of and under the control of the Iε promotor. The Iε/mLacZ fragment was then excised from pBluescript using the restriction enzymes *NotI* and *ClaI* and recloned into pGN at these sites in order to reconstitute the full length LacZ.

A20 cells were then electroporated with this DNA and grown in selection medium. After approximately three weeks, two separate cell populations grew which were resistant to G418, one from undiluted transfected cells named crude and the other from transfected cells diluted 1:3 with medium named 1:3. Genomic PCR was then performed in order to ascertain whether the foreign DNA had actually integrated. Primers binding to the Iε promotor region and the 5 prime region of the LacZ gene were used to demonstrate that both populations had the foreign DNA integration (Figure 9).

Cells were then incubated with or without IL-4 and LPS for 4 days, the time point at which a plateau of expression was found by PCR (data not shown), and expression of β-galactosidase detected either by blue staining of by FACS analysis. A clear up regulation of expression of β-galactosidase could be detected by FACS analysis with stimulated transfected cells when compared to unstimulated transfected and untransfected cells (Figure 10). These findings were supported by blue staining of the transfected cells

(figure 11). This experiment demonstrated that the mLacZ gene, under the control of the Iε promotor, could express detectable levels of β-galactosidase in murine B cells.

3.6 Construction of the Gene Targeting Vector

The vector pGN (43)was chosen for the gene targeting and contains the full length LacZ gene encoding β-galactosidase and a neomycin resistance gene under the control of a polyoma enhancer/RSV LTR promotor for selection in mammalian cells and the Tn5 transposon for selection in *E.coli*.

It was first modified by placing an oligonucleotide linker containing the *AscI* restriction site between the *SpeI/KpnI* restriction sites in the multiple cloning site. This allowed for the recloning of the partially characterised Sε genomic fragment into pGN from pBluescript. The upstream *LoxP* recognition site for the *Cre* recombinase was then inserted by oligonucleotide annealing and ligation between the unique *ApaI* and *AscI* restriction sites. The downstream *LoxP* site was introduced into the vector and the LacZ polyA signal removed simultaneously by digestion of the vector with the restriction enzymes *Bpu1102 I* and *XhoI* and insertion of the *LoxP* site by oligonucleotide annealing and ligation. The oligonucleotide sequence also contained a *PmlI* restriction site 5' of the *LoxP* site. The splice donor from the Iε exon was then excised from pBluescript containing the Iε promotor/exon region by digestion with the restriction enzymes *AvrII* and *NcoI* and the ends filled using the Klenow fragment of DNA polymerase I (Amersham Life Sciences). pGN was subsequently digested with the restriction enzyme *PmlI* and the splice donor fragment ligated in.

The Thymidine Kinase (TK) gene will be used to identify by negative selection clones which have undergone *Cre* recombination. The gene was excised from the vector pIC19R/MC1-TK (44) with the restriction sites *XhoI* and *SalI* and recloned into pGN at the unique *SalI* site upstream of the neomycin resistance gene. Colonies having the TK gene in the reverse orientation to that of the neomycin resistance gene were determined by restriction digestion of the vector. The 3 Kb DNA fragment containing the 1.1 Kb Iε promotor/exon region genomic DNA and the mLacZ was excised from pBluescript using the restriction sites *NotI* and *ClaI* and recloned into pGN digested with the same enzymes. The 4 Kb genomic DNA containing the Sε region was then excised from pBluescript with the restriction sites *AscI* and *NotI* and recloned into pGN at these sites (figure 12).

3.7 *Cre* Recombination of the Gene Targeting Vector in *E.coli*

The completed gene targeting construct was tested for its ability to undergo *Cre* recombination at the two *LoxP* sites in the *E.coli* strain Am-1. Am-1 *E.coli* have the gene encoding the Cre recombinase under the control of the Lac promotor. The bacteria were transformed with the gene targeting construct and then induced to express the *Cre* recombinase. The surviving colonies should contain plasmid DNA containing the neomycin resistance gene, pBR322 origin of replication and the TK gene only. The other plasmid resulting from the recombination should be lost due to the absence of the neomycin resistance gene and the pBR322 origin of replication.

24 Kanamycin resistant colonies were screened by PCR, and of these 24, only 3 colonies were non-recombinant, strongly amplifying a band of 350 bp. All other colonies amplified only weakly the 350bp band, suggesting recombination had taken place but

trace amounts of non-recombinant plasmid were present. 6 resistant colonies resulting from the transformation with pGN strongly amplified a band of 550 bp as expected. DNA from 8 of the colonies shown to be recombinant by PCR was analysed by restriction digestion using the restriction enzyme *PstI* and this confirmed that the recombination had occurred (data not shown).

3.8 ES Cell Transfection Positive Control

In order to optimise the conditions for the PCR screening of the gene targeting in the ES cells, a positive control was set up to mimic the homologous recombination. The 3 Kb DNA fragment containing the 1.1 Kb Iε promotor/exon region and the mLacZ was recloned into pGN along with a 100 bp *NcoI/PstI* restriction enzyme fragment which directly precedes the 1.1 kb genomic fragment in the genome but is not contained within the gene targeting construct. This represents exactly the configuration of the DNA following homologous recombination of the gene targeting construct (figure 13).

The DNA was linearised with the restriction enzyme *NotI* and transfected into the ES cell line D3. Following selection, 2 clones, called 2 and 5, were grown up, whilst the rest of the clones were pooled. 3 upstream primers, ESNCO-1, -2 and -3 were designed to hybridise to the 100 bp region upstream of the 1.1 Kb Iε genomic fragment, whilst the three downstream, ESEN-1, -2 and -3 were designed to hybridise to the En-2 splice acceptor DNA region. Pooled clone genomic DNA was used to find the best primer combination and $MgCl_2$ concentration in the buffer. Untransfected DNA was used as a negative control.

ESNCO-3 and ESEN-3 gave the best amplification with the least background using an MgCl2 concentration of 1 mM final. Of the two clones tested , only clone 5 gave a positive signal, and the amplification seemed to be much stronger than that given by the pooled clones. These results suggest that the full length DNA is not always amplified, and therefore amplification of a positive clone is more representative than that of a pooled clone.

3.9 Gene Targeting of the ES Cells

The gene targeting construct was linearized with the restriction enzyme *NotI* and transfected into Ethe ES cell line HM-1 (figure 12). Clones resistant to the selection medium were screened by genomic PCR for the presence of the desired genetic modification using the primers ESNCO-3 and ESEN-3. Two clones were identified, expanded and transiently transfected with the *Cre* recombinase expressing construct pGK-Cre pA and cells selected for the absence of the TK gene using FIAU. None of the resulting resistant subclones screened by PCR using the primers Lac3-5P and ESSE-2 were found to have undergone LoxP recombination. Figure outlines the steps involved in the gene targeting.

Cells from the original clones (without LoxP recombination) were subsequently injected into mouse blastocysts which were in turn injected into pseudo-pregnant females. Two male chimeric mice resulted, but numerous attempts to cross with non-chimeric females failed to result in the germline transmission of the desired genetic modification.

3.10 Identification of the Sterile ε Transcript in Tissues of Ova-Sensitized Mice

Total RNA (pooled in each case from 4 mice) was prepared from cells from the liver, thymus, spleen, lymph node or lung of either control or ova-sensitized mice at day 2, 5

and 8 post allergen challenge. RT-PCR from each tissue sample was performed in order to localise expression of the ε sterile transcript in different organs. Amplification of the HPRT housekeeping gene was used as a positive control for the reverse transcription. Expression of the ε sterile transcript was detected in the spleen as expected as well as in the lymph nodes. Interestingly, a signal was also detected in the lung of ova-sensitised mice at day 2, 5 and 8 after challenge of the allergen suggesting that class switching to IgE is occurring in this tissue. A signal from the lung of day 2 control mice was also detected, possibly being the consequence of pooling material from 4 mice in each case (figure 15).

4. DISCUSSION

Being an essential prerequisite in the B cell class switching to IgE, localisation of the expression of sterile ε transcripts is a useful tool in the identification of tissues in which B cells are undergoing such a switch. The sterile transcripts are expressed at relatively high levels per cell, allowing it to be detected by such techniques as *in situ* hybridisation with riboprobes using control and ova-challenged mice for example. The system chosen to localise sterile ε transcripts was in fact a reporter gene system, whereby the LacZ gene was placed under the control of the Iε promoter in the mouse genome by gene targeting. Cells undergoing the switch to IgE can therefore by detected by blue of fluorescent staining (Figure 2).

This system was chosen rather than the more conventional technique of *in situ* hybridisation for the following reasons: Firstly, the detection of blue or fluorescent cells in tissue sections is much easier than the use of riboprobes binding to RNA species, where problems of RNA degradation may exist. In addition, a reduction in non-specific background is likely to be observed. Secondly, the use of double staining will allow not only the detection of β-galactosidase, but also of cells types in which it is expressed, and cells co-localising with these cells to cause induction of the Iε promoter. Thirdly, this will be a useful tool to have in-house, allowing a number of crosses to be performed in order to assess the function of various immunoregulatory molecules on the induction of Iε promoter activity. For example, CD40L deficient mice, c-kit ligand deficient mice and CD28 deficient mice.

The first requirement of any gene targeting construct is genomic DNA to target the gene itself (45,46,47). A mouse genomic library was screened using two probes in parallel hybridising to different ends of the ε constant region of the Ig heavy chain locus (Figure 3). This ensured that clones positive for hybridisation with both probes would contain the complete ε constant region, and not a truncated form. The gene targeting vector itself requires two genomic arms, one of approximately 1 Kb and one of 4-6 Kb. The 1 Kb arm is

designed to facilitate the screening of the ES cell clones following transfection. The upstream primer is designed to hybridise to the genomic region directly upstream of the genomic arm, and the downstream is designed to bind directly downstream of the genomic arm in the gene targeting vector, (i.e. the first region of DNA foreign to the genome following homologous recombination). A DNA band of around 1 Kb amplified from any given clone suggests that the genome of this particular clone had undergone a homologous recombination with the gene targeting vector (figure 14).

