A Study of the Granulocyte-Macrophage Colony Stimulating Factor Receptor (GM-CSFR) in Leukaemia

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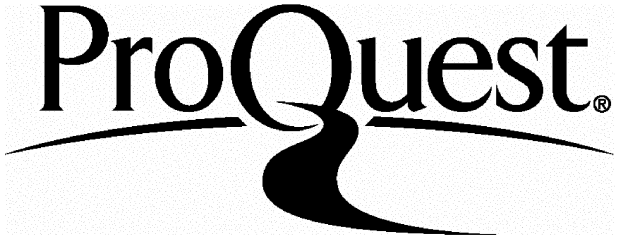

Abstract

Mutation of signaling molecules such as Ras have been shown to confer a growth advantage in blast cells from patients with acute myeloid leukaemia (AML) and may contribute to the pathogenesis of the disease. Alterations in other signaling molecules could also play a role in leukaemogenesis. GM-CSF is a key growth factor in haemopoiesis which exerts its influence on target cells via membrane bound receptors. The GM-CSF receptor (GM-CSFR) is composed of oligomers of ligand-specific α chains and β chains which are common to the GM-CSF/IL-3 and IL-5 receptors (β_c). The intracytoplasmic tail of the β_c chain is essential for the activation of several downstream signaling pathways and alterations in this region could deregulate normal signaling processes. RT-PCR-SSCP analysis was used to look for mutations in the β_c chain tail (nts 1281-2816) in RNA from 35 patients with acute myeloid leukaemia (AML) and 10 haematologically normal controls. Six nucleotide substitutions were detected, three of which were silent (Ser^{426}, Pro^{648} and Pro^{800}) and three which altered the amino acid residue at that position in the receptor sequence (Gly^{647}->Val, Val^{652}->Met and Pro^{603}->Thr). However all substitutions were detected in normal controls and were thought to be polymorphisms, with allele frequencies of 0.23 and 0.13 found for two of the most common silent substitutions. RNA from patients with juvenile chronic myeloid leukaemia (JCML) was also screened for mutations in the entire GM-CSFR α and β chain coding sequences using SSCP analysis as studies have shown progenitors from JCML patients have a hypersensitive growth response to GM-CSF in culture. Two nucleotide substitutions accounted for all α chain abnormalities (Ala^{17}->Gly and silent Val^{333}) with both previously detected in normal controls. Four base substitutions were detected in the β chain. Three were polymorphisms previously described in AML patients and normal controls. A further nucleotide mutation which resulted in a Glu^{249}->Gln substitution was also found but was not thought to be of pathological significance as a Gln residue is present at this amino acid in both mouse β chains. During the course of these studies a novel isoform of the β_c chain with a truncated intracytoplasmic tail (β_{IT}) was isolated by our group. Transcripts of this alternatively spliced isoform were shown to be present in relatively high levels in blast cells from AML patients. Polyclonal antibodies were raised against a novel 23 amino acid sequence in the tail of the β_{IT} chain and screened in COS-7 cells transfected with plasmids coding for β_c or β_{IT} chain before being used to demonstrate expression of the truncated receptor in a range of haemopoietic cells. The β_{IT}-specific antibodies were then used to investigate GM-CSFR α and β chain stoichiometry and demonstrated that β chain homodimerization occurred without ligand stimulation in primary haemopoietic cells or GM-CSFR α chain in transfected COS-7 cells.

ACKNOWLEDGEMENTS

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My final thanks go to my mum and dad who have always encouraged me in everything I've done and provided me with support when I most needed it. I hope that some day I'll be able to repay them for everything they have done for me.

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ABBREVIATIONS

AML	- acute myeloid leukaemia
APS	- ammonium persulphate
β_C	- beta common
β_{IT}	- beta intracytoplasmic truncation
bp	- base pair
C-terminal	- carboxy terminal
CNTF (R)	- ciliary neurotrophic factor (receptor)
CRH	- cytokine receptor homology
CSF	- colony stimulating factor
DNA	- deoxyribonucleic acid
ECL	- enhanced chemilumenescence
Epo (R)	- erythropoietin (receptor)
FAB	- French-American-British
FT III	- fibronectin type III
G-CSF (R)	- granulocyte colony stimulating factor (receptor)
GAP	- GTPase activating protein
GH (R)	- growth hormone (receptor)
GM-CSF (R)	- granulocyte-macrophage colony stimulating factor (receptor)
HCP	- haemopoietic cell phosphatase (or SHP-1)
HGF	- haemopoietic growth factor
HPLC	- high performance liquid chromatography
IFN	- interferon
IgG	- immunoglobulin G
IL- (R)	- interleukin (receptor)
IP	- immunoprecipitation
JAK	- janus kinase
JCML	- juvenile chronic myeloid leukaemia
kb	- kilobase
kDa	- kilodaltons
LIF (R)	- leukaemia inhibitory factor (receptor)
M-CSF (R)	- macrophage colony stimulating factor (receptor)
MAPK	- mitogen activated protein kinase
MPLV	- myeloproliferative leukaemia virus
mRNA	- messenger ribonucleic acid
N-terminal	- amino terminal
NF-1	- neurofibromatosis type 1

OSM (R)	- oncostatin M (receptor)
PAP	- pulmonary alveolar proteinosis
PBS	- phosphate buffered saline
PFCP	- primary familial and congenital polycythemia
pTyr	- phosphotyrosine
rpm	- revolutions per minute
RT-PCR	- reverse transcriptase polymerase chain reaction
SCF (R)	- stem cell factor (receptor)
SCID	- severe combined immunodeficiency diseases
SCN	- severe congenital neutropenia
SFFV	- spleen focus-forming virus
SH2 (SH3)	- Src homology-2 (3)
SSCP	- single strand conformation polymorphism
STAT	- signal transducer and activator of transcription
TBE	- tris borate EDTA
TEMED	- tetra-metylethylenediamine
TNFα	- tumour necrosis factor alpha
Tpo (R)	- thrombopoietin (receptor)
WSXWS box	- tryptophan-serine-X- tryptophan-serine box
DDW	- deionised distilled water
cpm	- counts per minute
Ac	- acetate
µg	- microgram

Chapter 1

Introduction

1.1 - Haemopoiesis

All the circulating blood cells in adult life are derived from a small pool of multipotential haemopoietic cells, located mainly in the bone marrow, known as stem cells [Metcalf *et al*, 1991]. Most cells in the blood have a limited life span and they need to be continuously replenished from progenitor cells, a process known as haemopoiesis. The exact requirements for blood cells obviously varies considerably from day to day and the bone marrow needs the ability to adapt to a changing environment and be able to increase production rate of all specific cell lineages as required [Lowenberg *et al*, 1993]. Haemopoiesis poses regulatory problems due to the dispersion of haemopoietic tissues in several bones throughout the body. As all blood cells originate from a small pool of stem cells a mechanism for achieving controlled commitment of progeny cells to the 8 major blood lineages is required, with cell proliferation in any one lineage requiring numerous cell divisions coupled with complex maturation changes to produce mature haemopoietic cells (Figure 1.1) [Metcalf *et al*, 1991].

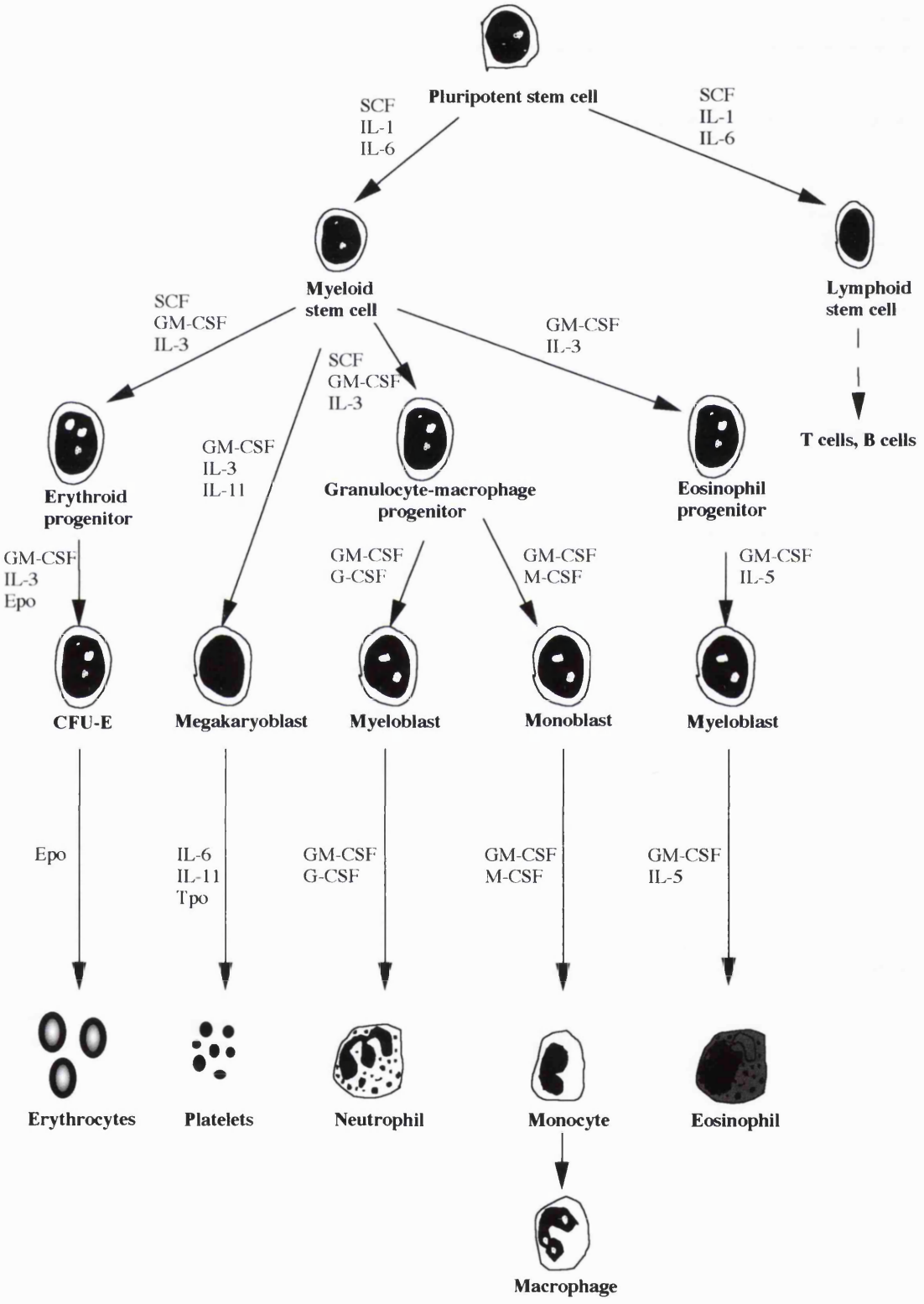
In order to produce fully differentiated mature cell populations, progeny of pluripotent stem cells become more irreversibly committed to specific lineages with each cell division. The first major step seems to be differentiation commitment to give progenitor cells committed to either the myeloid or lymphoid lineages. Committed progenitors are more restricted in the variety of cells they can produce and the further along the differentiation pathway cells proceed the more mature and morphologically identifiable the cells become (Figure 1.1) [Metcalf *et al*, 1991]. Studies on cultured haemopoietic cells have made clear the importance of the co-ordinated interaction of a group of regulatory molecules that stimulate the proliferation of progenitor cells and their progeny and which initiate maturation events necessary to produce fully mature cells [Metcalf*et al*, 1991]. Referred to as haemopoietic growth factors (HGFs) they are a group of glycoproteins which are highly biologically active at low picomolar concentrations.

1.2 - Haemopoietic Growth Factors

1.2.1 - Mechanism of haemopoietic growth factor action

Communication between cells of the haemopoietic system is mediated in part by soluble regulatory molecules. These HGFs exert their biological functions through specific receptors expressed on the surface of target cells [Kishimoto *et al*, 1994]. Interaction between a HGF and its receptor influences a series of cellular processes such as proliferation, differentiation, maturation and cell functions (Figure 1.2). An important role of HGFs is to initiate proliferation of target cells, thereby enabling the haemopoietic system to increase specific cell populations to meet environmental changes such as

Figure 1.1 - Current model of Haemopoiesis



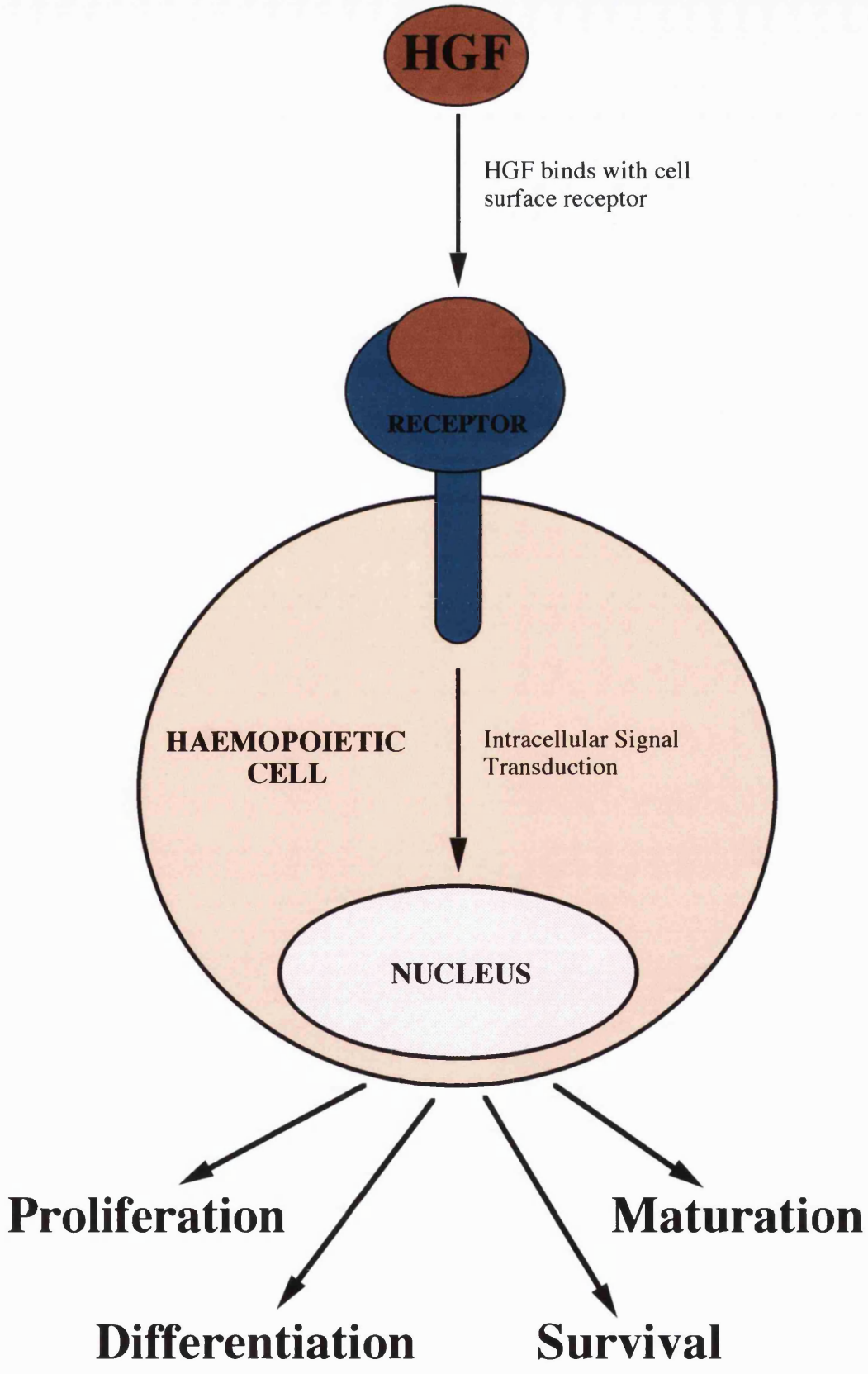
infection, or simply to maintain a constant cell number. HGFs are also involved in stimulation of the functional activity of mature cells, for example, phagocytosis or the synthesis of a variety of biologically active molecules including growth factors themselves. Although ligand-receptor interaction occurs at the cell membrane, as HGFs have the ability to induce cells to respond in a number of different ways their actions must ultimately involve complex and selective changes in gene transcription and expression (Figure 1.2) [Metcalf *et al*, 1989].

1.2.2 - Haemopoietic growth factor function

It was originally thought each HGF exerted a particular effect on its specific target cell but in fact most HGFs can influence more than one cell type (pleiotropy). Interleukin 6 (IL-6) is a typical example of a multifunctional cytokine having originally been identified as a B cell differentiation factor that induces the final maturation of B cells into antibody producing cells. However a series of subsequent studies revealed that this molecule functions not only in the immune system but also in the haemopoietic, endocrine, hepatic and even neural systems [Kishimoto *et al*, 1994]. HGFs also function in a redundant manner with several able to illicit similar effects on the same cell type, while a particular HGF can have different effects on a particular target cell depending on its maturation state [Nicola *et al*, 1991]. For example, leukaemia inhibitory factor (LIF) and IL-6 have been shown to act similarly to induce the differentiation of a murine cell line (M1) into macrophages [Kishimoto *et al*, 1994].

Several HGFs were first discovered due to their ability to stimulate the formation of colonies of granulocytes and macrophages in semi-solid bone marrow cultures and because of this are termed colony stimulating factors (CSFs). Others were first defined by their actions on lymphocytes and were consequently named interleukins (IL-) [Bradley *et al*, 1966, Ichikawa *et al*, 1966, Metcalf *et al*, 1991]. They are glycoproteins with a varying content of carbohydrate and have molecular masses in the range of 18 to 90 kDa [Metcalf *et al*, 1991]. Initially isolation of HGFs from natural tissue sources required the use of extensive sequential separative techniques, especially high performance liquid chromatography (HPLC) and although by 1983 erythropoietin (Epo), granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF), IL-1, IL-2 and IL-3 had all been purified, they were only available in minute quantities [Metcalf *et al*, 1992]. The isolation of cDNAs encoding the HGFs and subsequent production of active recombinant factors allowed widespread work on their biological effects [Metcalf *et al*, 1992]. The progressive use of molecular biology techniques such as cDNA expression cloning to

Figure 1.2 - Effects of Haemopoietic Growth Factors on cells via membrane receptor



further isolate new HGFs has now led to the situation where recombinant factors become available without any preceding framework of biological knowledge on their likely role in normal haemopoiesis.

1.3 - Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF)

1.3.1 - GM-CSF gene and protein structure

Granulocyte-macrophage colony stimulating factor was initially purified from medium conditioned by the Mo cell line [Gasson *et al*, 1984], and the cDNA for human GM-CSF (hGM-CSF) was cloned shortly after [Wong *et al*, 1985]. The hGM-CSF gene is located on chromosome 5q25-31 within 10kb of the gene for IL-3 [Huebner *et al*, 1985]. This region of chromosome 5 also encodes the genes for the HGFs IL-4, IL-5 and the receptor for M-CSF [Boultwood *et al*, 1994]. The GM-CSF gene is 2.5kb long and is composed of 4 exons and 3 introns [Arai *et al*, 1990] which code for a 144 amino acid polypeptide including a 17 amino acid signal peptide which is removed during secretion. Variable degrees of glycosylation give a molecular mass range of 14.5-34 kDa for the protein [Clark and Kamen, 1987]. However glycosylation of the protein does not seem to be essential for biological activity or cellular processing and in fact heavy glycosylation appears to reduce both activity and binding affinity with its receptor [Cebon *et al*, 1990, Kaushansky *et al*, 1992].

X-ray crystallography revealed that recombinant hGM-CSF consists of a two stranded anti-parallel β sheet and a bundle of four α helices which form an open barrel. Two intramolecular disulphide bonds between four cysteine residues stabilise the tertiary structure of GM-CSF and appear to be important for its biological activity. Studies on the structure and function of GM-CSF using hybrids of human and mouse sequence have shown that two regions, between amino acids 21-31 and 77-94 which lie in neighbouring helices, are critical for its haemopoietic activity [Kaushansky *et al*, 1989]. Mutation analysis has also indicated that many residues in the fourth helix especially Asp 112 are important for interactions with the low affinity receptor while the amino terminal helix, especially glutamic acid residue 21, plays a vital role in the high affinity binding of GM-CSF with its receptor [Shanafelt *et al*, 1991, Lopez *et al*, 1992, Hercus *et al*, 1994].

Expression of GM-CSF is regulated in the cell by a combination of transcriptional and post-transcriptional control mechanisms. Several conserved elements which appear to be important in regulating transcription have been identified in the 5' untranslated region of both the mouse and human GM-CSF genes. Post-transcriptional changes in mRNA half-life play an important role in the control of expression. A highly conserved AUUUA motif

is found in the 3' untranslated region of the mRNA for GM-CSF, G-CSF, stem cell factor (SCF) and IL-3 which is thought to influence the stability of mRNA transcripts and is probably a recognition motif for RNAses [Anderson *et al*, 1990, Akashi *et al*, 1991].

1.3.2 - Biological effects of GM-CSF

GM-CSF is produced in response to immunological and inflammatory signals in a range of cell types. It is produced in endothelial cells and fibroblasts when stimulated with tumour necrosis factor (TNF) or IL-1 and in macrophages activated by bacterial endotoxin or antigen presenting T cells [Gasson *et al*, 1991]. Various other cells including T and B lymphocytes, mast cells, monocytes, endothelial and mesothelial cells, fibroblasts and osteoblasts can be induced to accumulate GM-CSF mRNA and secrete the protein [Gasson *et al*, 1991].

GM-CSF is a multilineage cytokine and is thought to act at the intermediate progenitor level of normal haemopoiesis. It shows synergistic activity with early acting factors e.g. SCF [Bernstein *et al*, 1991], other intermediate factors e.g. IL-3 [Sieff *et al*, 1989] and also late acting factors e.g. Epo [Sieff *et al*, 1985]. GM-CSF is also able to stimulate the proliferation of a progressively broader spectrum of progenitor cells as concentrations are increased including macrophage, granulocyte, eosinophil, megakaryocyte progenitors and at highest concentrations multipotential progenitors [Metcalf *et al*, 1989].

GM-CSF also shows a broad range of activating effects on mature phagocytic cells (neutrophils, eosinophils and macrophages) such as increased secretion of IL-1, M-CSF and G-CSF, enhanced expression of adhesion molecules, enhanced antigen presentation and increased phagocytosis [Gasson *et al*, 1991, Rapoport *et al*, 1992, Khwaja *et al*, 1992]. The clinical administration of GM-CSF leads to a striking increase in neutrophil, eosinophil and monocyte numbers with a lesser effect on lymphocyte counts [Ganser *et al*, 1988].

1.4 - Haemopoietic Growth Factor receptors

1.4.1 - Types of haemopoietic growth factor receptor

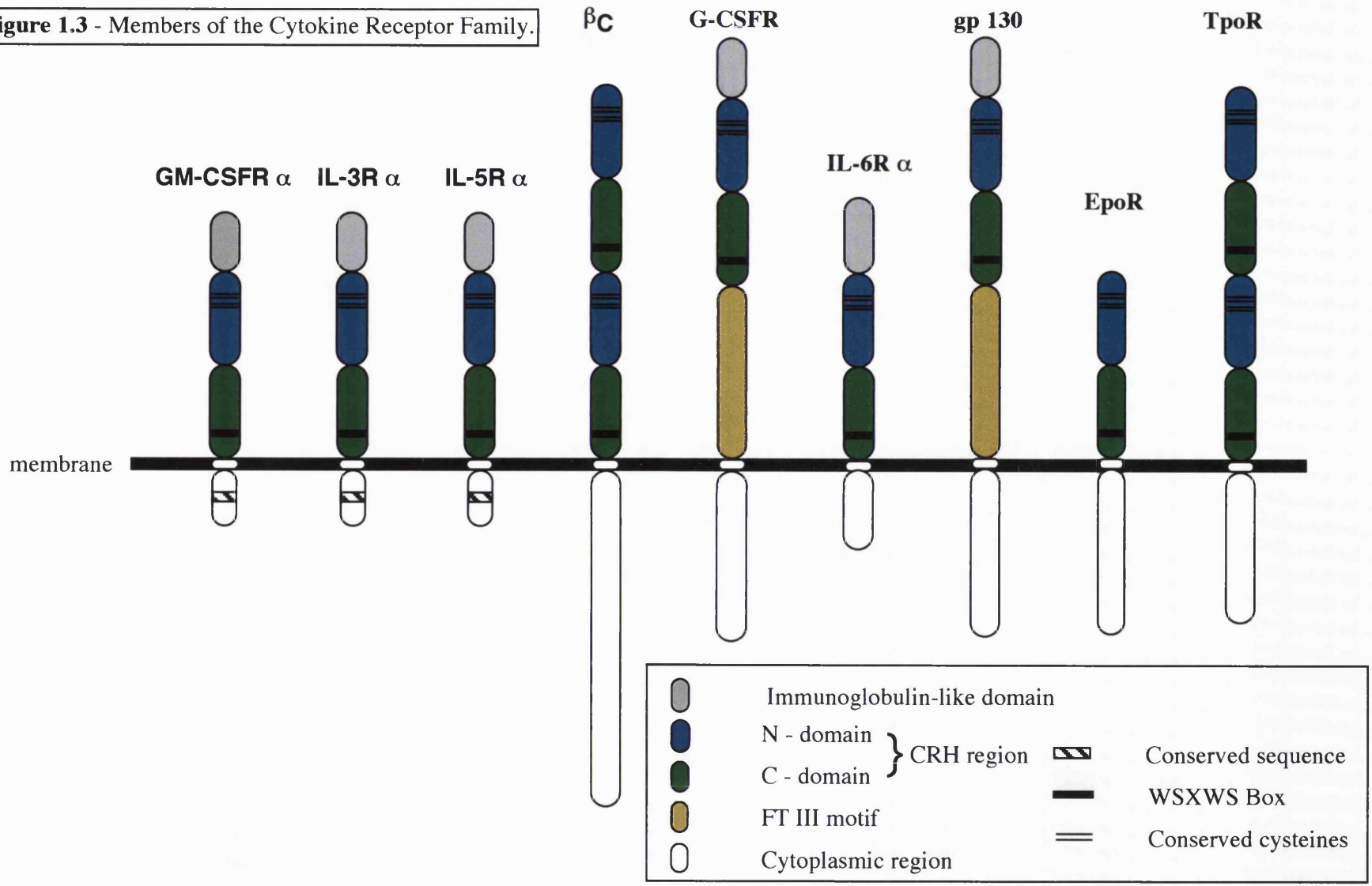
As described earlier HGFs exert their influence on target cells via binding to specific cell surface receptors. Most haemopoietic cells have only a few hundred receptors per cell for each regulator and low levels of ligand binding can elicit significant biological responses [Nicola *et al*, 1989]. The HGF receptors are glycoproteins and consist of a polypeptide chain with an extracellular ligand binding region, a single hydrophobic transmembrane segment and a cytoplasmic tail. In general the receptors are formed by more than one

subunit. The receptors for M-CSF, SCF, Epo, growth hormone (GH), Thrombopoietin (Tpo) and G-CSF homodimerize upon ligand binding [Blume *et al*, 1991, van Daalen Wetters *et al*, 1992, Alexander *et al*, 1995] while receptors for molecules like GM-CSF, IL-2, 3, 4, 5, 6, 7, 9, 11, 13, 15, LIF and ciliary neurotrophic factor (CNTF) are made up of more than one type of polypeptide chain [Ihle *et al*, 1996]. Some haemopoietic regulators such as SCF or M-CSF have classical transmembrane tyrosine kinase receptors, but the large majority of HGFs have glycoprotein transmembrane receptors which lack an intrinsic tyrosine kinase domain. These are now recognised to form a group of receptors which share significant homology in their extracellular domain and are known as the cytokine receptor family [Bazan *et al*, 1990]. Better understanding of the molecular biology of cytokine receptor systems has helped explain the functional pleiotropy and redundancy of various cytokines [Kishimoto *et al*, 1994].

1.4.2 - Cytokine Receptor family

Members of the cytokine receptor family can be divided into two groups. Class I includes the receptors for GM-CSF, G-CSF, IL-2, 3, 4, 5, 6, 7, 9, 11, 12, 13, 15, GH, LIF, Tpo and Epo which lack a tyrosine kinase consensus sequence and have several conserved characteristic features (Figure 1.3). The receptor chains have a motif of approximately 200 amino acids in their extracellular domain referred to as the cytokine receptor homology (CRH) domain which is required for ligand binding. The CRH domain can be subdivided into two modules of about 100 amino acids related to fibronectin type III domains (FT III). The N-terminal module includes a conserved tryptophan and a distinctive spatially conserved position of four cysteine residues, thought to form disulphide bonds which stabilise the structure, while the module proximal to the transmembrane region (C-terminal) contains the highly conserved Trp-Ser-X-Trp-Ser sequence (WSXWS box). Both the N- and C-subdomains contain seven anti-parallel β folds arranged to form a barrel-like structure with the WSXWS box located in a region which seems to act as a hinge between the two barrels [De Vos *et al*, 1992, Bazan *et al*, 1990]. In some receptor chains there are two repeats of this conserved motif, for example, the GM-CSFR β_C chain and TpoR, while other receptor chains e.g. the IL-6R and G-CSFR, have fibronectin-like motifs between the WSXWS box and the membrane region [Nicola *et al*, 1991, Bazan *et al*, 1990, Murata *et al*, 1992, Ihle *et al*, 1994]. Class II receptors, including receptors for interferon-α (IFN-α), IFN-γ and IL-10, share overall structural features but are more divergent. They share a conserved tryptophan and one cysteine pair with class I receptors but have an additional conserved cysteine pair, and several conserved prolines and tyrosines [Schindler and Darnell, 1995]

Figure 1.3 - Members of the Cytokine Receptor Family.



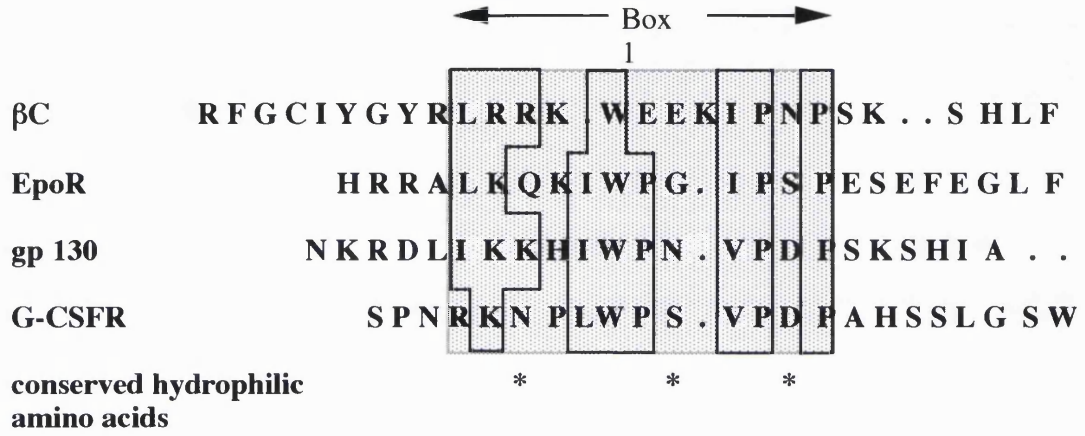
In contrast to the extracellular domain, the intracytoplasmic tails have relatively little homology between the receptors of the cytokine receptor family. The limited similarity that does exist among most receptor chains consists of a membrane-proximal region that contains motifs that have been referred to as the box 1 and box 2 motifs (Figure 1.4) [Murakami *et al*, 1991]. The box 1 motif or proline-rich motif resides within the first 20 amino acids of the cytoplasmic domain and consists of the sequence Al-Ar-Pro-X-Al-Pro-X-Pro or Ar-X-X-X-Al-Pro-X-Pro, where Al is an aliphatic amino acid, Ar is an aromatic amino acid and X is any amino acid [O'Neal *et al*, 1993]. The box 2 motif was initially defined as beginning with a cluster of hydrophobic amino acids and ending with 1 or 2 positively charged amino acids, but some of the receptors do show more specific sequence similarities as seen in Figure 1.4 [Ihle *et al*, 1996]. Another conserved region know as box 3 is found in some cytokine receptors, including the G-CSFR and gp130, and is thought to be important for differentiation [Fukunaga *et al*, 1993, Dong *et al*, 1993]

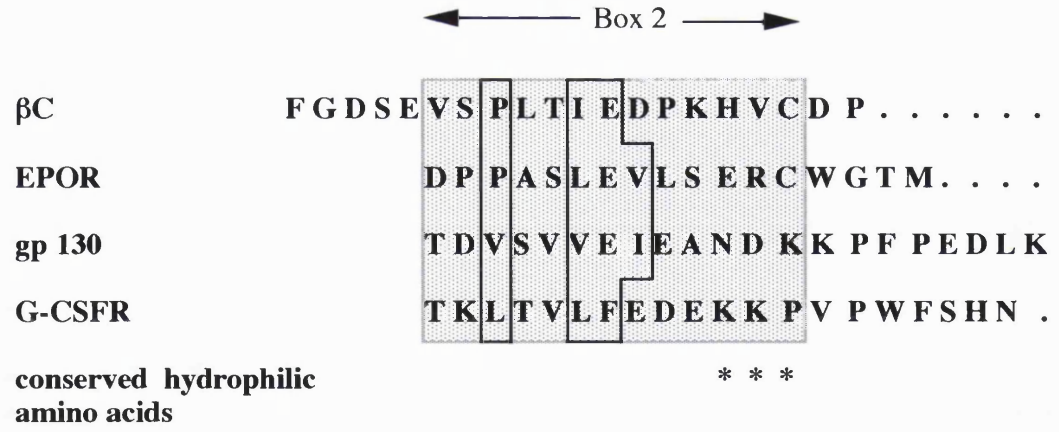
1.4.3 - Gene organisation of the cytokine receptors

Over the past few years many of the genes encoding members of the cytokine receptor family have been isolated and their genetic organisation elucidated. The cytokine receptor genes show remarkable homology in their intron and exon structure especially in the conservation of exon/intron boundaries in both position and phase of the introns (Figure 1.5A). Intron phases are defined as follows: Phase 0 introns interrupt the reading frame between codons, while Phase 1 and 2 introns interrupt the reading frame after the first and second nucleotides of codons, respectively. Comparison of cytokine receptor protein structures shows they all have the CRH domain in the extracellular region of the receptor, which can be subdivided into 2 fibronectin type III-like domains (FT III). All the boundaries of the FT III domains in the cytokine receptor family are defined by phase 1 introns, without exception, and each domain is roughly halved by intervening introns, of phase 2 for the N domain and of phase 0 for the C domain. Together this results in the sequential intron phases arranged in what has come to be regarded as the 1-2-1-0-1 rule [Nakagawa *et al*, 1994, Kosugi *et al*, 1995]. This intron/exon conservation means that each pair of conserved cysteines are coded for by separate exons, as are the WSXWS motif and the transmembrane region, as shown in Figures 1.5A and 1.5B.

Interestingly the gene structures of the cytoplasmic segment of the cytokine receptors are also similar to each other. The cytoplasmic region of most type I receptors is composed of 2 exons, with the exon next to the one encoding the transmembrane domain containing the Box 1 sequence in all receptors [Nakagawa *et al*, 1994]. In the case of the mouse AIC2A and AIC2B genes, which code for the mouse IL-3R β and β_C chains, and

Figure 1.4 - Amino acid sequence of the membrane-proximal cytoplasmic region of cytokine receptor subunits, showing highly conserved Box 1 and Box 2 sequences





Conserved amino acid residues are shown in outlined boxes

Box 1 and Box 2 motifs are contained in shaded areas

the human β_C chain the insertion of an additional exon of phase 0 means the intracytoplasmic tail is coded for by 3 exons.

The similarity in gene structure between cytokine receptors means it is likely they are derived from a common ancestral gene, while the conservation of the exon/intron boundaries, such as for the fibronectin type III-like domains, suggests they are important for the conservation of structurally and functionally important regions of the receptor [Kosugi *et al*, 1995]. Among the cytokine receptor family only the GM-CSFR, IL-3R and IL-5R α chains possess a third FT III domain in their N-termini (Figure 1.3), while the genes for the IL-3R and GM-CSFR α chains both have an additional intron in the C-terminal FT III domain (Figure 1.5). This may indicate an even closer evolutionary link between this subset of receptors, especially in view of the common β subunit they share [Kosugi *et al*, 1995].

1.4.4 - Receptor oligomerization

Although it has been shown cytokines exert their influences on target cells through membrane receptors, the stoichiometry of this interaction rarely involves just one ligand and one receptor chain. The binding of cytokines to surface receptors induces receptor clustering or oligomerization and leads to receptor activation, with the subsequent generation of intracellular signals. Ligand-dependent dimerization or oligomerization appears to be a general feature of the cytokine receptors with at least three patterns of subunit assembly (Figure 1.6). Examples of single subunit receptors include EpoR, GHR, which have been shown to dimerize following ligand binding, and the G-CSFR, although experimental evidence suggests the final stoichiometry of the G-CSFR involves two ligands and two receptor chains to form the preferred receptor complex [Horan *et al*, 1996].

Heterodimerization, with the sharing of a common subunit, is a characteristic of other cytokine receptor family members. Many multi-component receptors consist of two receptor chains as for the human IL-3R, IL-5R and GM-CSFR which have ligand specific α chains and a shared β chain (IL-3R α, IL-5R α, GM-CSFR α and β_C). Similarly the IL-2, IL-4, IL-7, IL-9 ,IL-13 and IL-15 receptors have a common receptor subunit, originally isolated as the γ chain of the IL-2R [Takeshita *et al*, 1992], although the IL-2R is thought to be a complex of three chains (IL-2R α, β and γ) with the α chain dispensable for signal transduction [Asao *et al*, 1993]. The γ chain is also utilised in the functional receptors for IL-4, IL-7 and IL-9, each of which also contains a ligand-specific α chain. The IL-15R utilises both the IL-2R β and γ chains as well as a distinct receptor chain related to the IL-

Figure 1.5A - Gene structure of Cytokine Receptor family - adapted from Kosugi *et al*, 1995.

