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**Structural and Functional Analysis of Interleukin-1 F5, a Novel
Member of the Interleukin-1 Family.**

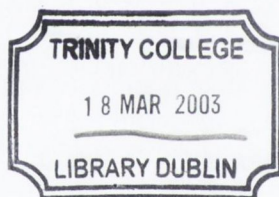
A thesis submitted to the University of Dublin for the Degree of Doctorate of
Philosophy

By

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THESIS
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Declaration

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Abstract

This study is an investigation into novel members of the Interleukin-1 (IL-1) family; IL-1F5 and IL-1F6. The classical members of the IL-1 family, including IL-1 α , IL-1 β and IL-18, are involved in immune responses to infection and injury, which they control by activating signalling pathways leading to NF κ B and MAP kinase activation. IL-1F5 has highest sequence identity to IL-1 receptor antagonist (IL-1Ra), a natural antagonist of the IL-1 system, with 44% identity at the amino acid level based on structural alignment derived from crystallographic data. IL-1F6, on the other hand, is 30% identical to IL-1Ra at the amino acid level. Both proteins are predicted to fold in a manner similar to IL-1 by forming a β -trefoil structure. However, the functions of the proteins are still uncertain. The sequence homology of IL-1F5 to IL-1Ra has been used to predict an antagonistic role for this molecule but detailed sequence analysis suggests that it does not have the features that have been shown to determine IL-1Ra's antagonistic activity.

Cloning and purification of murine IL-1F5 allowed functional and structural studies to be carried out. Functional assays, in terms of activation or inhibition of IL-1-like signal transduction pathways, have indicated that IL-1F5, as well as IL-1F6, is unable to act as novel IL-1 antagonists and is also unable to act as agonists in a range of tested cell types.

Presented here is a determination of the crystal structure of IL-1F5 using molecular replacement to a resolution of 1.6 Å. IL-1F5 has the same β -trefoil fold as the other determined IL-1 structures and the hydrophobic core is well conserved but it varies considerably in loop regions, which have been identified as receptor binding regions in IL-1 β and IL-1Ra. The structure shows that these loop regions are well ordered by extensive hydrogen bonding networks and that their unique conformations are likely to confer receptor specificity to the IL-1F5 ligand. The loop structures and electrostatic potential surface map also suggest that IL-1F5 could act as an agonist capable of recruiting an accessory protein rather than as an antagonist.

Abbreviations

AMoRe	A Molecular Replacement Program
ATP	Adenosine triphosphate
AP-1	Activator protein 1
bp	base pair
BSA	Bovine serum albumin
CCP4	Collaborative Computing Project 4
CD	Circular dichroism
CNS	Crystallographic and NMR system
DD	Death domain
DMSO	Dimethyl sulphoxide
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular regulated kinase
EST	Expressed sequence tag
FACS	Fluorescent activated cell sorter
FCS	Fetal calf serum
FGF	Fibroblast growth factor
HEPES	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid)
ICE	Interleukin-1 β converting enzyme
Ig	Immunoglobulin
I κ B	Inhibitor of NF κ B
IKK	I κ B kinase
IL	Interleukin
IL-1Ra	Interleukin-1 receptor antagonist
IL-1RAcP	Interleukin-1 receptor accessory protein
IL-1RAcPL	Interleukin-1 receptor accessory protein like
IL-1RI	Type I interleukin-1 receptor
IL-1RII	Type II interleukin-1 receptor
IL-1Rrp1/2	Interleukin-1 receptor related protein 1/2

IL-18BP	IL-18 binding protein
IPTG	Isopropyl- β -D-thiogalactopyranoside
IP ₃	Inositol triphosphate
IRAK	Interleukin-1 receptor associated kinase
JNK	c-Jun N terminal kinase
LB broth	Luria-Bertani broth
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
MAPK	Mitogen activated kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
MKK	MAPK kinase
MEK	<u>M</u> itogen activated/ <u>e</u> xtracellular regulated kinase <u>k</u> inase
MEKK	MEK kinase
MyD88	Myeloid differentiation primary response protein 88
NEMO	NF κ B essential modulator
NF κ B	Nuclear factor κ B
NIK	NF κ B inducing kinase
NMR	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDB	Protein data bank
PI3K	Phosphatidylinositol 3 kinase
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulphonylfouride
PVDF	Poly(vinylidene fluoride)
RT-PCR	Reverse transcriptase PCR
RMS	Root mean square
RMSD	RMS distance
SDS	Sodium dodecylsulphate
SIGGIR	Single immunoglobulin IL-1R related protein