The ε region of the IgH heavy chain locus has already been partially sequenced and characterised by restriction enzyme analysis (48). From this partial restriction analysis, three candidate *PstI* restriction sites were identified for the cloning of the genomic arms. Digestion with the restriction enzyme *PstI* produces a 1.1 Kb fragment containing the Iε promoter/exon region as well as 250 bp of DNA situated between the Iε exon and the Sε region. A 4 Kb fragment is also produced containing DNA from the Sε region itself. These two fragments were recloned into the vector pBluescript in order to both scale up the quantity of these DNA fragments increase the number of cloning sites flanking the two DNA fragments. This facilitated recloning of the fragments into expression vectors and the gene targeting vector (Figure 3).

In order to function in the reporter system proposed, the LacZ gene must be modified by insertion directly upstream of a murine SA, an IRES and an NLS. The vector pGT1.8IRESβ geo contains a LacZ gene with a murine SA and IRES. The insertion of a NLS between the IRES and the LacZ start site could not easily be performed using conventional recloning techniques due to the absence of any convenient restriction sites. The technique of bacterial *in vivo* cloning (IVC) (49,50) was chosen as a rapid and relatively simple alternative using PCR and 5' primer extensions to insert the NLS into the requires region and to obtain overlapping regions of homology (Figure 4). Fragments used for bacterial transformation had two regions of overlapping homology, one of 41 bp and the other of 466 bp. Of eleven colonies screened, 2 contained the desired recombined plasmid. It has been reported that recombination can been achieved with as little as 45 bp of total homology with up to 49 % of

the colonies being recombinant, and up to 64 % of the colonies recombinant with a total homology of 88 bp (49). Other groups have reported up to a 100 % efficiency of recombination with 88 bp of shared homology using the bacterial strain JC8679 with DNA purification by precipitation with ethanol (50). The lower percentage of recombinants in our hands may be due to the DNA purification procedure (purification by adsorption on glass beads) and the strain of bacteria used (DH5α).

Transient transfection in the cell line Cos 7 was chosen as the initial system to test the functioning of the mLacZ in mammalian cells. This system was chosen as the cell line has been transformed with the SV40 large T antigen, allowing episomal replication of plasmids containing the SV40 origin of replication and therefore transient but high level expression of foreign proteins. The vectors used were pCDNA3, containing the CMV, and pCDNA3/SRα, containing the SRα promoter and an SV40 splice donor. Both contain the SV40 ori and have strong promoters, allowing a potential high level expression of β-galactosidase. The mLacZ gene was tested under the control of the SRα promoter as it contains a complete splice junction, whereas mRNA produced from the CMV controlled mLacZ gene will have an exposed splice acceptor, possibly decreasing expression. This allowed us to examine whether the En-2 splice acceptor of the mLacZ was functional by looking for differences between the two transfections. Fluorescent antibody staining in parallel with the blue X-gal staining also gave us the opportunity to examine the nuclear localisation of β-galactosidase in transfected cells.

Results demonstrated that the SRα construct had a between a four and ten-fold higher efficiency of transfection (depending on the method of transfection employed) when compared to the CMV construct (figure 6). As the size of the vectors was the same, and in both cases the mLacZ was under the control a strong viral promoter, the reason for such a difference in efficiency of transfection is likely to be down to the difference in splicing. Expression of β-galactosidase was strong for both mLacZ constructs, with blue cells visible after approximately 10 minutes staining with X-gal (not shown). A much higher efficiency of transfection was observed for positive controls pCMV-βgal (CMV promoter controlled

containing a NLS) and pSV2-βgal (SV40 controlled and without a NLS), although the strength of expression was approximately equivalent (figure 6). Efficiency of transfection was between three and ten fold lower for the SRα constuct, and between 10 and 100 fold lower for the CMV construct. There is no obvious reason for these differences, but the most likely explanation is the difference in expression vector used for the positive controls and the expression of the mLacZ. Nuclear localisation was observed for the CMV SRα and pCMV-βgal constructs, seen most clearly by fluorescent antibody staining (figure 7) but also evident by blue staining (not shown). No difference was observed in terms of signal intensity however between LacZ constructs with and without the NLS.

Stable transfection of the murine B cell lymphoma line A20 was employed to investigate expression of the mLacZ under the control of the Iε promoter in a B cell environment. We felt that this was the best way to mimic the situation *in vivo*, in terms of induction of the Iε promoter and detection of expression of β-galactosidase. Firstly, IL-4/LPS induction of the sterile ε transcript was shown to occur in this cell line using RT-PCR (figure 8). In other murine B cell lines and B lymphoid cells, IL-4/LPS stimulation induces a relatively strong expression of the sterile ε transcript, shown to be detectable at day 2 post stimulation, peaking at day 4 and tailing off at around day 8 (23,51). RT-PCR was performed after a 4 day stimulation. B cells stably transfected with the mLacZ under the control of the Iε promoter were then stimulated with IL-4 and LPS and expression of β-galactosidase detected at day 4 post stimulation by FACS of blue staining. FACS analysis demonstrated that a clear difference in β-galactosidase activity could be detected between non-transfected, transfected non-stimulated and transfected stimulated A20 cells (figure 10). A strong β-galactosidase activity was only detected in a small proportion of stimulated cells (log fluorescence of more than 10^2), a finding supported by blue staining, whereby approximately 2.5% of the cells stained blue (figure 11). This again raises the question of a threshold of expression, below which cells are not visibly stained blue. A background level of β-galactosidase is also detectable by FACS when comparing the untransfected A20 cells with the transfected non-stimulated A20 cells. The most likely cause of this is a low level constitutive activity of the I ε promoter, which was weakly detected by RT-PCR (figure 8). A very low percentage of

transfected, non-stimulated cells (0.1-0.4%) express β-galactosidase strongly enough to stain weakly blue in the presence of X-gal (figure 11). This blue staining can be explained when one considers the integration of DNA into a genome. The transfected B cell populations which resulted from the selection were not clonal. That is to say that not all the cells contain transfected DNA integrated at the same region in the genome. Some DNA will have integrated into transcriptionally inactive regions, whereas some will have integrated into of transcriptionally active regions. Insertion of the DNA into a transcriptionally active region of genomic DNA may lead to transcription through the mLacZ. As the mLacZ has an IRES, transcription will result in expression of β-galactosidase, leading to its detection in non-stimulated transfected cells, some of which will be relatively high owing to the nature of the integration.

This transfected B cell line can be a useful tool when studying the effects of various immunoregulatory molecules, in conjunction of IL-4, on the regulation of expression of sterile ε transcripts. The transfected cell line will need to be more sensitive to molecules regulating expression of the transcript, however, to detect the effects of molecules less potent than LPS. It is for this reason that clones will be derived from the transfected cell populations in order to find those which have a low constitutive background expression of β-galactosidase, with a high expression following stimulation with LPS and IL-4. Attempts to optimise the IL-4 concentration for the strongest induction of the Iε promoter will also be made. As nuclear localisation of the β-galactosidase in the transfected B cells is not evident by blue staining, future experiments will also include investigating the nuclear localisation by fluorescent antibody staining.

Following completion of the gene targeting vector (figure 12), its ability to undergo recombination at the *LoxP* sites in the presence of the *Cre* recombinase was demonstrated using the E.coli strain Am-1. 87.5% of the resultant clones following induction of the *Cre* recombinase were shown to be recombinant by PCR screening, demonstrating the viability of the *LoxP* sites and the relatively high frequency of recombination. The *Cre/LoxP* system has already been shown to be capable of mediating site-specific recombination in both

Mammalian cells (37) and transgenic mice (52, 53). In conjunction with TK negative selection for the recombination, efficiency of recombination of up to 80% has been reported in ES cells uptaking the supercoiled *Cre*-expressing construct, with no interference in subsequent germline transmission of the genomic alteration (54).

Having tested *in vitro* as far as possible important components of the gene targeting, optimisation of the conditions and primers used for the screening of the positive clones for the gene targeting event was required. This is an important step in the gene targeting process, as it reduces not only the risk of missing a truely positive clone, but also of obtaining false positive clones. The vector (figure 13) was designed in order to give clones following transfection and selection which represented as closely as possible a clone arising from a homologous recombination event. Primer combinations and PCR conditions were tested in orderto give the strongest specific amplification form genomic DNA obtained from ES cell clones transfected with the positive control construct, with a complete absence of non-specific amplification from non-transfected ES cells.

Following the optimization of screening by PCR, the linearized gene targeting construct was transfected into ES cells, and following selection with G418, two clones identified as positive for the genomic integration of plasmid DNA by homologous recombination, a result subsequently confirmed by Northern blot analysis (not shown). We failed however to identify subclones which had undergone LoxP recombination following transient transfection of the two clones with a *Cre* recombinase expression construct using a PCR based screening approach. Due to time restraints involving the length of time of cells in culture, the two positive clones were subsequently injected into mouse blastocysts without having achieved the LoxP recombination (see below). The idea behind this was to cross subsequent prodigy heterozygous or homozygous for the genetic modification with transgenic mice having the Cre recombinase gene under the control of a promotor active at the very early stages of foetal development. This would have allow us to achieve the LoxP recombination event at a later step. The lack of detection of LoxP recombination could be due to one of two reason. Firstly, the transient transfection may have been too inefficient to result in any clones undergoing LoxP recombination, although previous observations would suggest this not to be the case (54). The second explanation is the possible inefficiency of the screening process, whereby primers were used which had been optimized on plasmid DNA. Testing on genomic DNA would have been

more appropriate, but this particular optimization approach was not practicably achievable. Indeed, our suspicions were confirmed at a later date when the two clones were re-transfected with the *Cre* recombinase expression construct and subclones analyzed LoxP recombination this time by Northern blot analysis. Three subclones out of the 15 tested had undergone LoxP recombination (not shown), suggesting that the approach was a viable one but the original PCR based-screening was flawed.

From the blastocysts injected with cells from the two clones which had undergone the homologous recombination, two chimeric male mice were generated, one of which died shortly after birth. The surviving male was used for breeding, but over a six month period, succeeded in producing only one litter, none of which where heterozygous for the genetic alteration (as detected by coat colour), suggesting that in this particular male, the genetic modification could not achieve germline transmission. At this stage, the project was terminated.