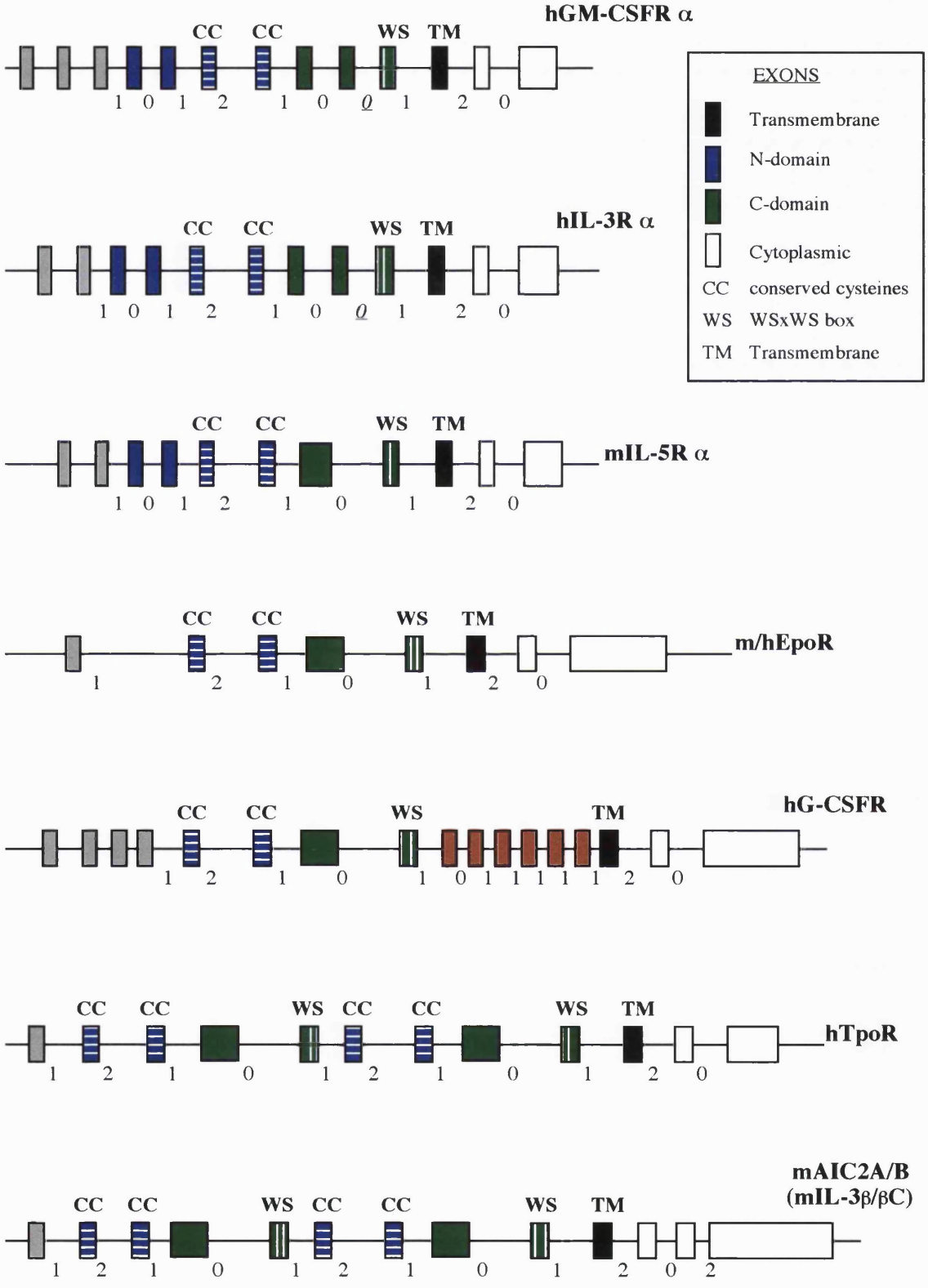
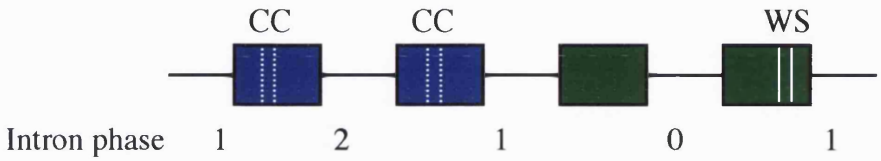
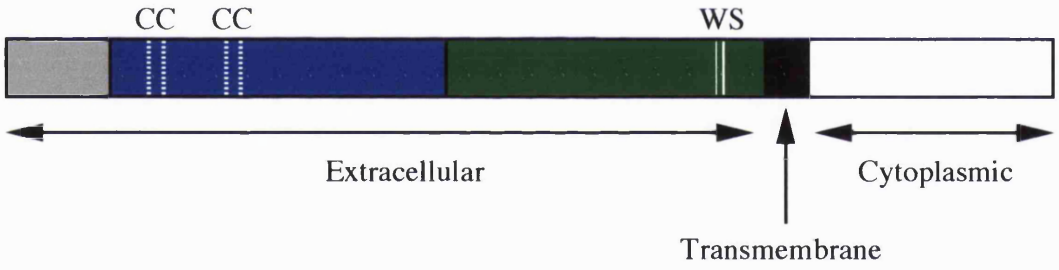


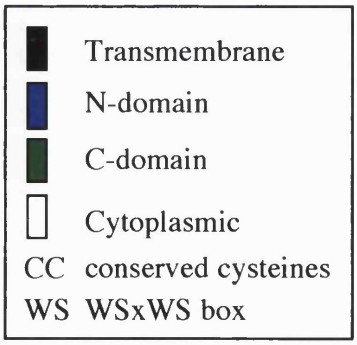
Figure 1.5B - Gene and protein structure of cytokine receptor family

Gene structure of two FT III-like domains



Protein structure





2R α chain, while the IL-13R associates with a novel chain functionally related to the IL-2R γ chain [Ihle *et al*, 1996].

The receptors for IL-6, IL-11, CNTF, LIF and oncostatin M (OSM) all utilise the gp 130 receptor subunit, which was first identified due to its association with the IL-6R α chain [Hibi *et al*, 1990]. The LIF and OSM receptors each bind one gp 130 subunit in their receptor complexes, while the CNTF receptor is formed by the CNTFR α chain, gp 130 and the LIF receptor chain [Bagley *et al*, 1997]. However the fully assembled IL-6R complex has been shown to be composed of two IL-6, two IL-6R α chains and two gp 130 chains, leading to proposals of a hexameric receptor complex which may be common to other receptors of the cytokine receptor family [Ward *et al*, 1994, Paonessa *et al*, 1995].

1.5 - Granulocyte-Macrophage Colony Stimulating Factor Receptor (GM-CSFR)

1.5.1 - Isolation of GM-CSFR chains

Early studies using ligand binding of radiolabelled GM-CSF revealed the existence of two classes of receptor for this cytokine in murine bone marrow, a low affinity receptor with a dissociation constant Kd of 1-10nM (500 receptors/cell) and a high affinity receptor with a Kd of 10-100pM (50 receptors/cell) [Walker *et al*, 1985]. Human neutrophils only express a single class of high affinity receptor (200-1000 per cell) whereas immature cells, monocytes and leukaemic myeloblasts express both classes of receptor [Rapoport *et al*, 1992, Chiba *et al*, 1990, Budel *et al*, 1989]. Some non-haemopoietic cells have been shown to have exclusively low affinity receptors [Nicola *et al*, 1991].

With the cloning and characterisation of the cytokine receptors it became apparent that high affinity binding of many cytokines required receptors consisting of several subunits. Two receptor chains have been cloned and identified for the GM-CSFR and are designated α and β. The α chain of the GM-CSFR was first isolated by expression screening of a cDNA library constructed from human placental mRNA. Expression of the cloned α chain in COS-7 cells revealed it specifically recognises and binds the GM-CSF ligand with low affinity [Kd 2-8 nM] [Gearing *et al*, 1989]. The second subunit of the GM-CSFR was cloned from a cDNA library of the haemopoietic cell line TF-1. When this cDNA was transfected into fibroblasts it expressed a protein of approximately 120 kDa which did not bind any of the human cytokines including GM-CSF and IL-3. However co-transfection with the low affinity binding α chain of the GM-CSFR resulted in the formation of a high affinity receptor (Figure 1.7) [Hayashida *et al*, 1990]. Further transfection studies in COS-7 cells showed that the GM-CSFR β chain was able to form a

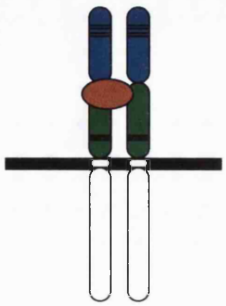
Figure 1.6 - Receptor chain oligomerization.

Single Receptor chain

EpoR
TpoR
G-CSFR
GHR
M-CSFR
SCFR

Homodimers

e.g. GHR



Two Receptor chains

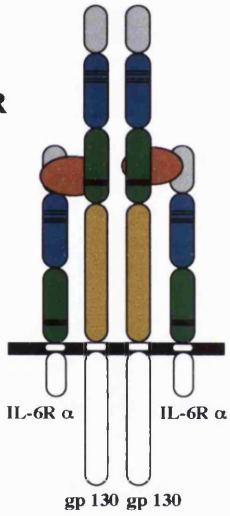
IL-3R	IL-6R	IL-4R
GM-CSFR	IL-11R	IL-7R
IL-5R	LIFR	IL-9R
	OSMR	IL-13R

Common subunit

βC	gp 130	IL-2R γ

Heterodimers

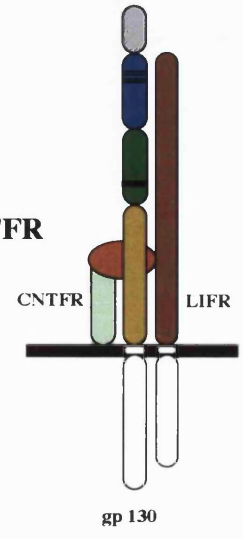
e.g. IL-6R



Three Receptor chains

IL-2R
CNTFR

e.g. CNTFR



high affinity receptor with the ligand binding α chain of the IL-3R [Kitamura *et al*, 1991] and that the chain was also shared with the IL-5R α chain [Tavernier *et al*, 1991]. This receptor chain is usually referred to as the β common chain (β_C) [Gorman *et al*, 1990, Sakamaki *et al*, 1992].

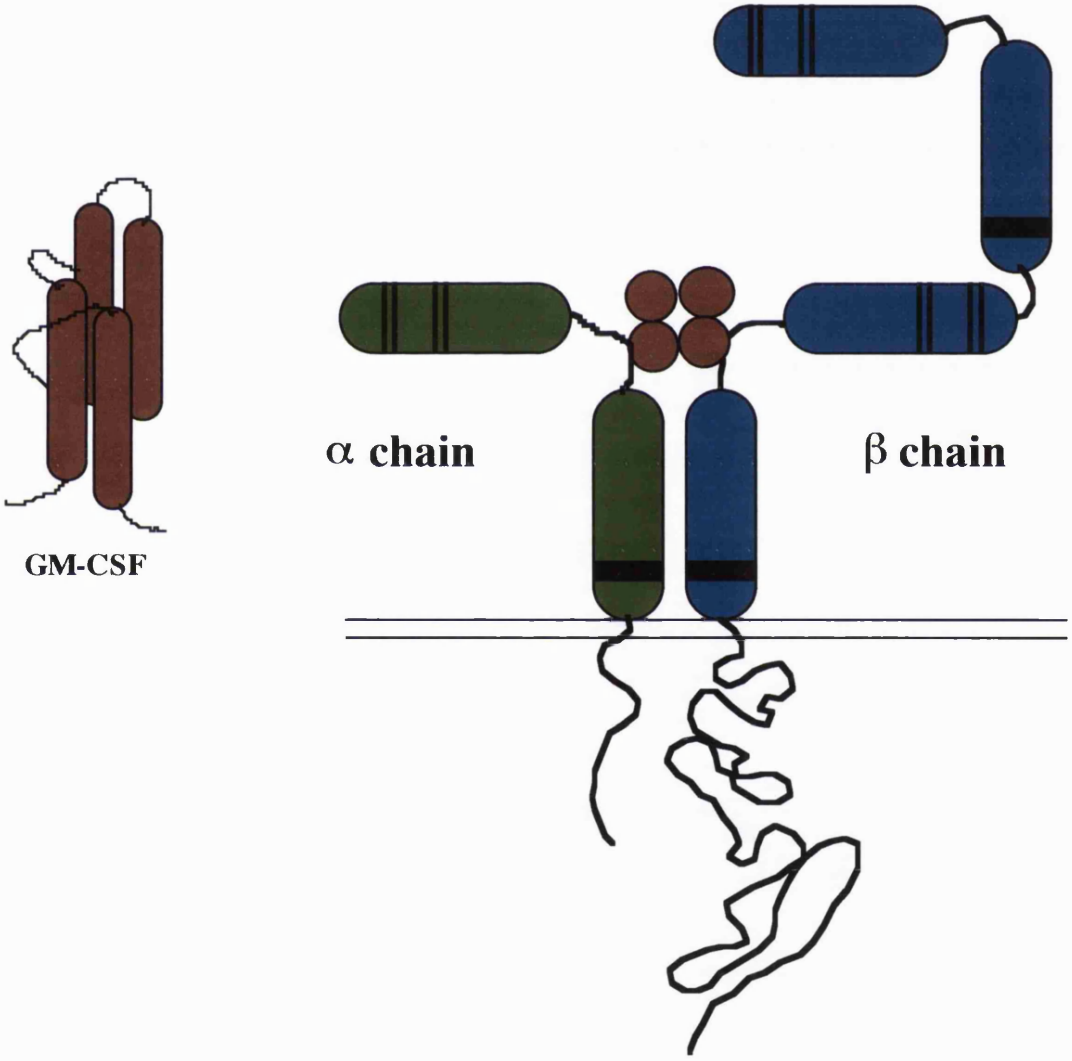
1.5.2 - GM-CSF Receptor alpha chain

The GM-CSFR α chain gene is located in the pseudo-autosomal region of the human X and Y chromosomes [Gough *et al*, 1990]. The gene covers approximately 44kb of DNA and is made up of 13 exons which code for a polypeptide of 400 amino acids [Nakagawa *et al*, 1994]. This is consistent with the low affinity binding 85kDa band seen in cross-linking studies using radiolabelled GM-CSF [Chiba *et al*, 1990].

As mentioned previously the α chains for the GM-CSF, IL-3 and IL-5 receptors are considerably more homologous in gene structure to each other than they are to other receptors of the cytokine family. Similarly at the amino acid level there is 54% homology between the GM-CSFR α chain and IL-3R α chain, with 43% homology between IL-3R α chain and IL-5R α chain. A region C-terminal to the four conserved cysteines and a short stretch of amino acids in the intracellular tail proximal to the transmembrane domain are highly conserved [Kitamura *et al*, 1991]. Similarly the N-terminal domains of the α chains share significant sequence similarity. The conservation of these sequences indicates they may be important in a functional role while the obvious relatedness of the α chains, including their gene structure, suggests they may be derived from a common evolutionary ancestor. [Metcalf *et al*, 1992, Barry *et al*, 1997]

The GM-CSFR α chain protein includes a 22 amino acid signal peptide, an extracellular domain of 297 amino acids, a membrane spanning region of 27 amino acids and a short intracytoplasmic tail of 54 amino acids (Figure 1.3) [Gearing *et al*, 1989]. At present eight other isoforms of the GM-CSFR α chain have been described, mostly due to alternative splicing at the 3' end of the originally described transcript. They are represented in Figure 1.8 and include two soluble forms of the receptor lacking the transmembrane and intracytoplasmic regions [Ashworth and Kraft, 1990, Hu *et al*, 1994], an isoform with a novel transmembrane and C-terminal end [Hu *et al*, 1994], an isoform with a variant intracytoplasmic tail which contains a non-homologous serine- and proline-rich 35 amino acid insertion at the 3'end [Crosier *et al*, 1991] and an isoform with a 34 amino acid insertion between the WSXWS box and the transmembrane domain [Devereux *et al*, 1993]. Alternative transcripts of the GM-CSFR α chain have also been described due to variable splicing at the 5' end of the coding sequence. These include transcripts with exon deletions in the 5' untranslated region of the α chain which do not affect protein structure

Figure 1.7 - Model of the High Affinity GM-CSF Receptor.



but may interfere with the translational efficiency, and another transcript which does not encode the signal peptide and may give a protein which is translated but unable to reach the cell surface [Chopra *et al*, 1996, Wagner *et al*, 1994].

1.5.3 - Common beta subunit (β_c) for the GM-CSF, IL-3 and IL-5 Receptors

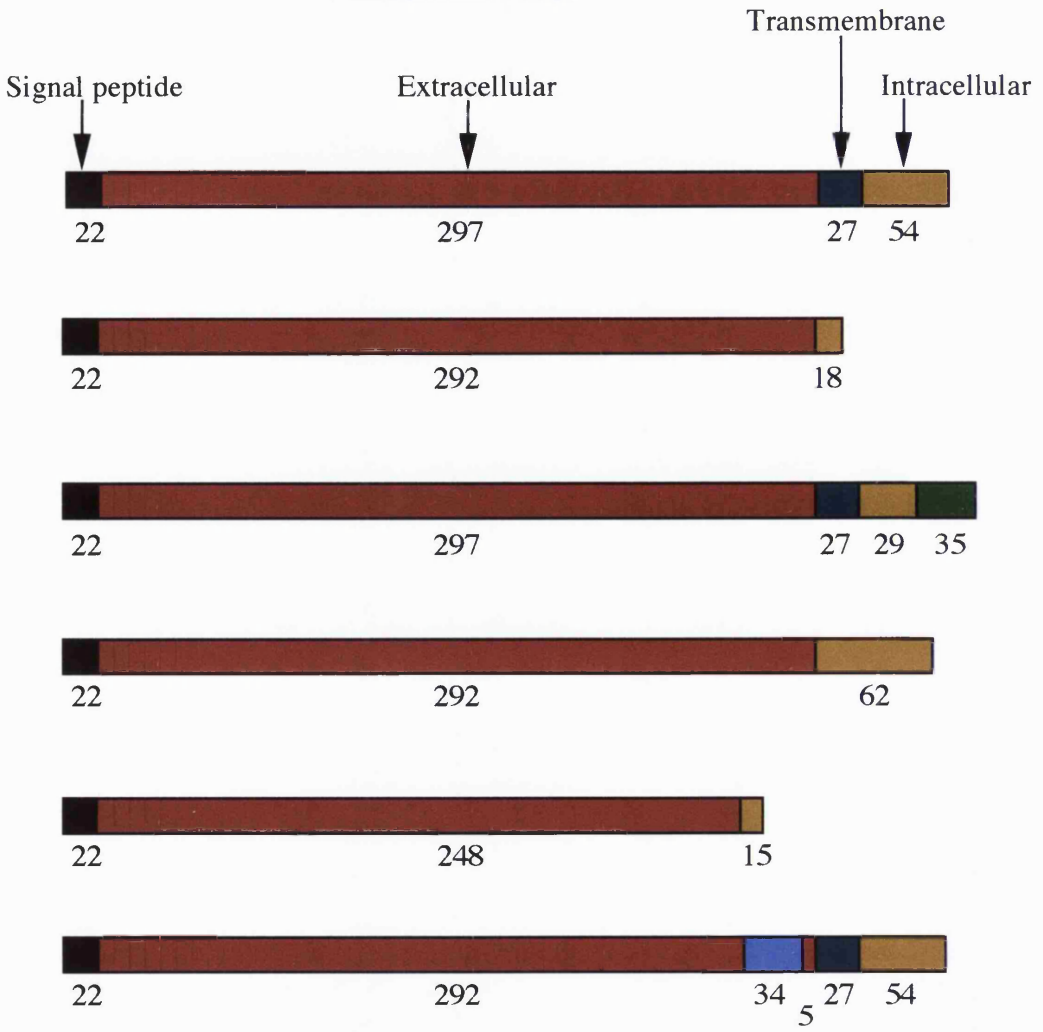
The gene for the human GM-CSFR β_c chain is located on chromosome 22q 12.3-13.1 [Shen *et al*, 1992] and codes for an 897 amino acid protein including a 16 amino acid signal peptide. The mature protein has an extracellular domain of 422 amino acids including 2 CRH domains, a membrane spanning region of 27 amino acids and a long intracytoplasmic tail of 432 amino acids (Figure 1.3) [Hayashida *et al*, 1990]. The β_c subunit corresponds to the 120 kDa band detected in cross-linking experiments with radiolabelled GM-CSF but has also been shown to be a subunit of the high affinity receptors for IL-3 and IL-5 [Kitamura *et al*, 1991, Tavernier *et al*, 1991]. The sharing of a common receptor chain explains how high affinity human GM-CSF binding is competed by IL-3 and vice versa [Miyajima *et al*, 1993]. Likewise the high affinity binding of IL-5 can be cross-competed by GM-CSF or IL-3 on human eosinophils and basophils [Lopez *et al*, 1991].

Interestingly the mouse has two distinct β chain genes, the first chain, AIC2A (mIL-3R β), is a specific β chain for the mouse IL-3R while the other chain, AIC2B (m β_c), is equivalent to the human β_c chain and is shared among the mouse GM-CSF, IL-3 and IL-5 receptors. The two mouse β subunits have a high degree of sequence homology (91% at the amino acid level) and are also quite homologous to the human β_c chain with about 56% homology at the amino acid level. The homology between the mouse and human GM-CSFR α chains is only 35% between the amino acid sequences [Miyajima *et al*, 1993].

1.5.4 - Residues involved in formation of the receptor-ligand complex

The presence of several conserved sequences in the members of the cytokine receptor family suggests that these are areas of the receptor which are important for ligand binding or biological activity. One of the most controversial subjects is the role of the highly conserved WSXWS box and several studies have focused on this motif in a variety of cytokine receptors including EpoR, IL-6R, GHR and the GM-CSFR α chain [Bagley *et al*, 1997]. Analysis in the WSXWS box of the GM-CSFR α chain demonstrated that mutation of the conserved serine residues does not affect the binding affinity of the receptor-ligand interaction. However it does have a profound effect upon the number of

Figure 1.8 - Isoforms of the GM-CSF receptor α chain due to 3' alternative splicing.



	Total length amino acids	
	400	Gearing *et al*,1 989
	332	Ashworth & Kraft, 1990
	410	Crosier *et al*, 1991
	376	Hu *et al*, 1994
	285	Hu *et al*, 1994
	434	Devereux *et al*, 1993

receptor molecules expressed at the cell surface suggesting it is important for protein stability and/or transport of the receptor to the plasma membrane [Yoshimura *et al*, 1990, Ronco *et al*, 1994]. Mutation of either the second or third conserved cysteine residues of the α chain results in the complete loss of low affinity binding to GM-CSF [Ronco *et al*, 1994]. Another study of three conserved sequences found in the area of the conserved cysteines and WSXWS box showed they are essential for surface expression and ligand binding [Doshi *et al*, 1994]. A similar set of experiments using site-directed mutagenesis of the WSXWS box in the EpoR showed that while insertion or deletion of a single amino acid in the WSXWS box was sufficient to abolish ligand binding, with the mutant receptors retained in the endoplasmic reticulum, substitution of amino acids in the WSXWS box had no effect on ligand binding [Yoshimura *et al*, 1992, Quelle *et al*, 1992]. It is therefore important to note that the nature of mutation can determine the severity of its effect on receptor function and in the case of the WSXWS box suggests that the appropriate spacing of the amino acids in the motif is essential for ligand binding and that the effect of amino acid substitutions depends on the severity of conformational change they induce.

Although the GM-CSFR β_C chain does not bind ligand by itself, studies have shown that the GM-CSF ligand and the β_C chain can be crosslinked. This indicates that they are in close proximity and indeed that the β_C chain may be involved in a direct interaction with ligand in the high affinity receptor complex. Most other receptors in the cytokine receptor family contain only a single copy of the CRH domain and while the β_C chain has two, it is thought the membrane proximal CRH domain is better positioned for interaction with ligand and the α chains [Bagley *et al*, 1997]. Further evidence comes from studies using GM-CSF, IL-3 and IL-5 which contain a substitution of a conserved glutamate in the first α helix of the ligand. In each case the substitution abolishes high affinity binding without affecting the ability of the mutated cytokines to bind to their respective α chains, suggesting interaction between specific domains of the ligands and the β_C chain [Bagley *et al*, 1997]. In the case of GM-CSF the effect of these mutants on biological activity is variable, with substitution of Glu21 by different amino acids giving analogues of varying potency depending on the substitution involved [Hercus *et al*, 1994]. It is thought that residual interactions with the β_C chain not detectable by binding experiments are present and may help explain the spectrum of biological activity seen with the various substitutions in what is supposedly low affinity binding only [Bagley *et al*, 1997].

1.5.5 - GM-CSFR complex stoichiometry and receptor activation

It has been amply demonstrated that the GM-CSF receptor α chain binds to the GM-CSF ligand with a low affinity and that in combination with the β_C chain forms a high affinity receptor, with the β_C unable to bind to the GM-CSF ligand by itself [Gearing *et al*, 1989, Hiyashida *et al*, 1990]. For both IL-3R and GM-CSFR, formation of high molecular weight complexes in the presence of ligand has been observed due to a disulphide linkage between the specific α chain and the β_C chain. The GM-CSFR, IL-3R and IL-5R α chains all contain an uneven number of cysteines and it is believed free cysteines in the N-terminal of the α chains form disulphide links with a free cysteine probably in the first CRH domain of the β_C chain [Bagley *et al*, 1997]. This is thought to play a critical role in the formation of ligand receptor complexes However for the GM-CSF receptor the stoichiometry of the ligand-receptor complex has not yet been defined, although it seems likely that more subunits than a single α and a single β_C chain are involved.

Although the GM-CSFR α chain is able to bind ligand by itself, evidence from experiments using mutated receptors and mutated GM-CSF analogues suggests that under certain conditions the GM-CSFR exists as a preformed complex in the absence of ligand [Bagley *et al*, 1997]. A set of experiments which looked at the effect of mutations in the conserved amino acids of the GM-CSFR α chain, expressed in COS-7 cells, demonstrated that mutation of the second conserved cysteine resulted in complete loss of low affinity binding but co-expression of the β_C chain yielded a high affinity receptor. As neither the mutated α chain nor the β_C can bind ligand alone the authors suggested the existence of preformed α and β_C heterodimers on the cell membrane [Ronco *et al*, 1994]. This is in contrast to the IL-3R and IL-5R where the requirement for IL-3 and IL-5 ligand is absolute for dimerization of the respective α chains with the βC [Bagley *et al*, 1997]. Similarly in the case of the IL-6 receptor, IL-6 ligand triggers the heterodimerization of the IL-6 receptor α chain, the major binding subunit, with gp 130, the major signalling subunit. The subsequent homodimerization of gp 130 with a second gp 130 molecule is important for initiation of signalling, with both gp 130 subunits being phosphorylated while the IL-6 α chain shows no evidence of phosphorylation [Murakami *et al*, 1993, Bagley *et al*, 1997]. Homodimerization has also been seen in the β_C chain and it now seems likely that at least two β_C chains are present in the GM-CSF receptor complex and is discussed further below [Bagley *et al*, 1997].

1.5.6 - Role of α chain in downstream signalling

The formation of complexes appears to be an integral part of receptor signal transduction. As shown earlier, the receptors of the cytokine receptor family have extensive homology

in their extracellular region but not nearly as much in their cytoplasmic domain, although shared signalling receptor subunits are a common occurrence. As the α chains for the GM-CSF, IL-3 and IL-5 receptors share a common β chain it has been suggested that the short cytoplasmic tail of each is involved in signal transduction and may play a role in interacting with intracellular signalling molecules, thus allowing for specific biological activities on target cells [Ronco *et al*, 1994, Muire-Sluis *et al*, 1995].

Although there are no sequences with homology to known signalling motifs, the GM-CSFR, IL-3R and IL-5R α chains do have a core sequence of 22 amino acids in the membrane proximal region of their cytoplasmic tails with extensive shared homology between the receptor chains, thought to be essential for signalling interaction with the βC [Kitamura *et al*, 1991, Weiss *et al*, 1993]. It has recently been suggested that two distinct cytoplasmic regions of the GM-CSFR α chain and IL-3R α chain are critical for all signalling events: a membrane proximal Box 1-like "FP"motif and an 8 amino acid "DIIWEEFT" sequence in the midportion of the cytoplasmic region. It has been demonstrated that in frame deletion of the FP motif and DIIWEEFT motif in the GM-CSFR and IL-3R α chains resulted in complete loss of all ligand-induced signalling in Ba/F3 cells [Zhang *et al*, 1996abs]

Previous studies have shown that mutations in the transmembrane domain or truncation of the cytoplasmic domain of the α chain do not effect ligand binding or receptor internalisation, however removal of the cytoplasmic tail of the hGM-CSFR α chain appears to inhibit its ability to stimulate growth of murine cells [Ronco *et al*, 1994, Doshi *et al*, 1994]. Deletion analysis demonstrated that amino acids 346-382 but not 383-400 (numbered including the signal peptide sequence) are necessary for GM-CSF mediated cell growth. Similar deletion analysis of the α chain by Muto *et al* again showed that 29 amino acids between 350-379, proximal to the transmembrane region, are required for various signalling events including activation of immediate early genes, induction of tyrosine phosphorylation of cellular proteins, and cell growth [Muto *et al*, 1995].

Further analysis of the role of the α chain cytoplasmic domain was carried out by constructing chimeric receptor subunits, designated α/β and β/α, by exchanging cytoplasmic domains of the α and βC subunits of the hGM-CSFR. Transfection of Ba/F3 and NIH3T3 cells with chimera α/β + β/α and α/β + β chains reconstituted high affinity receptors, with similar transduction of signals to the wild type high affinity receptor, while cells transfected with β/α + β, α + β/α, α + α/β, α/β or β/α chains did not. These results seem to indicate the cytoplasmic portion of the α chain is not essential for signal transduction, as the α/β + β transfected cells transduced signals through the intracytoplasmic tail of the β_C chain in an oligomeric form, without the cytoplasmic domain

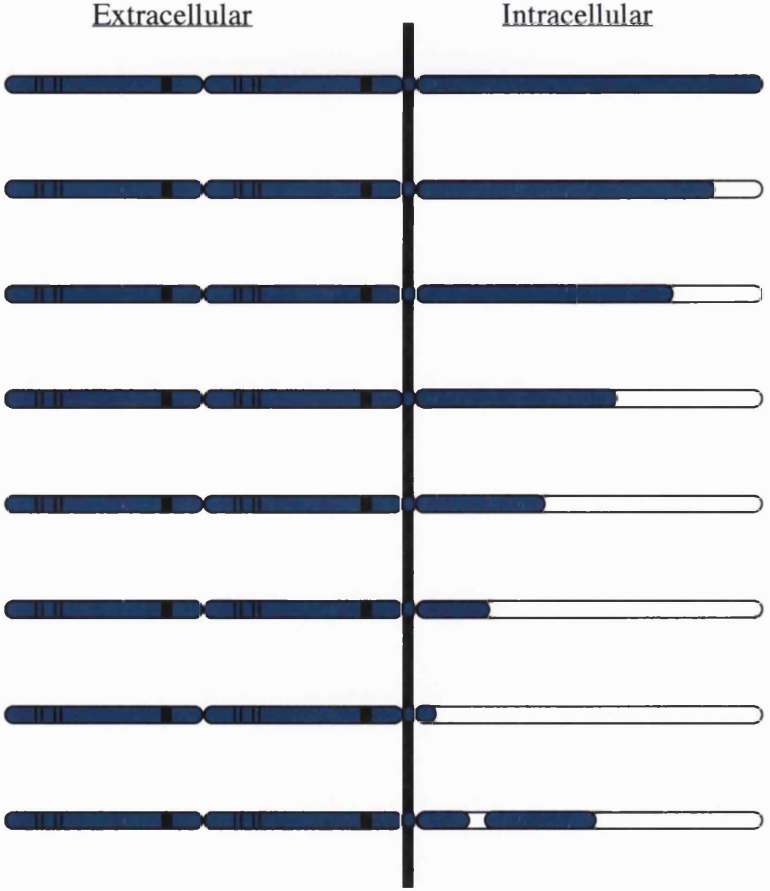
of the α chain [Muto *et al*, 1995]. Therefore it could be hypothesised that homodimerization of the intracytoplasmic region of the β_c chain is sufficient for the generation of receptor signal transduction. However, the existence of preformed β_c chain dimers has been demonstrated by immunoprecipitation in COS-7 cells transfected with the GM-CSFR α and β_c chains. The β_c homodimer was already formed in the absence of GM-CSF, and stimulation with the ligand brought both α and β_c subunits into a complex, with the result being tyrosine phosphorylation of the β_c homodimers. Tyrosine phosphorylation of the β_c subunit was impaired by deletion of the cytoplasmic domain of the α chain without interfering with the association of both subunits. This indicates that the β_c homodimer, which alone is insufficient for signalling, forms functional hGM-CSFR with the α chain in response to GM-CSF [Muto *et al*, 1996]. It is likely that the receptor signal transduction seen with the α/β + β hybrid receptor was due to the α/β hybrid in some way mimicking the role of the α chain in normal receptor activation [Muto *et al*, 1995]. Dimerization of the GM-CSFR β_c chain obviously plays a vital role in the GM-CSFR signal transduction pathway and will be examined further in this study.

1.5.7 - β_c chain signalling domains

Although the short cytoplasmic region of the α chain does seem to play a role in modulation of the growth signal, the β_c chain with its 432 amino acid long intracytoplasmic tail is thought to be responsible for initiation of most downstream signalling. Extensive work has been carried out to establish which regions of the cytoplasmic tail are involved in the various signalling pathways influenced by GM-CSF. The intracytoplasmic tail has no consensus sequences for signalling molecules such as kinases, phosphatases or nucleotide binding proteins, but can rapidly induce protein tyrosine phosphorylation with the binding of ligand to the high affinity receptor [Murata *et al*, 1990 Sakamaki *et al*, 1992].

Using a series of truncated β_c mutants transfected into the mouse pro-B cell line Ba/F3 and mouse T cell line CTLL2, several regions important in signalling have been identified (Figure 1.9). All the transfectants, regardless of the cytoplasmic deletions, exhibited high affinity GM-CSF binding confirming that the cytoplasmic domain is not necessary for high affinity ligand binding. A membrane proximal region of 32 amino acids between Arginine 456 and Phenylalanine 487 (numbering excludes the signal peptide sequence) was shown to be essential for proliferation. This domain contains Box 1 and the sequence is highly conserved between the human β_c chain and the mouse AIC2A (mouse IL-3Rβ) and AIC2B (mouse β_c) subunits. It is also well conserved in the EpoR [He *et al*,

Figure 1.9 - βC chain intracytoplasmic tail deletion mutants.



	Proliferation	JAK activation	Ras activation
Wild type βC 881 a.a.	+	+	+
β825 Mutant Δ 826 - 881	+	+	+
β763 Mutant Δ 764 - 881	+	+	+
β626 Mutant Δ 627 - 881	+	+	-
β544 Mutant Δ 545 - 881	+	+	-
β517 Mutant Δ 518 - 881	+/-	+(less)	-
β455 Mutant Δ 456 - 881	-	-	-
βINT Mutant Δ 488 - 517 + 683 - 881	-	-	-

Adapted from: Sakamaki *et al*, 1992, Sato *et al*, 1993, Quelle *et al*, 1994

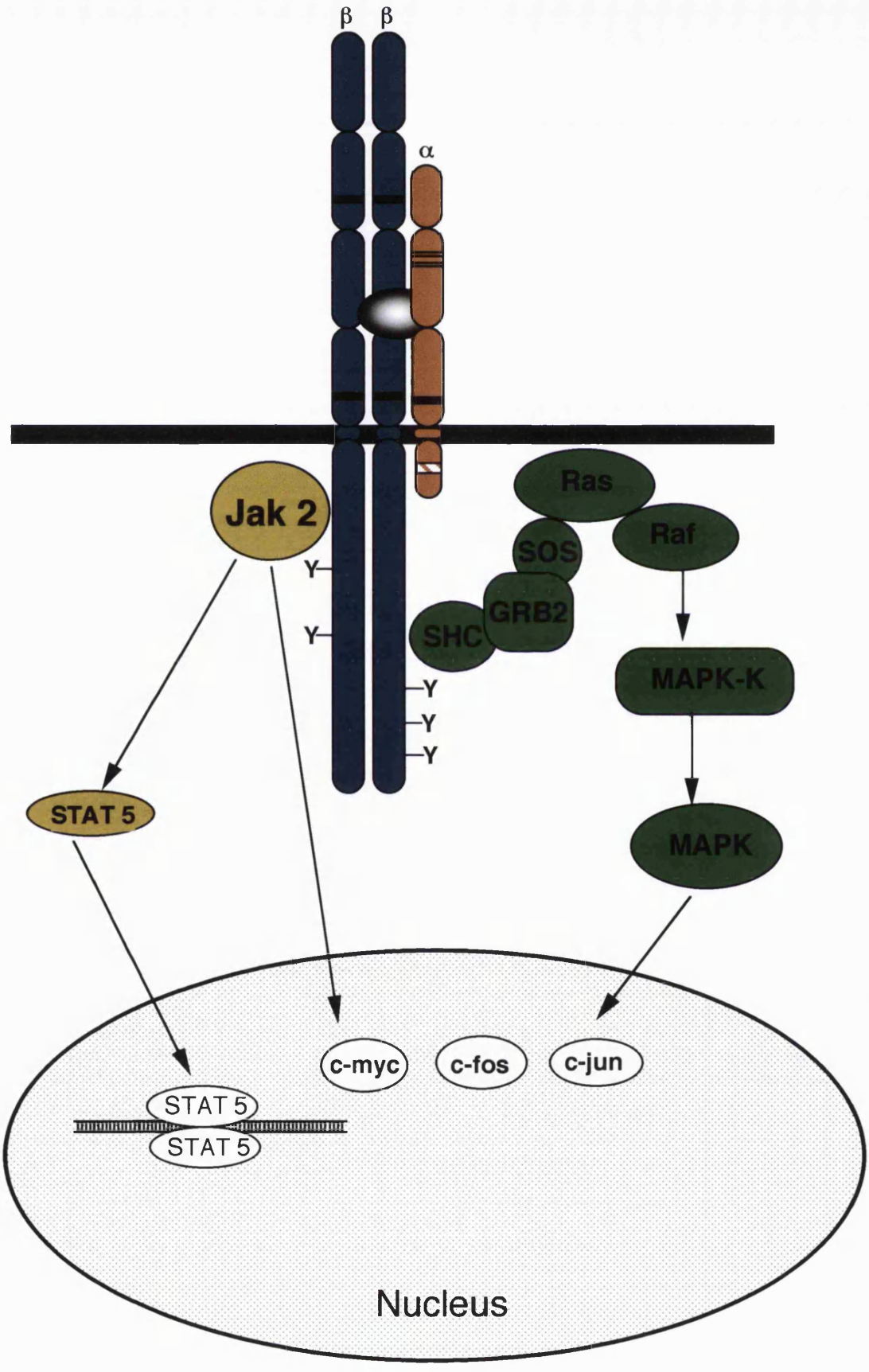
1994], gp 130 [Murakami *et al*, 1990] and the G-CSFR (Figure 4) [Fukunaga *et al*, 1990]. A second domain, consistent with the Box 2 containing region, was identified between Valine 518 and Asparagine 544 which enhanced the response to GM-CSF but was not absolutely required for proliferation. Again homologous sequences for this domain have been identified in the receptors for Epo, G-CSF and also in gp 130 (Figure 4) [Zeigler *et al*, 1993, Dong *et al*, 1993, Murakami *et al*, 1991]. Another region between Valine 518 and Leucine 626 was found to be responsible for major tyrosine phosphorylation of associated proteins. Since tyrosine phosphorylation is required for IL-3 and GM-CSF growth signal transduction, and truncated mutants without this region are sufficient for proliferation, other minor sites of phosphorylation must also have an important role in cell proliferation. [Sakamaki *et al*, 1992, Cleveland *et al*, 1989, Satoh *et al*, 1992]. The roles of the various regions of the β_C chain intracytoplasmic tail, identified by the use of the truncation mutants, in the induction of the major signalling pathways of the GM-CSFR are discussed below and summarised in Figure 1.10.

1.5.8 - GM-CSF and the JAK/STAT pathway

The hypothesis that tyrosine phosphorylation plays a critical role in cytokine receptor function came from the observation that activated oncogenic protein tyrosine kinases could abrogate the requirement for haemopoietic growth factors such as IL-3 [Ihle *et al*, 1996]. A variety of studies have demonstrated that members of a novel subfamily of cytoplasmic protein tyrosine kinases, termed Janus kinases (JAK), couple receptor-ligand binding to induction of tyrosine phosphorylation [Ihle *et al*, 1996]. The members of this family include Tyk 2, JAK 1, JAK 2 and JAK 3, with molecular weights ranging from 125-135 kDa. The kinases share an overall structural pattern with seven conserved domains and a sequence homology of 35-45%. Notable features of the JAKs include a carboxyl kinase domain and a second kinase-like domain but they lack readily detectable Src Homology-2 (SH2) or SH3 domains which are essential for the function of a variety of protein tyrosine kinases [Pawson *et al*, 1992].

At least one member of the JAK family is activated by each member of the cytokine receptor family. It has been shown using truncated mutants that binding of GM-CSF and IL-3 induces the tyrosine phosphorylation and activation of JAK 2 and that the membrane-proximal region between Arginine 456 and Glutamine 517 of the β_C subunit is essential for this phosphorylation (Figure 9). Ba/F3 cells transfected with the wild type GM-CSFR α chain and β_C, β763, β626, or β517 were all able to phosphorylate and activate JAK 2 in response to GM-CSF. However, the extent of JAK 2 phosphorylation seen in cells expressing the β517 mutant was consistently reduced. In contrast no JAK 2

Figure 1.10 - Diagram showing main downstream signalling pathways of the GM-CSF receptor.



phosphorylation or activation was seen in cells transfected with the β455 mutant, indicating the membrane-proximal region between amino acids 455 and 517 of the β_C is required for JAK 2 phosphorylation [Quelle *et al*, 1994].

Similarly, experiments using the α chain with its cytoplasmic domain truncated showed that Ba/F3 cells transfected with the truncated α chain, α328, and a wild-type β_C are incapable of proliferating in response to GM-CSF, nor is JAK 2 phosphorylated (Figure 1.9) [Quelle *et al*, 1994]. The cytoplasmic region of the α chain is also therefore required for JAK 2 activation. Among all the GM-CSFR mutants examined there was a complete correlation between the ability to activate JAK 2 and to support mitogenesis. The requirement of the membrane-proximal region of the β_C chain for mitogenesis and its requirement for JAK 2 activation suggests a role for JAK 2 in the initiation of cellular proliferation [Quelle *et al*, 1994].

It has been reported *in vitro* that JAK 2 can physically associate with the β_C chain and that this association is not dependent on the presence of the GM-CSF ligand or the GM-CSFR α chain. This is also the case for the β_C chain as demonstrated in polymorphonuclear leukocytes [Brizzi *et al*, 1996].