sIL-1Ra	secreted IL-1Ra
STAT	Signal transducer and activator of transcription
TAK	Transforming growth factor β activated kinase
TAB	TAK-1 binding protein
TBE	Tris borate EDTA
TEMED	Tetramethyl ethylene diamine
TLR	Toll like receptor
TIR domain	Toll/IL-1 receptor domain
TNF	Tumour necrosis factor
Tollip	Toll interacting protein
TRAF	TNF-receptor associated factor

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Chapter 1. Introduction

1.1 Interleukin-1

Interleukin 1 (IL-1) is a cytokine that is produced by, and acts on, many different cell types (Dinarello, 1996). It is classed as pro-inflammatory along with other cytokines such as tumour necrosis factor (TNF) (Beutler and Cerami, 1989) and interleukin-6 (IL-6) (Akira et al., 1990). It up-regulates the expression of many genes important in the initiation and development of the inflammatory state and other immune processes (reviewed in Dinarello, 1996). As such, IL-1 is critical in the management of host defence and important for maintaining the health of the organism. However, the potency of the cytokine is such that any imbalance in its functions can lead to a disease state. Indeed, IL-1 has been implicated in the development of many diseases including rheumatoid arthritis, inflammatory bowel disease, coeliac disease, septic shock, Alzheimer's disease and Parkinson's disease. Therefore, the understanding of the mechanism of IL-1 signalling and its regulation is a major area of investigation.

1.2 The interleukin-1 family

The interleukin-1 family of cytokines now consists of ten members. The classical members of the family, IL-1 α , IL-1 β , IL-1Ra and IL-18, which are also termed IL-1F1 to IL-1F4, have recently been joined by six novel members; IL-1F5 to IL-1F10 (reviewed in Dunn et al., 2001). They all share sequence identity and are all predicted to fold in a similar manner to form a β -trefoil fold. The well characterised members of the family, IL-1 α , IL-1 β and IL-18 also share common signalling pathways and bind proteins from the same receptor family. Little is yet known about any of the novel family members.

1.2.1 IL-1 α and IL-1 β

IL-1 α and IL-1 β were first cloned in the mid 1980's (Auron et al., 1984; March et al., 1985). Both IL-1 α and IL-1 β have identical properties *in vitro*, i.e. they can activate the same signals and both bind to the type I IL-1 receptor (IL-1RI) with similar affinities (Sims and Dower, 1994). Similarly, they produce identical effects when administered systemically in terms of production of fever and induction of acute phase proteins (Boraschi et al., 1990). The effects of IL-1 are wide ranging and include effects on the development of fever, invasion of infected tissue with macrophages and monocytes, bone metabolism and cartilage destruction (reviewed in Dinarello, 1996). These effects are mediated through changes in gene expression in target cells either through increased transcription or through stabilisation of mRNA (Table 1.2.1). So, for example, upon infection, IL-1 will up-regulate itself and other cytokines such as TNF, pro-inflammatory mediators such as inducible nitric oxide synthase (iNOS), cyclo-oxygenase-2 (COX-2) and adhesion molecules, which all work in concert to initiate and maintain the inflammatory response. Meanwhile it will down-regulate genes such as albumin and alkaline phosphatase that are not required for the inflammatory response (Dinarello, 1996). In a negative feedback response IL-1 will up-regulate genes such as IL-1Ra (Krzesicki et al., 1993) and IL-10 (Foey et al., 1998) to inhibit the inflammatory response and in this way help the system to return to homeostasis.