The hypothesis of B cell class switching to IgE in the lung following an airway challenge to ovalbumin is something which can be crudely studied using RT-PCR. Tissue samples taken from mice following an ovabumin challenge, or control mice challenged with a saline solution were subject to RT-PCR in an attempt to amplify a signal specific for the sterile ε transcript. Signals were found in the spleen of both control and ovalbumin challenged mice at days 2, 4 and 8 post allergen challenge, as well as in control mouse lung at day 2 post allergen challenge (figure 15). These results demonstrate that the detection of the sterile ε transcript is possible using this technique, and more intersetingly, the transcript is differentially expressed in the lung of challenged and non-challenged mice.

The identification of B cells undergoing the switch to IgE in organs other than secondary lymphoid tissue (i.e. lymph node and spleen) could have major clinical implications. The fact that B cell class switching could take place at the physical barriers where allergen uptake is occuring, such as the lung, skin and gut, would lead to the distinct possibility of a local treatment for allergy in easily accessible tissues by blocking B cell class switching to IgE.

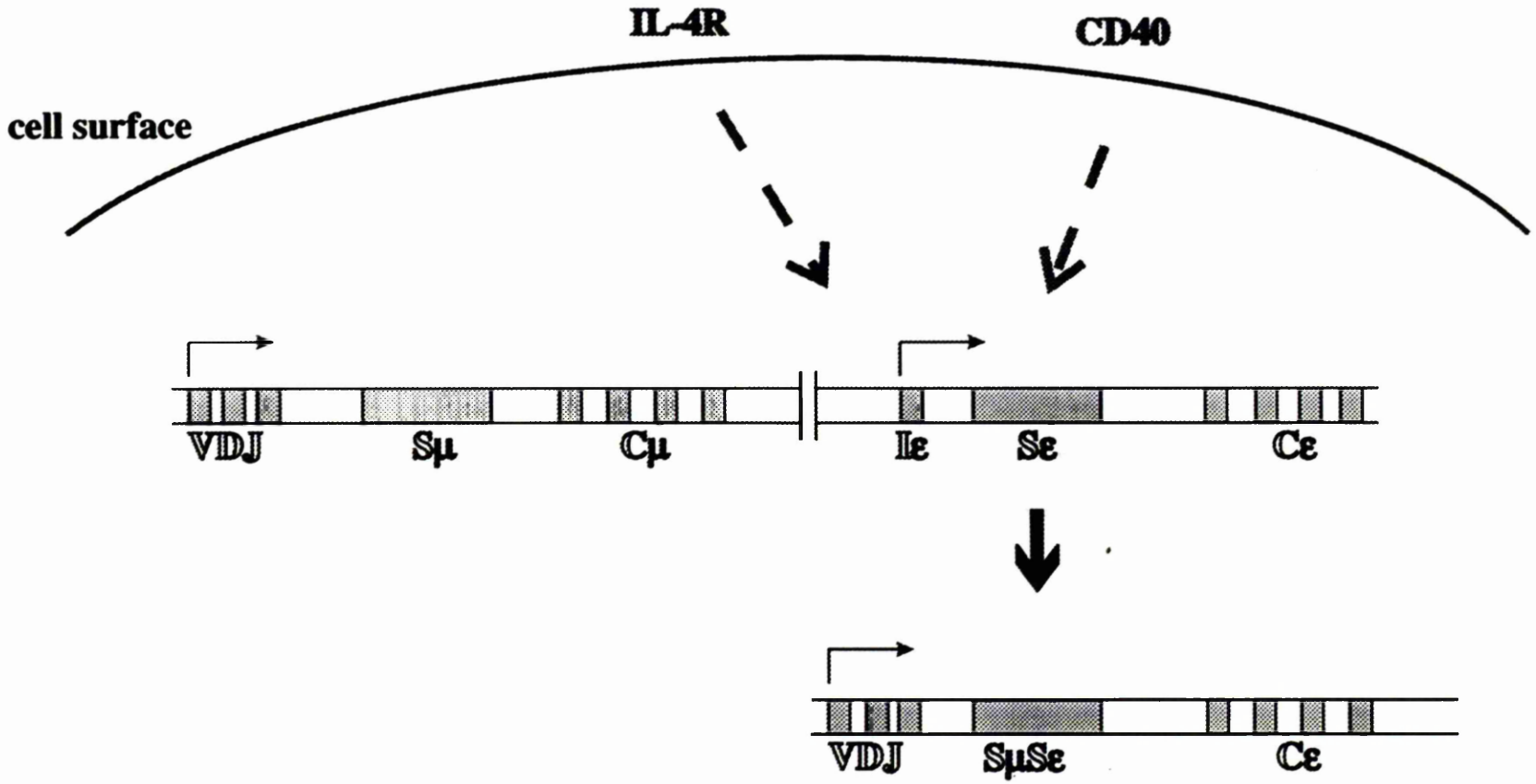


Figure 1. Schematic Representation of the signaling required and the genomic events occuring during the class switch to IgE.

Signalling through IL-4 induces expression of the sterile ε transcript, which, in conjunction with CD40L causes DNA recombination between the Sμ and the Sε. Following recombination, transcription from the variable region produces mature IgE.

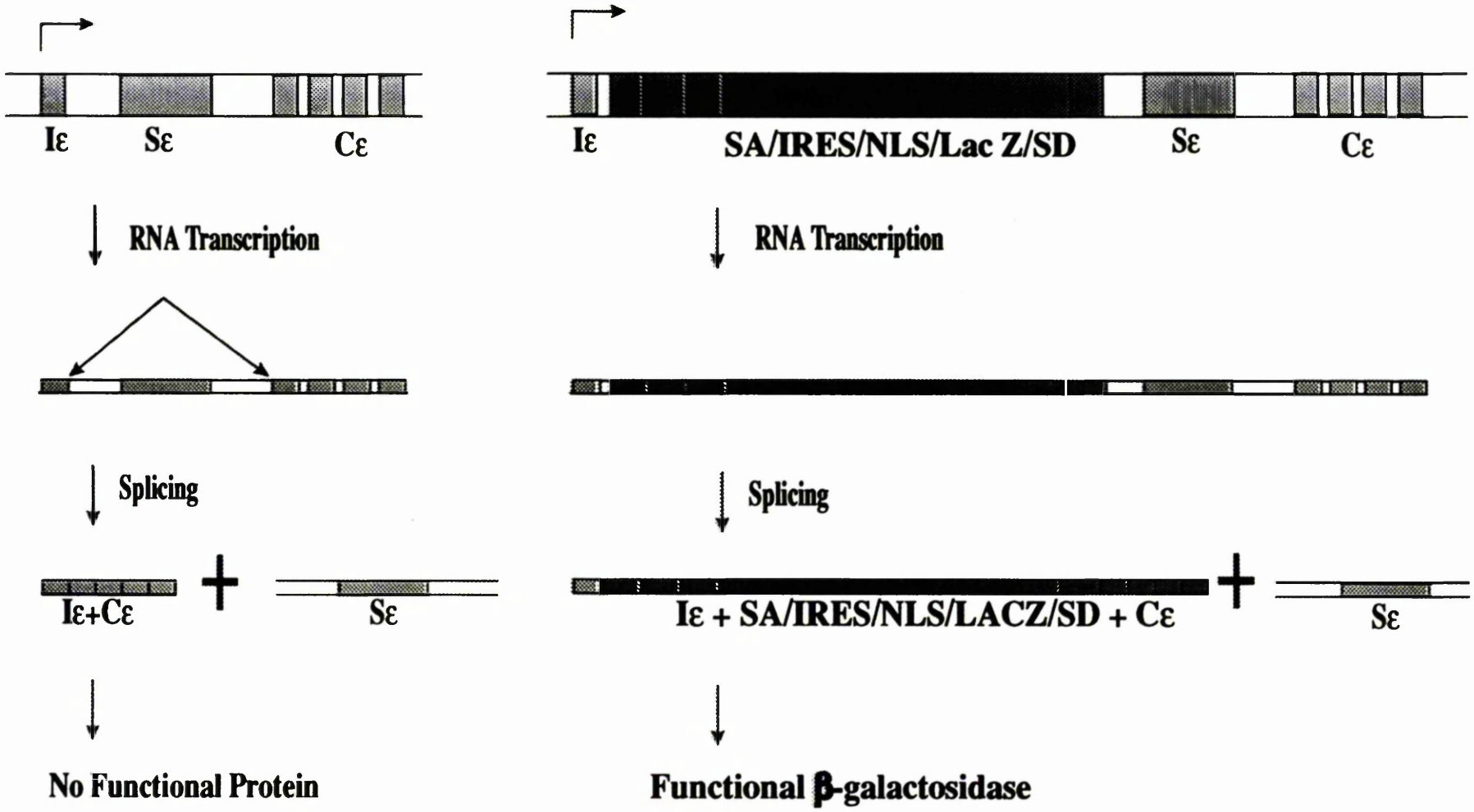


Figure 2. Transcription from the Iε promotor before and after Insertion of the mLacZ by Gene Targeting.

(a) Transcription from the Iε promoter of the sterile ε transcript following IL-4 induction. Mature transcripts resulting from RNA splicing do not express functional protein.

(b) Transcription from the Iε promoter of the mLacZ gene following IL-4 induction. Translation of processed transcripts results in the expression of β-galactosidase due to the presence of the IRES. Excsicion through splicing of RNA representing the Sε region has been retained.