It has been hypothesised that the JAK signalling cascade of the GM-CSFR is initiated when ligand binding of the α chain leads to formation of the GM-CSFR signalling complex, possibly as a hexamer of two ligands, two α chains and two β_C chains [Bagley *et al*, 1997]. This brings about aggregation of JAK kinases associated with the β_C chains which results in activation by reciprocal transphosphorylation. The kinases then phosphorylate a tyrosine (pTyr) in a distal region of the β_C chain leading to receptor recruitment of a specific member of the signal transducers and activators of transcription or STAT family. The STAT proteins are a family of transcription factors which bind specific pTyr sites through their SH2 domains and are subsequently phosphorylated at a tyrosine immediately C-terminal to the SH2 domain. This induces their dimerization through mutual pTyr-SH2 interactions and as a consequence they translocate to the nucleus, bind to specific promoter elements and induce gene expression (Figure 1.10) [Pawson, 1995]. SH2 and SH3 domains are sequences of 50-100 amino acids in length which can fold into a compact and functional module independently of surrounding sequences. SH2 and SH3 domains each recognise short peptide motifs bearing either phosphotyrosine (pTyr) in the case of SH2 or one or more proline residues in the case of SH3. The SH2 domain is localised in the carboxy terminal half of the STAT proteins and has a central GTFLLRFS-S sequence which is conserved in all known STATs. In vivo SH2-containing proteins bind pTyr-containing sites on activated receptors and cytoplasmic phosphoproteins [Pawson, 1995]. It is thought dimerization of specific STAT proteins plays a key role in

the ability of cytokine receptors to activate a unique set of STATs [Schindler and Darnell, 1995] and is hypothesised to occur by association of the phosphorylation site of one STAT molecule and the SH2 domain of a second [Ihle, 1996].

In the GM-CSF, IL-3 and IL-5 pathway STAT 5 is thought to be the major STAT involved with at least two different isoforms already identified in the murine system, but as with most components of signal transduction, the role of STAT 5 is likely to entail subtle differences dependent on the situation involved [Schindler and Darnell 1995].

1.5.9 - GM-CSF activation of the Ras pathway

Further signal pathways activated by GM-CSF have been examined using β_C chain truncated mutants. A C-terminal region distal to the membrane between Leucine 626 and Serine 763 has been shown to be responsible for the activation of the GTP-binding protein $p21^{RAS}$ in response to GM-CSF, leading to activation of a complex pathway that is responsible for transducing extracellular signals into activation of gene transcription. Ba/F3 cells transfected with wild-type GM-CSFR α chain and β_C, β825, or β763 activated Ras in response to GM-CSF stimulation while cells transfected with α chain and β626, β544, β517, or β455 mutants did not [Sato *et al*, 1993]. The membrane-distal region of the β_C chain contains the primary sites of tyrosine phosphorylation, and activation of the Ras pathway starts with receptor phosphorylation and recruitment of SHC through association of its SH2 domains with specific sites of tyrosine phosphorylation (Figure 1.10) [Sato *et al*, 1993]. It is thought SHC is subsequently phosphorylated and recruits GRB2 to the receptor complex. GRB2 is composed almost entirely of SH2 and SH3 domains and appears to function as a molecular adapter to nucleate the formation of protein complexes [Pawson, 1995]. GRB2 then recruits the Ras exchange factor SOS which mediates the conversion from GDP-Ras to GTP-Ras and eventually results in the recruitment of Raf-1 to the membrane and its activation. Activated Raf-1 then phosphorylates mitogen-activated protein kinase kinase (MAPKK), which in turn phosphorylates and activates the MAPKs. The MAPKs are then responsible for the phosphorylation of a variety of effector molecules including transcription factors, other serine/threonine kinases and cytoskeletal proteins (Figure 1.10) [Ihle, 1996].

In addition to the membrane-distal region, mutations or deletions of the membrane-proximal region, which affect JAK association and activation, also eliminate SHC phosphorylation and activation of the Ras pathway [Ihle, 1996]. This suggests that either the JAKs are responsible for SHC phosphorylation or that they are required to activate the kinases that associate with the more distal regions of the receptor. It has also been

demonstrated that the membrane-distal region of the receptor is required to suppress apoptosis under certain conditions [Kinoshita *et al*, 1995]. The potential role of the activation of the Ras pathway is indicated by the ability of an activated Ras allele to complement the receptor mutants. These results support the conclusion that GM-CSF can control both DNA synthesis and prevention of apoptosis through independent pathways [Ihle, 1996].

1.5.10 - Phosphatases

In addition to tyrosine kinases, protein tyrosine phosphatases play a critical role in cytokine signalling. Haemopoietic cell phosphatase (HCP) is a myeloid-specific enzyme also termed PTP1C [Shen *et al*, 1991], SHP-1 [Matthews *et al*, 1992], and SHPTP1 [Plutzky *et al*, 1992]. SHP-1 is a member of the group of intracellular protein tyrosine phosphatases which all contain a single catalytic domain with flanking regions that often contain amino acid sequences which direct the phosphatases to specific intracellular targets [Mourey *et al*, 1994]. In the case of SHP-1, it has two SH2 domains in the amino-terminal half of the protein through which it is thought to bind to the tyrosine phosphorylated β_c chain. It is thought that recruitment of SHP-1 to the receptor complex positions it to either dephosphorylate substrates of JAK 2 or alternatively dephosphorylate the activation site of JAK and assist downregulation of the receptor complex [Ihle, 1996]. It has recently been demonstrated that SHP-1 specifically binds to the tyrosine-phosphorylated derivatives of the β subunit of the IL-3R, and in the EpoR SHP-1 has been shown to associate with the distal region of the receptor and inactivate JAK 2 [Ihle *et al*, 1994, Klingmuller *et al*, 1995]. It is thought that the most likely site of GM-CSFR interaction with SHP-1 is in the membrane-distal region of the β_c chain and specifically around Tyr 806 or Tyr 866 [Itoh *et al*, 1996abs]. The potential significance of SHP-1 binding to the receptor complex comes from the phenotype of *moth-eaten* mice which do not express SHP-1 due to functional disruption of the SHP-1 gene [Schultz *et al*, 1993]. In these mice, all haemopoietic lineages proliferate at much higher levels than normal, resulting in death at 3-6 weeks after birth. Therefore SHP-1 is a potentially important negative regulator of cytokine signalling. Tyrosine phosphatases are also able to function as positive regulators of cytokine receptor signalling as seen with SHP-2 (PTPID, SHPTP2 or syp). Structurally related to SHP-1, phosphorylated SHP-2 has been shown to bind GRB2 through Tyr 577 and Tyr 612 of the β_c chain, leading to activation of the Ras signalling pathway [Ihle, 1996, Itoh *et al*, 1996abs].

1.5.11 - GM-CSF induction of gene expression

In haemopoietic cells several early response genes including c-*fos*, c-*jun* and c-*myc* are activated by GM-CSF and are assumed to play a role in proliferation and differentiation. The c-*myc* gene was originally identified as the cellular homologue of v-myc, a proto-oncogene which causes leukaemia in chickens, and codes for a protein transcription factor thought to play a key role in proliferation and differentiation. Analysis of the β_C using deletion mutants has shown the membrane-proximal Box 1 region is essential for the induction of c-*myc* while the more distal Box 2 domain has weak enhancing activity. The region between amino acids 544-589, has been shown to be essential for the activation of the c-*fos* promoter and c-jun induction, and also strongly enhances the activity of the c-*myc* promoter [Watanabe *et al*, 1995]. However the Box 1 region has also been demonstrated to be essential for c-*fos* promoter activation suggesting that JAK 2 is also involved in c-*fos* induction. This probably occurs via the Ras pathway and although divergent sites of tyrosine phosphorylation, including Tyr577, are thought to be involved in Ras activation they ultimately lead to transcriptional activation of c-*fos* [Sato *et al*, 1993, Itoh *et al*, 1996].

1.5.12 - Tyrosine Phosphorylation of the GM-CSFR

It is clear that even though the GM-CSFR contains no known tyrosine kinase domains, tyrosine phosphorylation of the receptor itself plays an important role in its signalling pathways. A mutant β_C chain in which all the intracytoplasmic tyrosine residues were substituted to Phenylalanine could still activate JAK 2 but was unable to activate SHC, SHP-2 or the c-*fos* promoter [Itoh *et al*, 1996abs]. However it has been demonstrated that Tyr 577 is essential for SHC phosphorylation and its subsequent association with GRB 2 while an intact tyrosine at any one of positions 577, 612 or 695 is sufficient to transduce signals for activation of the c-*fos* promoter [Itoh *et al*, 1996, Itoh *et al*, 1996abs]. The tyrosine phosphorylation of SHP-2 and its association with GRB 2 are mediated through Tyr 577 and Tyr 612 [Itoh *et al*, 1996abs]. It has also been shown that two tyrosine residues in the carboxy terminal of the β_C chain play a role in negative regulation of the receptor signals. Single mutations at either Tyr 806 or Tyr 866 result in augmented activation of the c-*fos* promoter and prolong the phosphorylation of JAK 2 by GM-CSF stimulation. Furthermore transfection of β_C receptor chains with either of these mutations into Ba/F3 cells allows the cells to proliferate in response to lower concentrations of hGM-CSF compared to cells transfected with the wild type receptor [Itoh *et al*, 1996abs].

1.5.13 - Other pathways of GM-CSF signalling

GM-CSF is also involved in several other downstream signalling pathways which include -

c-fps/c-fes

GM-CSF and IL-3 induce tyrosine phosphorylation and tyrosine kinase activity of the c-*fps/fes* proto-oncogene product $p92^{fes}$ (Fes), a non-receptor protein tyrosine kinase found to be expressed in high levels only in myeloid progenitor cells. It has been demonstrated in the erythroleukaemic cell line TF-1 that GM-CSF ligand binding induces the physical association of Fes with the β_C chain of the GM-CSFR, with Fes thought to be involved in the downstream signal transduction [Hanazono *et al*, 1993].

PI 3-kinase

The role of Phosphatidylinositol 3-kinase (PI 3-kinase) in haemopoietic cells is unclear though it has been shown that PI 3-kinase is activated after GM-CSF stimulation in Ba/F3 cells transfected with the β517 truncated receptor chain (Figure 1.9) [Rao and Mufson, 1995]. Some studies have linked it to the Ras pathway and upon phosphorylation it activates protein kinase B and also p70 S6 kinase [Downward *et al*, 1994]

Lyn and Syk

Activation of these protein tyrosine kinases is required for the anti-apoptotic effects of GM-CSF and IL-5 in eosinophils. Lyn is thought to be associated with the receptor through interaction of its SH3 domains with the proline rich region of the β_C chain and following tyrosine phosphorylation recruits Syk via mutual SH2 domains [Yousefi *et al*, 1996]. Lyn and Lck, a related kinase present specifically in lymphocytic cells, are capable of phosphorylating tyrosines residues in the membrane-proximal region of the β_C chain, although the major sites of β_C tyrosine phosphorylation are thought to be betwen Ser 683 and Ser 763, with serine/threonine kinase activity accounting for the majority of phosphorylation in the membrane-proximal domain of the β_C chain [Rao and Mufson, 1995].

Vav

Initially identified as a novel proto-oncogene the gene product has been shown to be phosphorylated by GM-CSF and functions as both a signal transducer and also as a transcription factor [Adams *et al*, 1992]. In the case of the EpoR it has been shown that the membrane-proximal region is sufficient for Vav phosphorylation and that it may be directly associated with the activated JAKs in the receptor complex [Ihle, 1996].

1.5.14 - Mice knockouts of GM-CSF function

Mice homozygous for a disrupted GM-CSF gene develop normally and show no major perturbation of major haemopoietic populations in blood or bone marrow up to 12 weeks of age. However all mice involved developed abnormal lungs, the features of which resemble the human disorder pulmonary alveolar proteinosis (PAP) [Stanley *et al*, 1994]. Similarly when gene targeting was used to create mice with a null mutation of the gene encoding the β_c chain of the GM-CSF, IL-3 and IL-5 receptors, the only haemopoietic abnormality observed was reduced numbers of eosinophils in the blood, less so in the marrow and tissues, though the mice again showed PAP-like disorders. It could be shown that high affinity binding of GM-CSF was abolished in the bone marrow cells of homozygous mice while an intermediate number of high affinity receptors was seen in heterozygotes, yet binding of IL-3 was unaffected. This demonstrated that the mouse IL-3R specific β chain (mIL-3Rβ) remained unaltered [Robb *et al*, 1995]. In order to exclude the possibility of redundancy between IL-3 and GM-CSF, mice deficient for the IL-3 ligand were crossed with the β_c null mice to give mice totally lacking in GM-CSF, IL-3 and IL-5 function. The severe lung abnormal development was again seen but the double mutant mice showed normal haemodynamic parameters except for reduced numbers of eosinophils and were even able to give a normal immune response to bacterial infection [Nishinakamura *et al*, 1996abs]. A similar study which looked at mice in which the mIL-3Rβ gene was homozygously disrupted again showed mice with no obvious haemopoietic abnormalities suggesting that in mice the functions of GM-CSF, IL-3 and IL-5 are dispensable in haemopoiesis and there must be alternative mechanisms which substitute for their roles when absent [Nicola *et al*, 1996].

1.6 - Molecular mechanisms in haemopoietic disorders

1.6.1 - Factors influencing leukaemogenesis

Genetic alterations play a central role in the development of cancer with tumour formation believed to be a multi-step process involving several genetic mutations [Bishop *et al*, 1987]. It is thought accumulation of mutations ultimately leads to deregulation of genes important for growth control giving malignant transformation and the clonal expansion of the transformed cell. The multi-step hypothesis of carcinogenesis is supported by epidemiological and experimental evidence. The increase in cancer incidence with age suggests the accumulation of genetic mutations with time [Cook *et al*, 1969], while overexpression of the oncogenes c-*myc* and H-*Ras* in transgenic mice leads to tumour development much earlier in life than in mice in which a single oncogene as been overexpressed [Stewart *et al*, 1984].

The leukaemias are stem cell disorders and are characterised by a malignant neoplastic proliferation and the accumulation of immature haematopoietic cells in the bone marrow [Russell *et al*, 1992, Lowenberg *et al*, 1993]. The development of leukaemia in humans seems to depend on an interplay between several factors with leukaemogenesis occurring by a process involving multiple steps. Factors implicated in leukaemogenesis include :

Ionising Radiation - based on the increased incidence of leukaemia in survivors of the nuclear explosions at Hiroshima and Nagasaki and in patients with ankylosing spondylitsis who had received repeated irradiation to the spine [Hughes-Jones *et al*, 1994].

Chemicals - evidence suggesting alkylating agents, benzene and cigarette smoking in the aetiology of leukaemia [Hughes-Jones *et al*, 1994].

Viruses - involved in leukaemogenesis in mice, rats, chickens and cats. The main virus implicated in a human leukaemia is the retrovirus human T-cell virus 1 (HTLV-1) which can be isolated from patients with adult T-cell leukaemia/Lymphoma (ATLL).

Oncogenes and Tumour suppressor genes - Genes involved in the pathogenesis of cancer are thought to act by two general mechanisms. The first involves the structural alteration of a normal gene (proto-oncogene) to generate a novel gene (oncogene) whose protein products acts on the host cell to induce characteristics of malignancy [Cline *et al*, 1994]. Activation of certain proto-oncogenes has been reported in some human leukaemias. The presence of the Philadelphia chromosome in virtually all cases of chronic myeloid leukaemia (CML) and in 5-25% of acute lymphoblastic leukaemia, is associated with activation of the abl proto-oncogene, while point mutations in the Ras proto-oncogene have been reported in up to 30% of patients with acute myeloid leukaemia (AML).

However these activating mutations of Ras are thought to promote the growth of a malignant subclone rather than initiate AML [Farr *et al*, 1988, Toksoz *et al*, 1989]. The second mechanism of genetic alteration involves the loss or inactivation of genes whose protein products suppress cancer. Genes of this class are known as tumour suppressor genes (anti-oncogenes). Inactivation of two such genes, p53 and retinoblastoma 1 (RB 1), by mutation or deletion sometimes appears to be involved in the evolution of certain leukaemias to a more aggressive course rather than their initiation, with abnormalities in p53 and RB 1 found in 10-30% of acute leukaemias [Hughes-Jones *et al*, 1994, Cline *et al*, 1994].

The activation of oncogenes or the loss of anti-oncogenes either endow the leukaemic cells with a proliferative advantage or prevent its normal differentiation and subsequent death. Several types of genes are potentially leukaemogenic in haemopoietic cells. They include genes involved in growth signalling pathways, genes which activate transcription, genes involved in the differentiation of cells, genes involved in apoptosis and genes whose normal function is to suppress tumour development, although obviously many genes have several effects in normal and abnormal haemopoiesis [Cline *et al*, 1994].

1.6.2 - The role of HGFs in leukaemia

Leukaemic blasts retain the ability to proliferate but have lost the capacity to differentiate and mature. They generally require exogenous HGF for survival, although a percentage of progenitors are capable of spontaneous proliferative activity *in vitro*. Frequently this spontaneous growth is mediated by autocrine growth stimulation and autocrine HGF production by leukaemic blasts may render them independent of exogenous HGF stimulation [Lowenberg *et al*, 1993]. Autocrine HGF production could allow neoplastic cells to become independent of the growth regulatory constraints placed upon normal cells, thus providing a major growth advantage to the malignant clone [Russell *et al*, 1992]. Two patterns of autocrine growth in tumour cells have been described. One is an extracellular autocrine loop involving the secretion of growth factors by cells expressing HGF receptors on their cell surface and thereby triggering cell proliferation. This autocrine loop can be interrupted by specific neutralising antibodies to the secreted factors as shown by Young and Griffin in 1985. In two cases of AML, autonomous growth of cells which secreted GM-CSF could be inhibited by the addition of neutralising antibodies to GM-CSF [Young *et al*, 1986]. Evidence has been found for a second intracellular autocrine loop involving non-secreted growth factors. Some AML blasts display characteristics of

autonomous growth which is not inhibited by neutralising antibodies but can be inhibited by anti-sense oligonucleotides to specific cytokines [Rogers *et al*, 1994].

The degree of influence which autocrine loops exert in the pathogenesis of leukaemia is unclear. However, transgenic mice which constitutively express GM-CSF mRNA, as a result of retroviral transfection, develop myeloproliferative syndromes but not acute leukaemias. This suggests that autocrine growth stimulation by itself is insufficient to induce leukaemia but must be accompanied by other genetic events which block differentiation [Lowenberg *et al*, 1993].

The importance of the role played in the regulation of normal haemopoiesis by HGFs makes them obvious candidates for investigation in haemopoietic disorders such as leukaemia.

1.6.3 - Cytokine receptors in leukaemia

Increased receptor expression and altered receptor affinities are potential mechanisms which could confer a growth advantage to a cell, and the function of HGF receptors in leukaemic cells is now an important focus of research. The use of radiolabelled cytokines has established that primary AML cells express high affinity receptors for GM-CSF, IL-3 and G-CSF, with the receptors for other factors such as IL-5 and IL-6 expressed less frequently. In most cases of AML the number of receptors for GM-CSF, IL-3 and G-CSF are quite low (about 50-300 high affinity GM-CSFR receptors per cell [Kelleher *et al*, 1988, Onnetto-Pothier *et al*, 1990], and while receptor overexpression has been implicated as one mechanism through which ligand independent activation of M-CSFRs can occur, this has not been described in AML patients [Sherr *et al*, 1990].

Certain retroviral oncogenes have been described which code for altered forms of HGF receptors, and are able to induce factor-independent growth of cell lines. For example, the oncogenic v-fms encodes for a receptor tyrosine kinase, analogous to the cellular M-CSFR, which is able to induce factor-independent growth of the murine FDC-P1 cell line [Wheeler *et al*, 1987]. Other viral encoded cytokine receptors which have been shown to confer factor-independent growth are discussed later in section 1.7.1.

Alternatively mutations in molecules involved in downstream cytokine signalling pathways may play a role in importing a growth advantage to haemopoietic cells, such as the Ras mutations mentioned previously (Section 1.6.1). It has been suggested that as STAT proteins are an important component of downstream signalling they may have a part to play in haemopoietic disorders. The use of bandshift analysis in a study of leukaemic cells from patients with ALL and AML showed the existence of distinct STAT related transcription factors in nuclear extracts. The authors concluded that the STAT proteins

detected possibly played a role in disease pathogenesis as they were constitutively activated in vivo, as under normal conditions STATs need to be tyrosine phosphorylated before they translocate to the nucleus but these cells had not been treated with cytokines prior to the extraction of the nuclear proteins and [Gouilleux-Gruart *et al*, 1996]. Also a JAK homologue has been identified in the fruit fly Drosophila [Binari *et al*, 1994], a dominant mutation which causes the abnormal proliferation and differentiation of the larval haemopoietic system (fly leukaemia) [Hanratty *et al*, 1993], again raising the possibility that mutations in JAKs may be associated with transformations in mammalian systems.

1.7 - Activating mutations in cytokine receptors

As the first step in the cytokine signal transduction pathway, cell surface receptors are obviously a potential site for mutations affecting cell proliferation, differentiation and maturation. Several mutant cytokine receptors have been identified due to their ability to induce ligand-independent survival in haemopoietic cells, which under normal conditions are factor-dependent.

1.7.1 - Virus encoded receptors

As briefly mentioned several viral oncogenes code for truncated growth factor receptors with transforming potential. For example, the Tpo receptor (c-mpl) was first identified as the transforming gene associated with the myeloproliferative leukaemia virus (MPLV), a murine leukaemia-inducing derivative of the Friend helper virus [Penciolelli *et al*, 1987]. Infection of adult mice with MPLV, in the presence of helper virus, induces an acute leukaemia with massive proliferation of haemopoietic cells in the erythroid and myeloid lineages. Cloning and sequencing of the transforming component (v-mpl) suggested a structure consistent with a truncated cytokine receptor [Souyri *et al*, 1990] which was confirmed by cloning of the proto-oncogene [Vigon *et al*, 1992]. Thus in v-*mpl* most of the Mpl extracellular domain has been deleted with just 43 amino acids of the extracellular plus the transmembrane and cytoplasmic regions derived from c-*mpl* with the remainder of the extracellular region composed of viral *env* sequences. The fusion protein probably functions as a constitutively activated receptor although the mechanism underlying the constitutive activity remains unclear [Gonda and D'Andrea, 1997].

The Friend erythroleukaemia retrovirus is a complex of a replication-defective spleen focus-forming virus (SFFV) and a replication-competent murine leukaemia virus (F-MuLV). The viral complex is responsible for a rapid and fatal erythroleukaemia when injected into adult mice. The SFFV env gene codes for a transmembrane protein called gp55 which can confer Epo-independent growth in murine cells. It is thought gp55 binds

to the EpoR principally via its transmembrane region and activates its proliferation signal, resulting in growth irrespective of Epo [Youssoufian *et al*, 1993]. Transport of gp55 to the cell surface is very inefficient with greater than 95% retained in the endoplasmic reticulum where it interacts with the EpoR, raising the possibility of signalling from the intracellular complex [Gonda and D'Andrea, 1997].

1.7.2 - Artificially created receptor mutants with altered protein structure

Experiments in murine cells by several groups using cytoplasmic truncations of the EpoR have shown two regions of opposing function: a membrane-proximal region of about 100 amino acids that is sufficient for transduction of proliferative signals and a second distal region that is important for negative regulation of growth. It was shown that loss of the distal domain renders transfected Ba/F3 cells hypersensitive to Epo, with deletion of the carboxy terminal 40 to 90 amino acids of the receptor allowing cells to proliferate in approximately 1/10th the concentration of Epo required with the full length receptor [Yoshimiura *et al*, 1990, D'Andrea *et al*, 1991, Youssoufian *et al*, 1993]. The negative regulatory domain found in the carboxy-terminal region deleted in these experiments contains 9 serine and 4 tyrosine residues, which includes a binding site for haemopoietic cell phosphatase (HCP or SHP-1). This suggests that dephosphorylation events mediated by an associated phosphatase may be involved in the downregulation of the EpoR. Loss of this regulatory region seems to confer a growth advantage and several C-terminal truncations in the EpoR have now been reported in patients with primary familial polycythaemia and are discussed below in section 1.8.1.

Although as yet no activating mutations have been shown in the β_C chain of the GM-CSF, IL-3 and IL-5 receptors in AML patients, it has been shown that duplication of a 37 amino acid sequence in the extracellular region of the human β_C chain is sufficient to confer ligand-independent growth when transfected into the murine myeloid cell line FDC-P1. This duplicated segment, which arose spontaneously following transfection with a retroviral $h\beta_C$ construct, includes the highly conserved WSXWS box and an adjacent conserved basic region (Y/HXXR/QVR) [D'Andrea *et al*, 1994, Patthy *et al*, 1990]. Similar experiments looking at extracellular truncations of the β_C chain identified this region of 37 amino acids between histidine 395 and alanine 431 as essential for factor-independent signalling in FDC-P1 cells and showed that β_C chain truncated mutants which only retained this membrane-proximal domain of the extracellular region were able to support factor-independent proliferation [D'Andrea *et al*, 1996]. It was suggested that the sequence defined by this region may be important in signalling because the altered receptor structure mimicked a ligand-induced signalling event and may lead to unmasking of an

interactive surface of the β_C chain not normally available (Table 1.1) [D'Andrea *et al*, 1994, D'Andrea *et al*, 1996].

An amino-terminal truncated β_C chain which arises from a 10kb deletion in the β_C chain gene was isolated from a murine myeloid cell line after retroviral insertional mutagenesis. Although both the predicted transmembrane and intracellular domains are identical to those in the normal β_C subunit, the entire extracellular domain was replaced by a new novel 34 amino acid sequence. It was demonstrated that this truncated receptor was able to mimic the activated GM-CSF/IL-3 receptor response in the absence of ligand, and was able to stimulate ligand-independent growth in FDC-P1 cells [Hannemann *et al*, 1993]. This truncated mutant does not appear to acting in the same manner as the truncated βC receptors described by D'Andrea et al, as in that study the most severely truncated mutants, with only seven amino acids of the original extracellular domain remaining, was inactive. The mutant described by Hannemann *et al* has none of the wild type extracellular sequence but the novel 34 amino acid sequence does contain a newly introduced cysteine residue, which may be involved in disulphide-linked receptor dimerization [D'Andrea *et al*, 1996, Hannemann *et al*, 1993].

These extracellular truncated β_C receptors are very similar in structure to the transforming receptor described earlier, coded for by the viral oncogene v-mpl, the cellular counterpart of which (c-mpl) codes for the TpoR (Table 1.1) [Vigon *et al*, 1992].

1.7.3 - Artificial point mutations with receptor activating potential

Several studies have shown that mutant cytokine receptors with structural alterations are able to confer ligand-independent proliferation or hypersensitivity to ligand. However constitutively activated cytokine receptors have also been described due to the presence of point mutations in the receptor gene.

Mutations engineered in the murine *mpl* gene have shown that the TpoR can be constitutively activated by the substitution of certain amino acids with cysteine residues in a region predicted to be the dimer interface domain of the receptor. Like the β_C chain of the human GM-CSF, IL-3 and IL-5 receptors, the TpoR has two CRH domains and each contains a cluster of amino acids homologous to sequences in the human GHR and EpoR, thought to be involved in stabilising subunit interactions. Substitution of specific amino acids (codons 117, 120, 368 and 369) in this region with cysteine residues led to constitutively activated receptors, presumably through the formation of disulphide-linked homodimers. Although cysteine substitutions in either CRH led to receptor activation, cysteine substitutions in the N-terminal CRH domain gave substantially less Tpo-independent growth in FDC-P1 and Ba/F3 cells, suggesting that for the TpoR the

Table 1.1 - Summary of activating mutations of the β_C chain

Mutation	Location	How Created	Possible effect	Reference
37 amino acid duplication	Extracellular membrane-proximal	Retroviral Transfer	May mimic ligand binding	D'Andrea *et al*, 1994
11 amino acid insertion	Extracellular membrane-proximal	-	May mimic ligand binding	Gonda and D'Andrea, 1997
Amino terminal truncated receptor	Entire extracellular region replaced by novel 34 amino acids	Retroviral Transfer	May induce disulphide-linked dimerization	Hannemann *et al*, 1993
Extracellular Truncations	Truncation of extracellular region with loss of N-terminal 394 amino acids	Site directed mutagenesis	May mimic ligand binding	D'Andrea *et al*, 1996
Val_{449} -> Glu	Transmembrane domain	Random mutagenesis	May mediate homodimerization	Jenkins *et al*, 1995
Ile_{374} -> Asp	Extracellular membrane-proximal	Retroviral Transfer	May mimic ligand binding	Jenkins *et al*, 1995

membrane-proximal CRH is more important for receptor dimerization [Alexander *et al*, 1995, Gonda and D'Andrea, 1997]. Using PCR-driven random mutagenesis an activating mutation was identified in the transmembrane region of the TpoR [Onishi *et al*, 1996]. This point mutation causes the substitution of the serine at codon 498 to an asparagine and was shown to abrogate the factor-dependency of all IL-3-dependent cell lines tested including Ba/F3, TF-1 and FDC-P1 cells. In addition it was shown that in Ba/F3 cells transfected with the mutant TpoR both the MAP kinase and JAK/STAT pathways were constitutively activated by the mutant receptor. Although the exact mechanism of constitutive activation was not clear, it was thought that the mutation induced constitutive homodimerization of the TpoR but this was not demonstrated [Onishi *et al*, 1996].

A retrovirally created mutant EpoR able to proliferate in the absence of ligand was isolated from transfected Ba/F3 cells. It was found to have a single point mutation involving a C to T transition at nucleotide 484, which caused the substitution of an arginine with a cysteine residue at codon 129 (R129C) in the exoplasmic region of the receptor [Yoshimiura *et al*, 1990]. The R129C mutant had similar physiological effects as seen with the gp55 subunit encoded by the erythroleukaemia retrovirus Friend spleen focus-forming virus (SFFV), which confered Epo-independent growth in murine cells [Youssoufian *et al*, 1993]. In addition to ligand-independent growth, the R129C mutant caused accumulation of the receptor in the endoplasmic reticulum (ER) and affected down-regulation of the receptor, leading to a longer half life for the mutant, although it did not affect binding affinity of ligand with the cell surface receptor [Watowich *et al*, 1992].

The R129C mutant receptor was shown to exist as a disulphide linked dimers on the cell surface as well as in the ER, an effect directly linked to the mutation, which is thought to mimic ligand-induced homodimerization of the EpoR [Watowich *et al*, 1992]. The R129C mutation is located in the extracellular membrane-proximal region of the EpoR, predicted to correspond to the dimer interface domain for the receptor, homologous to that of the GHR and the previously mentioned TpoR domains. Mutation analysis of other amino acids in this region of the EpoR showed that substitution of two other residues with cysteines (E132 and E133) resulted in ligand-independent growth in Ba/F3 and 32D cells, with disulphide-linked receptor dimers again present [Watowich *et al*, 1994]. Thus, disulphide-linked dimerization of receptors appears to correlate with constitutive activation suggesting an important role for ligand-induced dimerization in receptor activation and signal transduction [Gonda and D'Andrea, 1997].

Using a combination of retroviral expression cloning and PCR based random mutagenesis, two activating point mutations in the human β_c chain able to confer factor-independence in cells of the FDC-P1 line have been identified (Table 1.1). One mutation, a

valine to glutamic acid substitution at amino acid residue 449 (V449E) located in the transmembrane domain of the receptor chain, is analogous to an oncogenic mutation in the Neu receptor tyrosine kinase which is thought to induce constitutive receptor dimerization via its transmembrane region and also to the TpoR mutation discussed earlier. The other mutation, an isoleucine to asparagine substitution at residue 374 (I374N) which lies in the membrane-proximal region of the extracellular domain, also conferred ligand-independent growth in FDC-P1 cells [Jenkins *et al*, 1995]. However, these activating mutations appear to be cell-type specific as neither mutation was able to induce factor-independence in CTLL-2 cells and only the V449E could confer ligand-independence in Ba/F3 cells. This has led to suggestions of differences between the β_C extracellular mutations (I374N, 37 amino acid duplication and truncations) and the tranmembrane V449E mutation in terms of dimerization, quantitative activation or interaction with cell-specific molecules, but as yet the exact mechanism remains unknown [Jenkins *et al*, 1995].

1.8 - Cytokine Receptor mutations in haemopoietic disease (Table 1.2)

1.8.1 - EpoR alterations in erythroid disorders

Erythropoietin is a glycoprotein of about 34kDa which acts, through its receptor, as the main regulator of proliferation and differentiation in erythroid progenitor cells [Krantz *et al*, 1991]. The role of the EpoR in erythropoiesis has led to wide range of studies looking for receptor alterations in haemopoietic diseases affecting the erythroid lineage. At least five separate mutations have been described in the intracytoplasmic region of the receptor which lead to C-terminal truncations of the EpoR. These include three studies which looked at members of families with a history of primary familial and congenital polycythaemia (PFCP). In two of the three cases a point substitution, G to A at position 6002 [De la chapelle *et al*, 1993] and a C to T at position 5986 [Furakawa *et al*, 1996abs], introduced a premature stop codon, while in the third a single nucleotide insertion (G at nucleotide 5975) caused a shift in the reading frame leading to amino acid substitutions before a new stop codon [Sokol *et al*, 1995]. In each case truncated receptors, lacking the last 64-74 amino acids of the C-terminus, were produced which displayed a hypersensitive response to Epo stimulation. This hypersensitivity is caused by the loss of a negative regulatory domain known to contain an HCP binding domain, with the inability to recruit HCP resulting in a more active receptor complex [Klingmuller *et al*, 1995, Zhang *et al*, 1995]. These studies of EpoR mutations in patients with PFCP are in agreement with

Table 1.2 - Cytokine receptor mutations in haemopoietic disease

Receptor	Haemopoietic Disorder	Codon Change	Protein Alteration	Reference
EpoR	Primary and familial congenital polycythemia	Trp_{439} -> stop	70 amino acid truncation	De La Chapelle *et al*, 1993
		Gln_{435} -> stop	74 amino acid truncation	Furakawa *et al*, 1996
		Nucleotide insertion, frameshift, premature stop	64 amino acid truncation	Sokol *et al*, 1995
	Polycythemia	Pro_{488} -> Ser	missense mutation	Sokol *et al*, 1994
	Polycythemia	Pro_{319} -> Ser	missense mutation	White *et al*, 1995
		Trp_{340} -> Arg	missense mutation	
	Erythroleukaemia	Asp_{487} -> Ser	missense mutation	Le Couedic *et al*, 1996
G-CSFR	Severe congenital neutropenia	Glu_{716} -> Stop	98 amino acid truncation	Dong *et al*, 1994, Dong *et al*, 1995
		Glu_{718} -> Stop	96 amino acid truncation	Dong *et al*, 1997, Tidow *et al*,1997
		Glu_{720} -> Stop	94 amino acid truncation	
		Glu_{726} -> Stop	88 amino acid truncation	
		Glu_{731} -> Stop	83 amino acid truncation	
GM-CSFR α	Acute myeloid leukaemia	Ala_{17} -> Gly	missense mutation	Wagner *et al*, 1994
		Arg_{164} -> Glu	missense mutation	

experiments on artificially created mutant EpoRs described earlier, where truncated EpoRs gave a hypersensitive response to ligand [D'Andrea *et al*, 1991, Youssoufian *et al*, 1993].

In a study of three patients with an unusual variant of polycythemia involving thrombocytosis it was demonstrated that BFU-E colonies from peripheral blood or bone marrow grew without added Epo. Analysis of the EpoR revealed two unique point mutations which caused a Proline to Serine substitution at amino acid 319 and a tryptophan to arginine substitution at amino acid 340 both in the membrane-proximal region of the EpoR, but whether they played a part in the pathogenesis of the disease remains unclear [White *et al*, 1995abs].

Other point mutations involving single amino acid changes have been identified in the coding sequence of the EpoR. An asparagine to serine substitution at amino acid 487 was found in a patient with erythroleukaemia and again in one patient with polycythaemia [Le Couedic *et al*, 1996]. Although a previous proline to serine mutation at amino acid 488 was found in this area in another patient with polycythaemia [Sokol *et al*, 1994] there did not appear to be a molecular mechanism involved in abnormal growth. Mutations of the EpoR gene have also been found in the erythroleukaemic cell lines UT7 and TF-1, leading to upregulation of the receptor and expression of an abnormal receptor transcript respectively [Winkleman *et al*, 1995, Ward *et al*, 1992].

1.8.2 - G-CSF receptor in SCN

Similar to the situation with the EpoR several different point mutations in the granulocyte colony stimulating factor receptor (G-CSFR) have been reported in patients with severe congenital neutropenia (SCN). SCN is a rare paediatric disease characterised by decreased numbers of peripheral blood neutrophils and arrested maturation of myeloid progenitor cells in the bone marrow. In five cases of SCN, four of which later developed AML, C to T transitions at the codons of four different glutamine residues (716, 718, 720, 726 and 731) resulted in the introduction of a premature stop. This led to G-CSFRs with truncated intracytoplasmic tails lacking 83 to 98 amino acids at the C-terminal end [Dong *et al*, 1994, Dong *et al*, 1995, Dong *et al*, 1997, Tidow *et al*, 1997]. All the truncated receptors lack the distal region of the cytoplasmic tail, including the box 3 domain (also found in gp 130) required for differentiation, but all still contain the box 1 and box 2 domains which have been shown to be both necessary and sufficient for proliferation in the G-CSFR [Fukunaga *et al*, 1993, Dong *et al*, 1993]. It has therefore been suggested that, as they are found in a clonally expanded myeloid cell population, the presence of these mutations in SCN might disrupt the maturation signal of G-CSF and predispose the patients to leukaemia. Although it has also been suggested, the use of G-CSF in the treatment of

SCN may affect disease progression, with the possibility it may stimulate the accumulation of cells with a block in the maturation signal and hence influence the transformation of several SCN cases to acute leukaemia [Dong *et al*, 1994]. However studies looking for alterations of the G-CSFR in patients with de novo AML failed to show the presence of any mutations likely to be involved in the pathobiology of the disease, suggesting that abnormalities in the G-CSFR are uncommon in AML [Bernard *et al*, 1996, Dong *et al*, 1997]. Another study in which the G-CSFR was analysed in 11 patients with SCN, truncation mutants were detected in a minor percentage of mRNA transcripts from two patients. However the mutations were present at constant low levels and were not thought to be involved in the pathogenesis of SCN but were merely random mutations detectable due to clonal expansion. Importantly, in the one SCN patient who transformed to AML no mutations were detected in the G-CSFR, indicating that the development of G-CSFR mutations is not essential for leukaemic transformation [Bernard *et al*, 1996].

1.8.3 - IL-2 receptor in X-linked severe combined immunodeficieny disease

The severe combined immunodeficiency diseases (SCIDs) are a spectrum of immunological disorders characterised by dramatically compromised T-cell and B-cell function. X-linked SCID is the most common, accounting for about half the total cases diagnosed, and linkage analysis allowed the localisation of the gene for the interleukin-2 receptor (IL-2R) γ chain to the X chromosome at Xq13 [Nagouchi *et al*, 1993, Leonard *et al*, 1996]. Several subsequent studies have shown various types of mutations in the receptor gene including single amino acid changes, frame shifts, deletions, insertions and defects in splicing. Some of these mutations result in unstable γ chain mRNA or protein, while others result in expression of mutated forms of the receptor chain which have altered functions [DiSanto *et al*, 1994, Leonard *et al*, 1996]. For example, a mutation found in the cytoplasmic tail of the IL-2R γ chain substituted a leucine at amino acid 271 of the protein sequence to a glutamine. Although this mutation does not affect γ chain expression, it substantially inhibits the ability of the γ chain to associate with the protein tyrosine kinase Jak 3. It had been hypothesised that the defect in XSCID results from failure to activate signalling pathways such as Jak 3, and subsequently Jak 3-deficient patients have been described, including one female, who exhibited a classic XSCID phenotype [Leonard *et al*, 1996]. This provides an example of a mutation in a haemopoietic disorder which directly affects cytokine receptor interaction with downstream signalling molecules.

1.8.4 - GM-CSF receptor in leukaemia

It has been amply demonstrated that mutation of cytokine receptors can be sufficient to confer a growth advantage to haemopoietic cells as one of the hits in the multi-step pathogenesis of leukaemia. Expression of the GM-CSFR in myeloid cells has been shown to vary as they mature along the differentiation pathway. AML myeloblasts appear to express receptor complexes of differing binding affinities, with typical values of one hundred high affinity receptors and several thousand low affinity receptors per cell [Kelleher *et al*, 1988, Onnetto-Pothier *et al*, 1990]. Defects in the expression of the GM-CSFR β_C chain have been found in patients with pulmonary alveolar proteinosis (PAP), a heterogenous disorder associated with haemopoietic malignancy, and in the leukaemic blasts of AML patients with PAP symptoms. All patients failed to express normal levels of β_C chain protein while binding and proliferation assays showed striking reduced or absent function of the β_C chain [Dirksen *et al*, 1996abs]. However it has also been reported that the GM-CSFR is resistant to down-regulation in some patients with AML and that myeloblasts from these patients only express high affinity receptors, although to date no candidate mutations have been described [Cannistra *et al*, 1990].

Studies of the GM-CSFR α chain gene by restriction fragment length polymorphism analyses has shown that sequence of the receptor chain is not grossly rearranged in patients with AML [Bardy *et al*, 1992, Brown *et al*, 1993]. Polymerase chain reaction (PCR) based techniques have also been used to look for point mutations in the α chain gene. One study where the extracellular portion of the α chain was screened by direct sequencing, failed to find any abnormalities in 24 AML patients and three leukaemic cell lines [Decker *et al*, 1995]. Another study used single strand conformation polymorphism (SSCP) analysis to screen the entire coding sequence of the α chain, including the signal peptide, for mutations in 32 AML patients. Sequencing revealed four different base substitutions in the AML patients, two of which were conservative and did not alter the amino acid involved. One patient had an alteration located in the signal peptide of the receptor which involved a C to G base substitution and altered the alanine residue at amino acid 17 to glycine. However as the same alteration could be seen in a normal control and also in two of the patients kin, it was thought to be a constitutive polymorphism. A second base substitution changed an arginine residue into a glutamine at amino acid 164 in a region of the receptor not highly conserved in the cytokine receptor family. As the substitution was again found in a normal family member it was thought to be another polymorphism [Wagner *et al*, 1994]. So although nucleotide substitutions are quite common in the α chain sequence they do not appear to affect receptor function and are unlikely to contribute to the pathogenesis of AML.

1.9 - Aims

In this study I have examined the coding sequence of the GM-CSFR for mutations in patients with leukaemia and also looked at GM-CSF receptor complex formation. In Chapter 3 the intracytoplasmic tail and transmembrane regions of the GM-CSFR β_c chain were screened for mutations in RNA from patients with acute myeloid leukaemia (AML) using single strand conformation polymorphim analysis (SSCP). In Chapter 4 the entire coding sequence of the GM-CSFR α and β chains were examined for mutations in patients with juvenile chronic myeloid leukaemia (JCML), again using SSCP analysis. Antibodies specific to an alternatively spliced isoform of the GM-CSFR β_c chain were used in Chapter 5 to show expression of the isoform in primary haemopoietic cells and to investigate GM-CSFR stoichiometry.

CHAPTER 2

Materials and Methods

2. General Materials and Reagents

0.5, 1.5 ml polypropylene tubes	Eppendorf, Germany
25 ml universal tubes	Sterilin, Feltham, UK
50 ml polypropylene tubes	Falcon, Becton Dickinson, Oxford
1ml pipettes	LMG Smith, UK
5, 10, 25 ml pipettes	Sterilin, Feltham, UK
Tissue culture dishes	Greiner, Germany
Tissue culture flasks	Falcon, Becton Dickson, Oxford
Pipette tips	Gilson Anachem, Luton

10 x TBE Running Buffer (pH 8.3)	1L
Tris	108.9g
Orthoboric acid	55.7g
EDTA, disodium salt	7.4g

2.A General Molecular Biology methods

2.A.1 Agarose gels

Materials

Agarose	Sigma, Poole, UK
Low melting point Agarose	Gibco-BRL, UK
1 X TBE buffer	

Method

1 or 2% gels were regularly used to check PCR products for size and purity, while low melting point agarose gels were used to separate PCR products for direct sequencing and mutation specific digests. To the appropriate amount of agarose, 30ml of 1 x TBE was added, heated in a microwave until dissolved and allowed to cool before the addition of 3µl of ethidium bromide. The gel was poured into the mould and allowed to set. The PCR products were loaded and the gel electrophoresed in 1 x TBE before being visualised under UV light. Polaroid photographs were taken for permanent record.

2.A.2 Density Gradient separation of Cells

Materials

Ficoll-Hypaque	Pharmacia Biotech, Uppsala, Sweden
Phosphate buffered saline tablets	Sigma, Poole, UK
Dextran 10%	Pharmacia Biotech, Uppsala, Sweden
50 ml syringe	Terumo, Belgium

Method

10% dextran was added to whole blood to a final concentration of 1% before being mixed and left to sediment for 20 minutes. 10 mls of the non sedimented plasma was then gently layered onto 15mls of Ficoll in a 50ml Falcon tube and centrifuged at 800G for 20 minutes at room temperature. AML blasts and mononuclear cells are located at the interface between the Ficoll and plasma while neutrophils and red cells are pelleted at the bottom of the tube. Following removal of excess plasma and Ficoll, the required cells were pipetted out and transferred to a clean falcon tube. Cells were washed twice in sterile PBS with 5mM glucose added and centrifuged at 1200rpm for 5 minutes at room temperature each time before final resuspension in sterile PBS.

2.A.3 Preparation of RNA

In order to minimise the possible effects of RNAases, all materials used for RNA preparation were either sterile disposables or had been treated with diethyl pyrocarbonate (DEPC). DEPC is an RNAase inhibitor, and all solutions were prepared in glassware treated by immersion in DEPC (0.1% in water) at 37°C for 2 hours, rinsed several times in DEPC treated water and then autoclaved.

Materials

Guanidium thiocyanate	Fluka, Switzerland
Acrodisc filter 0.45 micron	Sigma, Poole, UK
Polypropylene Ultracentrifuge tubes	Beckman, UK
Syringes and 23 G needles	Terumo, Belgium

GTC

Guanidium thiocyanate	118 g
0.75M Na Citrate pH 7	8.35 ml
20% Na-lauryl-sarcosine	6.25 ml

made up to 250ml with DEPC treated water then filtered through a 0.45 micron acrodisc filter

1.8ml β Mercaptoethanol was added fresh before use

Caesium chloride ($CsCl_2$)

Caesium chloride	240 g
0.5M EDTA pH 7	5 ml

made up to 250ml with DEPC treated water and adjusted to a final density of 1.69g

Sodium acetate

2M Na acetate pH 8	1.5 ml
10% SDS	0.1ml

made up to 10ml with DEPC treated water

Method

Neutrophils, mononuclear cells and leukaemic blast cells were all separated by standard density gradient centrifugation. 50 x 10^6 purified cells were lysed in 3ml GTC. DNA present in the samples lowers total RNA yield so the sample was passed through a syringe with a 23G needle to shear the DNA. The GTC lysed sample was then carefully layered on top of 5ml $CsCl_2$ in a ultracentrifuge tube and centrifuged at 27000 rpm for 22 hours at 20°C. The supernatant was drained off and the bottom of each centrifuge tube, which contains the RNA pellet, was sliced off using a hot scalpel. 200µl of NaAc/ethanol solution (300µl NaAc in 800µl ethanol) was added to the pellet and left to precipitate for a few minutes. The precipitate was transferred to a 1.5ml eppendorf tube containing a further 0.9ml Na Ac/ethanol and left to precipitate at 4°C overnight. The sample was then microfuged at 13000rpm for 20 minutes at 4°C before discarding the supernatant. The pellet was washed in 70% ethanol before being resuspended in 50-1000µl sterile water.

To measure the total RNA concentration and gauge its purity the optical density of each sample was measured at 260nm and 280nm. An OD at 260nm of 1 is equivalent to an RNA concentration of 40ug/ml. The 260/280 ratio gives an indication of the sample purity with a ratio of greater than 1.8 indicating good RNA purity. The RNA samples were stored at -80°C.

2.A.4 Reverse Transcription - Polymerase Chain Reaction (RT-PCR)

Materials

10 x Reaction Buffer	Promega, UK
(1 x buffer - KCl 50 mM, Tris-HCl 10 mM, gelatin 0.01%, Triton 0.01%)	
Magnesium Chloride (25mM)	Promega, UK
Deoxynucleotides (10 mM dNTPs)	Promega, UK
RNAse inhibitor (40,000 U/ml)	Promega, UK
AMV reverse transcriptase (7,500 U/ml)	Promega, UK
Taq DNA Polymerase (500 U/ml)	Promega, UK
Oligo dT Primers (500µg/ml)	Promega, UK
PCR Primers (200ng/µl)	Oswell DNA, UK
Thermocycler	Hybaid, UK
Mineral Oil	Sigma, Poole,UK
α^{32} P-dCTP (6000 Ci/mmol, 10mCi/ml)	Amersham, UK

Reverse Transcription Reaction mix (20µl total)

	µl	Final concentrations
Sterile water	6.5	
$MgCl_2$	3	5.25 mM
10 x Buffer	2	1 x buffer
dNTPs	2	1mM
Oligo dT	0.5	250 ng
RNAse inhibitor	0.5	20 U
AMV RT	0.5	3.75 U
RNA template (1µg)	5	

Method

All pipetting was done on ice. A mastermix of all common reagents was made to ensure identical concentrations per tube and added to the total cellular RNA template. The tubes were incubated at 42°C for 60 minutes to allow reverse transcription of the oligo dT primed mRNA by the reverse transcriptase. The reaction was then heated to 99°C for 5 minutes to denature the RT enzyme which interferes with the PCR reaction. The reaction mix containing the cDNA was then stored at 4°C or used immediately.

Polymerase Chain Reaction mix (20µl total)

	µl	Final concentrations
Sterile water	13.9	

10 x Buffer	1.6	1 x buffer
3' Primer	0.2	80 ng
5' Primer	0.2	80 ng
Taq polymerase	0.1	0.5 U
cDNA template	4	

Method

On ice, a mastermix of reagents (except Taq polymerase) was added to microfuge tubes containing the cDNA. The reaction mix was then covered with a layer of mineral oil. To increase specificity and reduce the occurrence of spurious bands a hot start PCR protocol was used. In the thermocycler the tubes were heated to 95°C for 5 minutes before the temperature was lowered and held at 85°C. After the addition of Taq to each tube thirty amplification cycles were performed, each consisting of 95°C for 30 seconds, 64°C for 30 seconds and 72°C for 30 seconds, with a final extension step at 72°C for 5 minutes. PCR products were checked by electrophoresis through an ethidium bromide agarose gel, visualised by UV light.

2.A.5 Single Strand Conformation Polymorphism Analysis (SSCP)

Materials

40 % Acrylamide/Bis (37.5/1) stock solution	Biorad, Hemel Hempstead, UK
Glycerol	BDH, UK
Ammonium persulphate (APS)	Biorad, Hemel Hempstead, UK
TEMED	Biorad, Hemel Hempstead, UK
3MM Chromatography paper	Whatman , UK
Hyperfilm-MP	Amersham, UK
Denaturing solution	
0.1% SDS 10mM EDTA	

6% Non-denaturing Polyacrylamide Gel

10 xTBE	10 ml
40% Acrylamide/bis	15 ml
+/- Glycerol	10 ml
Made up to 100ml with DDW and polymerised with	
10% APS	1 ml
TEMED	100 µl

Denaturing solution

Formamide	95%
EDTA	20mM
Bromophenol blue	0.005%
Xylene cyanol	0.005%

Method

RT-PCR was performed as described above with the addition of 0.2µl ^{32}P-dCTP (6000 Ci/mmol Amersham, UK) to each PCR reaction. For each sample, 2µl of the radioactive PCR product was added to a mixture consisting of 10µl 0.1% SDS, 10mM EDTA and 11µl denaturing solution. Samples were heated to 95°C for 5 minutes to give single stranded DNA and then immediately placed on ice before loading 5µl on non-denaturing polyacrylamide gels with or without 10% glycerol. To maximise the probability of detecting mutations three different gel electrophoresis conditions were used,

(A) 10% glycerol run at room temperature, 17mA for 17 hours

(B) no glycerol, 4°C, 10mA for 16 hours

(C) no glycerol, room temperature, 15W for 5 hours.

After electrophoresis gels were dried on chromatography paper and exposed overnight at room temperature to Hyperfilm-MP.

2.A.6 Direct Sequencing of PCR products

Materials

Wizard™ PCR preps	Promega, UK
Urea	Sigma, Poole, UK
40% Acrylamide/bis (19:1) stock solution	Biorad, Hemel Hempstead, UK
Sequenase Version 2.0 sequencing kit	USB, USA
α ^{32}P-dCTP (6000 Ci/mmol, 10mCi/ml)	Amersham, UK

6% Denaturing Acrylamide Gel (0.5 x TBE)

Urea	46 g
10 x TBE	5 ml
40% Acrylamide/bis	15 ml

Made up to 100ml with DDW and polymerised with

10% APS	1 ml
TEMED	100 µl

Method

Non-radioactive PCR products were electrophoresed through a 1% low melting point agarose gel. The required band was excised and extracted using Wizard™ PCR preps before sequencing with a modified dideoxy chain termination method of the Sequenase version 2.0 sequencing kit.

Approximately 0.5µg of purified PCR product was used for each sequencing reaction. Four 0.5ml Eppendorf tubes were labelled G, A, T, C and 2.5µl of the appropriate dideoxy-termination mix added to each. A sequencing reaction was prepared for each template as below -

Template DNA	x µl (0.5µg)
5 x labelling mix	3 µl
primer (20ng/µl)	2.5 µl
α ^{32}P-dCTP	0.5 µl
made up to 13 µl with sterile water	

The reaction was heated at 99°C for 5minutes, then slowly cooled to 30°C. 2µl of Sequenase enzyme (diluted 1:8 in enzyme dilution buffer) was added and the reaction incubated at 30°C for 3 minutes. The tubes containing the dideoxy mix were heated to 37°C in the thermocycler and 3.3µl of the sequencing reaction added to each, and incubated at 37°C for 4 minutes before the addition of 4.2µl stop solution.

The completed sequencing reactions were boiled at 80°C for 3 minutes then electrophoresed on a 6% denaturing acrylamide gel at 1200V for 2 hours. The gel was then transferred to chromatography paper and suction dried for 50 minutes before being exposed overnight to Hyperfilm.

Alkali denaturation of a plasmid for Sequenase sequencing

Materials

Sepharose CL-4B

NaOH 1M, EDTA 1mM

Glass wool

Method

A hole was punched in the bottom of a 1.5ml Eppendorf tube and a small pearl of glass wool inserted to close the hole. Following the addition of 400ul of Sepharose CL-4B (2:1 in Tris-EDTA) the tube was inserted into another and centrifuged at 2000rpm for 1 minute to remove the liquid.

To 4ug of plasmid in a total of 22.5µl sterile water, 6µl of NaOh was added, mixed and left at room temperature for 5 minutes. The Sepharose column was placed in a new 1.5ml tube

and the alkali-plasmid solution was pipetted on top. The Sepharose column was then spun at 2000rpm for 2 minutes. The eluate was placed on ice immediately and 7µl used for each sequencing reaction as described above.

2.A.7 Cloning of PCR products

Materials

TA Cloning Kit	Invitrogen, USA
LB medium	Bio 101, California, USA
Bacto-Agar	Gibco, Paisley, UK
Ampicillin	Gibco, Paisley, UK
X- Gal	Sigma, Poole, UK
Mini-preps	Promega, USA

Method

PCR products were cloned using the TA cloning system which makes use of the single deoxyadenosine (A) overhang found at the 3' end of PCR products. The PCR products were ligated into a vector with deoxythymidine overhangs at the insertion site and then transformed into competent bacteria by heatshock. The cells were grown on LB-Agar plates with ampicillin and X-Gal. Transformed bacteria were selected by β-galactosidase activity (white colonies), picked and bulked up in LB medium. After extraction of the DNA by mini-prep, SSCP analysis was performed to select clones containing the abnormality by comparison with the patient SSCP pattern on a non-denaturing gel. The selected clones were then alkali denatured in a spun column and sequenced as described above.

2.A.8 Mutation specific enzyme digests

Method

PCR products were electrophoresed through low melting point agarose gels, the expected band excised and then cleaned with a Wizard™ PCR prep. 10µl of the purified PCR product was then digested overnight at 37°C with 10 units of the appropriate restriction enzyme in manufacturers buffer (Boehringer Mannheim, UK). Samples were electrophoresed through 1 or 2% agarose and bands visualised by ethidium bromide staining.

2.B Cell Culture and General Protein methods

2.B.1 Cell Culture

Materials

RPMI 1640 + L-Glutamine	Gibco, Paisley, UK
Dulbeccos modified Eagles medium (DMEM)	Gibco, Paisley, UK
Fetal Calf serum (FCS)	Gibco, Paisley, UK
Trypsin/EDTA	Gibco, Paisley, UK
Penicillin/Streptomycin (pen/strep)	Gibco, Paisley, UK

Growth Factors

Recombinant human GM-CSF (rhGM-CSF) produced in E.Coli with a specific activity of 5 x 10^7 units/mg protein donated by Behringwerke, Germany

Cell lines

Cell line	Tissue of origin	Reference
TF-1	erythroleukaemia	Kitamura *et al* 1989
COS-7	kidney- African Green monkey SV-40 transformed	Gluzman et al, 1981
HL-60	acute promyelocytic leukaemia	Collins *et al* 1977

TF-1 cells were maintained in suspension in RPMI 1640 medium with 10% FCS (heat inactivated at 55°C for 30 minutes) and 5ng/ml rhGM-CSF.

COS-7 cells were grown adhered to 750ml tissue culture flasks in DMEM with 10% FCS and 5µg/ml pen/strep at a constant 37°C and 5% CO_2.

2.B.2 COS-7 Transfections

Materials

Gene Pulsar™	Biorad, Hemel Hempstead, UK
Gene Pulsar cuvettes (0.4cm gap)	Biorad, Hemel Hempstead, UK
Sterile PBS	Gibco, Paisley, UK
Dulbeccos modified eagles medium	Gibco, Paisley, UK
Fetal Calf serum	Gibco, Paisley, UK
Penicillin/Streptomycin	Gibco, Paisley, UK

Method

5 x 10^6 COS-7 cells were suspended in 0.8ml PBS and placed in chilled cuvette. 10μg DNA was added and the cuvette vortexed gently and left for 15 minutes on ice. The cuvette was vortexed again before being electroporated at 500μF and 0.4KV. After cooling on ice for 15 minutes the transfected cells were pipetted into tissue culture dishes containing 22ml DMEM, 10%FCS and 5μg/ml pen/strep and grown for 72 hours at 37°C.

2.B.3 Cell lysates and Immunoprecipitations

Materials

NP-40	Sigma, Poole, UK
Phenylmethylsulfonylfluoride (PMSF)	Sigma, Poole, UK
Aprotinin	Sigma, Poole, UK
Leupeptin	Sigma, Poole, UK
Pepstatin	Sigma, Poole, UK
Protein A Sepharose	Sigma, Poole, UK

Lysis Buffer

137mM NaCl
20mM Tris pH 8
1mM $MgCl_2$
1mM $CaCl_2$
1% NP-40
10% Glycerol
1mM Na orthovanadate
1mM β glycerophosphate
1mM NaF
5mM pyrophosphate
2mM EDTA
1mM PMSF
Aprotinin 10μg/ml
Leupeptin 10μg/ml
Pepstatin 10μg/ml

Sample Buffer (8ml)

DDW	4.0 ml
0.5M Tris-HCl pH 6.8	1.0 ml

10% SDS	1.6 ml
Glycerol	0.8 ml
Dithiothreitol	0.1 g
0.05% Bromophenol blue	0.2 ml

Cell lysates

Method

Transfected COS-7 cells were washed in PBS while still adhered to the tissue culture plates before being lysed using a cell scraper in 1ml of ice cold lysis buffer with protease inhibitors. The lysed cells (about 2 x 10^6) were transferred to a 1.5ml microfuge, left on ice for 30-60 minutes and then centrifuged at 13000 rpm for 10 minutes. The supernatant was transferred to a new tube with an equal volume of 2 x sample buffer was added and then boiled at 100°C for 10 minutes. Lysates were stored at -20°C.

Immunoprecipitation

Method

Samples were centrifuged at 1200 rpm for 5 minutes and then resuspended in 1ml of ice cold lysis buffer with protease inhibitors. Samples were transferred to ice and incubated in the cold room at 4°C for 30-60 minutes. All subsequent steps were performed on ice at 4°C. In a benchtop microfuge, samples were centrifuged at 13000 rpm for 10 minutes and the supernatant transferred to a fresh microfuge tube containing the antiserum of interest. After incubation for 4-14 hours with end over end rotation, 60µl of 50% Protein A Sepharose (in lysis buffer) was added and the tube rotated for a further 2-4 hours. The Sepharose was spun down and washed 3 times in 1ml of lysis buffer before being resuspended in 60µl of 2 x sample buffer and boiled at 100°C for 10 minutes. Samples were stored at -20°C.

2.B.5 Western blotting

Materials

30% Acrylamide/bis (37.5:1) stock solution	Biorad, Hemel Hempstead, UK
Ammonium persulphate (APS)	Biorad, Hemel Hempstead, UK
TEMED	Biorad, Hemel Hempstead, UK
Protein Molecular weight markers	Gibco, Paisley, UK
Nitro-cellulose membrane (Hybond-C extra)	Amersham, Bucks, UK
Bovine serum albumin (BSA)	Sigma, Poole, UK
Fat free milk	

5 x Running Buffer (pH 8.3)	1L
Tris base	15 g
Glycine	72 g
Sodium dodecyl sulphate (SDS)	5 g

Western Transfer Buffer	1L
Tris	3 g
Glycine	14.8 g
Methanol	200ml

Separating Gel (8%)	
DDW	4.71 ml
Tris-HCl 1.5M, pH 8.8	2.50 ml
Acrylamide/bis 30% solution	2.64 ml
10% SDS	100 µl
10% APS	100 µl
TEMED	10 µl

Stacking Gel (4%)	
DDW	3.05 ml
Tris-HCl 0.5M, pH 6.8	1.25 ml
Acrylamide/bis 30% solution	0.65 ml
10%SDS	50 µl
10% APS	50 µl
TEMED	5 µl

Method

The gel apparatus (Mini-Protean II, Biorad, UK) was set up according to manufacturers instructions and the separating gel with polymerizing agents added poured in. An overlay of water saturated butanol was added and the gel left to polymerize for 30 minutes. The butanol was removed and the gel washed with distilled water. The stacking gel was then poured in, a 10 well comb inserted and left for 30 minutes. After the gel had set the comb was removed and the wells gently washed out with running buffer to remove unpolymerised acrylamide. Samples were loaded and after electrophoresis (typically 130V for 2 hours) the gel was transferred to nitro-cellulose membrane (Hybond-C extra) using transfer apparatus (Milliblot, Millipore, USA) at 0.45mA for 50 minutes. The membrane was then placed in 10% fat free milk, 3% BSA in PBS at 4°C overnight to block non specific binding sites.

Exact methods for the probing of Western blots varied depending on the antibodies used and more precise details for each experiment are given in Chapter 5.

Chapter 3

SSCP Analysis of the Intracytoplasmic domain of the GM-CSFR β_C chain in patients with Acute Myeloid Leukaemia

3.1 - Introduction

Acute myeloid leukaemia (AML) is a clonal disorder characterised by discordant proliferation and differentiation of primitive myeloid cells leading to excessive accumulation of malignant blast cells. AML incorporates a range of heterogeneous leukaemias distinguishable by the stage of maturation at which the cells become arrested. The acute leukaemias can be assigned to subgroups based on morphological and cytochemical characteristics, known as the FAB (French-American-British) classification system [Bennett *et al*, 1985]. The majority of AML patients are either adults or infants younger than 1 year, with a sharp increase in incidence of AML in adults over 50 years of age. Common features of the disease include bone marrow failure, bleeding, bruising, fatigue and thrombocytopenia. Laboratory findings characteristic of AML include anaemia, nucleated red cells in the peripheral blood, monocytosis, neutropenia, dysplastic neutrophils, decreased platelets and the presence of blast cells in peripheral blood, while the bone marrow is typically hypercellular with greater than 30% blasts. It is estimated that approximately two thirds of AML patients have detectable nonrandom cytogenetic abnormalities with the most common being trisomy 8, monosomy 7, deletion of the long arm of chromosome 7 (7q-), monosomy 5 and deletion of the long arm of chromosome 5 (5q-) [McKenzie *et al*, 1994].

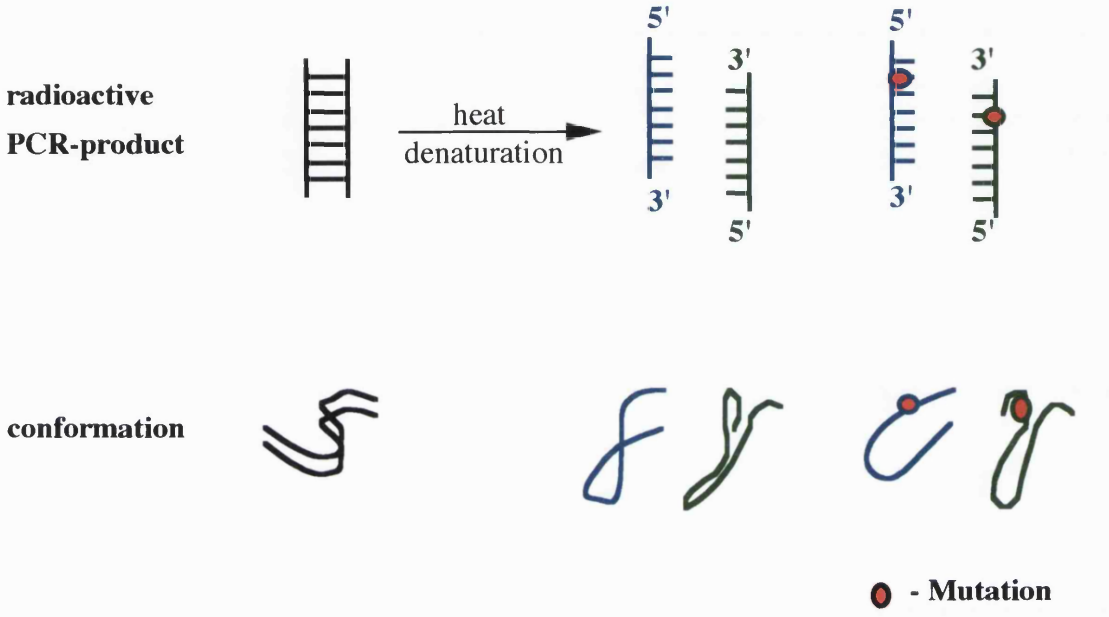
A number of mutant cytokine receptors have been isolated due to their ability to induce ligand-independent growth in factor dependent cell lines while point mutations which lead to constitutively activated receptors have been demonstrated for the several cytokine receptors including the EpoR, TpoR and GM-CSFR β_c chain [Yoshimiura *et al*, 1990, Jenkins *et al*, 19 95, Onishi *et al*, 1996]. The possible involvement of cytokine receptor mutations as one of the hits in the multi-step pathogenesis of leukaemia was reviewed earlier in Chapter 1 (section 1.3) and although to date no activating mutations of the GM-CSFR have been described in patients with AML, the receptor has been shown to be resistant to downregulation in some cases [Cannistra *et al*, 1990].

An earlier study reported four different base substitutions when the coding sequence of the α chain of the GM-CSFR was analysed in patients with AML although all were thought to be polymorphisms and were not expected to contribute to the pathogenesis of the disease [Wagner *et al*, 1994]. As the entire coding sequence of the β_c chain is over 2.5 kilobases long, in the present study RNA from 35 AML patients and a series of haematologically normal controls was screened for mutations in the transmembrane region and intracytoplasmic tail of the β_c receptor chain. This portion of the β_c was screened as extensive work has established that several domains of the β_c are essential for the initiation of various signalling pathways with vital roles in the proliferation and differentiation of haemopoietic cells [Sakamaki *et al*, 1992, Sato *et al*, 1993]. The

intracytoplasmic tail of the β_C chain includes the highly conserved Box 1 and Box 2 domains and studies using truncated mutants have shown the membrane proximal region of the β_C chain is essential for activation of the JAK/STAT pathway while a membrane-distal region was shown to be responsible for activation of the Ras pathway [Sato *et al*, 1993, Quelle *et al*, 1994] The transmembrane domain and highly conserved WSXWS box were also included in the area of the β_C chain studied.

To detect sequence changes in the intracytoplasmic tail of the GM-CSFR a technique known as Single Strand Conformation Polymorphism (SSCP) was used. SSCP was used as it is relatively rapid way of screening large areas of sequence for mutations and is sensitive enough to detect single nucleotide changes. The area of sequence covered in the present study was just over 1500 bp and with a total of 45 samples initially screened, direct sequencing of the entire region for each sample was not feasible. SSCP relies on the fact that under non-denaturing conditions single stranded DNA has a folded conformation that is stabilised by intrastrand interactions and consequently the conformation adopted by the DNA is sequence-dependent. Changes in the nucleotide sequence alter the conformation of the single stranded DNA and such changes can be detected by the use of gel electrophoresis (Figure 3.1) [Orita *et al*, 1989]. The conformation of single stranded DNA is thought to be determined by the balance between thermal fluctuation and weak local stabilising forces such as short intrastrand base pairings and base stackings. Therefore the electrophoretic mobility of single stranded DNA in polyacrylamide gels is strongly dependent on environmental conditions. Factors which influence the mobility shift of single strands include temperature, ionic strength and gel composition including the addition of glycerol. It is therefore essential to optimise electrophoretic conditions in order to achieve maximum sensitivity and in this study three different gel conditions were used to increase the chance of detecting mutations [Glavac *et al*, 1994]. One of the most important factors affecting the detection of mobility shifts in SSCP gels is the size of the DNA fragment involved. Previous studies using SSCP to look for mutations in the CFTR gene in Cystic Fibrosis, Ras genes and the factor IX gene in haemophilia, have determined that the optimum size of PCR product is between 200 and 400 base pairs (bp) [Hayashi *et al*, 1991, Glavac *et al*, 1994]. Therefore all primer pairs used in this and subsequent SSCP studies were designed to give PCR products of about 300 bp (Table 3.1).

Figure 3.1 - Schematic diagram of RT-PCR-SSCP analysis



Heat denaturation of the PCR product creates 2 single strands which migrate differently in a non-denaturing polyacrylamide gel according to their conformation. Mutations are detected by altered mobilities.

3.2 - Materials and Methods

3.2.1 - Patients

Bone marrow or peripheral blood was obtained from 35 patients with AML aged from 18 to 85 years old (Table 3.2). The 14 female and 21 male patients were classified according to the FAB (French-American-British) group criteria: M1:5 patients, M2:6, M3:3, M4:13, M5:2, M6:1 and 5 unknown. Abnormal karyotypes were found in 13 patients, but none involved chromosome 22q13.1 where the β_c chain gene is located [Takai *et al*, 1994]. Twelve of the AML patients had normal karyotypes while 10 were of unknown karyotype. Ten haematologically normal individuals were also studied in detail, either bone marrow samples from seven allogeneic bone marrow transplant donors or purified neutrophils from peripheral blood of three laboratory staff. Limited analysis was also carried out on 30 additional peripheral blood samples, all from haematologically normal women attending the antenatal clinic.

3.2.2 - Sample preparation

Leukaemic blasts were separated from patient samples (either peripheral blood or bone marrow) by density gradient centrifugation using Ficoll-Hypaque. Purified cells were lysed in GTC and RNA extracted by ceasium chloride ultracentifugation as described in Chapter 2 (section 2.4). In SSCP analysis RNA extracted from the haemopoietic cell line TF-1 was used as a control [Kitamura *et al*, 1991].

3.2.3 - Reverse Transcription - Polymerase Chain Reaction (RT-PCR)

Total cellular RNA (1μg) was used in a 20μl reverse transcription reaction incubated at 42°C for 60 minutes and then 99°C for 5 minutes. To confirm the RT had worked, 4μl of the RT was used for a PCR of 30 cycles consisting of denaturation at 95°C for 30 seconds, primer annealing at 64°C for 30 seconds and extension at 72°C for 30 seconds. PCR products were checked by agarose gel electrophoresis.

The sequence analysed covered nucleotides 1281-2816 of the published β_c chain sequence which included the WSXWS box, the transmembrane domain and the intracytoplasmic tail (Figure 3.2) [Hayashida *et al*, 1990]. The seven pairs of overlapping PCR primers were designed to produce PCR products of approximately 300 bps in length (Table 3.1).

3.2.4 - Single Strand Conformation Polymorphism Analysis (SSCP)

For SSCP analysis RT-PCR was performed as described in Chapter 2 (section 2.4) with the addition of 0.2μl ^{32}P-dCTP (6000 Ci/mmol, Amersham, UK) to each PCR reaction. 2μl of each radioactive PCR product was added to 21μl of SSCP solution

Table 3.1 - PCR Primers used to screen intracytoplasmic tail of β_C chain

Fragment No.	5' Primer (5' -> 3')	3' Primer (5' -> 3')	Nucleotides Amplified [a]	Fragment Size (bp)
1	CTCGCTCCGGAGTAAACCTTC	GCTGCCCCTTGGCTTTGCAGA	2527 -> 2816	290
2	CAGGCCCTGTGAAGTCAGGGT	GAGCTGAATGACGGGCACCTG	2295 -> 2647	353
3	CCTGTGGCTATACCCATGAGC	CAGGACAGGGCTTTTGGCCTC	2102 -> 2410	309
4	AGCCAGAAGTCCCCACCTCCA	ACTAGGGAGACAGACGAGGCC	1877 -> 2220	344
5	CTGACACGACTCCAGCTGCCT	TGGCTCGGCCTTCTCTCCACT	1683 -> 2007	325
6	CAGAACGGGAGCGCAGAGCTT	CAGGTAGGGCCCATTGAAGTC	1490 -> 1810	321
7	GCACCGGCTACAACGGGATCT	TCCCCGAATCCTACAGGGAAC	1281 -> 1620	340

[a] Numbered according to sequence published by Hayashida *et al*, 1990

Table 3.2 - Patient details

Patient	Sex	Age	Source	FAB Type
1	M	19	PB	6
2	F	59	PB	4
3	M	41	PB	3
4	M	UN	PB	UN
5	M	21	BM	UN
6	F	50	PB	1
7	F	56	PB	4
8	M	58	PB	4
9	M	73	PB	UN
10	M	46	PB	4
11	F	62	PB	4
12	M	44	PB	4
13	F	57	BM	UN
14	M	48	PB	4
15	M	UN	PB	UN
16	M	55	BM	2
17	M	29	BM	2
18	F	53	BM	UN
19	M	70	BM	1
20	F	67	PB	4
21	M	58	PB	UN
22	M	64	PB	4
23	F	23	PB	3
24	F	52	BM	4
25	F	27	PB	4
26	F	72	PB	1
27	M	18	BM	2
28	M	69	PB	2
29	F	50	PB	2
30	F	50	PB	4
31	M	78	PB	1
32	M	40	BM	2
33	M	61	PB	5
34	F	85	PB	UN
35	M	60	PB	5

F - female
M - male
BM - bone marrow
PB - peripheral blood
UN - unknown

(11µl denaturation solution and 10µl 0.1% SDS, 10mM EDTA) before being heated for 5 minutes at 95°C. 5µl of the denatured samples were loaded on 6% non-denaturing polyacrylamide gels and run under three different gel electrophoresis conditions optimized for maximum band separation in these PCR fragments.

(A) 10% glycerol run at room temperature, 17mA for 17 hours

(B) no glycerol, 4°C, 10mA for 16 hours

(C) no glycerol, room temperature, 15W for 5 hours.

3.2.5 - Sequencing of samples abnormal in SSCP analysis

Where abnormal SSCP patterns were detected non-radioactive PCR products were agarose gel purified then directly sequenced using a modified dideoxy chain termination method (section 2.5). If this failed to reveal any abnormalities the relevant PCR fragment was then cloned into the PCRII plasmid of the TA Cloning Kit (Invitrogen, USA). Clones containing the abnormality were selected by comparision of their SSCP patterns with that of the patient before being denatured and sequenced, as described in Chapter 2 (section 2.6)

3.2.6 - Confirmation of Substitutions

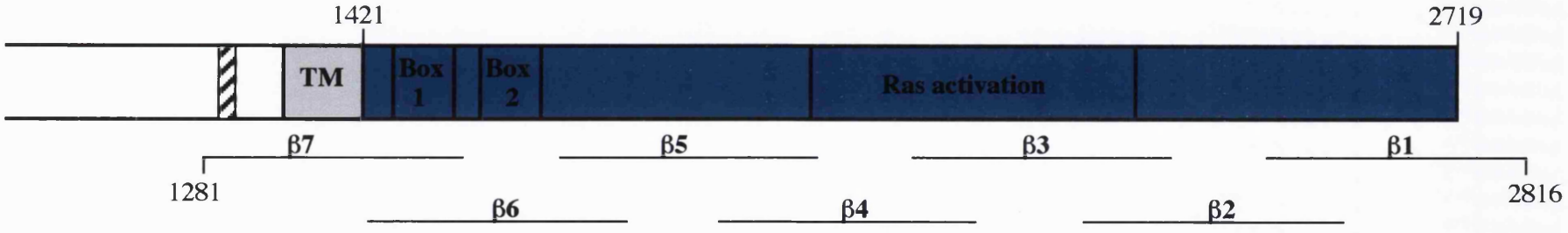
If a substitution destroyed or created a cutting site for a restriction enzyme, a gel-purified PCR product from the patient was digested with the appropriate enzyme and compared to a digested wild type product by agarose gel electrophoresis (section 2.A.9). If no suitable mutation specific digest could be found, three independent gel-purified PCR products were sequenced to confirm the abnormalities presence.

3.3 - Results

3.3.1 - SSCP analysis of AML patients and haematologically normal controls

RT-PCR-SSCP analysis was carried out on RNA from 35 AML patients using seven primer sets covering the transmembrane domain and intracytoplasmic tail of the GM-CSFR β_c chain and the patterns were compared with those from TF-1 cell RNA. Twenty five patients (71%) were found to have variant SSCP patterns in one or more fragments. The differences detected were not evenly distributed along the chain (Table 3.3). No abnormal patterns were found in fragments 1 (nucleotides 2527-2816), 3 (2102-2410) and 6 (1490-1810). Abnormal SSCP bands were detected in two patients (6%) in fragment 2 (nucleotides 2295-2647), nine patients (26%) in fragment 7 (1281-1620) which includes the transmembrane region, 11 patients (31%) in fragment 4 (1877-2220) and 14 patients (41%) in fragment 5 (1683-2007). Ten haematologically

Figure 3.2 - Position of PCR primers used for SSCP analysis of β_C chain intracytoplasmic tail and transmembrane region



TM - transmembrane region
Box 1 - box 1 containing region
Box 2 - box 2 containing region
Ras activation - Ras activation region
- WSXWS box
- intracytoplasmic region

1281 - nucleotide numbering according to Hayashida et al, 1991

Table 3.3 -Results of SSCP analysis in AML patients

Patient No.	β_C chain PCR fragment						
	β1	β2	β3	β4	β5	β6	β7
1	N	N	N	Abnorm	Abnorm	N	N
2	N	N	N	N	N	N	Abnorm
3	N	N	N	N	N	N	Abnorm
4	N	N	N	Abnorm	Abnorm	N	N
5	N	N	N	N	N	N	N
6	N	N	N	N	N	N	N
7	N	N	N	Abnorm	Abnorm	N	N
8	N	N	N	Abnorm	Abnorm	N	N
9	N	N	N	N	Abnorm	N	N
10	N	Abnorm	N	N	N	N	N
11	N	N	N	N	N	N	Abnorm
12	N	N	N	Abnorm	Abnorm	N	N
13	N	N	N	N	N	N	N
14	N	N	N	N	N	N	Abnorm
15	N	N	N	N	N	N	N
16	N	N	N	N	N	N	N
17	N	N	N	N	N	N	Abnorm
18	N	N	N	N	N	N	Abnorm
19	N	N	N	N	N	N	N
20	N	N	N	N	N	N	N
21	N	N	N	Abnorm	Abnorm	N	N
22	N	N	N	N	N	N	Abnorm
23	N	N	N	Abnorm	Abnorm	N	Abnorm
24	N	N	N	N	N	N	N
25	N	N	N	N	N	N	Abnorm
26	N	N	N	Abnorm	Abnorm	N	N
27	N	N	N	N	N	N	N
28	N	N	N	N	N	N	N
29	N	N	N	N	Abnorm	N	N
30	N	N	N	N	Abnorm	N	N
31	N	Abnorm	N	Abnorm	Abnorm	N	N
32	N	N	N	N	N	N	Abnorm
33	N	N	N	Abnorm	Abnorm	N	N
34	N	N	N	Abnorm	Abnorm	N	N
35	N	N	N	N	N	N	N

N - normal SSCP pattern

Abnorm - abnormal SSCP pattern

normal individuals were also screened. As before no abnormal patterns were similarly observed in fragments 1, 3 or 6. One individual had a different pattern in fragment 2 and two individuals in fragment 7. In fragments 4 and 5, seven individuals had variant SSCP patterns for both fragments.