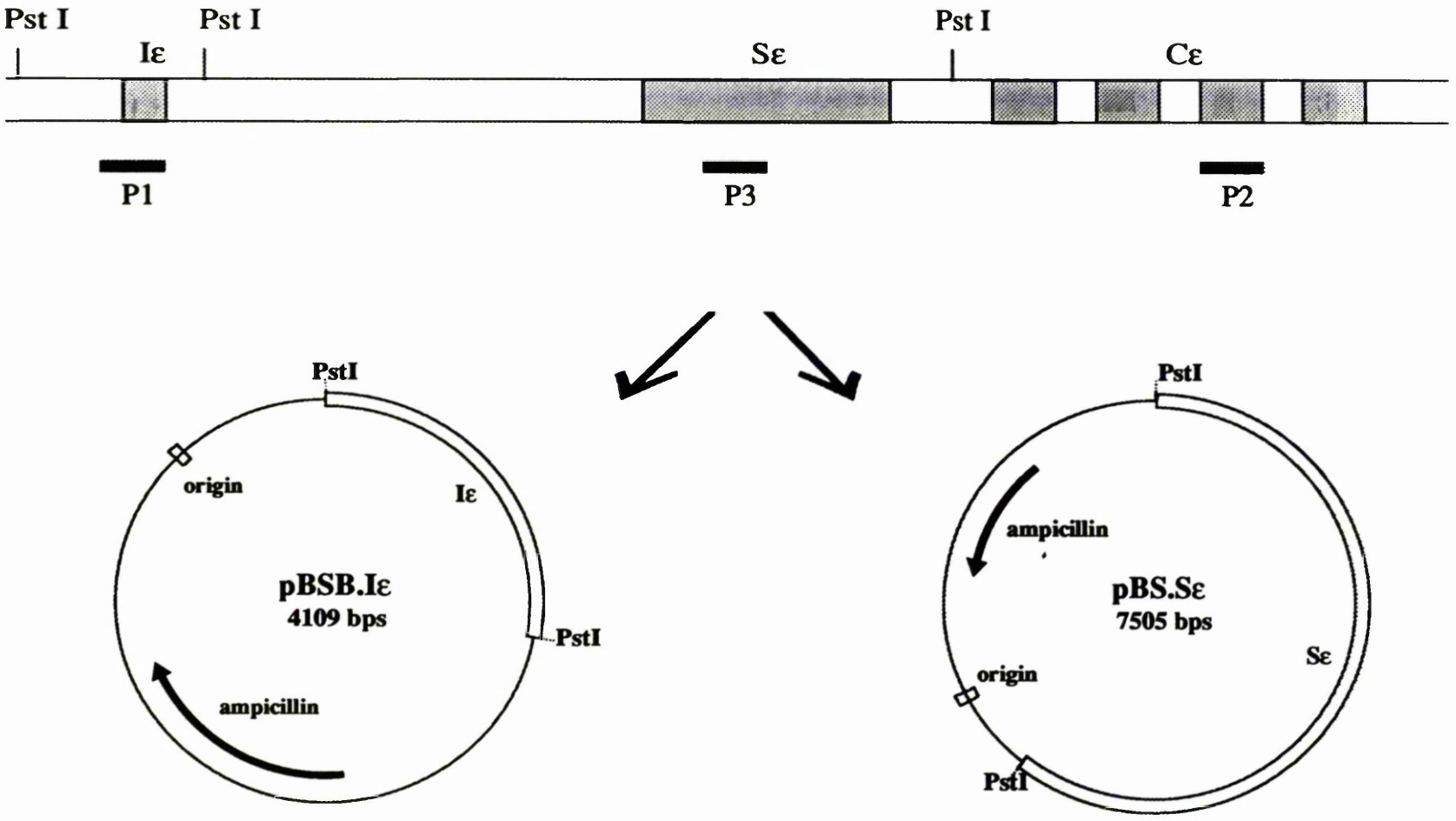


Figure 3. Diagram showing the Strategy for Genomic Library Screening and Recloning of the Genomic Arms for Gene Targeting.

A mouse genomic library was screened using the probes P1 and P2 in parallel. The probes hybridise to opposite ends of the genomic region, ensuring that the entire region was cloned.
Genomic DNA was subcloned with PstI and colony hybridisation was used to screen for the appropriate fragments using probes P1 and P3.

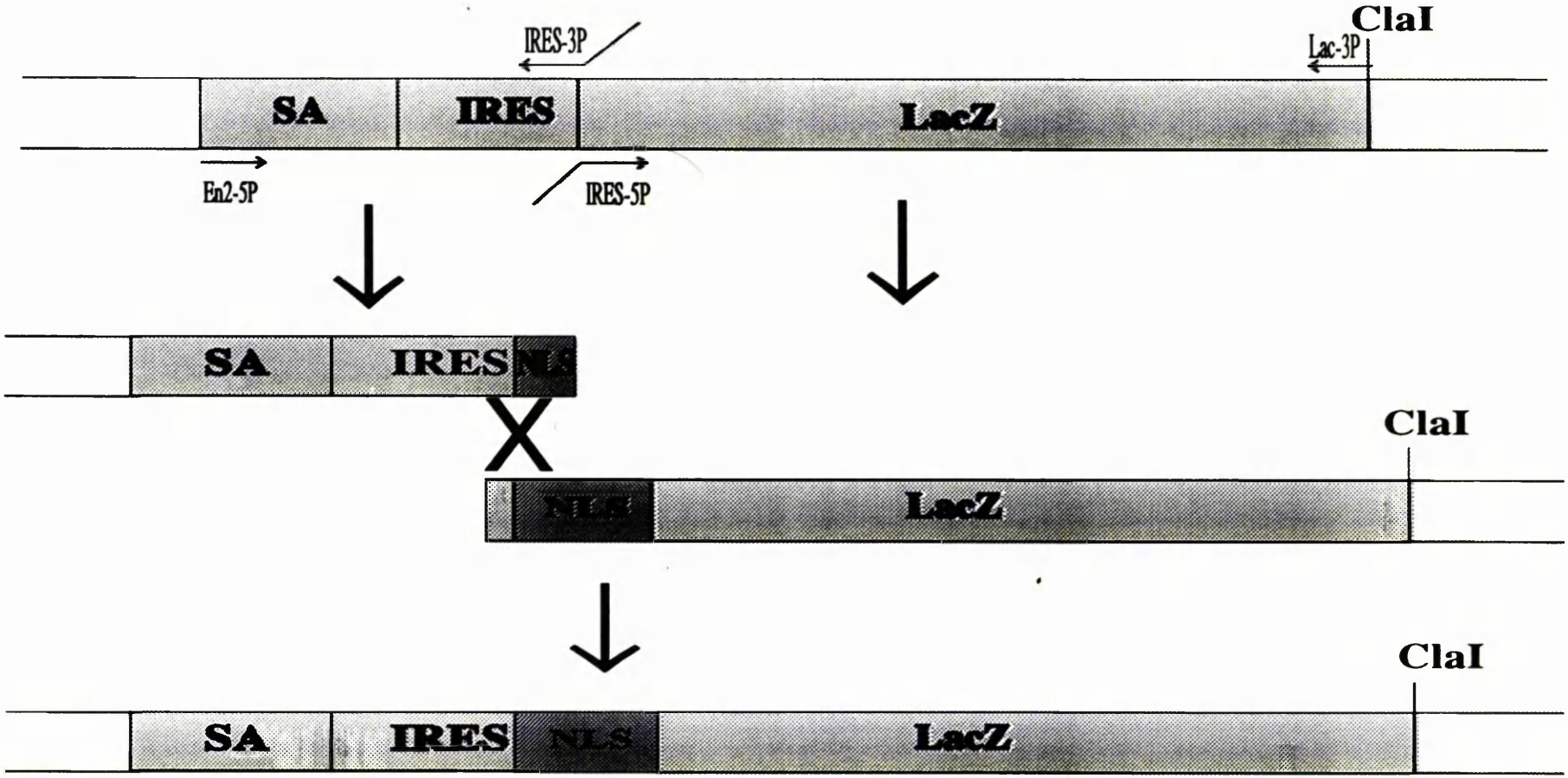


Figure 4. Diagram showing the Technique Employed for the Insertion of the NLS.

The NLS was introduced between the IRES and the LacZ gene by PCR amplification using 5' primer extensions. Fragments amplified using primers EN2-5P and IRES-3P or IRES-5P and LacZ-3P shared 41 bp of homology between the IRES and NLS. Combination of the fragments was achieved by bacterial homologous recombination.