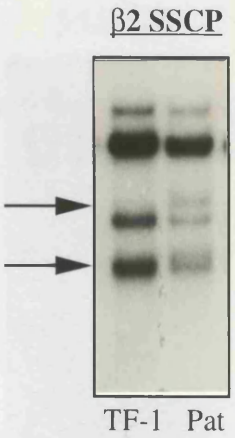
Fragment 2 (nucleotides 2295-2647)

SSCP analysis showed that two of the 35 AML patients had extra bands in fragment 2 in gel condition C (Figure 3.3). Direct sequencing of PCR products showed they were both heterozygous for a G->A substitution at nucleotide position 2427. The presence of this substitution could be confirmed by restriction enzyme digestion of a fragment 2 PCR product as it destroys a cutting site for the restriction enzyme Nci I (CC|C/GGG). Digestion of the wild type 353bp PCR product with Nci I produced six fragments of 130, 90, 79, 35, 12 and 7bps. The substitution removed the cutting site between the 130 and 79bp fragments and a new fragment of 209bp was observed (Figure 3.3). This heterozygous substitution was also found in one normal control and does not alter the proline residue at amino acid position 800.

Fragments 4 and 5 (nucleotides 1683-2220)

Eleven patients had SSCP patterns in fragment 4 (nucleotides 1877-2220) that differed from the TF-1 control. Seven of these gave the same divergent pattern under electrophoretic condition C, with extra bands present in addition to the normal bands and a double band visible in the region where the double stranded DNA migrates (Figure 3.4). The same seven patients all had an extra band in SSCP conditions A and B in the overlapping fragment 5 (nucleotides 1683-2007). Direct sequencing of PCR products showed these patients were heterozygous for a G->A substitution at nucleotide position 1972, which, as expected, lies in the overlapping region between these fragments. This alteration does not change the proline residue at position 648 in the amino acid sequence. The presence of the substitution was confirmed by restriction enzyme digestion of fragment 4 (or 5) as it destroys a cutting site for the enzyme Msp I (C|CGG). In the wild type 344bp fragment 4 there are two Msp 1 sites and digestion results in three fragments of 184, 93 and 67bps. The G->A substitution led to a new fragment of 160 bp (93 + 67bp) (Figure 3.4). Msp I digestion of the seven AML patients with this SSCP pattern showed all had the three wild type fragments and the extra 160bp fragment, confirming they were heterozygous for this substitution. Another two patients had abnormal SSCP patterns in fragments 4 and 5 similar to those seen in the other seven patients but these patients only had one band in the double stranded region of fragment 4 and lacked some of the normal single stranded bands in fragments 4 and 5 (Figure 3.4). Sequencing of these patients showed they were

Figure 3.3 - SSCP and mutation specific digest of fragment β2 abnormality (nt 2295-2647)

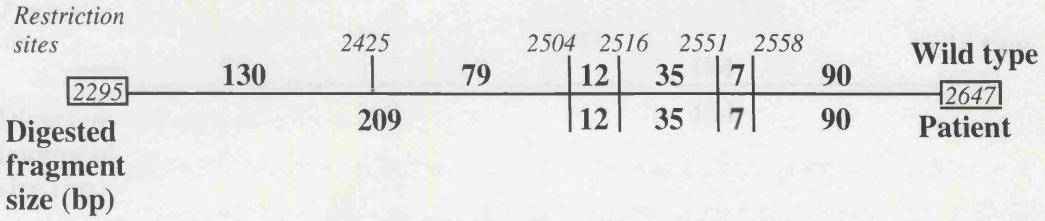


Substitution

G_{2427} -> A

unchanged Pro_{800}

Nci 1 (CC|C/GGG) digest of β2 PCR products



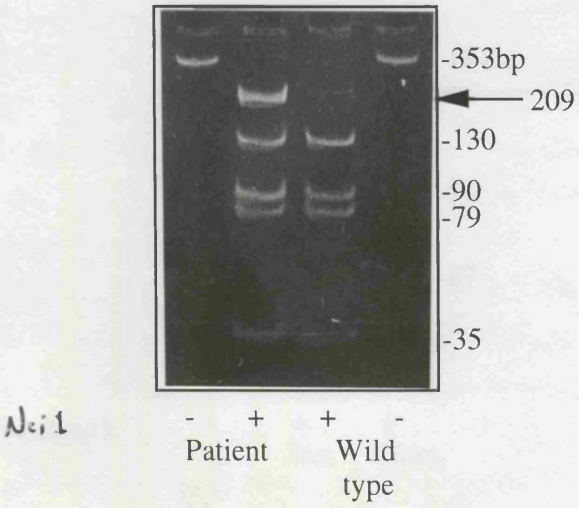
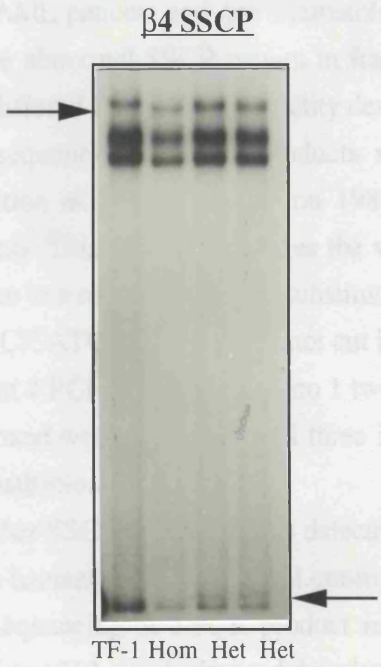


Figure 3.4 - SSCP and mutation specific digest of β4/5 abnormality (nt 1683-2220)

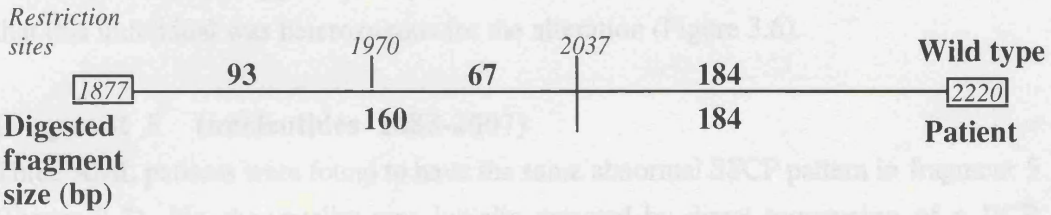


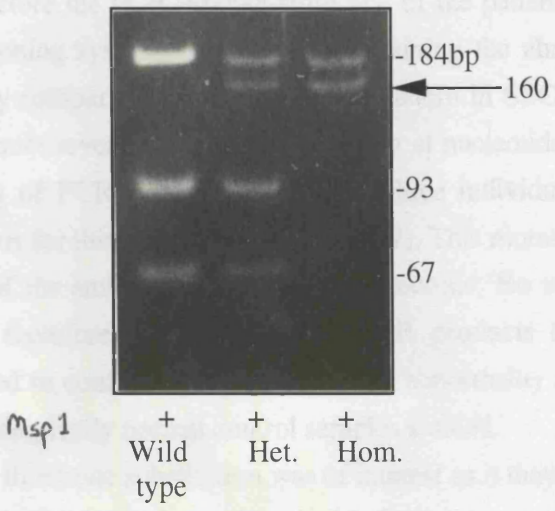
Mutation

G_{1972} -> A

unchanged Pro_{648}

Msp 1 (ClCGG) Digest of β4 PCR products





homozygous for the G->A substitution and this was confirmed by Msp I digestion of fragment 4 which produced only the 184 and 160bp fragments (Figure 3.4). Further SSCP analysis and restriction digests showed that seven of the 10 haematologically normal controls were also heterozygous for this substitution.

Two AML patients and one haematologically normal individual were found to have the same abnormal SSCP pattern in fragments 4 and 5 in electrophoretic condition A, which differed from the abnormality described above for these fragments (Figure 3.5). Direct sequencing of PCR products showed they were heterozygous for a G->A substitution at nucleotide position 1982 in the overlapping region between the two fragments. This mutation changes the valine residue at position 652 in the amino acid sequence to a methionine. The substitution introduced a restriction site for the enzyme Nco 1 (ClCATGG) which does not cut in the wild type fragment 4. After digestion of a fragment 4 PCR product with Nco 1 two extra bands of 104 and 240bp plus the uncut 344bp band were observed in all three individuals, confirming their heterozygosity for this substitution (Figure 3.5)

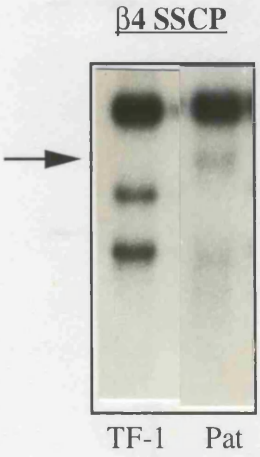
A further SSCP difference was detected in the overlapping region of fragments 4 and 5 in one haematologically normal control in all electrophoretic conditions (Figure 3.6). Direct sequencing of a PCR product revealed the presence of a G->T substitution at nucleotide 1968 which changed the glycine at position 647 in the amino acid sequence to a valine. The alteration created a new restriction site for the enzyme Kpn 1 (GGTAClC), and digestion of a fragment 4 PCR product from this individual showed the uncut 344bp wild type fragment and two extra bands of 94 and 250bp, confirming that this individual was heterozygous for the alteration (Figure 3.6).

Fragment 5 (nucleotides 1683-2007)

Three AML patients were found to have the same abnormal SSCP pattern in fragment 5 (Figure 3.7). No abnormality was initially detected by direct sequencing of a PCR product and therefore the PCR product from one of the patients was cloned using the Invitrogen TA cloning system. Four clones containing the abnormality were selected for sequencing by comparison with the patients' pattern in SSCP analysis. Sequencing of the selected clones revealed a C->A substitution at nucleotide 1835, and subsequent direct sequencing of PCR products from these three individuals confirmed that they were heterozygous for this substitution (Figure 3.7). This mutation changed the proline at position 603 of the amino acid sequence to threonine. No suitable restriction digest was found and therefore three independent PCR products from each patient were directly sequenced to confirm the alteration. This abnormality was not detected in any of the 10 haematologically normal control samples studied.

This proline -> threonine substitution was of interest as it may alter the function of the receptor chain. Firstly, loss of a proline could change the conformational shape of the

Figure 3.5 - SSCP and mutation specific digest of abnormality in fragment β4/5 (nt 1683-2220)

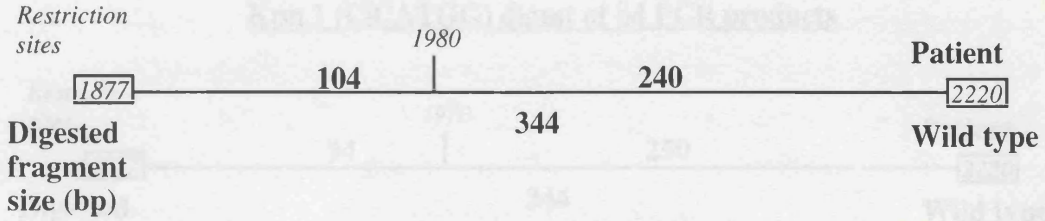


Substitution

G_{1982} -> A

val_{652} -> met

Nco 1 (C|CATGG) digest of β4 PCR products



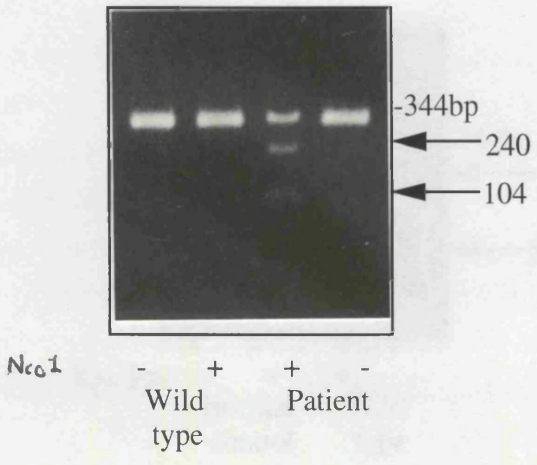
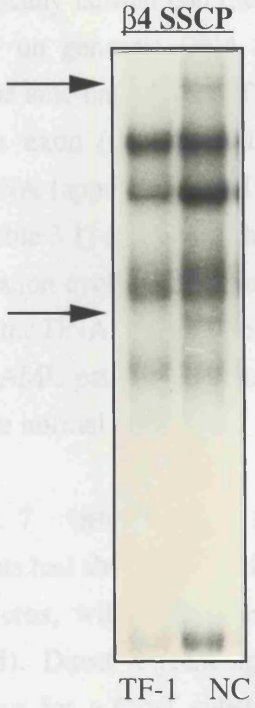


Figure 3.6 - SSCP and mutation specific digest of abnormality in fragment β4/5 (nt1877-2220)

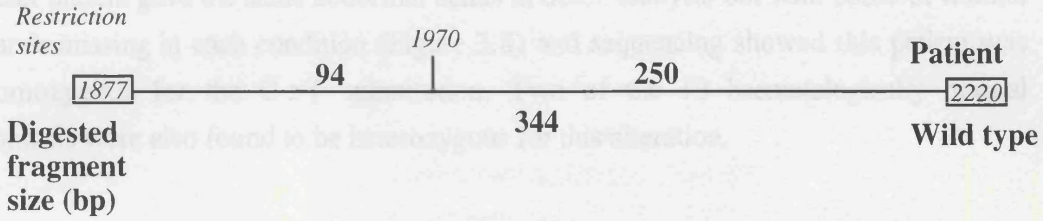


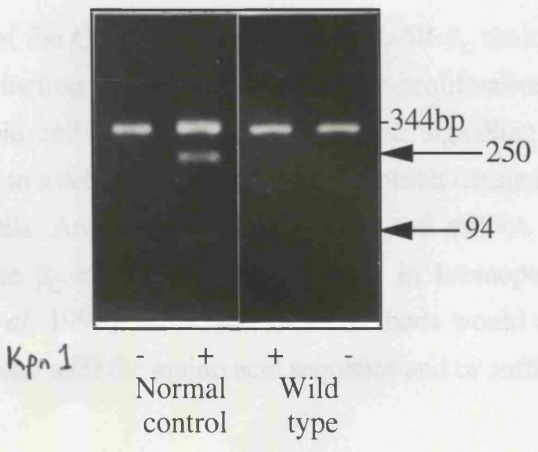
Substitution

G_{1968} -> T

Gly_{647} - > Val

Kpn 1 (C|CATGG) digest of β4 PCR products





protein in this region and secondly, the introduced threonine may potentially act as a phosphorylation site. The three patients carrying the substitution were all dead and family studies were not possible. In order to exclude the possibility that this substitution was a constitutive polymorphism that had not occurred in any of the haematologically normal controls studied, we therefore carried out SSCP analysis of fragment 5 on genomic DNA from a further 30 normal controls (all individuals attending the ante-natal clinic). The sequence covered by this fragment occurs within a single large exon (unpublished observation) and therefore it was possible to use genomic DNA (approximately 1μg) as the starting template with the same fragment 5 primers (Table 3.1) and obtain the expected 325bp fragment (Figure 3.7). The number of amplification cycles was reduced to 25 to decrease non-specific bands due to mis-priming of the DNA. Five of the 30 individuals had the same abnormal SSCP pattern as seen in the AML patients and the identical heterozygous substitution was confirmed in two of these normal controls by direct sequencing of PCR products.

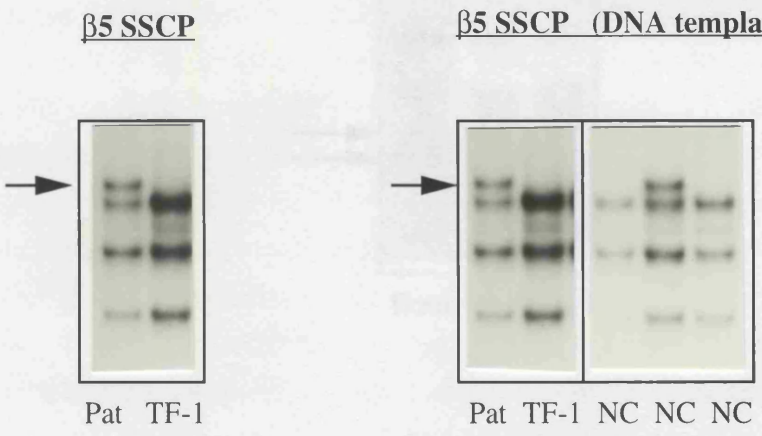
Fragment 7 (nucleotides 1281-1620)

Nine patients had abnormalities in fragment 7, of which eight gave the same abnormal SSCP patterns, with extra bands visible under all three electrophoretic conditions (Figure 3.8). Direct sequencing of a PCR product showed the eight patients were heterozygous for a C->T substitution at nucleotide position 1306. This substitution does not alter the serine residue at position 426 in the amino acid sequence. No suitable restriction enzyme digest was found to verify the alteration so three separate PCR products from each individual were sequenced as confirmation of its presence. One other patient gave the same abnormal bands in SSCP analysis but with some of normal bands missing in each condition (Figure 3.8) and sequencing showed this patient was homozygous for the C->T substitution. Two of the 10 haematologically normal controls were also found to be heterozygous for this alteration.

3.4 - Discussion

The intracytoplasmic tail of the GM-CSFR, IL-3R and IL-5R β_c chain is an essential component of signal transduction pathways involved in the proliferation, differentiation and functioning of myeloid cells. Any disruption of these signalling pathways may confer a growth advantage to a cell or alternatively might inhibit terminal differentiation, as is seen in leukaemic cells. Analysis of genomic DNA and mRNA has shown that gross rearrangement of the β_c chain gene is infrequent in haemopoietic neoplasms including AML [Brown *et al*, 1993]. However, these methods would not detect single nucleotide changes which can alter the amino acid sequence and be sufficient to result in

Figure 3.7 - SSCP and sequencing of fragment β5 abnormality (nt 1683-2007)



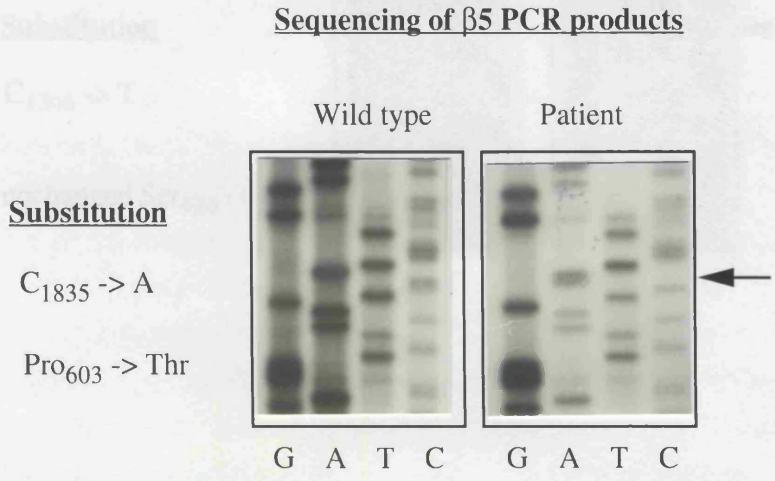
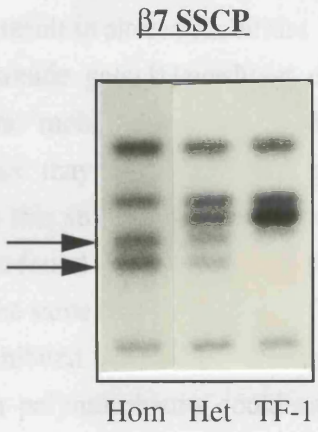
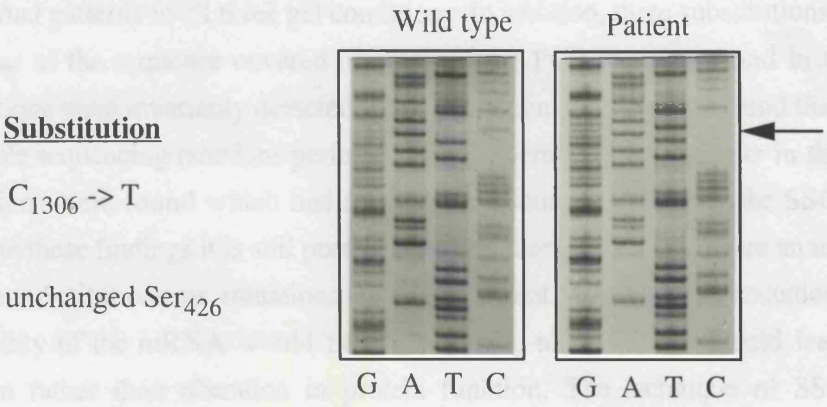


Figure 3.8 - SSCP and Sequencing of fragment β7 abnormality (nt 1111-1620)





a protein with abnormal function. Therefore RNA from blast cells of 35 AML patients was screened for point mutations in the membrane spanning region and intracytoplasmic tail of the β_c chain, using SSCP analysis.

SSCP analysis exploits the fact that nucleotide mutations change the conformation of single stranded DNA and result in altered mobilities of heat-denatured PCR products in non-denaturing polyacrylamide gels [Hayashi *et al*, 1991]. The effect of sequence changes on electrophoretic mobility is unpredictable and it is possible that some alterations to the sequence may not appreciably affect the mobility. However the mobility shifts detected in this study were consistent for each substitution, with repeat analysis of patient samples found to be abnormal giving the same altered pattern in each experiment. Also, when the same abnormality was found in more than one patient, the patient samples always exhibited the same variant SSCP pattern for that mutation, and in fact the more common polymorphisms could easily be recognised by their SSCP pattern alone. A potential disadvantage of the technique is that efficiency of mutation detection has been shown to vary from 35 to 98% when known mutations have been screened [Hayashi *et al*, 1991, Sarkar *et al*, 1992]. It is thought that the change in mobility of fragments in gel electrophoresis depends on both the nucleotide change involved and its position in the PCR fragment [Hayashi *et al*, 1991, Sarkar *et al*, 1992]. In order to maximise detection efficiency three different gel conditions were used, varying the temperature, glycerol concentration and electrophoretic parameters. However in practice, five of the six alterations detected in this study gave rise to abnormal patterns in all three gel conditions. In addition, three substitutions were found in areas of the sequence covered by overlapping PCR fragments and in all cases the alterations were invariably detected in both fragments. It was also found that despite the multiple sequencing reactions performed on numerous PCR products in this study, no mutations were found which had not been previously detected by the SSCP analysis. Despite these findings it is still possible that the alterations detected are an underestimate of the substitutions or mutations that are present. In addition, mutation leading to instability of the mRNA would not be detected, although this would lead to loss of protein rather than alteration in protein function. The technique of SSCP is quite sensitive and it has been shown using artificially created mixtures of mutant and wild type template, it is possible to detect as little as 6% mutant template in a sample [Wagner *et al*, 1994]. This was important in the current study of acquired alterations where leukaemia-specific clonal abnormalities may only be present in a proportion of the cells and alterations might only be present in one allele.

A total of five substitutions were detected in the intracytoplasmic tail and membrane-proximal region of the β_c chain in the AML patients. All the identified substitutions could be found when normal control samples were screened by SSCP and in addition another substitution, not previously seen, was discovered in one of the normal

controls. Three of the alterations were conservative and did not change the amino acid involved, a serine at position 426, a proline at position 648 and another proline at position 800 of the protein sequence. The other SSCP abnormalities resulted in three amino acid alterations of which the glycine -> valine substitution at position 647 in the protein was detected only in a normal control and is therefore unlikely to be of any pathological significance. A valine -> methionine substitution at position 652 found in two AML patients was of interest as the mouse AIC2A (mIL-3Rβ) and AIC2B ($m\beta_C$) beta chains, which are highly homologous to the β_C, both contain a methionine residue at this position in their amino acid sequence [Hayashida *et al*, 1990]. This together with the later discovery of the substitution in a normal control suggests that it is unlikely to be a pathogenic mutation.

Three of the 35 patients (9%) had a nucleotide change which resulted in the substitution of a proline residue at position 603 to a threonine and this alteration was not detected in the 10 haematologically normal samples initially analysed. This substitution was interesting due to a possible change in protein shape with the loss of a proline and also because the threonine introduced a potential new phosphorylation site. Therefore an additional 30 normal controls were screened for this alteration. The substitution was found in five individuals of the new normal controls giving an overall frequency 5/40 (13%) similar to that found in the AML patients, and again suggesting that this alteration was not directly involved in the pathogenesis of AML. These results suggest that all six substitutions detected probably represent polymorphisms at the nucleotide level and are not leukaemia-specific mutations.

The number and frequency of polymorphisms detected in this region of the β_C chain is striking, although perhaps not surprising as the human genome is thought to contain, on average, one polymorphism every few hundred base pairs [Orita *et al*, 1989]. The six different polymorphisms were identified within a 1121 bp region of the coding sequence with three substitutions clustered within 15 bp of each other at nucleotides 1968, 1972 and 1982. Similar clustering has been observed in the G-CSFR with at least four mutations occurring within a 46 bp area, all leading to a truncated receptor due to the substitution of a premature stop codon for the amino acid involved, interestingly a glutamine residue in each case [Dong *et al*, 1994]. Over 73% of samples analysed in this study had one or more of the polymorphisms and in particular two of the nucleotide substitutions appeared to be very common. The G -> A substitution at position 1972 of the nucleotide sequence had an allele frequency of 0.23 and the C -> T substitution at position 1306 had an allele frequency of 0.13, although neither alteration changed the amino acid sequence. A similar incidence of polymorphic sites has been demonstrated in the GM-CSFR α chain with five different substitutions identified in 1326 bp of the coding sequence, but allele frequencies were much lower than found here, with the most frequent substitution being detected in just two of the 47 individuals

studied [Wagner *et al*, 1994]. Other cytokine receptors do not apparently display such a high level of polymorphisms. For example, in a study of the EpoR coding sequence using SSCP analysis, no mutations were found in a total of 24 bone marrow and peripheral blood samples from patients with polycythaemia vera [Hess *et al*, 1994]. Similarly, although a number of truncated G-CSFR mutants have been reported in AML patients, either due to point mutations that substitute glutamine residues with stop codons or to splice variants [Dong *et al*, 1994, Dong *et al*, 1995], in a total of 110 individuals analysed by either SSCP or direct sequencing (11 normal controls, 87 patients with leukaemia and 12 with congenital neutropenia), only two substitutions that might represent polymorphisms were detected, each in a single patient [Guba *et al*, 1994, Sandoval *et al*, 1995, Bernard *et al*, 1995, Carapeti *et al*, 1995].

Four of the six base substitutions detected in the β_c chain involved either a C -> T or G -> A transition where the C or G is part of a CpG doublet. CpG doublets are usually found in areas known as CG islands which are often located around the promoter region of housekeeping genes. Methylation of the cytosine to 5-methylcytosine is thought to play a role in the regulation of transcription, and this is a key process in the control of development and differentiation [Bird *et al*, 1992]. These CG doublets are well known hotspots of mutation in the genome [Cooper *et al*, 1989] and in fact over one third of all point mutations giving rise to human genetic disease are caused by C to T transitions (or G -> A depending on which strand is involved) [Bird *et al*, 1992]. This is thought to arise from spontaneous deamination of methylated cytosine residues. Accidental deamination of cytosine to uracil will be recognised as a mismatch, excised and repaired back to the original sequence. However deamination of 5-methylcytosine produces thymidine which is indistinguishable from the normal thymidine and the sequence is therefore permanently altered. The β_c coding sequence is very G + C rich (63%), which may partially explain the high frequency of polymorphisms detected.

The intracytoplasmic tail of the β_c chain is therefore highly polymorphic but mutations of this region do not appear to contribute to the pathogenesis of AML. It is still possible that mutation in the extracellular domain of the receptor could be of functional significance and this warrants further study. A number of other genes or loci of interest have been mapped to this region of the genome, for example the neurofibromatosis 2 gene, which is inactivated in patients with the autosomal dominant disease neurofibromatosis type 2, and the chromosome breakpoint frequently associated with Ewing sarcoma and peripheral neuroepithelioma both map to the chromosome region 22q12 [Sanson *et al*, 1993, Sutherland *et al*, 1989]. The polymorphisms detected in the β_c chain may therefore be useful as a linkage marker for this region of the chromosome.

Chapter 4

SSCP Analysis of the GM-CSF receptor in patients with Juvenile Chronic Myeloid Leukaemia (JCML)

4.1 - Introduction

Juvenile chronic myelogenous leukaemia (JCML) is a rare myeloproliferative disorder in which 95% of cases are found in children less than four years old at the time of diagnosis, with a slight predominance of male patients [Pui *et al*, 1995, Busque *et al*, 1995, Hess *et al*, 1996]. The median survival from presentation is approximately ten months, with death usually resulting from infection, organ failure or haemorrhage due to infiltration of monocytic cells into pulmonary or gastrointestinal tracts [Freedman *et al*, 1992, Busque *et al*, 1995]. JCML is an extremely aggressive disease in which conventional chemotherapy has not been shown to improve overall survival and allogeneic bone marrow transplant is currently the therapy of choice for JCML patients [Hess *et al*, 1996]. Cytogenetic studies are important in establishing the diagnosis of JCML, primarily to exclude the presence of the Philadelphia chromosome and although patients usually have a normal karyotype, a variety of cytogenetic abnormalities have been reported without a consistent pattern of abnormalities emerging. Raised levels of fetal haemoglobin are found in approximately 60% of JCML cases and other typical findings include prominent monocytosis, thrombocytopenia and moderate leukocytosis [Altman *et al*, 1974, Brodeur *et al*, 1979, Pui *et al*, 1995, Hess *et al*, 1996].

It has been shown *in vitro* that peripheral blood granulocyte-macrophage progenitors (CFU-GM) from patients with JCML are able to proliferate spontaneously at low cell densities in the absence of exogenous growth factors [Emanuel *et al*, 1991, Gaultieri *et al*, 1989]. However, this spontaneous proliferation can be abolished by prior depletion of the adherent layer (monocytes) from the culture suggesting that proliferation depends on growth factors produced endogenously [Gaultieri *et al*, 1988]. Several growth factors have been implicated in the regulation of this proliferation in JCML, with the addition of granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin 1 (IL-1) or tumour necrosis factor alpha (TNFα) to JCML cultures sufficient to stimulate colony proliferation *in vitro* [Gaultieri *et al*, 1989, Freedman *et al*, 1992, Attias *et al*, 1995]. Conversely, the use of antagonists to these factors is able to inhibit colony growth [Gaultieri *et al*, 1989, Freedman *et al*, 1992, Schiro *et al*, 1994, Attias *et al*, 1995].

The exact mechanism of GM-CSF, IL-1 or TNFα action is unclear, but it would seem that in the case of GM-CSF and TNFα both factors act directly to stimulate the JCML colonies [Freedman *et al*, 1992, Gaultieri *et al*, 1989]. IL-1 stimulation appears to act by directly stimulating colony growth whilst also stimulating the production of other cytokines from accessory cells [Attias *et al*, 1995, Schiro *et al*, 1994]. Although elevated secretion of IL-1 and GM-CSF from monocytes has been suggested in some cases of JCML based on *in vitro* proliferation assays, it is not a consistent finding and therefore does not offer a

plausible explanation for the spontaneous proliferation observed [Gaultieri *et al*, 1989]. *In vitro* studies have also shown that myeloid and erythroid progenitors from JCML patients demonstrate approximately a ten fold selective hypersensitivity to GM-CSF as measured by colony growth of CFU-GM and BFU-E, whereas responses to other cytokines such as granulocyte-colony stimulating factor (G-CSF) and interleukin-3 (IL-3) were identical to control dose-response curves [Emanuel *et al*, 1991].

Hypersensitivity to GM-CSF seems to be a consistent finding in JCML with all 38 patients tested in one study showing a hypersensitive response to GM-CSF, while neutralizing antibodies to GM-CSF were shown to inhibit colony growth in all cases [Emanuel *et al*, 1994]. There also appears to be no difference between progenitors isolated from peripheral blood and those isolated from bone marrow. Although the precise nature of this hypersensitivity is unclear, it does appear therefore to be an exaggeration of the normal response to GM-CSF [Emanuel *et al*, 1991] .

Molecular analysis of genes that undergo X-chromosome inactivation in female JCML patients indicate that JCML is a clonal disorder and arises from a cell as primitive as a myeloid stem cell and potentially from a pluripotent stem cell [Busque *et al*, 1995]. The persistence of fetal haemoglobin and the occasional spontaneous growth of CFU-E and BFU-E in the absence of Epo is further evidence for this stem cell derivation, as is the fact that CFU-GM and BFU-E both show selective hypersensitivity to GM-CSF [Freedman *et al*, 1988]. One case of JCML has been described in identical twins but this does not necessarily mean that there is a genetic predisposition to the disease, as the development of leukaemia in utero by one twin can lead the disease spreading to the other through their common circulation in the placenta. In the above case this theory is supported by the fact that while one twin showed an aggressive course the other remained stable without therapy [Castro-Malaspina *et al*, 1984].

It is not known whether the hypersensitivity to GM-CSF occurs at the receptor, cytoplasmic or nuclear signalling level, but fluorescence-labelled binding studies have shown apparently normal GM-CSFR numbers and affinity on JCML cells [Hess *et al*, 1996]. It has been hypothesised that mutations of the GM-CSFR may play a role in dysregulation of the GM-CSF pathway seen in JCML [Pui *et al*, 1995, Emanuel *et al*, 1991abs]. Hypersensitive responses of receptors to their ligands have been reported in both the G-CSFR and the EpoR. When a retrovirally created mutant EpoR lacking the C-terminal 42 amino acids was transfected into Ba/F3 cells, the cells were able to proliferate in 0.01 units/ml of Epo, one-tenth of the concentration required to support growth in cells expressing the wild type receptor [Yoshimura *et al*, 1990]. Similarly, in a Finnish family carrying a heterozygous mutation resulting in loss of the terminal 70 amino acids from the

receptor tail, increased numbers of erythroid colonies were obtained in vitro at low levels of Epo, <1 unit/ml, from affected family members [Juvonen *et al*, 1991, De La Chapelle *et al*, 1993]. A point mutation in the G-CSFR has been described in a patient with severe congenital neutropenia (SCN) which introduced a premature stop codon resulting in a receptor with truncation of 98 amino acids from the intracytoplasmic tail [Dong *et al*, 1994]. BAF3 cells transfected with this mutant receptor could grow maximally at one tenth of the concentration of G-CSF (0.1ng/ml) required for cells transfected with the wild type receptor [Dong *et al*, 1994]. These mutant receptors indicated that negative growth regulatory areas may be located in the C-terminal region of these cytokine receptors which when removed led to increased sensitivity to ligand concentration. Recent studies have demonstrated that this may result from loss of specific tyrosine residues within this region which when phosphorylated bind haemopoietic phosphatases, thought to be involved in the down regulation of signalling molecules such as JAK kinase. Loss of such a regulatory element may lead to increased signalling following ligand stimulation of the receptor and hence increased growth [Klingmuller *et al*, 1995].

The consistent hypersensitivity of JCML progenitors to GM-CSF suggests that an abnormal response to the cytokine is a significant factor in disease progression. As the first step in the signalling pathway the GM-CSFR is an obvious site for disruption of the normal growth signal. As previously described (chapter 1 section 1.5) the GM-CSFR consists of a ligand specific alpha chain (α) and a beta chain (β_C) which is shared with the IL-3R and IL-5R [Sakamaki *et al*, 1992]. In the present study RNA from six patients with JCML was analysed for mutations in the entire coding sequence of both α and β_C receptor chains. The patient samples were screened with the same RT-PCR-SSCP method as was used to examine the cytoplasmic and transmembrane region of AML patients in Chapter 3.

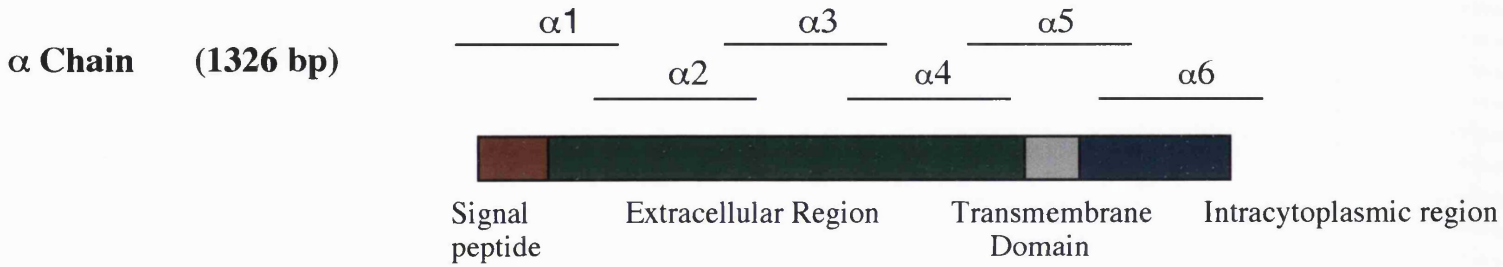
4.2 - Materials and Methods

4.2.1 - Patient samples

Bone marrow or peripheral blood was obtained from 6 patients with JCML. Mononuclear cells were separated by standard density gradient centrifugation (Nycomed, Norway). Total cellular RNA was prepared by lysis in GTC and ultracentrifugation in caesium chloride according to a standard protocol as described in sections 2.A.2 and 2.A.3. As a control, RNA was extracted from the haemopoietic cell line TF-1 [Kitamura *et al*, 1991].

4.2.2 - Reverse Transcription - Polymerase Chain Reaction (RT-PCR)

Figure 4.1 - Primers Sets for SSCP Analysis of GM-CSF Receptor



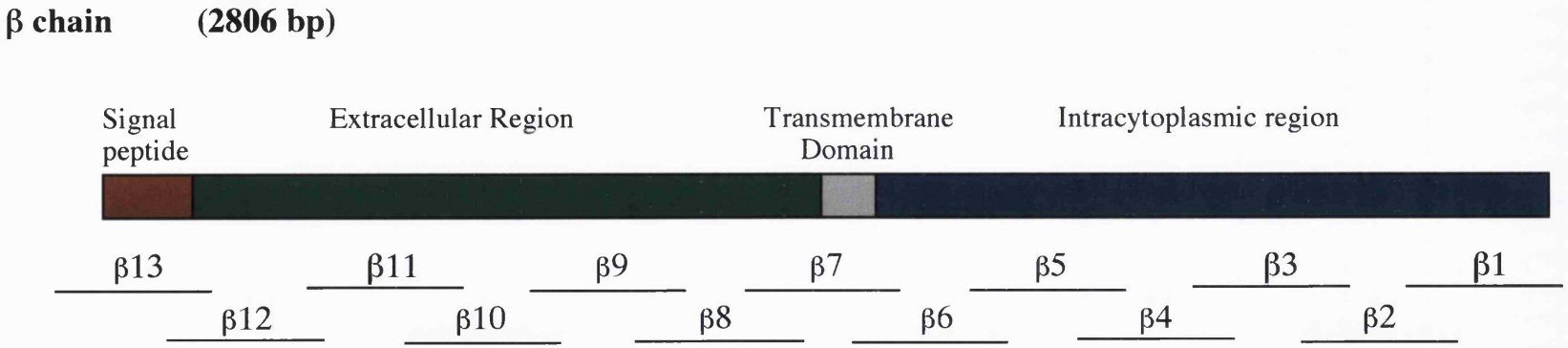


Table 4.1A **GM-CSF receptor α chain PCR Primers**

Fragment No.	5' Primer (5' -> 3')	3' Primer (5' -> 3')	Nucleotides Amplified [a]	Fragment Size (bp)
1	GTAGAACCCTGTACGTGCTT	GCACGAACATTCGTTGTTAC	90 - 392	303
2	TTAAGCTGGGACTGCCAAGA	AAAAATACTGGACGTCACGG	288 - 603	316
3	CTGCTCAGAATTTCTCCTGT	AGTGCGTCGTGTTGCAACGT	508 - 846	339
4	GCCGAGAAATTGGCATCCAA	CTGCAGCTCTGATCTTCACA	742 - 1047	306
5	CACGGAAAACCTACTGATTA	CTTTGATCTGTGGAACTGGC	944 - 1239	296
6	CATTTATGTGCTCCTAATCG	AGAAAACAGTTCCCCCGTGT	1127 - 1416	290

[a] Numbered according to sequence published by Gearing *et al*

Table 4.1B β chain PCR primers

Fragment No.	5' Primer (5' -> 3')	3' Primer (5' -> 3')	Nucleotides Amplified [a]	Fragment Size (bp)
1	CTCGCTCCGGAGTAAACCTTC	GCTGCCCCTTGGCTTTGCAGA	2527 -> 2816	290
2	CAGGCCCTGTGAAGTCAGGGT	GAGCTGAATGACGGGCACCTG	2295 -> 2647	353
3	CCTGTGGCTATACCCATGAGC	CAGGACAGGGCTTTTGGCCTC	2102 -> 2410	309
4	AGCCAGAAGTCCCCACCTCCA	ACTAGGGAGACAGACGAGGCC	1877 -> 2220	344
5	CTGACACGACTCCAGCTGCCT	TGGCTCGGCCTTCTCTCCACT	1683 -> 2007	325
6	CAGAACGGGAGCGCAGAGCTT	CAGGTAGGGCCCATTGAAGTC	1490 -> 1810	321
7	GCACCGGCTACAACGGGATCT	TCCCCGAATCCTACAGGGAAC	1281 -> 1620	340
8	CGTGACCAAGGATGGAGACAG	CAGGAGCACAGCAGTGGTGAG	1066 -> 1402	337
9	GGCCTATTCTACAAGCCCAGC	CTTGCTGTCCTTCCACGTGGC	851 -> 1189	339
10	TCTCCAACACCTCCCAGGCCA	AATCTGGCAGTGGTGCCTGGT	594 -> 952	359
11	TCACCGTCACTCTGACCCAGC	CTCCACTTGCTGGGACGTCCT	396 -> 717	322
12	GCTCGTCAACGTGACCCTCAT	GACTCCCAAGGGCCACACTC	193 -> 503	311
13	CCAGAGCTGACCAGGGAGAT	GGAATGACACATCTCCTGGGC	11 -> 324	314

[a] Numbered according to sequence published by Hayashida *et al*, 1990

As before, 1μg of total cellular RNA from each of the six patients was reverse transcribed using an oligo dT primer and the cDNA used as template in a PCR reaction to confirm the RT had worked. Figure 4.1 shows the position of the PCR fragments obtained and Table 4.1 gives a list of PCR primers and annealing temperatures used in order to cover the coding sequence of both receptor chains. PCR products of approximately 300 bps in length were used as this is optimal for the detection of abnormalities in the system used.

4.2.3. - Single Strand Conformation Polymorphism Analysis (SSCP)

Radioactive PCR products were obtained as described in section 2.A.4 and were screened by SSCP analysis on non-denaturing polyacrylamide gels. As before, to maximise the probability of detecting mutations three different gel electrophoresis conditions were used,

(A) 10% glycerol run at room temperature, 17mA for 17 hours

(B) no glycerol, 4°C, 10mA for 16 hours

(C) no glycerol, room temperature, 15W for 5 hours.

After electrophoresis gels were vacuum dried and exposed overnight at room temperature to Hyperfilm-MP (Amersham, UK).

4.2.4 - Direct Sequencing of PCR products

Non-radioactive PCR products were electrophoresed through low melting point agarose. The required band was excised and extracted using Wizard™ PCR preps before sequencing using a modified dideoxy chain termination method as described in section 2.A.6.

4.2.5 - Cloning of PCR products

In order to identify some of the SSCP abnormalities PCR products were directly cloned using the TA Cloning Kit (Invitrogen, USA). Clones containing the abnormality were selected using SSCP analysis by comparison with the patient SSCP pattern, alkali denatured and sequenced as above.

4.2.6 - Mutation specific enzyme digests

When possible the presence of mutations were confirmed by restriction enzyme digest. PCR products were purified through agarose gel electrophoresis then digested overnight at 37°C with 10 units of the appropriate restriction enzyme in manufacturers buffer. Samples were electrophoresed through 1 or 2% agarose and bands visualised by ethidium bromide staining.

4.3 - Results

4.3.1 - Overall SSCP results from GM-CSF receptor

In this study RNA from six patients with JCML was screened for mutations in the coding sequence of the GM-CSFR. Only one patient had normal SSCP patterns for the entire coding sequences of both receptor chains, a total of 19 PCR fragments (primer table). The other five patients showed at least one abnormal SSCP pattern for each receptor subunit when screened, with an overall total of 17 altered patterns seen (Table 4.2).

4.3.2 – α chain analysis

The entire coding sequence of the α chain was screened using six sets of overlapping primers numbered one to six, beginning at the 5' end with fragment one which includes the signal peptide (Figure 4.1). Five abnormal SSCP patterns were seen in the six patients, with one patient having an abnormal SSCP pattern in fragment one while four patients gave abnormal bands in fragment six when compared to the control pattern from TF-1 RNA.

Fragment α 1 (nt 90-392)

One patient gave an altered SSCP pattern in fragment α1 with additional downward shifted bands under all gel conditions (Figure 4.2). Direct sequencing of a gel purified PCR product failed to indicate the abnormality responsible so an α1 PCR product from the patient was cloned using the TA Cloning Kit (Invitrogen). Sequencing of several clones from this patient, selected by comparison with the patient's original SSCP analysis, revealed a C -> G base substitution at nucleotide position 199. The presence of this base substitution could be confirmed by enzyme digestion as it creates a new cutting site for the restriction enzyme Hinf 1 (GANT'C). Digestion of the 303bp α1 PCR fragment from this patient gave the expected wild type bands of 180, 82 and 41bp plus two additional bands of 109 and 71bp created by the new cutting site (Figure 4.2). The presence of the wild type bands plus the mutation created bands confirmed that the patient was heterozygous for the substitution. The C -> G substitution alters the codon at position 17 in the amino acid sequence and results in the alteration of the alanine residue at this position to a glycine.

Fragment α 6 (nt 1127-1416)

Four of the six JCML patients analysed gave similar abnormal SSCP patterns for fragment α6. Analysis revealed the presence of extra bands clearly visible under gel condition A, although one individual lacked one of the bands seen in the normal controls (Figure 4.3). Direct sequencing of gel purified PCR products showed that all patients had a G -> A

Table 4.2 - Summary of SSCP results for JCML patients.

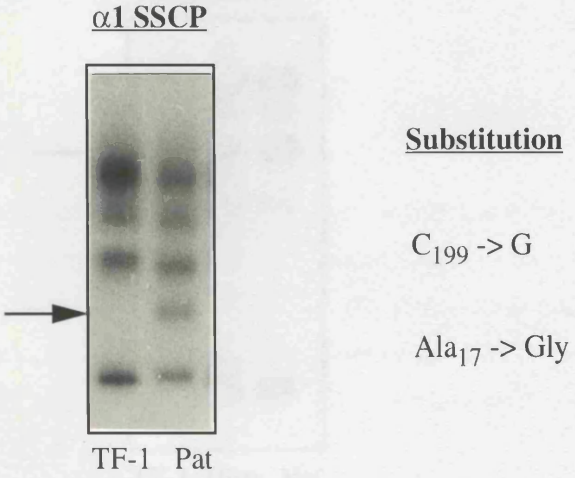
JCML Patient

	1	2	3	4	5	6
α chain fragments						
α1	N	N	N	Abnorm	N	N
α2	N	N	N	N	N	N
α3	N	N	N	N	N	N
α4	N	N	N	N	N	N
α5	N	N	N	N	N	N
α6	Abnorm	Abnorm	Abnorm	N	Abnorm	N
β chain fragments						
β1	N	N	N	N	N	N
β2	N	N	N	N	N	N
β3	N	N	N	N	N	N
β4	N	Abnorm	N	Abnorm	Abnorm	N
β5	Abnorm	Abnorm	N	Abnorm	Abnorm	N
β6	N	N	N	N	N	N
β7	N	N	Abnorm	N	Abnorm	N
β8	N	N	Abnorm	N	Abnorm	N
β9	N	N	N	N	N	N
β10	N	N	N	Abnorm	N	N
β11	N	N	N	N	N	N
β12	N	N	N	N	N	N
β13	N	N	N	N	N	N

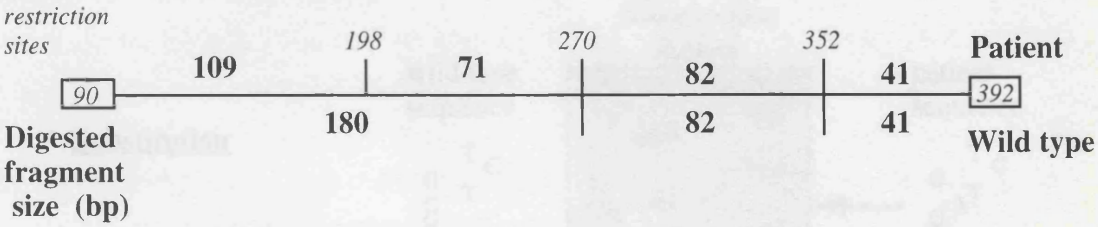
N - normal SSCP pattern compared to TF-1 control

Abnorm - abnormal SSCP pattern compared to TF-1 control

Figure 4.2 - SSCP abnormality in fragment α1 (nt 90-392)



Hinf 1 (GANT|C) digest of α1 PCR product



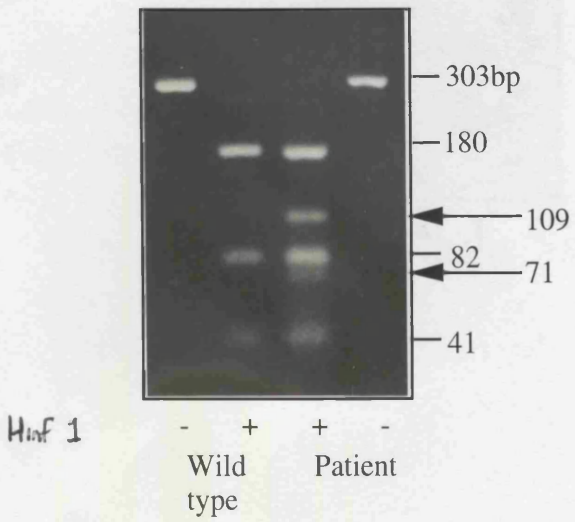
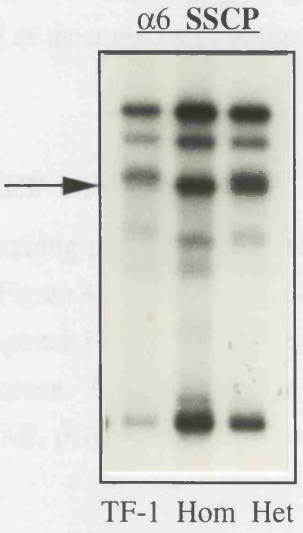
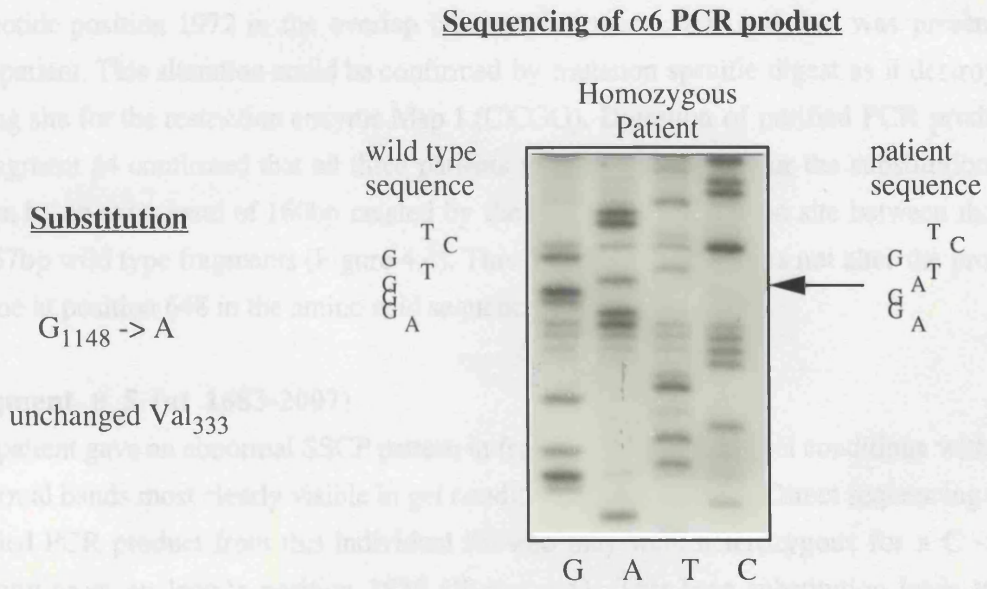


Figure 4.3 - SSCP abnormality in fragment α6 (nt 1127-1416)





substitution at nucleotide 1148 of the coding sequence (Figure 4.3). As was expected after analysis of the SSCP results, three of the patients were heterozygous for the substitution and the one patient which lacked a normal band in the SCCP proved to be homozygous for the abnormality. The G -> A substitution in the coding sequence does not alter the valine residue at position 333 of the amino acid sequence.

4.3.3 – β_C chain SSCP analysis

Thirteen sets of overlapping primers were used to study the entire β_C coding sequence an area of over 2800bp (Figure 4.1). The orientation of fragment numbering was the opposite of the α chain with fragment β1, which includes the termination codon, being at the 3' end of the β_C coding sequence. A total of twelve abnormal SSCP patterns were detected in analysis of the six JCML patients but four base substitutions accounted for all the variant SSCP patterns seen.

Fragments β 4 and β 5 (nt 1683-2220)

Three patients showed abnormal bands under all gel conditions in fragment β4, while the same three patients also displayed abnormal SSCP bands in fragment β5 (Figure 4.4). Direct sequencing of gel purified PCR products revealed that a G-> A substitution at nucleotide position 1972 in the overlap between fragments four and five was present in each patient. This alteration could be confirmed by mutation specific digest as it destroys a cutting site for the restriction enzyme Msp 1 (C'CGG). Digestion of purified PCR products of fragment β4 confirmed that all three patients were heterozygous for the substitution, as shown by an extra band of 160bp created by the loss of the restriction site between the 93 and 67bp wild type fragments (Figure 4.4). This base substitution does not alter the proline residue at position 648 in the amino acid sequence.

Fragment β 5 (nt 1683-2007)

One patient gave an abnormal SSCP pattern in fragment β5 under all gel conditions with the abnormal bands most clearly visible in gel condition A (Figure 4.5). Direct sequencing of a purified PCR product from this individual showed they were heterozygous for a C -> A substitution at nucleotide position 1835 (Figure 4.5). This base substitution leads to an alteration in the protein with a threonine residue replacing a proline at position 603 of the amino acid sequence.

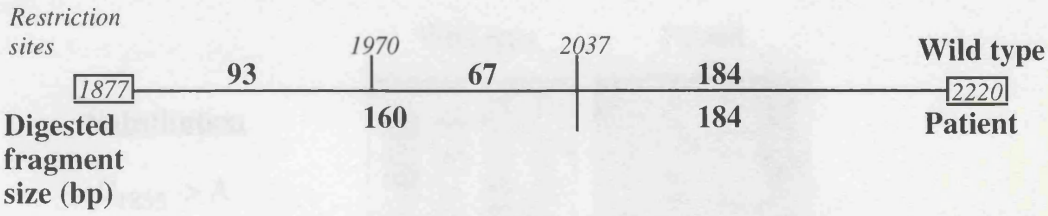
Figure 4.4 - SSCP and mutation specific digest of β4/5 abnormality (nt 1683-2220)

Substitution

G_{1972} -> A

unchanged Pro_{648}

Msp 1 (C|CGG) Digest of β4 PCR products



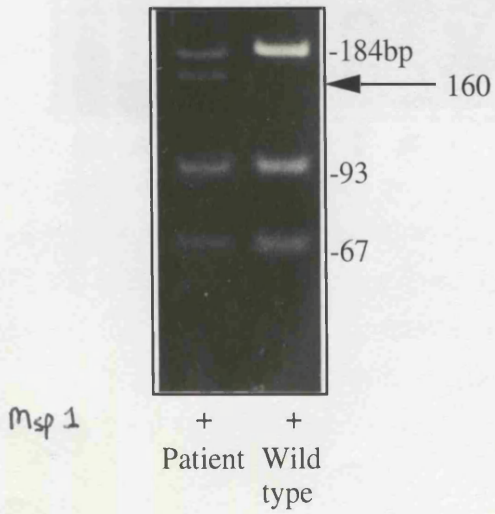
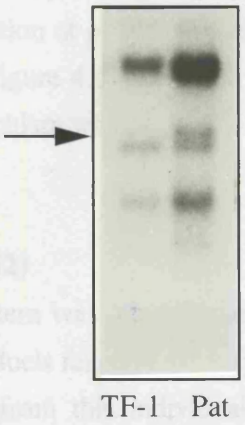


Figure 4.5 - SSCP and Sequencing of β5 abnormality (nt 1683-2007)



Fragment β 7 and β 8 (nt 1066-1620)

Two patients which gave variant SSCP patterns under all gel conditions in fragment β7 also had abnormal bands of the same mobility present in fragment β8 (Figure 4.6). Although both displayed abnormal bands, one individual lacked one of the wild type bands seen in the control TF-1 sample. Direct sequencing of gel purified PCR products showed that both patients had a C -> T substitution at position 1306 which as expected falls in the overlap between the two fragments (Figure 4.6). One individual was heterozygous and the other homozygous for this polymorphism which does not alter the serine residue at position 426 in the protein sequence.

Fragment β 10 (nt 594-952)

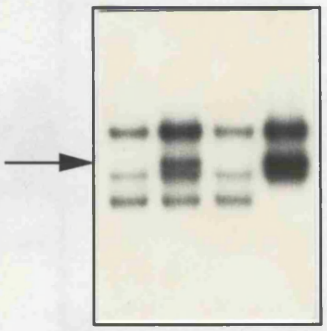
A further abnormal SSCP pattern was seen in one patient in fragment β10 (Figure 4.7). Direct sequencing of PCR products failed to identify an obvious nucleotide alteration in this fragment so a PCR product from this individual was cloned using the Invitrogen TA cloning kit. Clones containing the abnormality were selected by comparison with SSCP patterns from the original patient sample and a wild type control. Sequencing of the selected clones showed they had a G -> C substitution at nucleotide position 773. This alteration could be confirmed in the patient by restriction enzyme digestion as it creates a cutting site for the enzyme Pst 1 (CTGCA$^{|}$G). Digestion of a β10 PCR product indicated that the patient was heterozygous for the substitution as in addition to the wild type 359bp fragment there were two new fragments of 181 and 178bp, which appeared as one band on electrophoresis (Figure 4.7). The G -> C alteration changes glutamic acid at position 249 in the amino acid sequence to a glutamine.

4.4 - Discussion

Two characteristic features of JCML seen *in vitro* are the ability of haemopoietic progenitors to spontaneously proliferate at low cell densities in the absence of exogenous stimuli and a selective hypersensitivity to GM-CSF by these progenitor cells [Gaultieri *et al*, 1989, Emanuel *et al*, 1991]. The spontaneous proliferation is growth factor dependent while the hypersensitivity appears to be an exaggeration of the normal response to GM-CSF [Emanuel *et al*, 1991], suggesting that both features of the disease may be linked. Since formation of a receptor-ligand complex is the first step in the transduction of GM-CSF signalling and therefore an obvious point at which disruption to the normal growth factor response could occur, SSCP analysis was used to look for point mutations in both GM-CSFR chains in JCML patients. As previously reported by Wagner *et al*, 1994 and

Figure 4.6 - SSCP and Sequencing of β7/8 abnormality (nt 1066-1620)

β7 SSCP



TF-1 Het N Hom

Sequencing of β7 PCR product

Substitution

C_{1306} -> T

unchanged Ser_{426}

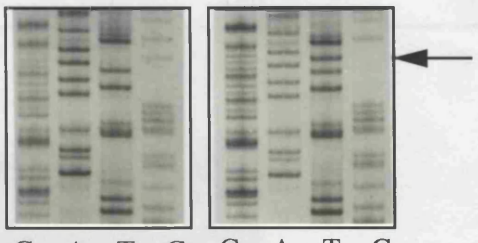
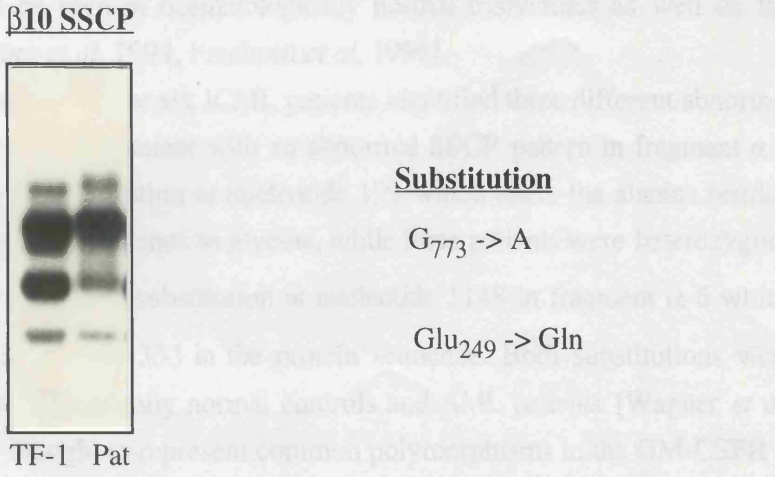
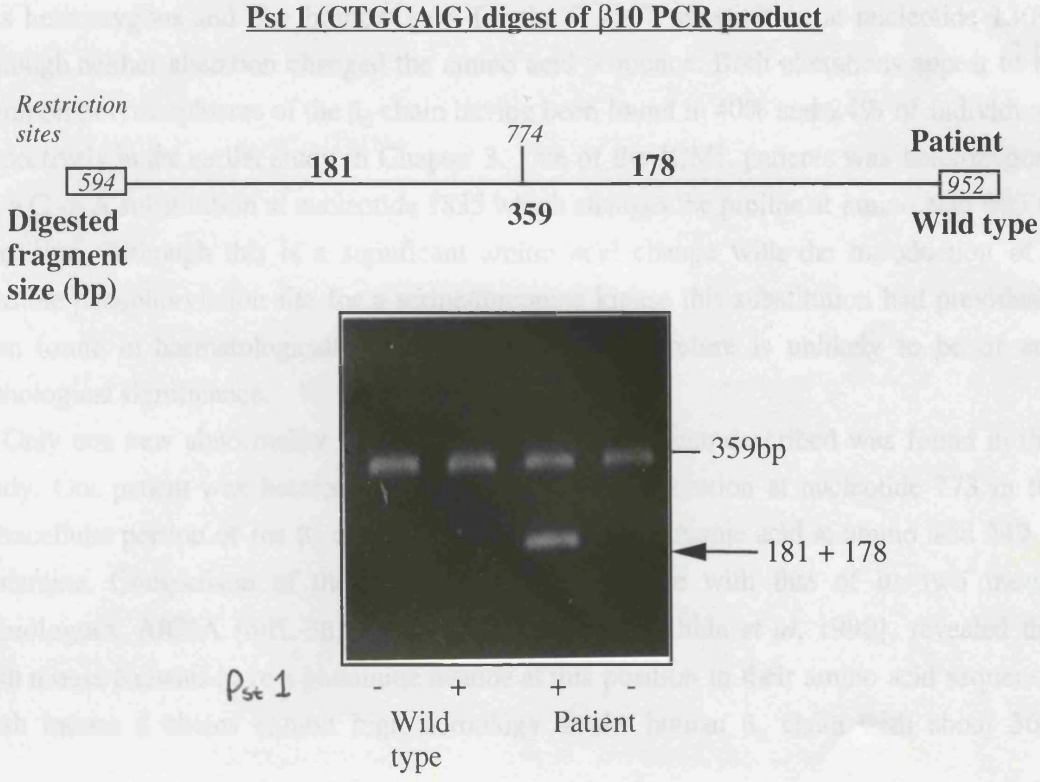


Figure 4.7 - SSCP and mutation specific digest in fragment β10 (nt 594-952)





earlier in chapter 3 this technique to be an effective method for identifying point mutations in the GM-CSFR α and β_c chains in two studies on AML patients, although all base changes detected were thought to be constitutive polymorphisms rather than pathogenic mutations, as they could be seen in haematologically normal individuals as well as the patients with AML [Wagner *et al*, 1994, Freeburn *et al*, 1996].

SSCP analysis of the α chain in the six JCML patients identified three different abnormal patterns in the six fragments. One patient with an abnormal SSCP pattern in fragment α 1 was found to have a C -> G substitution at nucleotide 199 which alters the alanine residue at position 17 in the amino acid sequence to glycine, while three patients were heterozygous and one homozygous for a G -> A substitution at nucleotide 1148 in fragment α 6 which does not alter the valine at position 333 in the protein sequence. Both substitutions were previously detected in haematologically normal controls and AML patients [Wagner *et al*, 1994] and were therefore thought to represent common polymorphisms in the GM-CSFR α chain.

Following SSCP analysis, sequencing revealed that four base substitutions accounted for all abnormalities seen in the β_c chain of the GM-CSFR. Three of these abnormalities had previously been found in the study of the intracytoplasmic tail of the β_c chain in haematologically normal controls and patients with AML described in Chapter 3. Three patients were heterozygous for the G -> A substitution at nucleotide 1972 whilst one patient was heterozygous and one homozygous for the C -> T substitution at nucleotide 1306, although neither alteration changed the amino acid sequence. Both alterations appear to be common polymorphisms of the β_c chain having been found in 40% and 24% of individuals respectively in the earlier study in Chapter 3. One of the JCML patients was heterozygous for a C -> A substitution at nucleotide 1835 which changes the proline at amino acid 603 to threonine. Although this is a significant amino acid change with the introduction of a possible phosphorylation site for a serine/threonine kinase this substitution had previously been found in haematologically normal controls and therefore is unlikely to be of any pathological significance.

Only one new abnormality which had not previously been described was found in this study. One patient was heterozygous for a G -> A substitution at nucleotide 773 in the extracellular portion of the β_c chain which changed the glutamic acid at amino acid 249 to glutamine. Comparison of the human β_c chain sequence with that of its two mouse homologues, AIC2A (mIL-3β) and AIC2B ($m\beta_c$) [Hayashida *et al*, 1990], revealed that both mouse β chains have a glutamine residue at this position in their amino acid sequence. Both mouse β chains exhibit high homology to the human β_c chain with about 56%

homology at the amino acid level. It was thought unlikely this alteration was of functional significance.

Although only a small number of patients were screened in this study, the failure to find any novel alterations in the coding sequence of either receptor chain indicates that point mutations of the GM-CSFR are not frequent in JCML and are unlikely to be responsible for the hypersensitivity to GM-CSF seen in JCML progenitors. However GM-CSFR signalling obviously plays an important role in JCML and studies of GM-CSFR down-regulation and of the signalling pathways downstream of the receptor may prove more fruitful at identifying the pathogenesis of this rare malignancy.

It is an interesting observation that children with Neurofibromatosis type 1 (NF-1) have a greatly increased risk of developing JCML. NF-1 is an autosomal dominant disorder affecting 1 in 3,500 people making it one of the most common human genetic diseases. The NF-1 gene, one of a small number of cloned human tumour suppressor genes, spans 350 kb and encodes a messenger RNA of 12-13kb made up of more than 50 exons [Largespada *et al*, 1996]. It codes for neurofibromin, the mutant protein in NF-1, which is one of 2 known human GTPase activating proteins (GAPs) that negatively regulate the p21Ras family of proto-oncogenes by accelerating the hydrolysis of GTP to GDP. [Hess *et al*, 1996]. Studies looking at Ras mutations found 20-30% of JCML patients had mutations in either N-Ras or K-Ras but no Ras mutations were found in JCML patients with NF-1 mutations. This suggests that the abnormal Ras and neurofibromin proteins were functionally redundant as they act in the same signal transduction pathway [Kalra *et al*, 1994, Largespada *et al*, 1996]. Ras proteins regulate cell growth and differentiation by cycling between an active (GTP-bound) and inactive (GDP-bound) form. Neurofibromin is essential for myeloid growth control of GM-CSF induced activation of the Ras pathway, and the lack of Ras mutations in JCML patients with NF-1 supports the hypothesis that Ras activation plays a central role in the development of JCML [Brodeur *et al*, 1994, Shannon *et al*, 1994, Bollag *et al*, 1996].

In vitro culture of fetal haemopoietic cells from mice with a null mutation in the NF-1 gene demonstrated a pattern of selective and enhanced sensitivity to GM-CSF which in the homozygous mutant cells was 10 times more sensitive to GM-CSF than control cells, a similar hypersensitivity to that of JCML cells. However no difference in sensitivity was observed when the cells were cultured in IL-3 or IL-5 [Largespada *et al*, 1996]. The IL-3R shares a common chain (β_C) with the GM-CSFR and levels of Ras-GTP increase in response to both ligands yet JCML cells respond normally to IL-3 in colony growth assays. This suggests either a quantitative difference in signalling between the IL-3R and GM-CSFR or the involvement of the unique α chains in differential regulation.

GM-CSF hypersensitivity is a consistent finding in JCML cells although only 40% of cases show alterations of Ras or NF-1. It is likely that other proteins involved in the Ras signalling pathway are mutated in the remaining patients. These findings raise the possibility of anti-GM-CSF or anti-Ras therapies for the treatment of JCML. One potential therapeutic use of anti-GM-CSF treatment has recently been described using an mutated analogue of GM-CSF [Iverson *et al*, 1996]. This GM-CSF analogue is referred to as E21R as it was created by substitution of the glutamate (E) at amino acid 21 in the protein sequence of GM-CSF with an arginine (R). This residue in the first α helix of GM-CSF is critical for high affinity but not low affinity binding which strongly suggests residue 21 is involved in interaction of GM-CSF with the β_C chain of the GM-CSFR complex. Biological assays have shown this residue is important for GM-CSF functions including proliferation, differentiation and mature cell function [Lopez *et al*, 1992].

E21R binds only to the α chain of the GM-CSFR and is devoid of classical GM-CSF activity, behaving as a complete antagonist of GM-CSF in binding and biological assays [Lopez *et al*, 1992]. Like anti-GM-CSF antibodies, E21R is able to inhibit the spontaneous growth of JCML colonies in culture. However unlike anti-GM-CSF blocking antibodies, E21R can directly induce generalised apoptosis of JCML cells. E21R initiation of apoptosis requires phosphorylation, transcriptional activation and protein synthesis. Interestingly, specific tyrosine kinase inhibitors did not affect the apoptotic effects of E21R unlike protein kinase C inhibitors which did, suggesting serine/threonine kinases may be central to the apoptotic mechanism of E21R. The apoptotic effect of E21R was found to be dominant over the survival effects of G-CSF and SCF in culture but not those of IL-3, suggesting an important role for the β_C in survival of haemopoietic cells. Since E21R binds selectively to the α chain it is possible that the GM-CSFR α chain, or E21R-α chain complex, triggers signals leading to cell death. Alternatively E21R may induce apoptosis by disrupting a pre-existing α-β_C interaction important for cell survival, a view supported by evidence indicating the existence of preformed GM-CSFR complexes while in contrast IL-3R α and β_C association seeming to be ligand dependent [Iverson *et al*, 1996]. Importantly E21R induced apoptosis even in the presence of the cytokines TNFα and IL-1β, both of which have been implicated as stimulators of JCML growth. Given the hypersensitivity of JCML to GM-CSF these results offer a new therapeutic approach to control JCML cell load [Iverson *et al*, 1996] and further study of the complex interactions involved in ligand and receptor coupling in GM-CSF signalling may provide additional insights into the role of GM-CSF in JCML.

Chapter 5

Analysis of a GM-CSFR β chain isoform in haemopoietic cells and its role in receptor complexes

5.1 - Introduction

Although many of the receptors in the cytokine receptor family have only been isolated and sequenced in the last decade, already numerous receptor chain isoforms have been described. Soluble receptors are one of the most frequently found receptor isoforms and have been described for the majority of cytokines. Soluble receptor chains are generated either by proteolytic cleavage, as for the IL-1, IL-2 and M-CSF soluble receptors, or by alternative splicing of mRNA as seen in the G-CSF, IL-5, Epo and Tpo soluble receptors [Heaney *et al*, 1996]. The various isoforms of the GM-CSFR α chain were reviewed in Chapter 1 (section 1.5.2), with most arising from alternative splicing of the 3' end of the chain. They include soluble receptor chains lacking the transmembrane domain [Ashworth *et al*, 1990, Hu *et al*, 1994], an isoform with an altered 3' end [Crosier *et al*, 1991] and one with an insertion 5' of the transmembrane domain [Devereux *et al*, 1993]. Other isoforms created by alternative splicing of the 5' end of the GM-CSFR α chain do not alter the mature protein but may affect translation efficiency and transport of the receptor to the cell surface [Chopra *et al*, 1996, Wagner *et al*, 1994].

Although in the murine system the IL-3R has two distinct β receptor chains, one specific to the mouse IL-3R (AIC2A) and the other a common chain shared with the mouse GM-CSFR and IL-5R (AIC2B), only one common subunit has been described for the human system, the β_C chain shared by the GM-CSFR, IL-3R and IL-5R [Hayashida *et al*, 1990]. The full length β_C chain is 881 amino acids long and as reviewed in Chapter 1 (section 1.5.7), is thought to be responsible for most downstream signalling of the GM-CSFR complex via its long intracytoplasmic tail (432 amino acids). During analysis of the intracytoplasmic tail of the β_C chain by PCR based techniques our laboratory observed that RT-PCR of the β7 fragment (nucleotides 1281-1620), which includes the WSXWS box, transmembrane and box 1 sequences, using mRNA extracted from TF-1 cells, consistently gave two products, the expected 339bp band and a smaller fragment of approximately 240bp (Figure 5.1). This smaller band was again seen when mRNA from other cell lines, AML patients and haematologically normal individuals were amplified in an RT-PCR of fragment β7.

Sequencing of a purified PCR product revealed that the smaller fragment was due to a 104 bp deletion from nucleotides 1493 to 1596 of the β_C chain coding sequence [Gale *et al*, 1993abs]. Further analysis of the β_C chain genomic sequence using DNA from TF-1 cells showed that the 104 bp deletion was in fact a complete exon (Figure 5.2), and the intracytoplasmic tail of the human β_C chain has a similar intron/exon structure to the mouse AIC2A/AIC2B genes (Figure 1.5), with three exons coding for the entire cytoplasmic tail

Figure 5.1 - RT-PCR of Fragment β7 using RNA from haemopoietic cell lines and AML patients

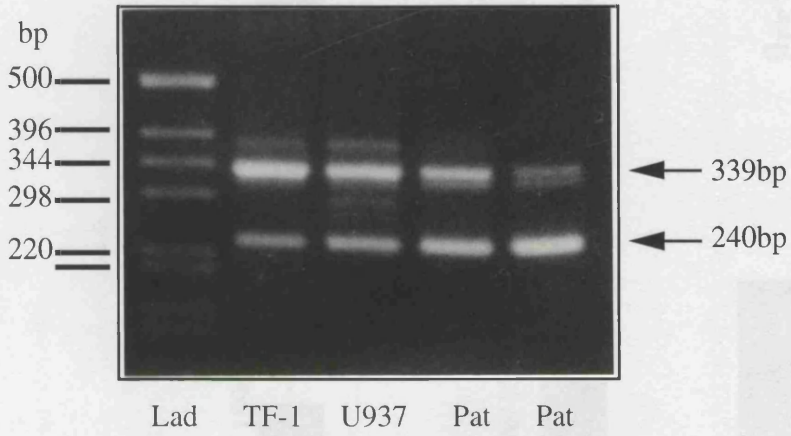


Figure 5.2 - Diagram showing how β_{IT} arises by alternative splicing

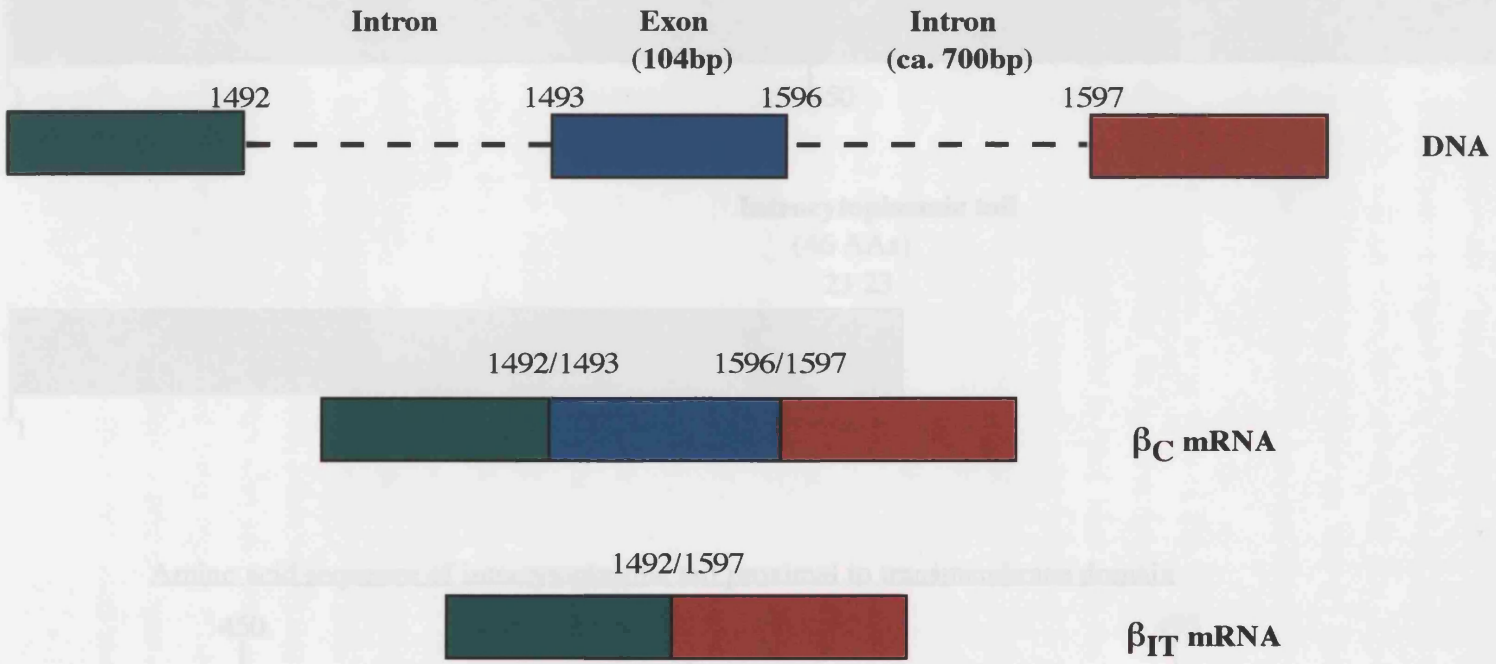
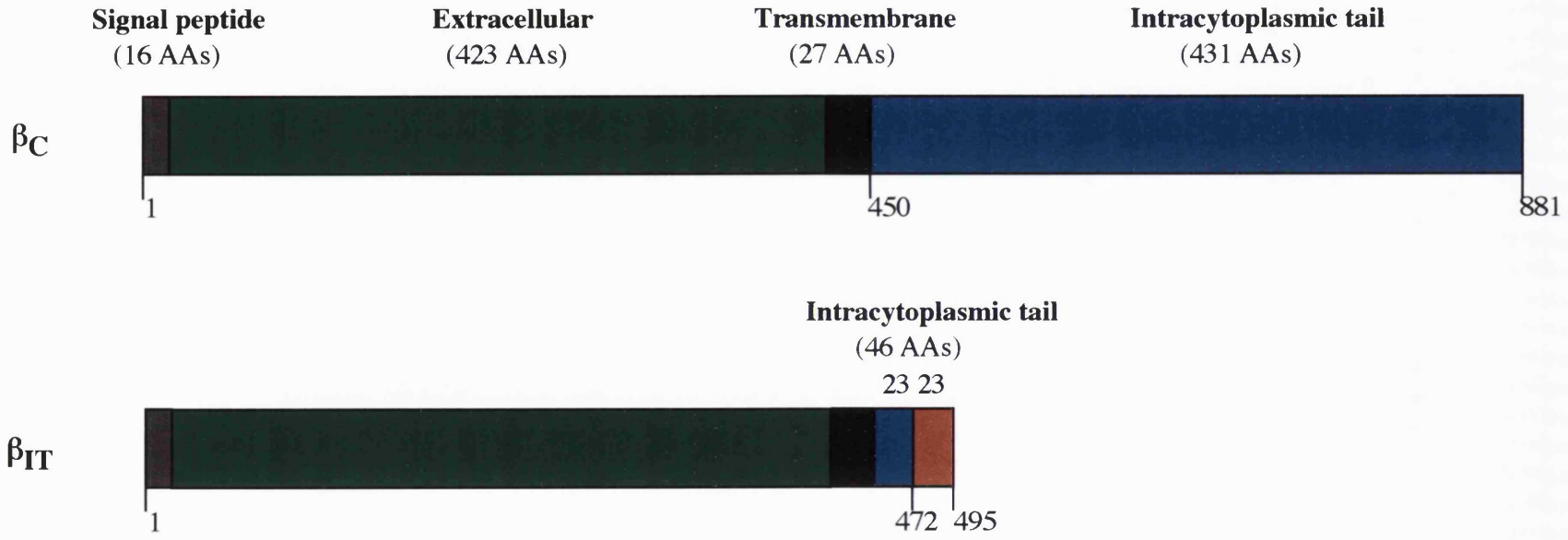


Figure 5.3 - Protein structures of β_C and β_{IT} chains



Amino acid sequence of intracytoplasmic tail proximal to transmembrane domain

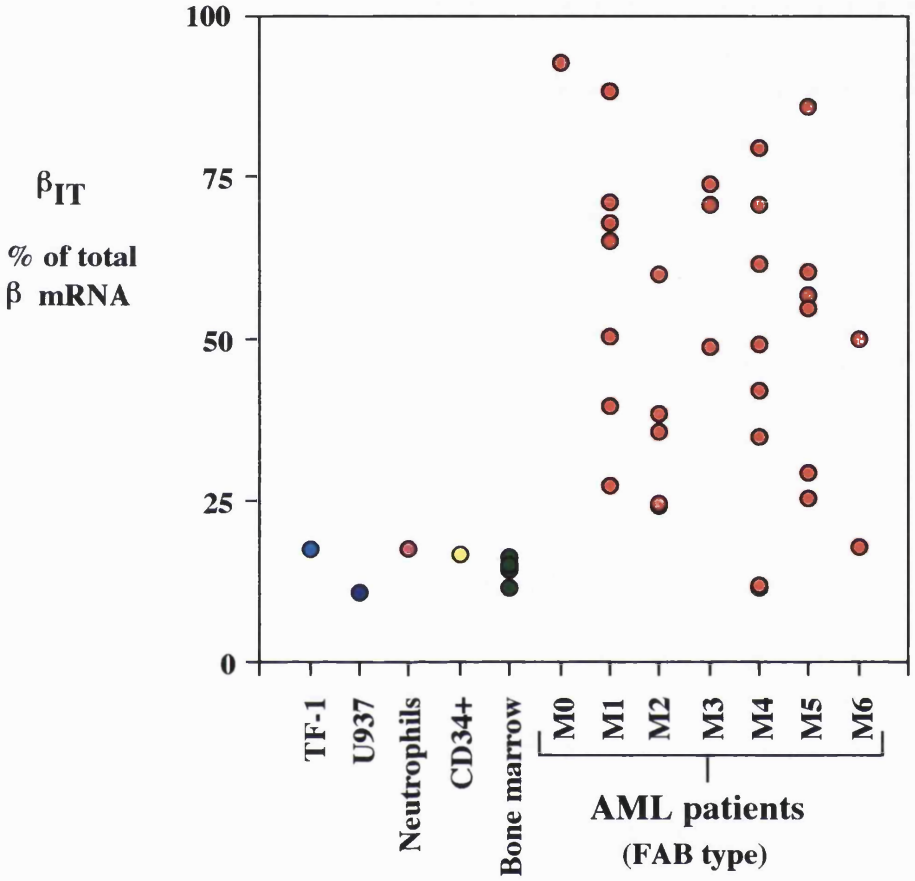
	450	472	495
β_C	YGYRLRRKWEEKIPNPSKSHLFQ	NGSAELWPPGSMSAFTSGSPPHQGP....	
β_{IT}	YGYRLRRKWEEKIPNPSKSHLFQ	GVPCRIRGQRGVTSHHRGPQACL	

[Kosugi *et al*, 1995]. Deletion of the second intracytoplasmic exon causes a frameshift alteration which results in the introduction of a premature stop codon, giving rise to a truncated intracytoplasmic tail of just 46 amino acids, compared to the 432 amino acids of the full length β_C chain. Referred to as the β_{IT} chain for β **I**ntracytoplasmic **T**runcated chain, the first 23 amino acids of the cytoplasmic domain of the β_{IT} are identical to those of the β_C chain followed by a new 23 amino acid sequence (Figure 5.3). The first 23 amino acids of the intracytoplasmic tail of the β_{IT} chain still contains the proline rich box 1 sequence necessary for phosphorylation of the tyrosine kinase Jak 2, while the new 23 amino acid sequence contains two cysteines, a serine and a threonine but does not have any known consensus binding sequences.