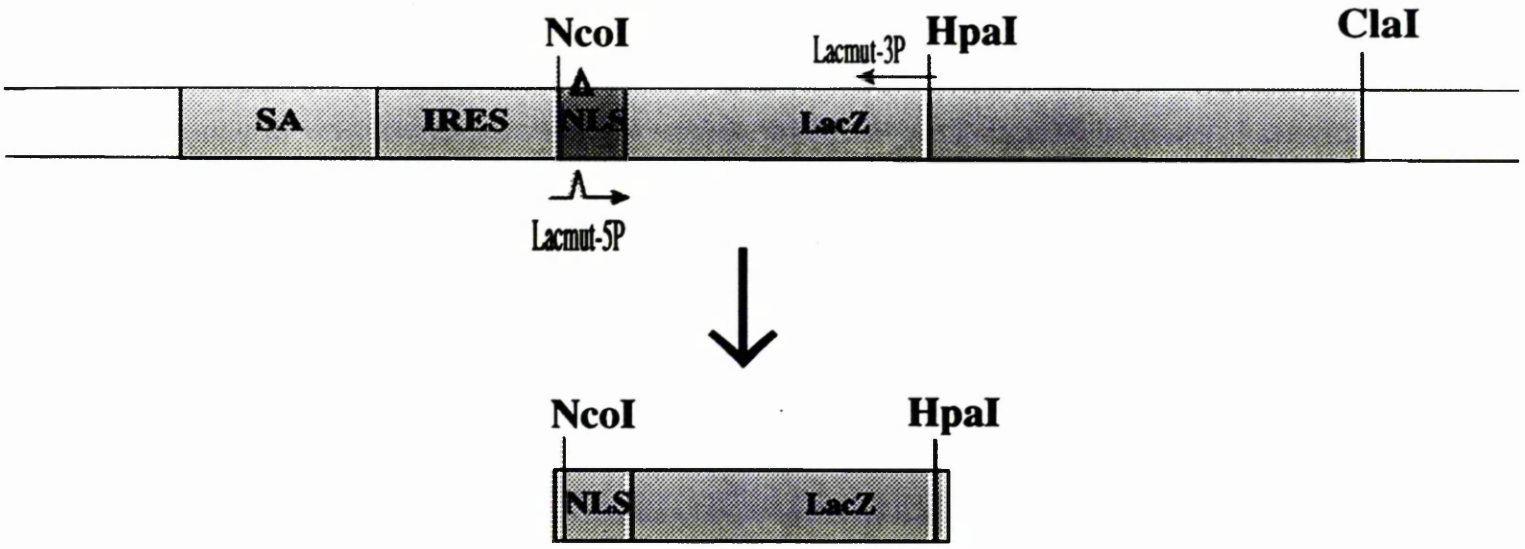
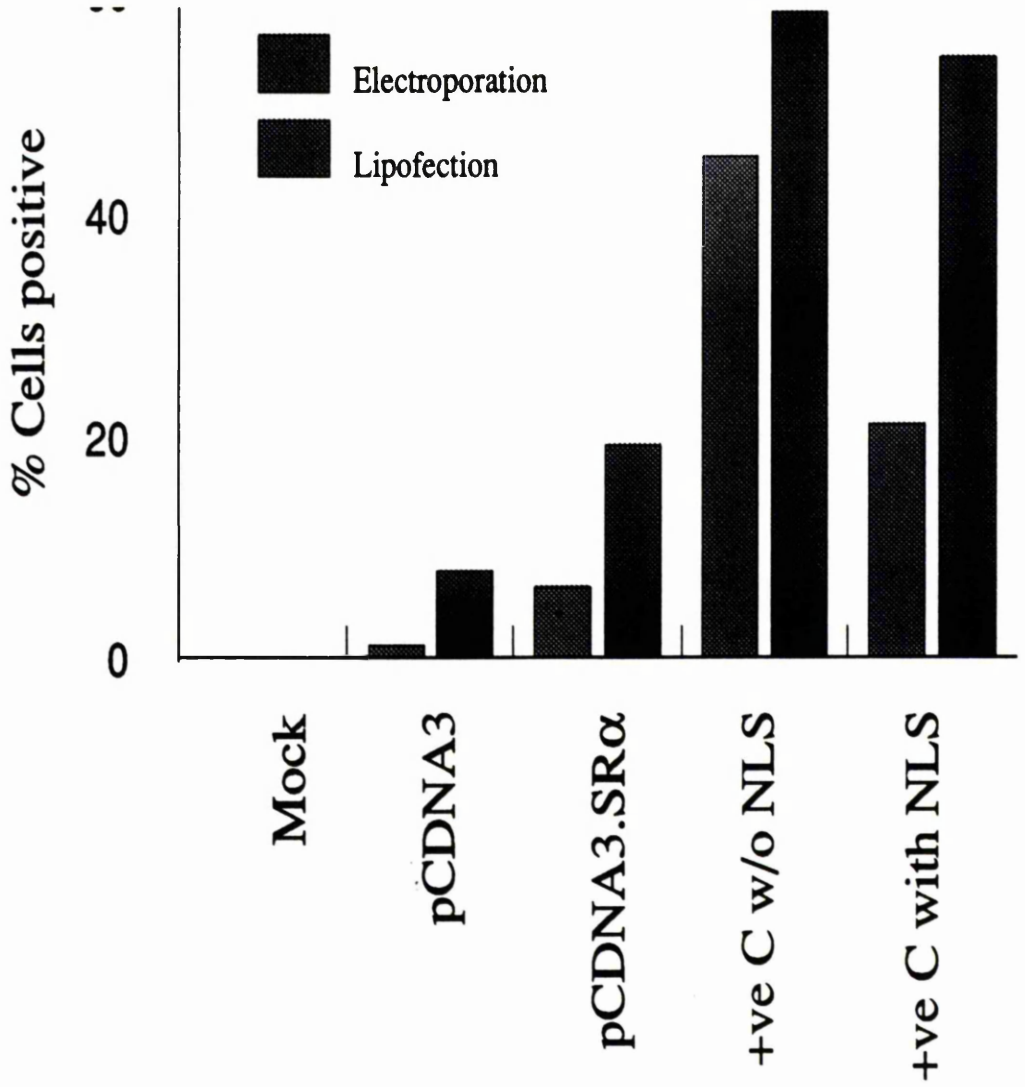


Figure 5. PCR Mutagenesis of the mLacZ

The primer Lacmut-5P contained the single base mismatch and its region of hybridisation spanned the *NcoI* site. Primer Lacmut-3P spanned the *HpaI* site. Amplification products were directly digested and recloned into pBluescript containing the mutant mLacZ.



→ **Positive cells visible after 10 minutes incubation with X-Gal**

→ **Strong blue staining after 1 hour**

Figure 6. Transient transfection of Cos 7 cells with the mLacZ gene.

72 hours post-transfection, cells were fixed and incubated with the X-gal reaction mixture for 16 hours and the % of blue staining cells calculated. Mock transfections were performed with empty pCDNA3. (See materials and methods for the other constructs transfected).

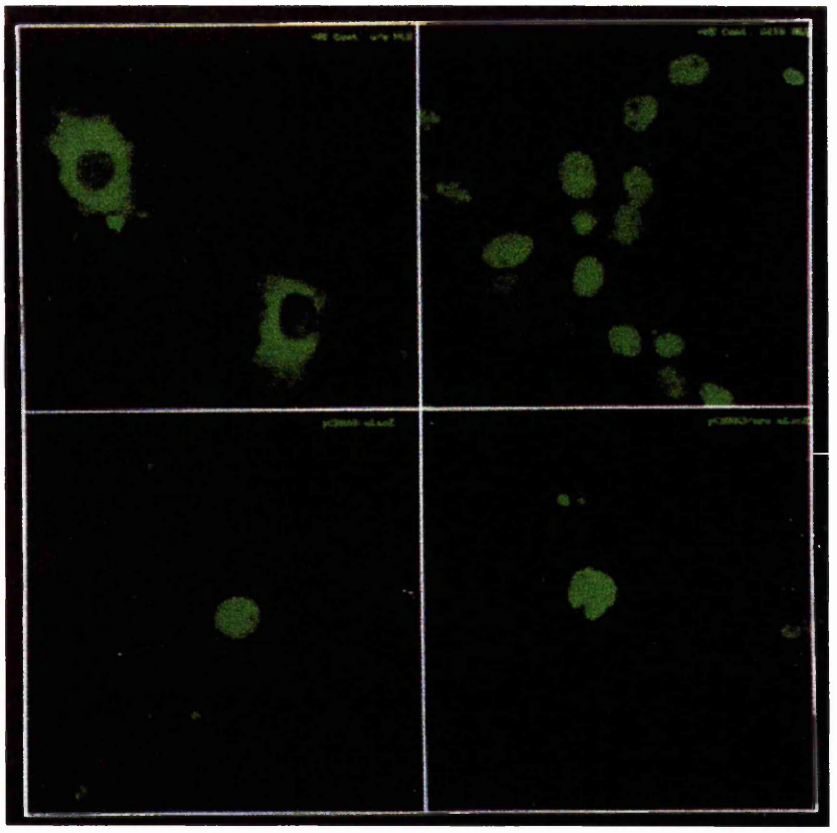


Figure 7. Picture Demonstrating Nuclear Localization of the β-Galactosidase.

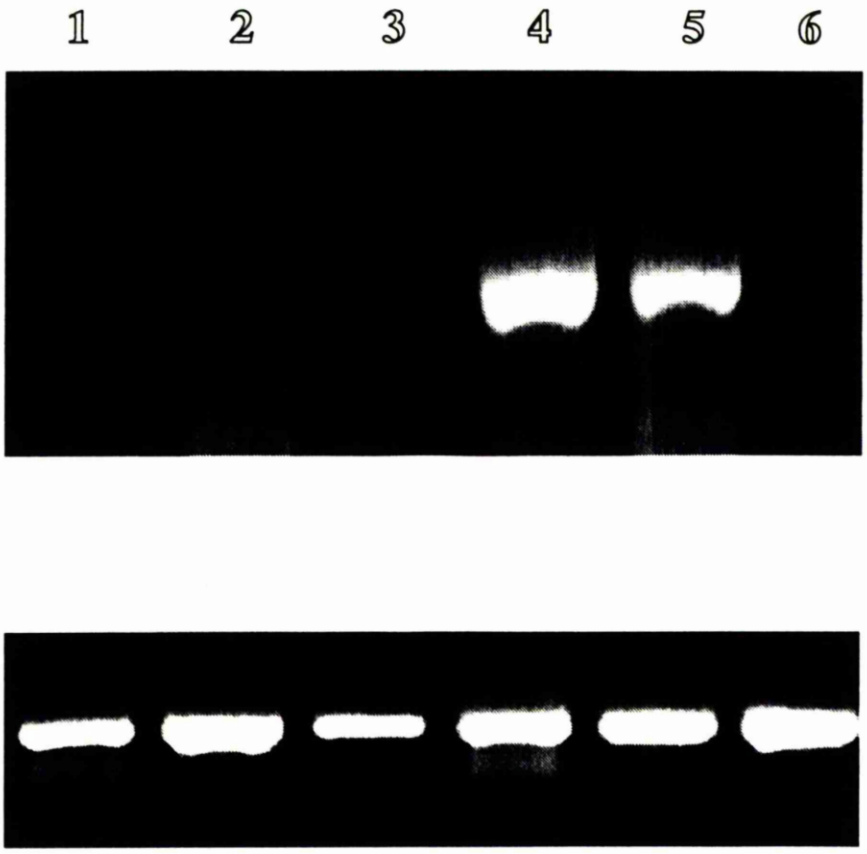
72 hours post-transfection, β-galactosidase was localised by fluorescent antibody staining and detected on a confocal microscope.