Analysis of β_{IT} mRNA levels by RNAse protection assay showed that in haemopoietic cell lines, normal bone marrow and purified neutrophils, the β_{IT} transcript accounts for 10-20% of the total β chain message in these cells. However in AML blast cells the level of β_{IT} message varied from 10-90% of the total β chain message, with levels of > 30% β_{IT} message found in over 75% of the AML patients analysed (Figure 5.4) [Gale *et al*, 1993abs]. Transfection of β_{IT} DNA into COS-7 cells demonstrated that the β_{IT} protein could be expressed at the cell surface, while Scatchard analysis of COS-7 cells co-transfected with GM-CSFR α and β_{IT} DNA showed the β_{IT} chain was able to form a high affinity receptor with a binding affinity K_D of 200pM similar to that reported for the full length β_C chain (Figure 5.5) [Hayashida *et al*, 1990, Gale *et al*, 1993abs].

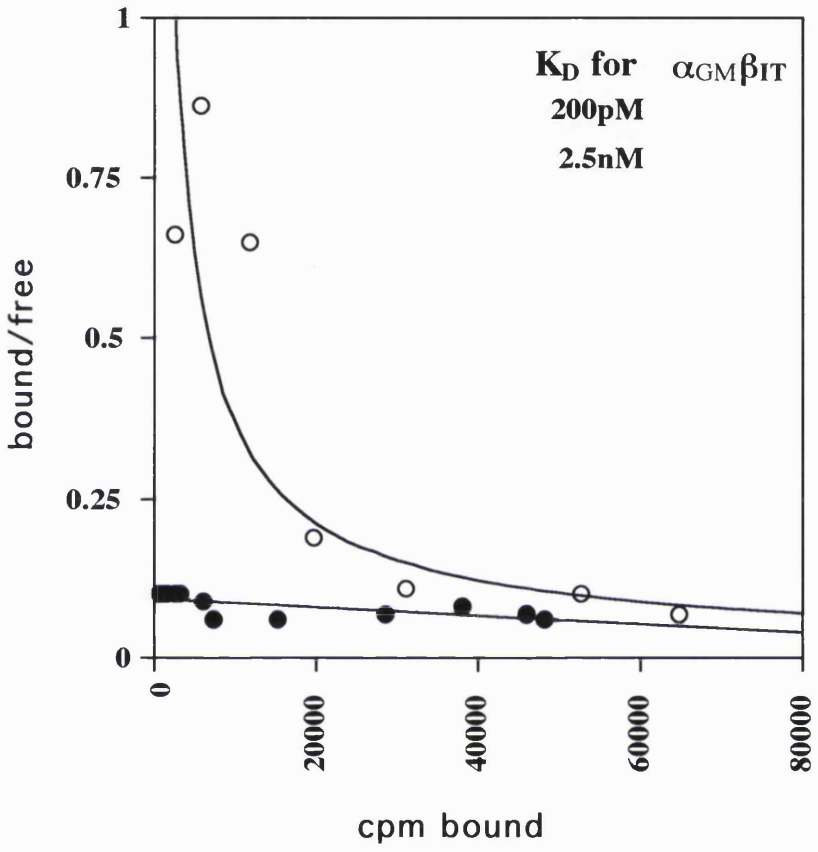
RT-PCR was used to demonstrate that full length transcripts of the β_{IT} chain could be obtained from TF-1 RNA. Therefore, antibodies to the β_C chain which recognise sites in the extracellular region could also be used to probe for the β_{IT} chain. To demonstrate this plasmids containing the full coding sequence for the β_C chain and the full coding sequence of the β_{IT} chain were constructed and used to transfect COS-7 cells. Whole cell lysates of COS-7 cells transfected with either the β_C or β_{IT} chain containing plasmids were probed in western blotting using an antibody (DC-9) which recognises the extracellular domain of the β chain. On a western blot probed with DC-9 the β_C chain protein was visible in the expected 130kDa size region while the β_{IT} chain protein was seen in the 70-80kDa region. However, in the lysate from cells transfected with the β_C plasmid, bands which migrated in the 80-90kDa region, thought to be degradation products, were also seen and interfered with detection of β_{IT} chain proteins which are found in the same region [Gale *et al*, 1993abs]. To further study expression of the β_{IT} chain, especially in primary haemopoietic cells, and avoid confusion with the β_C degradation products, polyclonal antibodies were

Figure 5.4 - Relative expression of β_{IT} mRNA as detected by RNAse protection assay on total RNA from haemopoietic cell lines and primary haemopoietic cells *



* - from Gale *et al*, 1993

Figure 5.5 - Scatchard analysis of ^{125}I-GM-CSF binding to COS-7 cells transfected with GM-CSFR α chain or GM-CSFR α + β_{IT} chain DNA *



● - GM-CSFR α chain

○ - GM-CSFR α + β_{IT} chain

* - From Gale *et al*, 1993

therefore raised against the novel 23 amino acid sequence of the intracytoplasmic tail of the β_{IT} chain.

5.2 - Materials and Methods

5.2.1 - Cell culture and Transfections (electroporation)

All transfection experiments were carried out using cultured COS-7 cells. 10μg of plasmid encoding the receptor chain required was transfected into 5 x 10^6 cells in a final volume of 0.8ml as described in Chapter 2 (section 2.B.2). Experiments examining glycosylation of the β chain receptors were carried out using COS-7 transfected by the standard method described previously but following transfection the cells were cultured in medium with or without 3μg/ml of tunicamycin (Sigma, UK) for 72 hours at 37°C.

5.2.2 - Cell Lysates and Immunoprecipitations

Whole cell lysates were prepared using the standard method described in Chapter 2 (section 2.B.3), with cells lysed in 1ml of lysis buffer on ice, centrifuged at 13000rpm for 10 minutes, an equal amount of 2 x sample buffer added and the samples boiled for 10 minutes at 99°C.

Immunoprecipitations

Up to 40 x 10^6 cells were lysed in 1ml of lysis buffer (with protease inhibitors) and left on ice for 30 minutes, then centrifuged at 13000rpm for 10 minutes at 4°C. The supernatant was removed and immunoprecipitated for 4-14 hours at 4°C using the following antibodies

Anti-β (DC-9) - 2μg per ml of lysed cells

Anti-β_{IT} (purified G1, G2, R1, R3) - 50μl per ml of lysed cells

Anti-α (S-50 Santa-Cruz Biotech, USA) - 4μg per ml of lysed cells.

After incubation with the antibody of interest, 60μl of Protein A Sepharose was added and the samples rotated end over end at 4°C for 2-4 hours. The Sepharose was then centrifuged, washed and boiled in 2 x sample buffer as described in section 2.B.3.

2-15μl of the immunoprecipitate were loaded on polyacrylamide gels for western blotting.

5.2.3 - Immunisation of Rabbits with β_{IT} specific peptide

Two peptides for immunisation were selected from the novel intracytoplasmic sequence of the β_{IT} chain. These two peptides only differed in sequence by an additional cysteine residue in peptide 2 although this peptide was partially oxidised prior to conjugation so

that both cyclic and linear forms were present to mimic a possible conformation introduced by the two additional cysteine residues of the novel β_{IT} chain sequence.

Peptide 1 - H-Arg-Ile-Arg-Gly-Gln-Arg-Gly-Val-Thr-Ser-His-His-Arg-Gly-Pro-Gln-Ala-Cys-Leu-OH

Peptide 2 - H-Cys-Arg-Ile-Arg-Gly-Gln-Arg-Gly-Val-Thr-Ser-His-His-Arg-Gly-Pro-Gln-Ala-Cys-Leu-OH

Peptide 1 was conjugated to keyhole limpet haemocyanin (KLH) C-terminally via the cysteine sulphydryl moiety, whilst Peptide 2 was N-terminally conjugated to KLH using glutaraldehyde. Three New Zealand white rabbits were immunised with peptide 1 and three with peptide 2. Peptide preparation, rabbit immunisation and serum harvesting were all carried out by Affiniti Research Products Ltd. Pre-immune serum was collected 7 days prior to the first immunisation injection and the final harvest bleed collected seven injections and 91 days later. The harvest sera were called R1, 2 and 3 for rabbits immunised with peptide 1 and G1, 2 and 3 for those immunised with peptide 2.

5.2.4 - Western Blots

Gels

Samples were electrophoresed on polyacrylamide gels, transferred to nitro-cellulose membrane and non-binding specific sites blocked in 3% non-fat milk/PBS as given in general materials and methods (Chapter 2, section 2.B.5).

Primary antibodies

Several different blotting conditions including concentration, blotting solution, temperature and time were tested for each of the antibodies used in this study before the optimised conditions given below were decided upon :

Anti-β (DC-9) - 0.2μg per ml in 3% BSA (in PBS) for 4 hours at room temperature.

Anti-β_{IT} (G1,2,3, R1,2,3) - 1 in 100 in 5% non-fat milk, 3% BSA for 4 hours at room temperature.

Anti-α chain (S-50, Santa Cruz Biotech, USA) - 1μg per ml in 3%BSA overnight at 4°C.

Following primary antibody probing the blots were washed 3 times for 5 minutes each in 0.05% Tween 20 in PBS.

Secondary antibodies

Horse radish peroxidase conjugated Goat anti-Mouse or Goat anti-Rabbit antibodies were used at a concentration of 1 in 2000 in 5% non-fat milk. Blots were probed for 30 minutes at room temperature with constant rotation before being washed 3 times for 5 minutes each in 0.05% Tween 20 in PBS.

Enhanced Chemiluminescence (ECL)

ECL was performed as per manufacturers protocol (Amersham, UK) and blots exposed for varying lengths of time to Hyperfilm-MP (Amersham, UK).

5.3. - Results

5.3.1 - Western blot of whole cell lysates from transfected COS-7 cells

To confirm that the β_C chain and β_{IT} chain proteins could be expressed, COS-7 cells were transfected with plasmids which contained either the β_C or β_{IT} chain coding sequence. 10μg of plasmid was transfected into the COS-7 cells which were cultured at 37°C for 72 hours before being lysed and made into whole cell lysates. Western blots were then performed to examine expression of the β_C and truncated β_{IT} receptor chains in the transfected COS-7 cells. Figure 5.6 shows COS-7 cells transfected with either water, β_C or β_{IT} in a western blot probed with an anti-β antibody (DC-9) to the extracellular domain. No bands were visible in the water transfection, while the β_C transfected cells gave a band of the expected size (130kDa). The β_{IT} transfection gave at least three distinguishable bands of between 70-80kDa in size, the relative intensities of which were seen to vary between experiments. In addition to the expected 130kDa band, β_C transfected cells also gave bands of 80-90kDa, thought to be degradation products. These broad bands migrated through the gel in approximately the same area as the β_{IT} bands, and in cells co-transfected with both β_C and β_{IT} the degradation bands partially obscured the β_{IT} bands. To avoid possible confusion between this degradation product and the β_{IT} chain proteins and to confirm the 70-80kDa bands really were β_{IT} chain proteins, polyclonal antibodies were raised against the novel C-terminus sequence of the β_{IT} chain.

5.3.2 - Rabbit antiserum - Pre-immune and Harvest bleed

Antisera were obtained from six rabbits immunised with one of two β_{IT}-specific peptides. The antisera from animals immunised with peptide 1 were named R1, 2 and 3 while those immunised with peptide 2 were called G1, 2 and 3. Figure 5.7 shows western blots of whole cell lysates from COS-7 cells transfected with either β_C or β_{IT} chains. The top six

Figure 5.6 - Western blot of whole cell lysates from transfected COS-7 cells

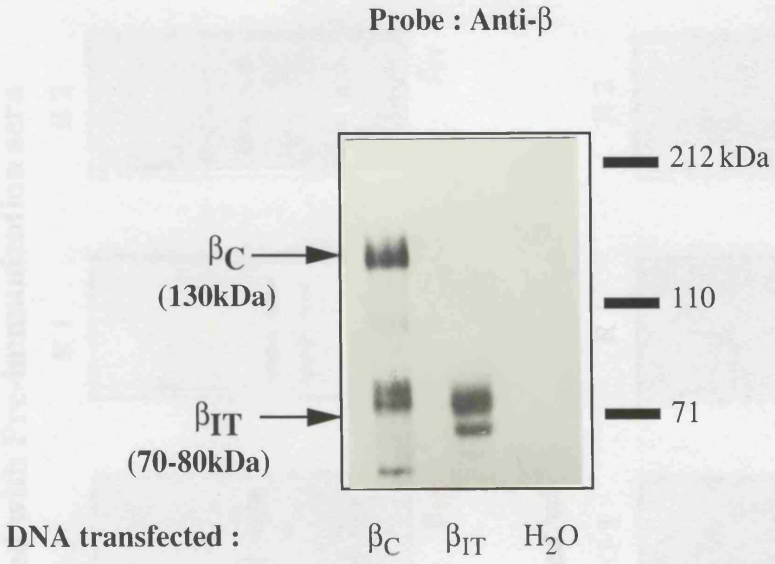
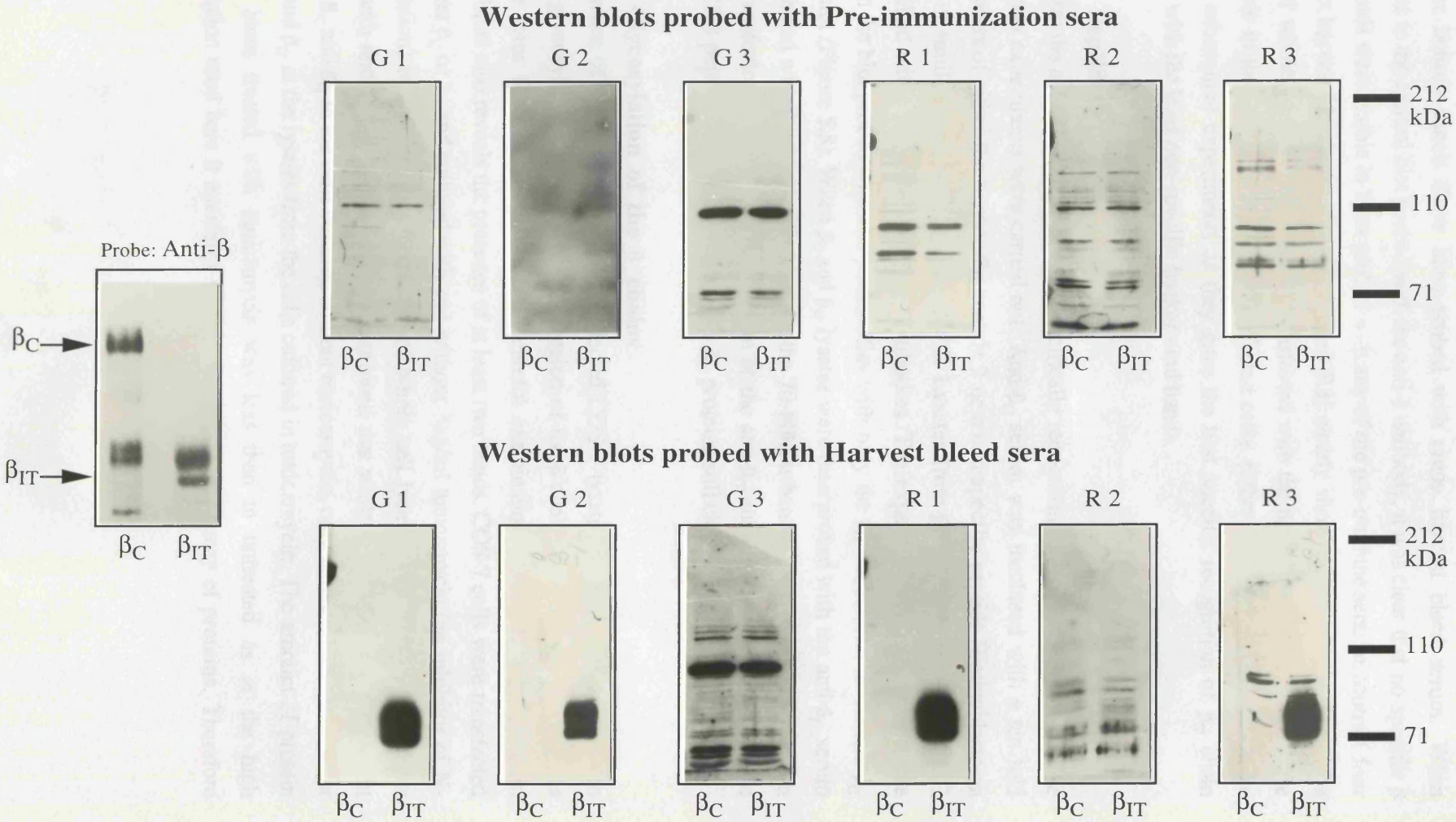


Figure 5.7 - Western blots of transfected COS-7 lysates probed with Anti-sera of Rabbits immunized with β_{IT} peptide



panels show blots probed with pre-immune serum from each of the immunised rabbits while the bottom panels show blots probed with crude harvest bleed serum. When compared to the control blot probed with the anti-β antibody, it was clear that no specific β chain bands were visible in blots probed with any of the pre-immune sera. In contrast four of the six harvest bleed sera (G1, G2, R1 and R3) clearly showed bands in the 70-80kDa region of whole cell lysates from cells transfected with the β_{IT} chain plasmid which were not visible in the lysates from the β_C transfected cells. Either the G2 or R3 antisera were used in subsequent experiments as they gave the best specific recognition of β_{IT} chain proteins with the least non-specific background bands.

5.3.3 - Peptide competition

To confirm the anti-β_{IT} antibodies were specifically recognising β_{IT} chain proteins, peptide competition experiments were carried out. Anti-β_{IT} serum was incubated with a ten fold molar excess of either the β_{IT} specific peptide 2 or a non-specific peptide (Retinoblastoma peptide) of similar size for four hours at 4°C. Lysates from β_C and β_{IT} transfected COS-7 were probed with the anti-β and anti-β_{IT} antibodies. The expected β_C and β_{IT} bands were visible in the blot probed with anti-β antibodies with only the β_{IT} bands seen in the anti-β_{IT} probed blot (Figure 5.8). When β_C and β_{IT} lysates were then probed with the anti-β_{IT} serum pre-incubated with the β_{IT}-specific peptide, the 70-80kDa bands were no longer visible in the β_{IT} transfected cells. Similar competition of the anti-β_{IT} antibodies with a non-specific peptide (RB peptide) had no effect, with the β_{IT} proteins still clearly visible.

5.3.4 - Glycosylation of the β chains

The presence of several bands in β_{IT} transfected COS-7 lysates was thought to be due to variable glycosylation as the extracellular region of the β chains contains three consensus sequence sites for N-glycosylation, and careful examination of β_C COS-7 lysates in western blots also reveals the presence of at least two bands. COS-7 cells were transfected with either β_C or β_{IT} and cultured with and without 3μg/ml tunicamycin (an inhibitor of N-glycosylation) for 3 days. A western blot of whole cell lysates from these transfections probed with anti-β antibodies (Figure 5.9) showed that while several bands were present for both β_C and β_{IT} in the cells cultured without tunicamycin, only one band was present for both β_C and β_{IT} in the lysates from the cells cultured in tunicamycin. The amount of protein seen in lanes treated with tunicamycin was less than in untreated as at the high concentration used here it interferes with the translation efficiency of proteins. Therefore

Figure 5.8 - Western blot of COS-7 lysates probed with Rabbit anti-sera to confirm specificity of antibodies

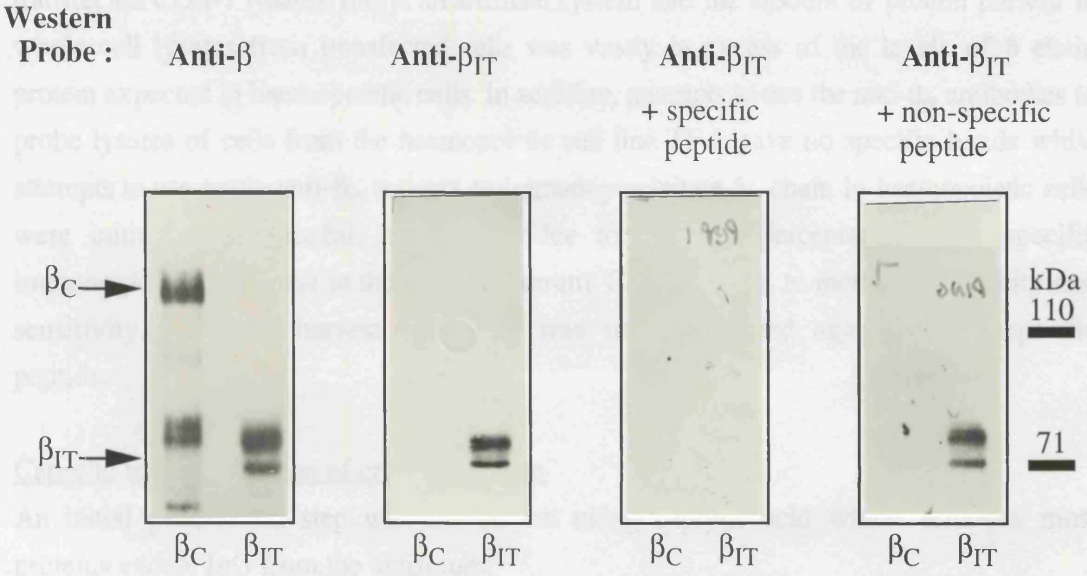
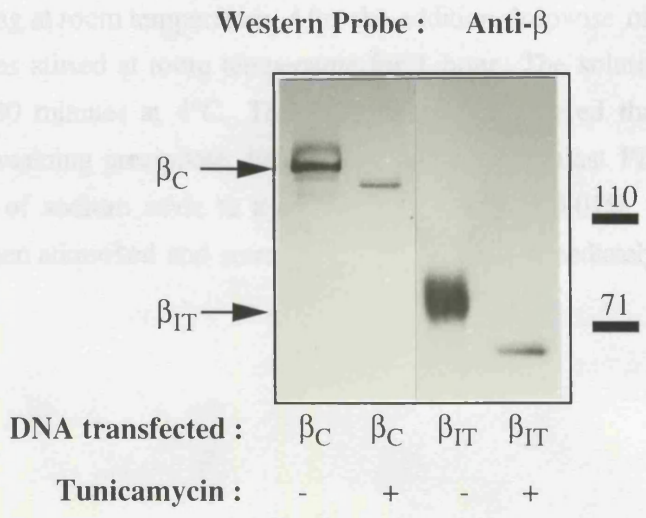


Figure 5.9 - Western blot of transfected COS-7 cells cultured with or without tunicamycin



N-glycosylation is at least partly responsible for the different sized protein bands seen for both the β_C and β_{IT} chains.

5.3.5 - Immunopurification of Rabbit antisera

Although the anti-β_{IT} antibodies were able to specifically recognise β_{IT} chain proteins from transfected COS-7 lysates, this is an artificial system and the amount of protein present in whole cell lysates from transfected cells was vastly in excess of the levels of β chain protein expected in haemopoietic cells. In addition, attempts to use the anti-β_{IT} antibodies to probe lysates of cells from the haemopoietic cell line TF-1 gave no specific bands while attempts to use crude anti-β_{IT} antisera to immunoprecipitate β_{IT} chain in haemopoietic cells were initially unsuccessful, presumably due to the high percentage of non-specific immunoglobulins present in the crude antiserum. Consequently, to increase specificity and sensitivity, the crude harvest bleed sera was immunopurified against the β_{IT}-specific peptide.

Caprylic acid purification of crude antiserum

An initial purification step was carried out using caprylic acid which removes most proteins except IgG from the antiserum.

Materials

Caprylic acid	Sigma, Poole, UK
Phosphate buffered saline pH 7.2 (PBS)	Gibco-BRL, Paisley, UK
60mM Sodium acetate pH 4.0 (NaOAc)	
Glass wool	
Crude antiserum	Affiniti Research Products, UK

Method

In a conical flask, 20ml 60mM NaOAc was added dropwise to 10ml crude antiserum with continual stirring at room temperature. After the addition dropwise of 700µl caprylic acid, the solution was stirred at room temperature for 1 hour. The solution was then spun at 3000rpm for 30 minutes at 4°C. The supernatant was filtered through glass wool to remove any remaining precipitate, before being dialysed against PBS overnight at 4°C. After addition of sodium azide to a final concentration of 0.01%, the immunoglobulin solution was then aliquoted and stored at -20°C or used immediately in the next stage of purification.

Actigel immunopurification column

Following initial purification, a β_{IT}-specific Actigel column was used to immunopurify antibodies specific to the β_{IT} peptide 2 from the part purified antiserum.

Materials

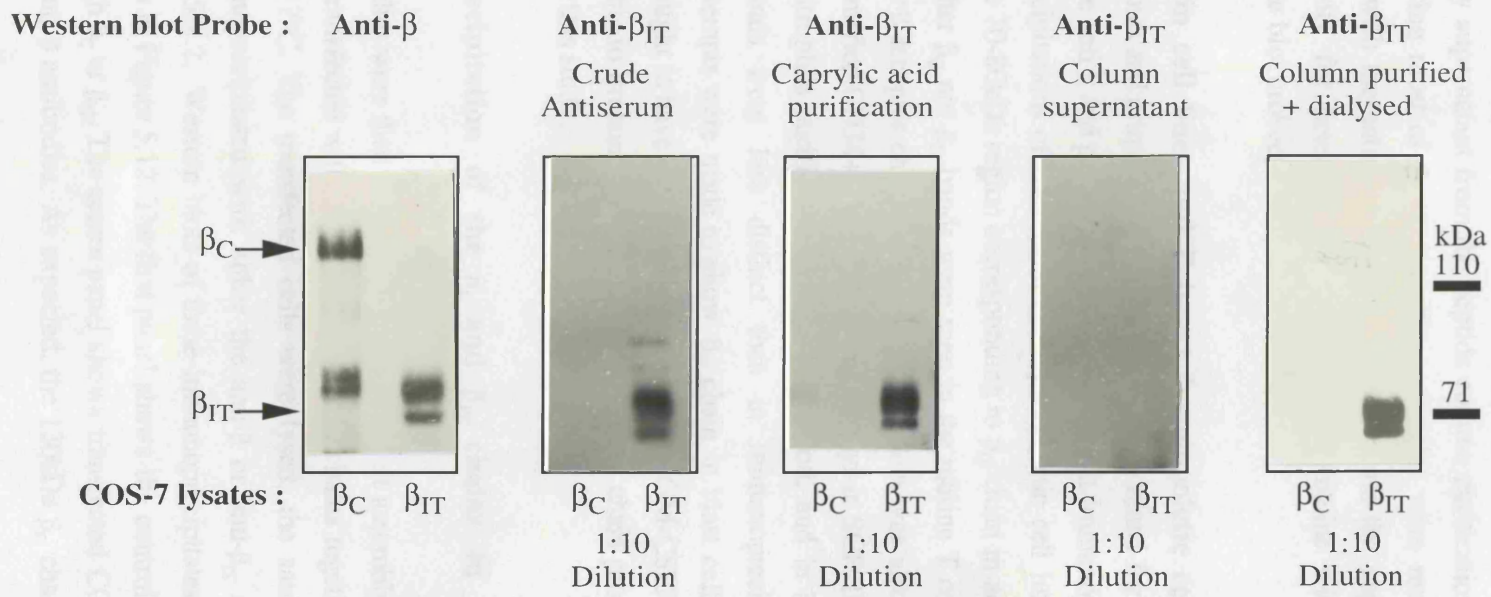
Actigel-ALD	Sterogene, Belgium
Coupling buffer (1M $NaCNBH_3$)	Sterogene, Belgium
Immunisation peptide solution (peptide 2)	Affiniti Research Products, UK
20ml barrel syringe	
Glass wool	
0.5M Sodium chloride	
PBS, pH 7.2	Gibco-BRL, Paisley, UK
100mM Glycine pH 2.5 / 1.8	
1M phosphate buffer pH	
Slide-A-Lyzer™ cassette	Pierce, USA

Method

Using a vacuum manifold, 5ml of Actigel-ALD resin was suctioned dry in a 20ml syringe plugged with glass wool to remove the storage buffer before being washed 3 times in 10ml PBS. Next, the coupling solution consisting of 0.8ml coupling buffer plus 1mg of the immunisation peptide 2 made up to a final volume of 5ml with sterile water, was added to the resin, the top of the syringe sealed and rotated slowly at 4°C for 4-12 hours. After filtering off the coupling solution the resin was washed twice in 2 volumes of PBS and twice in 2 volumes of NaCl. (The efficiency of the coupling reaction was gauged from measurement of the peptide concentration in the filtrate by mass spectrometry). 10ml of the caprylic acid purified antiserum was added to the resin column and rotated overnight at 4°C. The serum was filtered off and the column washed 3 times in 2 volumes of PBS. Peptide specific antibodies were then eluted from the resin initially with 1ml aliquots of 100mM glycine pH 2.5, then aliquots of 100mM glycine pH 1.8. The aliquots were collected directly into 1.5ml Eppendorf tubes containing 50µl 1M phosphate buffer pH 8 to neutralise the low pH elutant. The aliquots were then dialysed against PBS at 4°C in the Slide-A-Lyzer cassette which are efficient for dialysis of small volumes. Aliquots with 0.01% sodium azide added were stored at -20°C.

Figure 5.10 shows western blots of β_C and β_{IT} transfected COS-7 lysates which had been diluted 1:10 in sample buffer, thereby requiring increased sensitivity for detection of

Figure 5.10 - Immunopurification of Anti-β_{IT} antibodies



specific bands. The blot was probed with crude anti-serum and showed β_{IT} chain bands in the expected region although a long exposure time was required and hence the level of background in the blot was very high. Following caprylic acid purification of the anti-serum, the β_{IT} bands were clearer with less non-specific background present. The blot probed with the preliminary supernatant from the peptide column purification showed no specific bands, demonstrating most of the β_{IT}-specific antibodies were retained on the column. The blot probed with the anti-β_{IT} antibodies purified from the peptide column showed increased sensitivity for detection of the β_{IT} chain proteins with very low background compared to the blot probed with crude antiserum.

5.3.7 - Presence of β_{IT} in cell lines and Primary haemopoietic cells

Several haemopoietic cell lines and primary myeloid cells were examined for the presence of β_{IT} chain protein using the anti-β and purified anti-β_{IT} antibodies in immunoprecipitations (Figure 5.11). Immunoprecipitations of cells from the haemopoietic cell lines TF-1 and HL-60 showed bands in the 70-80kDa region corresponding to β_{IT} chain in addition to the full length β_C protein. Neither β_C nor β_{IT} bands were seen in the murine T cell line CTLL which does not express any β receptor chains. The β_{IT} chain protein was also clearly seen in immunoprecipitates of purified CD34+ cells, cultured for 9 days in SCF, IL-3 and IL-6. It was again present in neutrophils purified from peripheral blood, and in bone marrow cells although the β_{IT} bands were less distinct than in immunoprecipitates from haemopoietic cell lines. Attempts were made to show β_{IT} chain in blast cells from AML patients. These cells are thought to have less than 100 high affinity GM-CSFR per cell and it was not technically possible to immunoprecipitate either β_{IT} or β_C chain protein from the AML samples examined in this study.

5.3.8 - Co-immunoprecipitation of the β_C and β_{IT} chains in transfected COS-7 cells

The purified anti-β_{IT} antibodies were then used to investigate subunit assembly of the GM-CSFR. COS-7 cells were transfected with either β_C, β_{IT} or both chains together (β_{CIT}) and cultured for 72 hours at 37°C. The transfected cells were lysed, the samples divided equally in two and immunoprecipitated with either the anti-β or anti-β_{IT} antibodies as described above in section 5.2.2. Western blots of these immunoprecipitates probed with anti-β antibodies are shown in Figure 5.12. The first panel shows the control lysates from COS-7 cells transfected with β_C or β_{IT}. The centre panel shows transfected COS-7 samples immunoprecipitated with anti-β antibodies. As expected, the 130kDa β_C chain bands and

Figure 5.11 - Western blots showing presence of β_{IT} receptor chain in primary haemopoietic cells and cell lines

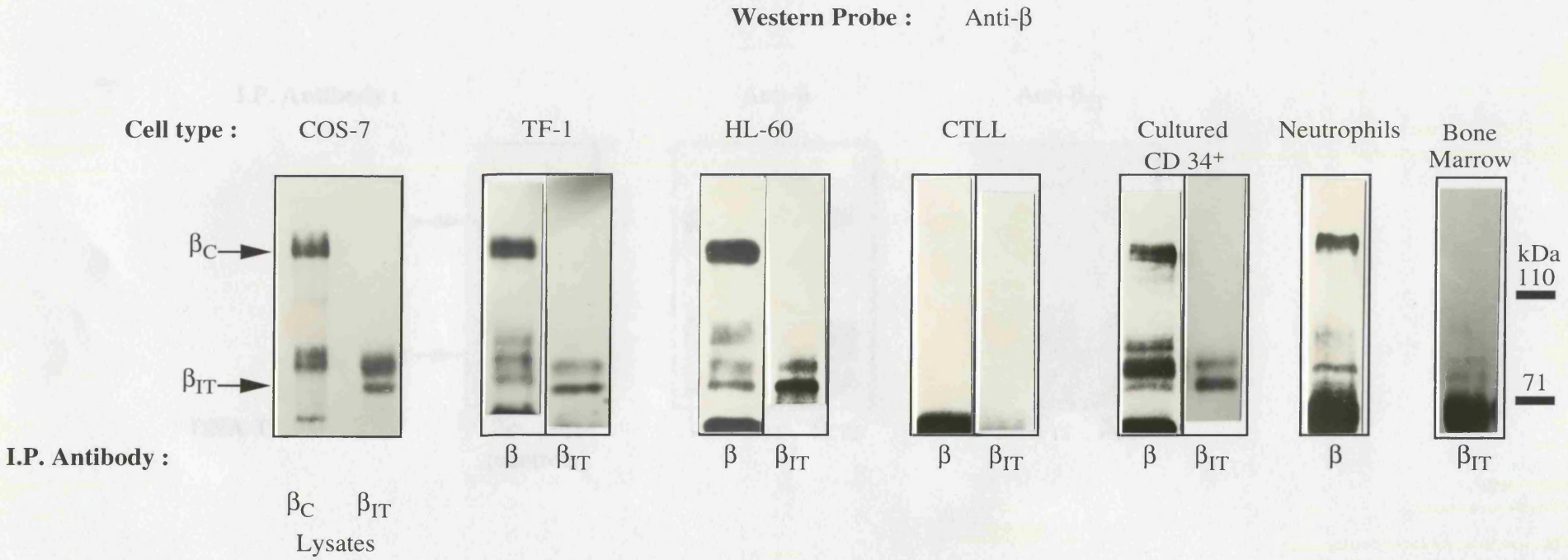
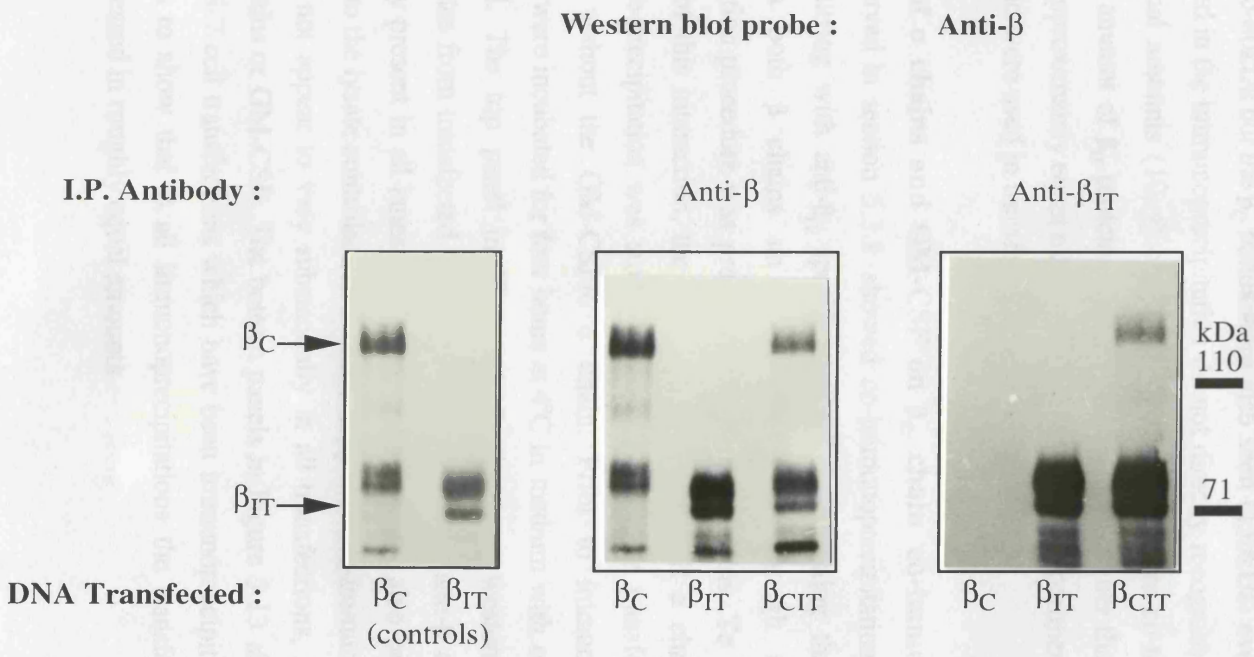


Figure 5.12 - Co-immunoprecipitation of β_C and β_{IT} chains in transfected COS-7 cells

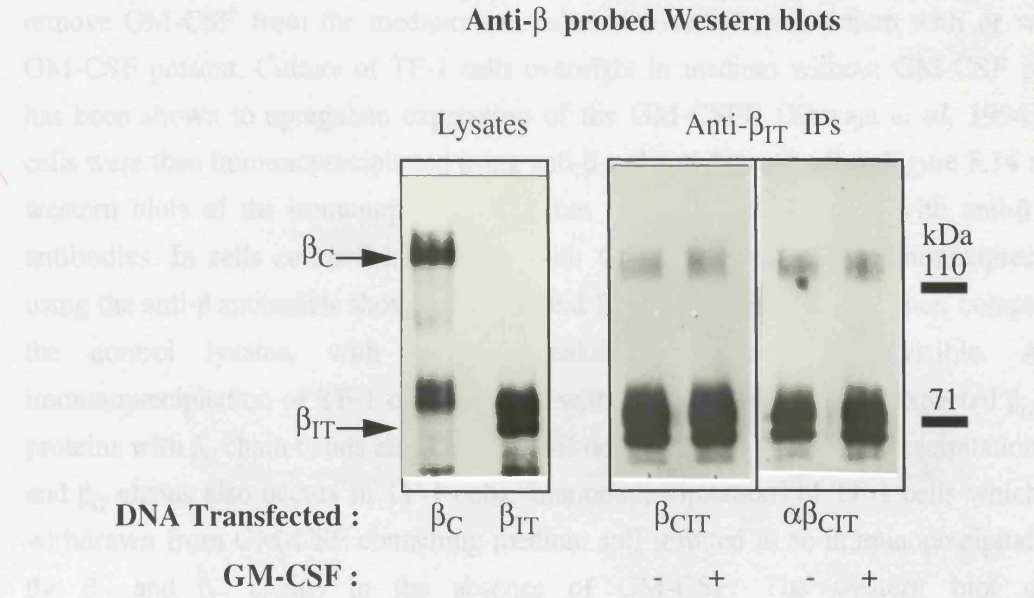


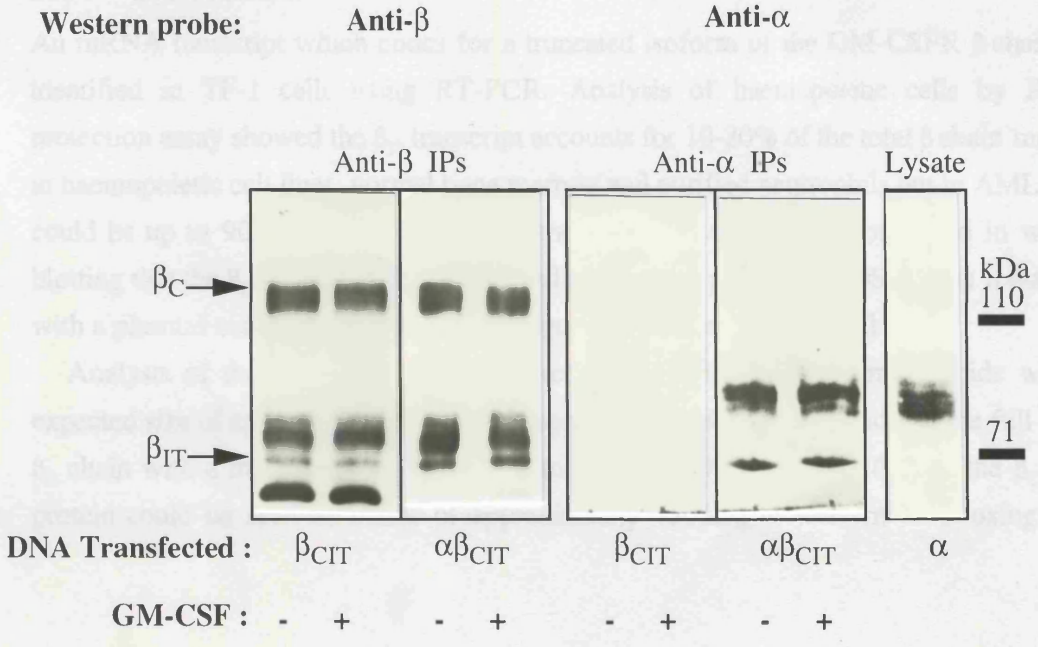
degradation products (80-90kDa) were clearly visible in cells transfected with the β_C chain alone, the β_{IT} chain proteins were clearly seen in the immunoprecipitate of cells transfected with the β_{IT} chain alone, while both receptor proteins could be seen in the immunoprecipitate of the cells co-transfected with both chains (β_{CIT}). The panel on the right shows a western blot of the same transfected cells immunoprecipitated with the anti-β_{IT} antibodies. As expected, no bands were visible in the β_C transfected cells as the anti-β_{IT} antibodies do not cross react with the β_C chain proteins (Figure 5.7). The β_{IT} bands were clearly seen in the β_{IT} transfected cells. In the β_C and β_{IT} co-transfected cells the β_{IT} bands were visible at 70-80kDa but the β_C bands were also seen (130kDa) even though the anti-β_{IT} antibodies used in the immunoprecipitation do not directly recognise β_C chain proteins. Even though equal amounts (10μg) of β_C and β_{IT} plasmids were co-transfected into the COS-7 cells the amount of β_{IT} protein seen was consistently higher than the β_C and in an attempt to give approximately equal expression in subsequent experiments 10μg of β_C and 5μg of β_{IT} plasmid were used in transfections.

5.3.9 - Effect of α chains and GM-CSF on β_C chain co-immunoprecipitation

The results observed in section 5.3.8 showed co-immunoprecipitation of the β_C and β_{IT} chains was occurring with anti-β_{IT} specific antibodies suggesting that in COS-7 cells transfected with both β chains an interaction strong enough to withstand the immunoprecipitation procedure was present between the β chains. To further investigate the mechanism of this interaction, the effect of the GM-CSFR α chains and GM-CSF ligand on this co-precipitation was studied. COS-7 cells were transfected with both β chains with or without the GM-CSFR α chain. Prior to immunoprecipitation the transfected cells were incubated for four hours at 4°C in medium with or without 15ng/ml GM-CSF added. The top panel in Figure 5.13 shows a western blot of anti-β_{IT} immunoprecipitates from transfected COS-7 cells, probed with anti-β antibodies. The β_{IT} chain was clearly present in all lanes, while the β_C chain could also be seen in all lanes when compared to the lysate controls. The amount of β_C chain co-immunoprecipitated with the β_{IT} chain did not appear to vary substantially in all transfections, irrespective of the presence of α chains or GM-CSF. The bottom panels in Figure 5.13 show western blots of the same COS-7 cell transfections which have been immunoprecipitated with anti-β or anti-α antibodies to show that in all immunoprecipitations the transfected chains were present and expressed in roughly equal amounts.

Figure 5.13 - Effect of α chain and GM-CSF on β_C β_{IT} Co-immunoprecipitation





5.3.10 - Effect of GM-CSF on β chain co-immunoprecipitation in haemopoietic cells

Cells from the haemopoietic cell line TF-1 were also examined for co-immunoprecipitation of the β_C and β_{IT} chains. The cells are growth factor dependent and were cultured in 5ng/ml GM-CSF. To investigate any effect of GM-CSF on co-precipitation, cells were washed to remove GM-CSF from the medium and cultured overnight in medium with or without GM-CSF present. Culture of TF-1 cells overnight in medium without GM-CSF present has been shown to upregulate expression of the GM-CSFR [Khwaja *et al*, 1994]. The cells were then immunoprecipitated using anti-β and anti-β_{IT} antibodies. Figure 5.14 shows western blots of the immunoprecipitates from the TF-1 cells probed with anti-β chain antibodies. In cells cultured in medium with GM-CSF present, the immunoprecipitate using the anti-β antibodies showed both β_C and β_{IT} chains were present when compared to the control lysates, with several breakdown products also visible. Anti-β_{IT} immunoprecipitation of TF-1 cells cultured with GM-CSF showed the expected β_{IT} chain proteins with β_C chain bands also visible. This demonstrated co-immunoprecipitation of β_C and β_{IT} chains also occurs in TF-1 cells. Immunoprecipitations of TF-1 cells which were withdrawn from GM-CSF containing medium still resulted in co-immunoprecipitation of the β_C and β_{IT} chains in the absence of GM-CSF. The western blot of the immunoprecipitates from these cells shows both chains were still present in similar levels in the anti-β immunoprecipitates and a comparable level of β_C co-immunoprecipitation was seen with the anti-β_{IT} antibodies.

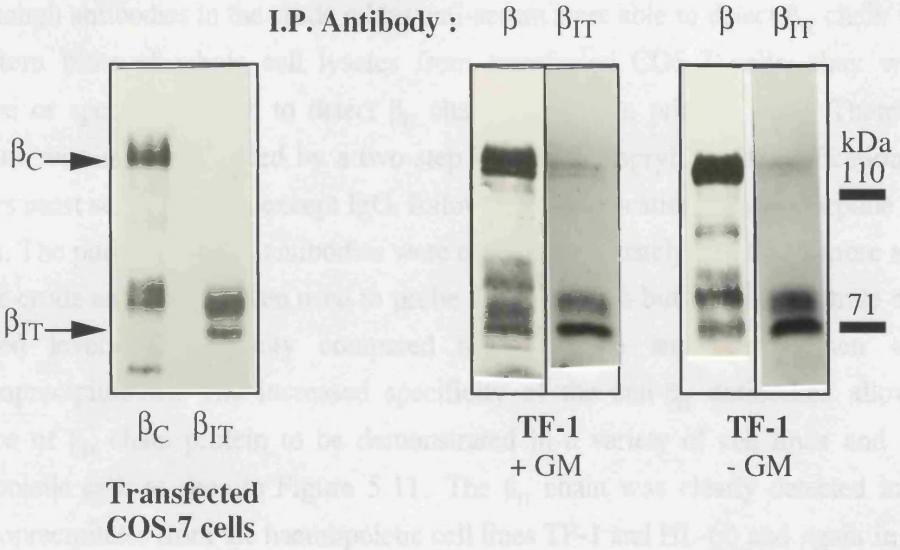
5.4 - Discussion

An mRNA transcript which codes for a truncated isoform of the GM-CSFR β chain was identified in TF-1 cells using RT-PCR. Analysis of haemopoietic cells by RNAse protection assay showed the β_{IT} transcript accounts for 10-20% of the total β chain message in haemopoietic cell lines, normal bone marrow and purified neutrophils but in AML blasts could be up to 90% of the total β chain message. It was also demonstrated in western blotting that the β_{IT} chain could be expressed as a mature protein in COS-7 cells transfected with a plasmid containing the β_{IT} coding sequence [Gale *et al*, 1993abs].

Analysis of the β_{IT} coding sequence predicts a protein of 495 amino acids with an expected size of approximately 75kDa compared to the 881 amino acids of the full length β_C chain with a mature protein of approximately 130kDa in size. Although the β_C chain protein could be seen as bands of approximately 130kDa in western blots using anti-β

Figure 5.14 - Effect of GM-CSF on Co-Immunoprecipitation of β_C β_{IT} chains in Haemopoietic cells

Western blot probed with Anti-β Antibody



antibodies, degradation products of 80-90kDa were also seen in both lysates from β_C transfected cells and in immunoprecipitates. These bands interfered with detection of β_{IT} chain proteins which migrated in the 70-80kDa region of gels. Therefore rabbit polyclonal antibodies were raised against two peptides selected from the novel 23 amino acid C-terminus of the β_{IT} chain. These peptides only differed by an additional cysteine residue in peptide 2 which was partially oxidised to give both cyclic and linear forms. A total of four anti-sera, two from rabbits immunised with peptide 1 and two from rabbits immunised with peptide 2, recognised the β_{IT} receptor chain in western blots of whole cell lysates from transfected COS-7 cells (Figure 5.6). The specificity of the antibodies was demonstrated in peptide competition experiments. Pre-incubation of the rabbit anti-sera with either of the β_{IT} peptides was sufficient to abolish binding to the β_{IT} proteins when used to probe a western blot whilst incubation of the anti-sera with a non-specific peptide had no effect (Figure 5.7).

Although antibodies in the crude rabbit anti-serum were able to detect β_{IT} chain proteins in western blots of whole cell lysates from transfected COS-7 cells, they were not sensitive or specific enough to detect β_{IT} chain proteins in primary cells. Therefore the antiserum was immunopurified by a two-step process; a caprylic acid purification, which removes most serum protein except IgG, followed by purification on a β_{IT}-peptide specific column. The purified anti-β_{IT} antibodies were only approximately five times more sensitive than the crude anti-serum when used to probe western blots but did demonstrate a greatly increased level of specificity compared to the crude anti-serum when used in immunoprecipitations. The increased specificity of the anti-β_{IT} antibodies allowed the presence of β_{IT} chain protein to be demonstrated in a variety of cell lines and primary haemopoietic cells as seen in Figure 5.11. The β_{IT} chain was clearly detected in anti-β_{IT} immunoprecipitates from the haemopoietic cell lines TF-1 and HL-60 and again in CD34+ cells cultured in growth factors for 9 days. In TF-1 cells, the β_{IT} would only be expected to be 10-20% of the total β chains present from mRNA levels but as several thousand high affinity receptors per cell have been reported for TF-1 cells, immunoprecipitaion of the β_{IT} chain proved to be within the technical limits of this study. Detection of the β_{IT} chain in neutrophils and bone marrow proved to be difficult perhaps due to the low number of β_{IT} receptor chains expected in these cells (10-20% of the total β chains), with approximately 250-1000 high affinity receptors per cell for neutrophils while studies of murine bone marrow suggest more primitive haemopoietic progenitors have less than 500 high affinity receptors per cell. The β_{IT} message is only 10-20% of the total β chain message in these populations, which combined with difficulty in purifying sufficient cell numbers, meant

only faint bands were seen for the β_{IT} chain protein in neutrophils and bone marrow. In AML blasts studies have reported approximately one hundred high affinity receptors per cell for GM-CSF and although the β_{IT} message can be up to 90% of the total β chain message, the β_{IT} protein was not clearly demonstrated in the AML samples studied [Chiba *et al*, 1990, Onetto-Potheier *et al*, 1990, Khwaja *et al*, 1994, McKinstry *et al*, 1997].

One of the most noticeable features of western blots on lysates of β_{IT} transfected COS-7 cells was the presence of at least three bands in the 70-80kDa range. The β_C protein was consistently seen as at least two bands of approximately 130kDa even though this is substantially greater than the expected 96kDa protein calculated on the basis of the β_C chain amino acid sequence alone [Ding *et al*, 1995]. The difference in molecular mass was thought to be mainly due to N-glycosylation, a co-translational modification initiated in the endoplasmic reticulum which is found in most cell surface proteins although the precise function of the carbohydrate on these proteins is not known. N-glycosylation occurs on asparagine residues in the consensus sequence Asn-X-Ser/Thr, where X is any amino acid except proline or aspartic acid. The extracellular region of the β_C chain has three such consensus sequences [Ding *et al*, 1995]. The antibiotic tunicamycin inhibits N-glycosylation but previous experiments in GM-CSFR α chain transfected COS-7 cells showed it does not affect cell surface expression of receptors [Ding *et al*, 1995]. In the present study treatment of β_C and β_{IT} transfected COS-7 cells with tunicamycin resulted in just one smaller band for both receptors as seen in Figure 5.6. These single bands are still larger than the size predicted on amino acid sequence alone and the difference in size may be due to other post-translational modifications such as O-glycosylation and phosphorylation.

The high affinity binding GM-CSFR is comprised of at least two subunits, the ligand specific GM-CSFR α chain and the β_C chain common to the IL-3 and IL-5 receptors, but the exact stoichiometry of the complex is not clear [Hayashida *et al*, 1990]. However it is unlikely only a single α and single β_C chain are involved in the final receptor complex and β_C chain dimerization is thought to be involved in initiation of downstream signalling. It has been shown using chimeric molecules expressing the extracellular GM-CSFR α chain domain and cytoplasmic β_C chain domain (α/β), that in the presence of wild type β_C chain and ligand, dimerization of the α/β + β_C chain is sufficient for receptor activation [Muto *et al*, 1995]. In one study β_C dimers were demonstrated by crosslinking irrespective of human GM-CSF stimulation and these dimers were phosphorylated only after association with the α subunit in response to ligand, suggesting the existence of preformed β chain dimers irrespective of GM-CSFR α chain or ligand [Muto *et al*, 1996]. However these

studies have used either chimeric receptors or tagged receptor chains in artificial systems to examine β_C dimerization and may not represent the situation found in primary haemopoietic cells.

In the present studies anti-β_{IT} antibodies were used to investigate the role of the β chains in the formation of the GM-CSFR. It was shown that in immunoprecipitations using anti-β_{IT} antibodies of COS-7 cells transfected with both β_C and β_{IT} chains, the β_C chain was co-immunoprecipitated with the β_{IT} chain (Figure 5.12). This was not due to cross-reactivity of the anti-β_{IT} antibodies with the β_C chain as immunoprecipitation of cells transfected with β_C chain alone with the anti-β_{IT} antibodies does not immunoprecipitate any β_C chains. The co-immunoprecipitation of the β chains does not appear to be affected by the presence of either the GM-CSFR α chain or ligand as seen in Figure 5.13, where co-immunoprecipitaion of the β_C chain occurred at a similar level for all transfections with or without GM-CSFR α chain and ligand. This suggests that in this system the β chains exist as pre-formed oligomers, most probably as dimers, in the GM-CSFR as co-transfection of GM-CSFR α chain with the β chains followed by ligand stimulation did not increase the amount of β chain oligomerization detected. Formation of both the GM-CSFR and IL-3R has been shown to involve a disulphide-linkage between the α chains and the β_C and in the case of IL-3 prevention of this disulphide-linkage has been shown to inhibit receptor phosphorylation [Stomski *et al*, 1996]. It has been demonstrated in COS-7 cells that IL-3 induced formation of IL-3R α and β_C chains in a heterodimeric complex and co-immunoprecipitation of IL-3R α chain and β_C chain only occurred in the presence of IL-3 with the ligand needing to be in contact with both receptor chains in order to trigger α and β_C association [Stomski *et al*, 1996]. Similarly in NIH3T3 cells it has been shown that GM-CSFR α and β_C only co-immunoprecipitate after GM-CSF binding [Eder *et al*, 1994]. However other experiments using mutated receptors and mutated GM-CSF analogues have suggested the existence of preformed GM-CSFR α and β_C chain heterodimers [Hercus *et al*, 1994]. In a mutated GM-CSFR α chain where the second conserved cysteine was replaced, GM-CSF was unable to bind to this receptor alone but bound with high affinity when this mutant was co-expressed with the β_C chain. Also, a mutated GM-CSF analogue which showed no detectable binding to the GM-CSFR α chain alone exhibited nearly full wild type activity in cells expressing both α and β_C chains [Hercus *et al*, 1994]. Both cases suggest that the presence of β_C chains in a preformed complex with the GM-CSFR α chains compensates for losses in the GM-CSF binding with the α chain.

The existence of β_C oligomers as demonstrated by this study and as previously published gives further support to the theory that a GM-CSFR complex is composed of

more than one ligand, one α chain and a β_C chain. A study of a GM-CSFR α chain truncation mutant showed that in NIH3T3 cells which expressed the wild type and truncated GM-CSFR α chains in approximately equal stoichiometric proportions the truncated α chain had a dominant negative effect over the wild type α chain as measured by colony formation and cell growth [Lia *et al*, 1996]. When combined with evidence from cross-linking studies this indicated that the GM-CSFR complex contains at least two α chains. Similarly it has been suggested that the existence of β_C homodimers in the absence of ligand may favour the formation of hexameric complexes necessary for receptor activation, analogous to the IL-6R system [Bagley *et al*, 1997]. Hexameric complexes have already been shown for the IL-6R consisting of two IL-6 ligands, two IL-6R α chains and two gp130 molecules by analytical centrifugation, IL-6 mutagenesis and IL-6R immunoprecipitation experiments [Ward *et al*, 1994, Paonessa *et al*, 1995]. A similar stoichiometry has been proposed for the GM-CSFR complex but has not yet been defined [Bagley *et al*, 1997].

When β chain co-immunoprecipitation was examined in the haemopoietic cell line TF-1 the β_C chain was again seen in immunoprecipitates using anti-β_{IT} antibodies and the level of co-immunoprecipitation did not vary considerably in the presence or absence of GM-CSF. These experiments demonstrate the β chains exist as preformed oligomers in primary haemopoietic cells and that this homodimerization does not require the presence of ligand. These results agree with published data which has suggested homodimerization of the β_C chain in absence of ligand in haemopoietic cells [Stomski *et al*, 1996, Muto *et al*, 1996, Bagley *et al*, 1997]. Also the existence of covalently linked β_C homodimers in the absence of ligand in primary cells (leukaemic cells) has been reported and these β_C dimers were not phosphorylated and phosphotyrosine reactivity was only seen when ligand was added to these cells [Bagley *et al*, 1997]. It was not possible to show any β chain protein in the AML blast cells examined in the present study. This was thought to be due to the technical difficulties associated with the low GM-CSFR numbers expected in these cells.

It has been suggested from experiments using chimeric receptors that under certain conditions dimerization of the β_C chain was sufficient for receptor activation and downstream signalling [Muto *et al*, 1995, Shikama *et al*, 1996]. This is in contrast to the evidence from experiments using truncated mutants which demonstrated that cytoplasmic domain of the α chain, especially 29 amino acids near the transmembrane region, were absolutely required for various signalling events by the GM-CSFR including cell growth, although this region is not required for reconstitution of a high affinity GM-CSFR [Sakamaki *et al*, 1992, Weiss *et al*, 1993, Muto *et al*, 1995]. This seems to suggest that

under normal conditions the α chain is required to activate the receptor in addition to binding ligand [Bagley *et al*, 1997].

Although no other naturally occurring isoforms of the β_C chain have been described, a series of C-terminal truncated mutants have been created and extensively studied as reviewed previously in Chapter 1 (section 1.5.7). The intracytoplasmic tail of the β_{IT} chain is 46 amino acids long, 23 of which are identical to the first 23 membrane proximal amino acids of the β_C chain. This region contains the highly conserved Box 1 sequence found in other cytokine receptors including gp130, G-CSFR, EpoR and IL-2Rβ [Murakami *et al*, 1991]. Studies of the β_C deletion mutants demonstrated that the Box 1 containing region was essential for proliferation and is also required for the binding and activation of JAK 2 [Quelle *et al*, 1994]. The β_{IT} chain lacks the Box 2 containing region which, although not essential for proliferation, may enhance the proliferative response to GM-CSF. In addition the β_{IT} chain obviously lacks the membrane distal regions of the β_C intracytoplasmic tail responsible for activation of the Ras pathway and also the major sites of β_C chain tyrosine phosphorylation [Itoh *et al*, 1996]. Although the new 23 amino acid sequence of the β_{IT} chain intracytoplasmic tail contains a serine and a threonine it does not contain any known consensus binding sequences.

As reviewed earlier in Chapter 1 (section 1.8.2) several mutations which lead to truncated G-CSFRs lacking 83 to 93 C-terminal amino acids have been reported in patients with SCN [Dong *et al*, 1994, Dong *et al*, 1995, Dong *et al*, 1997]. All receptors still have the Box 1 and Box 2 containing sequences necessary for proliferation but lack the Box 3 domain thought to be involved in differentiation [Fukunaga *et al*, 1993, Dong *et al*, 1993]. When one of these truncated receptors, the result of a nonsense mutation, was transfected into murine myeloid cells the mutant receptor transduced a strong growth signal but in contrast to the wild type G-CSFR was defective in maturation signal and it was suggested by the authors that the truncated receptor may act in a dominant negative manner to block granulocytic maturation [Dong *et al*, 1994] Similarly several EpoRs with C-terminal truncations have been found in patients with PFCP. In each case loss of 64-74 amino acids at the C-terminal gave receptors which had a hypersensitive response to Epo, thought to be due to loss of a phosphatase binding region [De la Chapelle *et al*, 1993, Sokol *et al*, 1995, Klingmuller *et al*, 1995]. A truncated EpoR (EpoR-T) which has only 56 amino acids in the intracytoplasmic domain has been isolated from human bone marrow cells [Nakamura *et al*, 1992]. The EpoR-T was predominantly expressed over the full length EpoR (EpoR-F) in immature progenitor cells while the EpoR-F was predominant in late stage progenitors [Nakamura *et al*, 1992]. Transfection experiments in Ba/F3 cells

revealed no difference in binding affinity to Epo between the two receptors and showed that both the EpoR-T and EpoR-F were capable of transducing mitotic signals. Ba/F3 cells transfected with the EpoR-T were more prone to apoptose in culture and when co-transfected with the EpoR-F exhibited a dominant negative effect, probably by the formation of EpoR-F/EpoR-T heterodimers [Nakamura *et al*, 1994].The discovery of a similar EpoR truncated in the mouse suggest that the EpoR-T may be important in the regulation of erythropoiesis [Nakamura *et al*, 1994].

The exact role the β_{IT} chain plays in haemopoiesis remains unclear as it does not appear able to support proliferation when stably co-transfected with the GM-CSFR α chain in CTLL-2 cells [Gale *et al*, 1993abs]. Evidence from experiments in the present study suggests that the formation of β_{IT}/β_C dimers occurs in primary haemopoietic cells (TF-1). Such interaction of the β chains could have several implications. The β_{IT} chain may have a dominant negative effect on β_C signalling as seen with the EpoR-T by heterodimerization with the wild type receptor or the β_{IT} chain may act to block differentiation.

CHAPTER 6

Conclusions

Genetic alterations are thought to play a central role in the development of cancer, with tumour formation believed to be a multi-step process involving several genetic mutations [Bishop *et al*, 1987]. The leukaemias are stem cell disorders and are characterised by a malignant neoplastic proliferation and the accumulation of immature haematopoietic cells in the bone marrow [Russell *et al*, 1992, Lowenberg *et al*, 1993] and it is possible that mutations in cytokine receptors could be one of the hits in the multi-step pathogenesis of leukaemia. Receptors constitutively activated by artificially created point mutations have been reported for several cytokines including the TpoR and EpoR [Yoshimiura *et al*, 1990, Alexander *et al*, 1995, Onishi *et al*, 1996]. In each case a point mutation in the extracellular or transmembrane region of the receptor was thought to induce constitutive activation by mimicking ligand-induced dimerization of receptor subunits.

Studies presented in this thesis used the PCR based technique of single strand conformation polymorphism analysis (SSCP) to screen the genes of the GM-CSFR subunits for such mutations. Chapter 3 reports the results from analysis of the intracytoplasmic tail and transmembrane region of the GM-CSFR β chain in patients with AML. This region was studied as it is believed to be responsible for initiating most of the downstream signalling of the GM-CSFR and contains sequences conserved throughout the cytokine receptor family such as the box 1 and box 2 containing regions, the transmembrane domain and the highly conserved WSXWS box. A total of six nucleotide substitutions were identified in the 35 AML patients and 10 normal controls studied. Three of the substitutions were silent and did not alter the amino acids involved at Ser^{426}, Pro^{648} and Pro^{800}. Three substitutions which altered amino acids at Gly^{647}->Val, Val^{652}->Met, and Pro^{603}->Thr were also found but were thought to be polymorphisms, as all were detected in samples from haematologically normal controls, while the Val^{652}->Met substitution results in a methionine at amino acid position 652, which is the same amino acid as found at this position in both mouse β chains, AIC2A and AIC2B [Hayashida *et al*, 1990]. The frequency of polymorphisms was quite high, with one of the silent mutations (Pro^{648}) detected in over 35% of the samples studied and this high incidence may in part be due to methylation related substitutions. Four of the six base substitutions detected involved a C to T (or G to A) transition in a so called CpG doublet. These sequences are known hotspots of mutation in the genome, where methylation of the cytosine followed by spontaneous deamination can lead to a C to T substitution. Over 1/3 of all point mutations giving rise to human genetic disease are caused by similar substitutions and the high GC content (63%) of the β chain may partially explain the number of polymorphisms detected [Bird *et al*, 1992]. Similar studies of the GM-CSFR α chain coding sequence in patients

with AML have shown that this gene is highly polymorphic, although they also failed to find any mutations which might be pathogenic [Wagner *et al*, 1994, Decker *et al*, 1995].

SSCP proved to be a very reliable technique for this study, with consistent mobility shifts and good reproducibility seen for each of the PCR fragments. This technique was also sensitive, with previous studies demonstrating that SSCP analysis could detect as little as 6% abnormal template in a sample [Wagner *et al*, 1994]. The abnormal SSCP patterns were consistent for each polymorphism and no 'false' abnormal SSCP patterns were seen, as sequencing of products from each patient with an abnormal SSCP pattern revealed the presence of at least one substitution. Therefore, SSCP analysis was a reasonably efficient method for the identification of abnormalities in AML patients, and although it is possible some mutations may not have been detected, it appears that point mutations of the transmembrane and intracytoplasmic regions of the GM-CSFR β chain are not a major contributing factor in the pathogenesis of AML.

The failure to find any pathogenic mutations in the AML patients studied was disappointing but is consistent with other studies. Artificially created activating mutations due to point mutations or more major structural alterations have been described for several cytokine receptors but studies to identify mutations in cytokine receptors involved in malignant haemopoietic disease have been largely disappointing. Mutations which lead to truncations of the C-terminus and hypersensitivity to Epo have been identified in the EpoR in patients with primary familial and congenital polycythemia (PFCP) but other studies of the EpoR in patients with erythroleukaemia or sporadic polycythemia have failed to reveal any somatic mutations proven to be pathogenic [De la Chapelle *et al*, 1993, Sokol *et al*, 1994, Le Couedic *et al*, 1996]. Similarly studies of the G-CSFR in de novo AML patients have shown only 2 mutations likely to be involved in the pathogenesis of AML [Bernard *et al*, 1996, Dong *et al*, 1997]. One study showed that a novel isoform of the G-CSFR, which has an altered C-terminus downstream of the box 2 region and is unable to transduce a proliferative signal in transfected Ba/F3 cells, was significantly elevated in the cells from an AML patient and it was suggested this may result in altered signalling properties [Dong *et al*, 1995]. This patient was shown to have a mutation in a splice donor site of the G-CSFR gene which may have led to increased splicing efficiency for that site [Dong *et al*, 1995]. Another study reported an AML patient found to have a threonine to asparagine amino acid substitution in the transmembrane region of the G-CSFR [Bernard *et al*, 1996]. Although originally thought to represent a polymorphism this alteration does show homology to an artificially created mutation in the transmembrane region of the TpoR which was shown to confer factor-independent growth in transfected IL-3 dependent cell lines [Onishi *et al*, 1996]

An activating mutation of the GM-CSFR β chain has been described in the transmembrane region of the receptor and similar homologous mutations have been reported in the TpoR and also in the Neu receptor tyrosine kinase [Jenkins *et al*, 1995, Onishi *et al*, 1996]. As this region of the GM-CSFR β chain was screened in the 35 AML patients of this study with no functional abnormalities detected, it seems unlikely that such mutations are commonly involved in the pathogenesis of the disease. However other point mutations in the extracellular domain of the GM-CSFR β chain have been shown to confer ligand-independent growth and it is still possible that point mutations in the extracellular region of the β chain may contribute to the pathogenesis of AML. Further analysis is required to discover if pathogenic mutations are present in the extracellular region of the GM-CSFR β chain of patients with AML.

RNA from six patients with the rare paediatric disorder JCML was screened for mutations in the coding sequence of both the α and β chain of the GM-CSFR using SSCP analysis and the results are presented in Chapter 4. The receptor sequence was analysed in these patients as studies of peripheral blood granulocyte-macrophage progenitors (CFU-GM) from patients with JCML have provided evidence for an abnormal response to GM-CSF stimulation. It has been demonstrated *in vitro* that CFU-GM from JCML patients are able to proliferate spontaneously at low cell density in the absence of exogenous growth factors, but prior depletion of monocytes from the culture abolishes this spontaneous proliferation suggesting a role for endogenously produced growth factors [Gualtieri *et al*, 1989, Emanuel *et al*, 1991]. Addition of GM-CSF, TNFα or IL-1 to JCML progenitor cells is sufficient to stimulate proliferation *in vitro*. However, JCML progenitors demonstrate approximately a ten fold hypersensitive response to GM-CSF *in vitro* as measured by colony growth of CFU-GM and BFU-E [Emanuel *et al*, 1991]. Binding studies have shown apparently normal GM-CSFR number and affinity on JCML cells, leading to suggestions that mutations of the GM-CSFR may play a role in the dysregulation of the GM-CSF pathway seen in JCML [Emanuel *et al*, 1991, Pui *et al*, 1995, Hess *et al*, 1996].

Two nucleotide substitutions, one silent for Val^{333} and the other a Ala^{17}->Gly, were identified in the GM-CSFR α chain of the 6 JCML patients studied. In the β chain, four base substitutions were found, two silent for the Pro^{648} and Ser^{426} involved and two which altered the amino acid at Pro^{603}->Thr and Glu^{249}->Gln. All substitutions, except for the Glu^{249} substitution in the extracellular region of the β chain, had previously been detected in haematologically normal controls as presented in Chapter 3 or in a previous study of the GM-CSFR α chain and they were thought to represent non-pathological polymorphisms of the coding sequence [Wagner *et al*, 1994]. The one substitution found in this study

which had not been previously described involved the substitution of a glutamic acid with a glutamine residue at position 249 of the amino acid sequence. However the presence of a glutamine at this position in both mouse β chains, which are highly homologous to the human GM-CSFR β chain, suggests this alteration is not of functional significance. Although only a small number of samples were analysed in this study, mutations of the GM-CSFR do not appear to be a common feature of JCML.

The data in Chapter 4 suggests that mutations of the receptor are not responsible for the hypersensitivity to GM-CSF seen in JCML progenitors. However, it is possible that dysregulation of signalling molecules downstream of the GM-CSFR may be involved in the hypersensitive response. A similar ten fold hypersensitivity to GM-CSF has been demonstrated in fetal haemopoietic cells from mice with a homozygous null mutation in the NF-1 gene, a GTPase activating protein which negatively regulates Ras [Largespada *et al*, 1996]. In total, mutations in either Ras or NF-1 are found in approximately 40% of patients with JCML and examination of other signalling molecules of the Ras pathway may reveal further mutations [Brodeur *et al*, 1994, Bollag *et al*, 1996, Ihle, 1996]. An obvious molecule for study is p120-GAP, which is similar in function to NF-1, as it is the other known human GTPase activating protein involved in negative regulation of Ras [Trahey *et al*, 1987]. Other potential molecules in which mutations may be involved in the hypersensitive response of JCML progenitors to GM-CSF include son of sevenless (Sos) and Ras-GRF (Ras-guanine nucleotide release factor) which are both thought to activate Ras through promotion of guanine nucleotide exchange on the Ras protein [Feig, 1993]

Another possible group of molecules for study are haemopoietic cell phosphatases and associated substrate molecules. Mice homozygous for the moth-eaten mutation have a functional loss of SHP-1 and die from pulmonary accumulation of macrophages. The macrophages have an enhanced proliferative response to GM-CSF, suggesting that SHP-1 plays a critical role in down-regulation of GM-CSFR signalling in these macrophages [Jiao *et al*, 1996Abs]. It was also demonstrated that in macrophages from the moth-eaten mutant, GM-CSF induced phosphorylation of the β_C chain, Jak 2, STAT 5 and MAPK did not differ significantly from that seen in normal controls, indicating these molecules are not major SHP-1 substrates in GM-CSF signalling [Jiao *et al*, 1996Abs]. It was therefore suggested that SHP-1 plays a role in the hypersensitivity to GM-CSF of macrophages from the moth-eaten mutant and that SHP-1 may dephosphorylate novel substrates critical in GM-CSF signalling [Jiao *et al*, 1996Abs].

Analysis of mRNA from AML samples revealed the presence of an alternatively spliced isoform of the β chain common (β_C) to the GM-CSF, IL-3 and IL-5 receptors which codes for a receptor chain with a truncated intracytoplasmic domain of just 46 amino acids

compared to the 432 of the β_C chain. Analysis of mRNA levels by RNAse protection assay showed that this isoform accounts for approximately 10-20% of the total β chain message in haemopoietic cell lines, normal bone marrow and neutrophils but can be up to 90% of the total β chain message in patients with AML [Gale *et al*, 1993]. Referred to as the β_{IT} chain, the first 23 amino acids proximal to the transmembrane region are homologous to the β_C chain followed by a novel 23 amino acid sequence before a premature stop codon.

To demonstrate the presence of the β_{IT} chain protein in primary haemopoietic cells, polyclonal anti-peptide antibodies specific to the novel 23 amino acid sequence of the β_{IT} were raised. Although specific recognition of the β_{IT} chain was demonstrated using anti-serum from four of six immunised animals, attempts to show the β_{IT} chain protein in primary cells using the anti-sera were unsuccessful. However, following immunopurification of the anti-sera, the β_{IT} chain protein was demonstrated in a variety of primary haemopoietic cells including the cell lines TF-1 and HL-60 and also in neutrophils, normal bone marrow and cultured peripheral blood progenitor cells. It proved much harder than anticipated to demonstrate the β_{IT} chain protein in blast cells from AML patients considering that in some patients β_{IT} mRNA is up to 90% of the total β message. However the low β chain numbers in blast cells, with approximately 100 high affinity receptors per cell [Onnetto-Pothier *et al*, 1990], means that in this heterogeneous population of cells even if the β_{IT} chain was 50% of all the β chains present, the antibodies would be trying to detect approximately 50 β_{IT} chains per cell and this proved beyond the technical limitations of this study.

Several studies have described data which suggests the GM-CSFR complex is composed of at least two β chains and possibly two α chains [Jenkins *et al*, 1995, Stomski *et al*, 1996, Muto *et al*, 1996, Lia *et al*, 1996]. Crosslinking experiments have shown the presence of β_C homodimers irrespective of GM-CSF which were only phosphorylated following GM-CSFR α chain association and ligand stimulation [Muto *et al*, 1996]. It has also been suggested that the β_C chain can homodimerize in haemopoietic cells in the absence of ligand [Bagley *et al*, 1997]. However, these studies have used artificially created systems to look for β chain dimerization. In Chapter 5, a COS-7 transfection system was used to optimise experimental conditions before examination of receptor chain oligomerization in primary haemopoietic cells. Co-precipitation of the β_C and β_{IT} chains in co-transfected COS-7 cells was shown using β_{IT}-specific antibodies and this co-precipitation did not require the presence of GM-CSFR α chains or ligand. Similarly in TF-1 cells co-precipitation of the β_C and β_{IT} chains was shown and again this oligmerization did not appear to be ligand-dependent. Results from Chapter 5 provide further evidence of preformed β oligomers, probably as homodimers, but importantly have

shown the presence of preformed β chain oligomers in primary cells using antibodies to a naturally occurring isoform of the receptor chain. Whether β chain dimers and GM-CSFR α chains exist in a preformed complex prior to ligand stimulation remains unclear. Studies to examine co-precipitation of the GM-CSFR α chain with either or both of the β chains, combined with the effect of ligand stimulation on any co-precipitation, may help resolve these issues.

Scatchard analysis of COS-7 cells co-transfected with the GM-CSFR α chain and β_{IT} chain has demonstrated that β_{IT} is able to form a high affinity binding receptor complex [Gale *et al*, 1993]. However the exact role of the β_{IT} chain remains unclear. The first 23 amino acids of the β_{IT} chain intracytoplasmic tail are identical to those of the β_C including the box 1 sequence essential for Jak 2 phosphorylation, while the novel 23 amino acid sequence of the β_{IT} chain contains no known consensus binding sequences. The β_{IT} chain does not appear able to support proliferation in response to GM-CSF, as shown when co-transfected with the GM-CSFR α chain into CTLL cells [Gale *et al*, 1993]. A similar intracytoplasmic truncated receptor has been described for the EpoR which exhibited a dominant negative effect when co-transfected with the full length EpoR in Ba/F3 cells, while a C-terminal truncated GM-CSFR α chain has been shown to have a dominant negative effect in NIH3T3 cells when co-transfected with the wild type receptor [Nakamura *et al*, 1992, Lia *et al*, 1996].

Future work is required to elucidate the role of the β_{IT} chain in haemopoiesis and whether this role varies with the differentiation and maturation changes that occur as cells progress along the haemopoietic pathway. Interestingly it was observed in COS-7 cells that even though equal amounts of plasmid DNA were transfected for both the β_C and β_{IT} chains, levels of β_{IT} protein expression were consistently higher compared to the levels of β_C chain protein. Whether this was a cell specific effect or whether the β_{IT} chain was preferentially translated remains to be seen but comparisons of β_{IT} mRNA and protein levels in other cells may help explain the differences seen.

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Publications from work presented in this thesis:

Freeburn RW, Gale RE, Wagner HM, Linch DC. (1997) - Analysis of the coding sequence for the GM-CSF receptor α and β chains in patients with juvenile chronic myeloid leukaemia (JCML). *Experimental Haematology* 25:306-311.

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