Top left; Cells transfected with pCMV-βgal (expressed without NLS)

Top right; Cells transfected with pSV2-βgal (expressed with NLS)

Bottom left; Cells transfected with pCDNA3-mLacZ (expressed with NLS)

Bottom right; Cells transfected with pCDNA3-mLacZ (expressed with NLS)

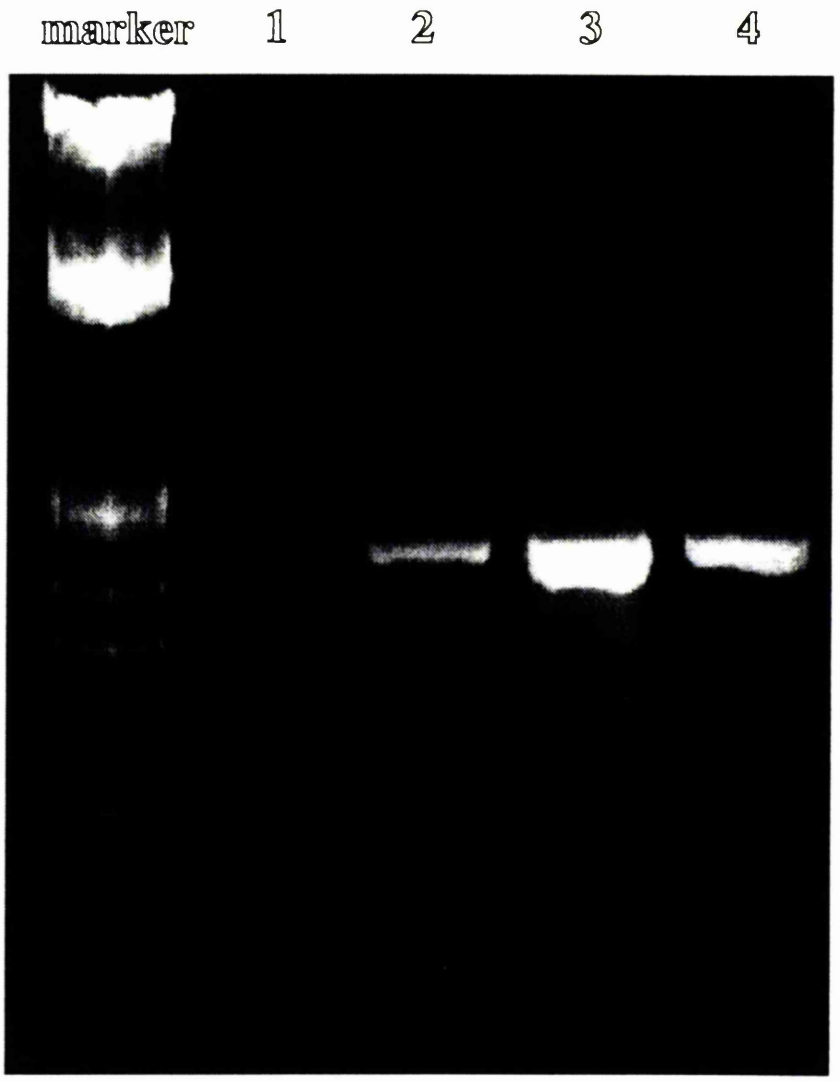


1. A20 Unstim. crude
2. A20 Unstim. 1:10
3. A20 Unstim. 1:100
4. A20 Stim. crude
5. A20 Stim. 1:10
6. A20 Stim. 1:100

Figure 8. Detection of Sterile ε Transcript expression in A20 B Cells by RT-PCR.

First-strand cDNA for the sterile ε transcript was amplified using the primers 5P-CEI and 3PCE. As a positive control for the reverse transcription, cDNA for the HPRT housekeeping gene was amplified using the primers HPRT-5P and HPRT-3P.

The amount of cDNA template used in each PCR amplification is demonstrated above by Crude, 1:10 and 1:100 which represent 5, 0.5 and 0.05μl of the reverse transcription reaction respectively (from 20μl total).



1. **Untransfected population**
2. **'Crude' population**
3. **'1:3 dilution' population**
4. **+ve Control**

Figure 9. Genomic PCR of Transfected A20 B cell Populations.

Transfected DNA was identified in the genome by amplification of genomic DNA using the primers 5PIE and OHR-2. The positive control for the PCR was purified plasmid DNA used for the B cell transfection itself.

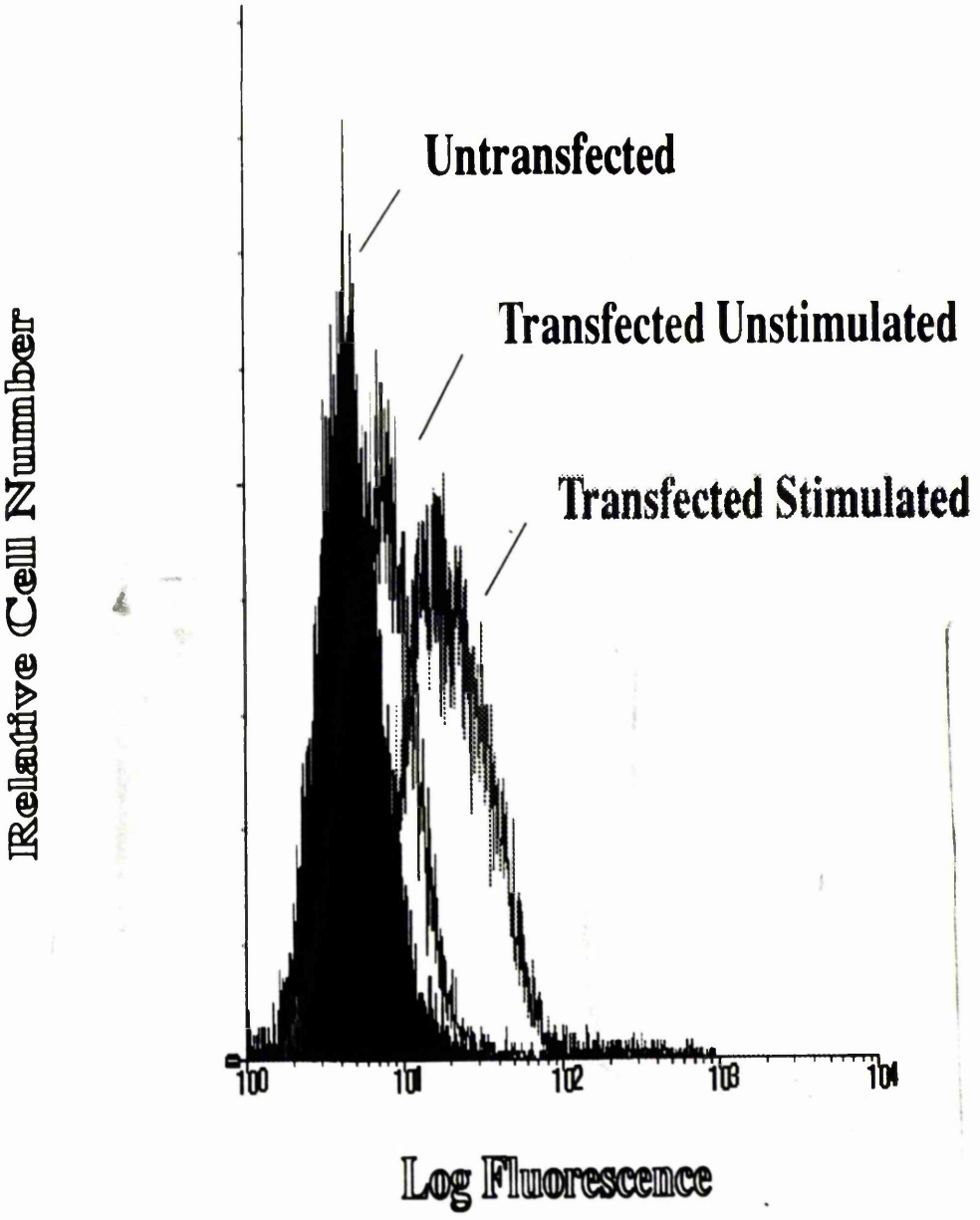
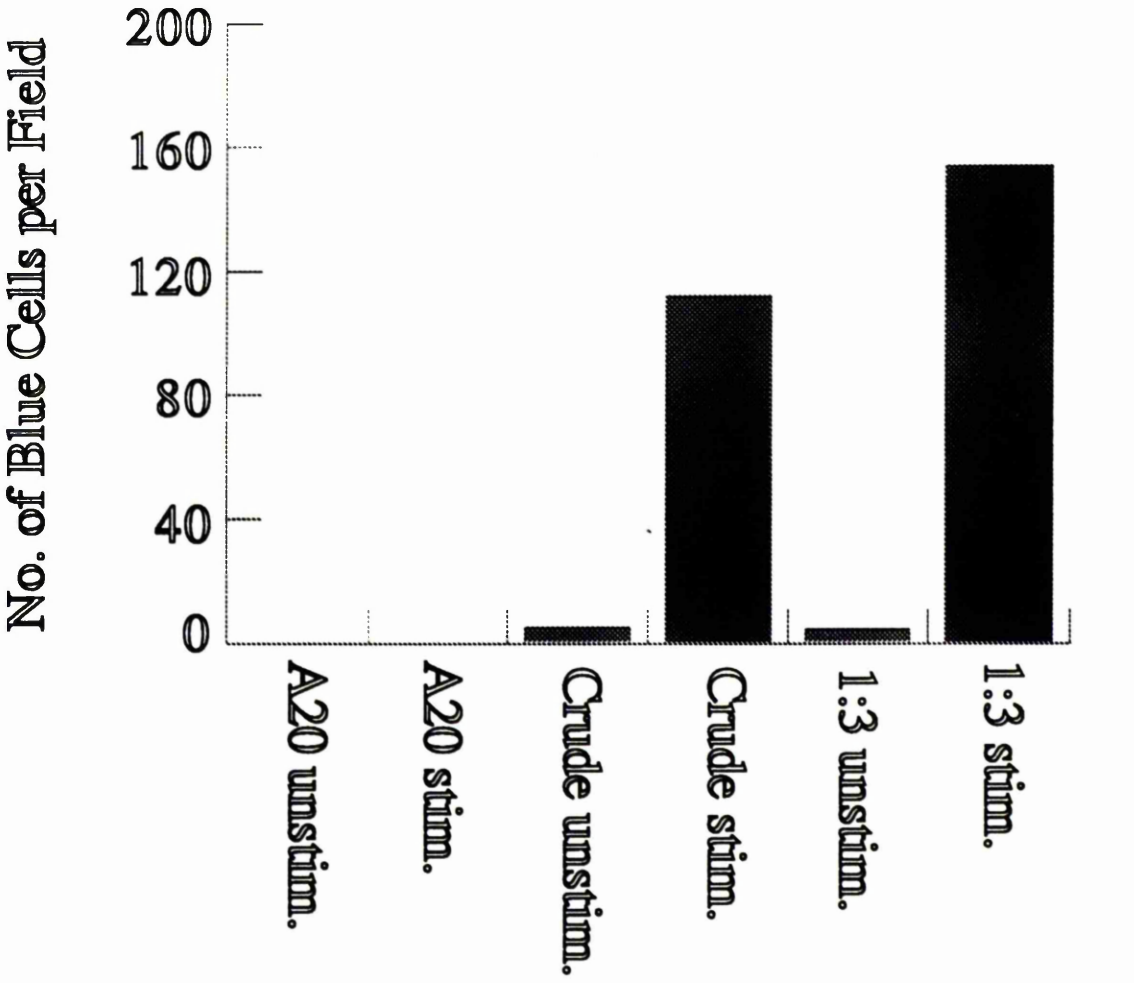


Figure 10. FACS analysis of β-galactosidase activity in Transfected B cells.

Transfected B cell population 1:3 was incubated for 4 days in the absence (unstimulated) or presence (stimulated) of IL-4 and LPS. β-galactosidase activity was detected using the fluorescent substrate FDG and FACS analysis.



→ **Blue cells visible after two hours incubation with X-Gal**

→ **Stimulated transfected cells strongly stained after 4 hours**

Figure 11. β-galactosidase activity in Transfected B Cell Populations following IL-4/LPS Stimulation.

Cell populations were incubated in the absence (unstim.) or presence (stim.) of IL-4 and LPS for 4 days. β-galactosidase activity in each cell population was assayed by X-gal staining and presented as the number of cells staining blue per field of 10 000 cells.

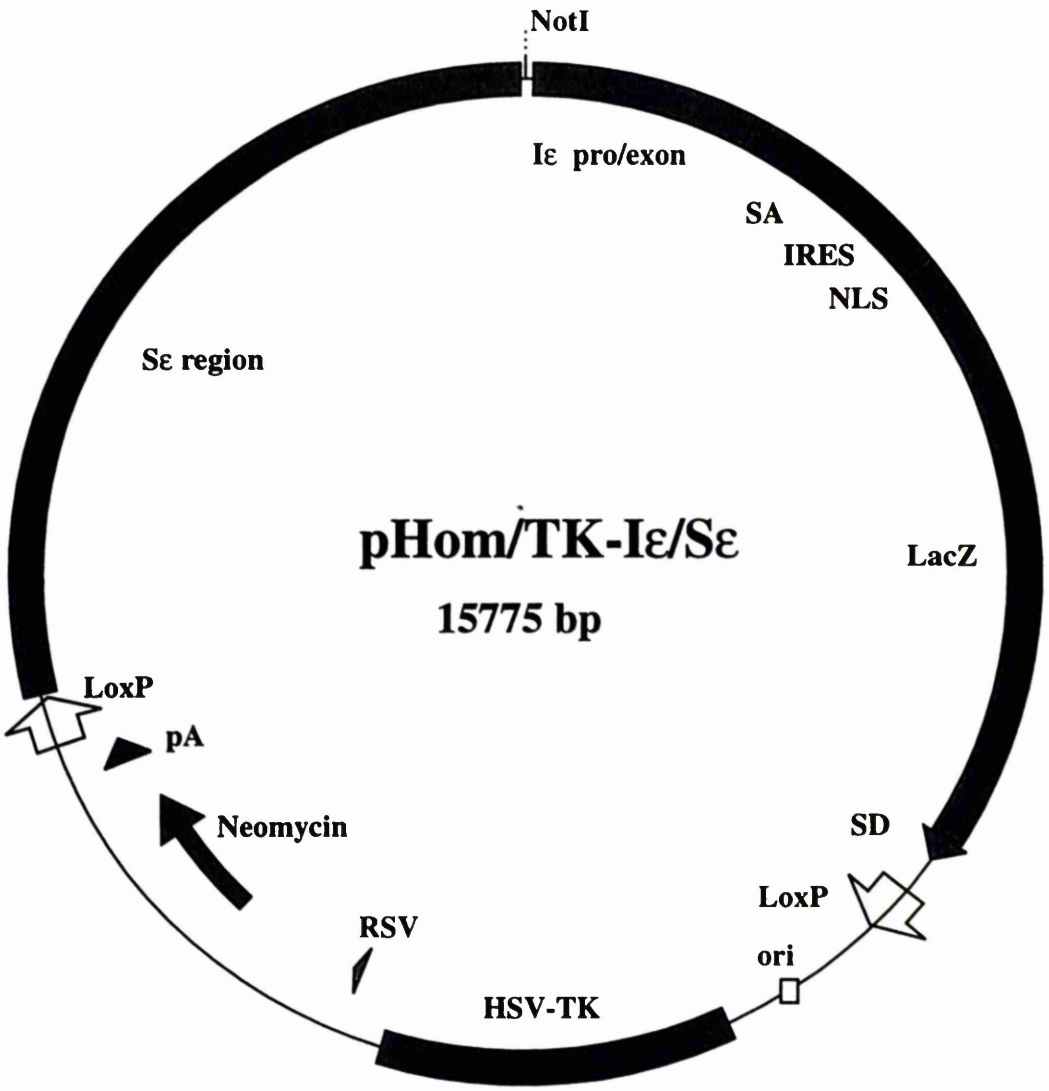


Figure 12. Map of the Final Vector Construct used for the Gene Targeting.

The genomic arms containing the Iε and Sε regions will serve to target the mLacZ to the ε constant region in the mouse genome, the LoxP sites will be used to remove the NeoR and TK expression cassettes and the splice donor (SD) will allow splicing to exon Cε1. Prior to its transfection the vector was linearised with *NotI*.

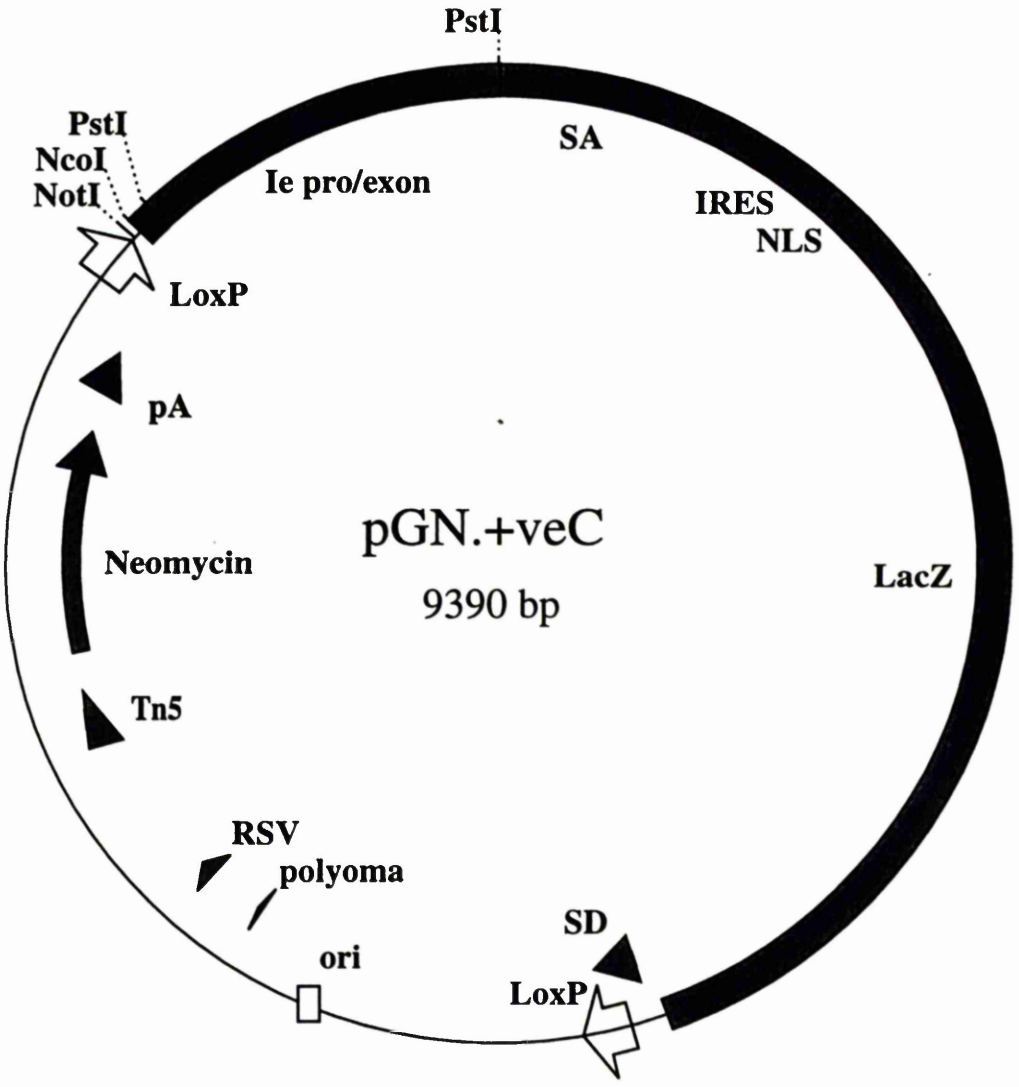


Figure 13. Map of the vector used for the ES Cell Transfection Positive Control.

The 1.1Kb *PstI* genomic DNA fragment containing the Iε promotor/exon region was cloned, along with an upstream100bp *NcoI/PstI* fragment (also situated directly upstream of the 1.1Kb PstI fragment in the genome and marked as a black box) into the vector pGN. The vector was linearised prior to transfection by digestion with *NotI*.

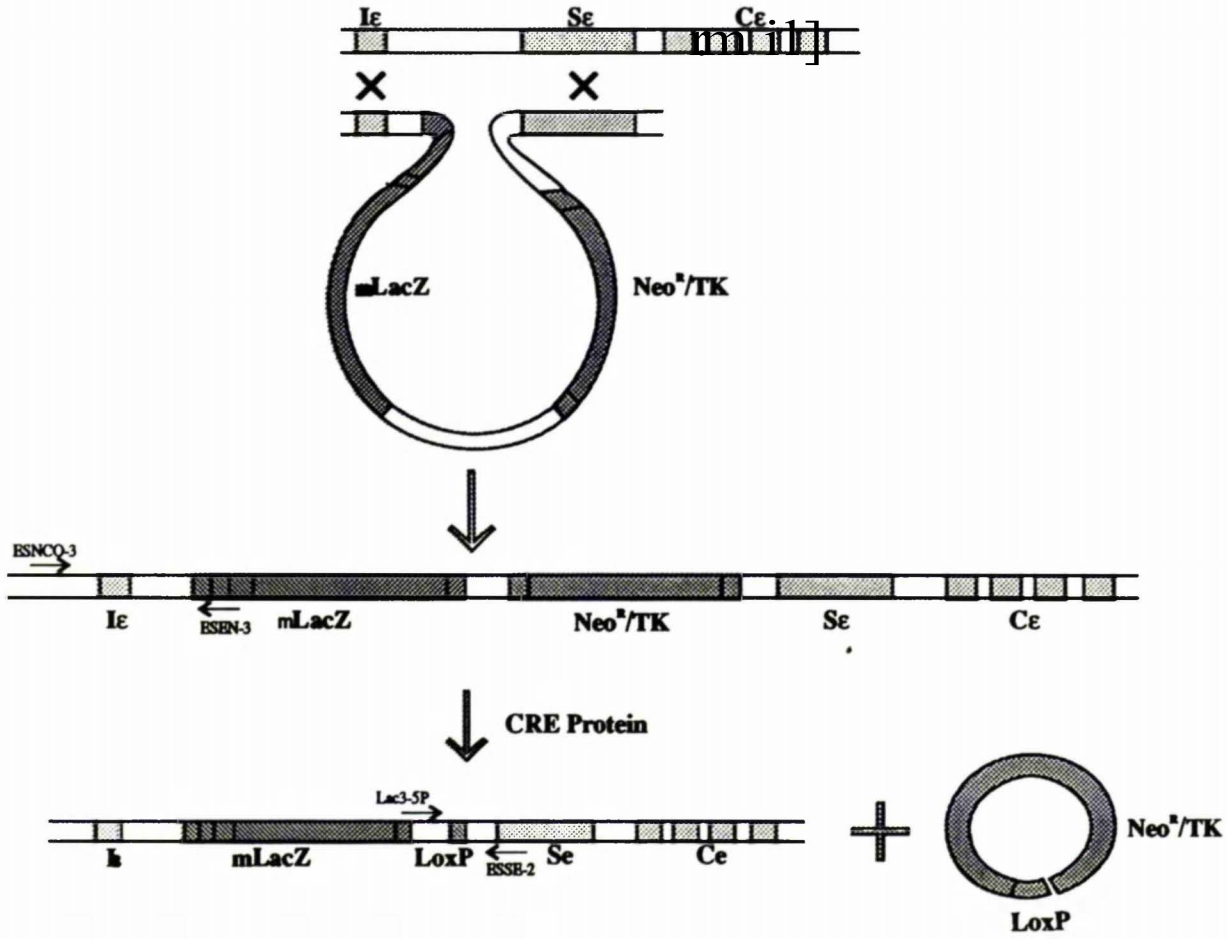


Figure 14. Scheme of Events Occuring During and After Gene Targeting in the ES Cells.

Homologous recombination occurs between the gene targeting vector and the genomic DNA within the region covered by the genomic arms of the vector. This causes the insertion of the mLacZ into the DNA between the Iε promotor/exon and the Sε region, placing it under the control of the Iε exon. Primers ESNCO-3 and ESEN-3 will be used to screen clones by PCR for the homologous recombination.

Expression of the *Cre* recombinase in cells containing the desired genomic alteration causes recombination of the *LoxP* sites and the subsequent loss of the Neo^R/TKcassette. Primers Lac3-5P and ESSE-2 will be used to screen for the *LoxP* recombination.

Primer binding sites are indicated by arrows.

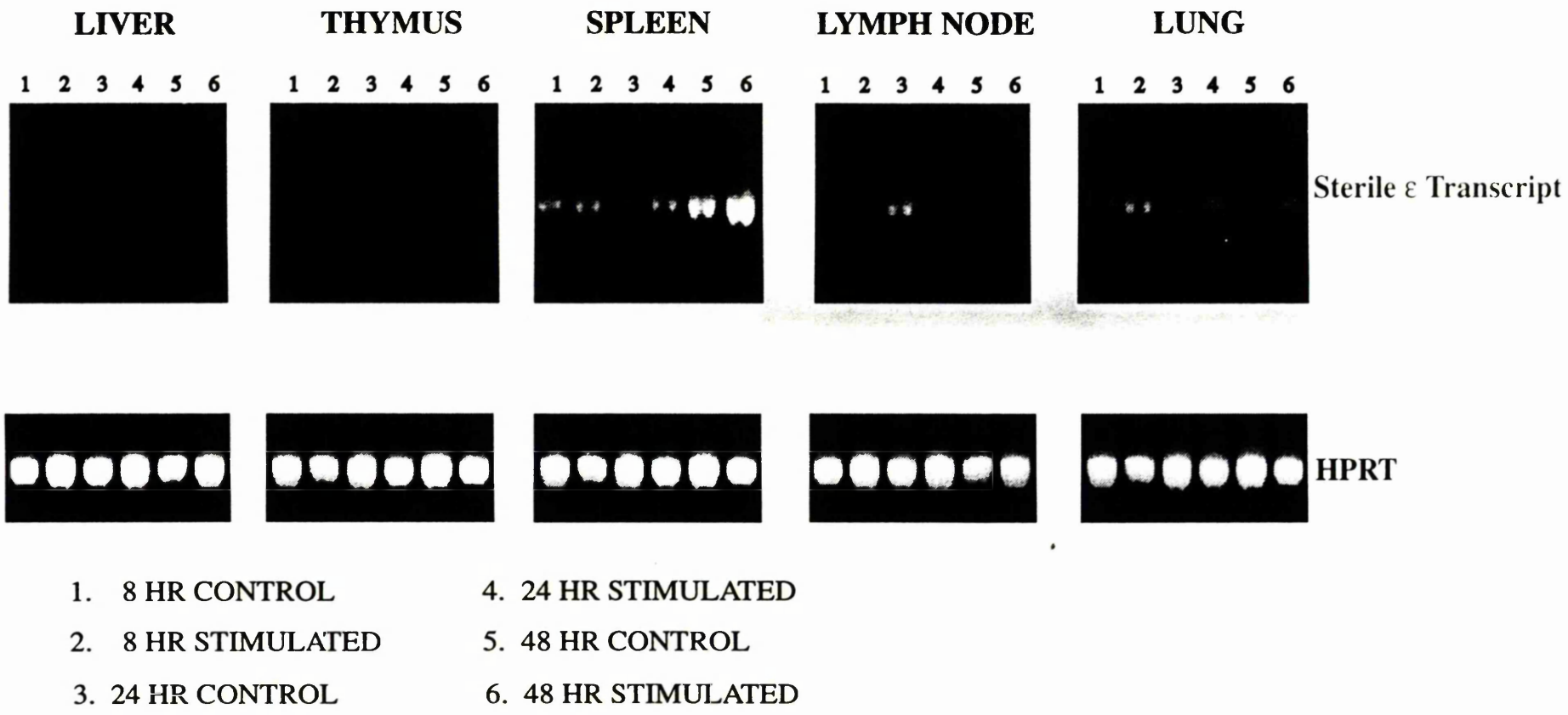


Figure 15. Detection of the Sterile ε Transcript in Tissues from Mice undergoing an Allergic Response.

First-strand cDNA for the sterile ε transcript was amplified by PCR following reverse transcription of RNA obtained from the tissues shown above. cDNA from the HPRT housekeeping gene was amplified as a positive control for the reverse transcription. Primers used were RTIE-2 and 3PCE for the sterile ε transcript and HPRT-5P and HPRT-3P for the HPRT gene.

Mice were either challenged with NaCl (control above) as a control or with ovalbumin (stimulated above) in order to induce an allergic response. The time points represent time after final challenge.

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Appendix 4

Buffers and Solutions

TE (1x)

10 mM Tris-HCl (pH 7.4)

1 mM EDTA (pH 8.0)

PBS (1x)

150 mM NaCl

2.5 mM KCl

10 mM Na_2HPO_4

1.75 mM KH_2PO_4

Klenow Buffer (10x)

0.5 M Tris-HCl (pH 7.6)

100 mM $MgCl_2$

T4 DNA Ligase Buffer (10x)

200 mM Tris-HCl (pH 7.6)

50 mM $MgCl_2$

50 mM Dithiothreitol

0.5 mg/ml BSA

PCR Buffer (10x)

0.5 M KCl

0.1 M Tris-HCl (pH 9.0)

15 mM $MgCl_2$

1% Triton X-100

MOPS Gel Running Buffer (5x)

0.1 M MOPS (pH 7.0)

40 mM Na acetate

5 mM EDTA (pH 8.0)

TBE Gel Running Buffer (5x)

450 mM Tris-Borate

10 mM EDTA (pH 8.0)

SSC (20x)

3 M NaCl

300 mM Na citrate

SSPE (20x)

3 M NaCl

200 mM NaH_2PO_4

25 mM EDTA

Luria Broth (For 1 litre):

10 g bacto-tryptone

5 g bacto-yeast extract

10 g NaCl

(adjust to pH 7.0 with NaOH)