However, subtle differences in the regulation of IL-1 α and IL-1 β , in terms of receptor affinities, cellular localisation and sites and regulation of expression, may allow for divergence of functions *in vivo*. Indeed, there are reports that show divergence of function between IL-1 α and IL-1 β in terms of nerve growth factor secretion from astrocytes (Juric and Carman-Krzan, 2001), co-mitogenic effects on Schwann cells (Lisak et al., 1994) and Leydig cell function (Calkins et al., 1990). In addition, knockout studies indicate that IL-1 β but not IL-1 α is required for T-cell dependent antibody production (Nakae et al., 2001a) and similarly IL-1 β , but not IL-1 α is required for fever development (Alcami and Smith, 1992; Horai et al., 1998). IL-1 α however, has been shown to be essential to T-cell

Table 1.2.1 Increased Expression of Various genes by IL-1 β .

(After Dinarello, 1996)

Cytokines: IL-1α, IL-1β, IL-1Ra, TNF, IL-2, IL-3, IL-6, IL-12, GM-CSF, TGFβ-3, G-CSF, m-CSF, stem cell factor, LIF, IFNα,β,γ, IL-8, MIP-1α. Cytokine receptors: IL-2, IL-3, IL-5, GM-CSF.	Tissue remodeling: Stromelysin, gelatinase, elastase, collagenases, tissue inhibitor of metalloproteinases.
Pro-inflammatory mediators: COX-2, PLA₂-2, iNOS, endothelin-1, γglutamyl transferase.	Adhesion molecules: ICAM-1, ELAM, VCAM-1, Lymphocyte L-selectin, E-selectin.
Hepatic acute phase proteins (APP): Mn superoxide dismutase, C reactive proetin, serum amyloid A, complement C2, C3 factor B, metallothioneins, lysozymes.	Extracellular matrix: Aortic smooth muscle decorin, collagen type IV, laminin B1, B2.
Growth Factors: PDGF ,FGF, Keratinocyte GF, Hepatocyte GF, NGF, IGF-1.	
Clotting Factors: Fibrinogen, tissue factor, Urokinase plasminogen activator, plasminogen activtor inhibitor, protease nexin-1.	

priming in contact hypersensitivity responses, unlike IL-1 β (Nakae et al., 2001b). In this text the term IL-1 is used where the two may be used interchangeably.

Both IL-1 α and IL-1 β are made as precursor molecules of 31 kDa. Unlike pro-IL-1 β , pro-IL-1 α is biologically active and since it lacks a leader sequence it is retained largely in the cytoplasm and little is released into the circulation (Mosley et al., 1987). IL-1 α can be released from cells, but with a delayed kinetics as compared with IL-1 β (Hazuda et al., 1988). Upon release from the cell, the precursor molecule can become cleaved by extracellular proteases (Kobayashi et al., 1990). About 10%-15% of IL-1 α can become myristoylated (Stevenson et al., 1993) and associate with the membrane to become what is termed 'membrane IL-1' (Kurt-Jones et al., 1985).

IL-1 β , on the other hand, is readily and rapidly exported from the cells, although it does not contain a recognised signal sequence in its amino acid sequence (Hazuda et al., 1988). The mechanism of secretion may involve packaging of IL-1 β into specialised vesicles and subsequent ATP-driven exocytosis (Andrei et al., 1999; Rubartelli et al., 1990). The proform of IL-1 β is cleaved by caspase-1 (formerly ICE) to form mature IL-1 β , which is biologically active (Black et al., 1988).

1.2.2 IL-1 Receptor Antagonist

IL-1 Receptor Antagonist (IL-1Ra) is the third member of the IL-1 family (reviewed in Arend et al., 1998). It is structurally similar to IL-1 α and IL-1 β , and can bind IL-1RI with near equal affinity (Dripps et al., 1991a), but it is incapable of inducing intracellular responses (Hannum et al., 1990). As such this molecule acts as a true antagonist, competitively blocking IL-1 from binding its receptor. Four isoforms of the molecule have been identified which result from differential splicing and use of alternative translational start sites on the mRNA (Figure 1.2.1). Secreted IL-1Ra (sIL-1Ra), which was the first isoform identified (Carter et al., 1990), is synthesised with a leader sequence which targets it for secretion. Meanwhile the 18 kDa type I intracellular IL-1Ra (type I

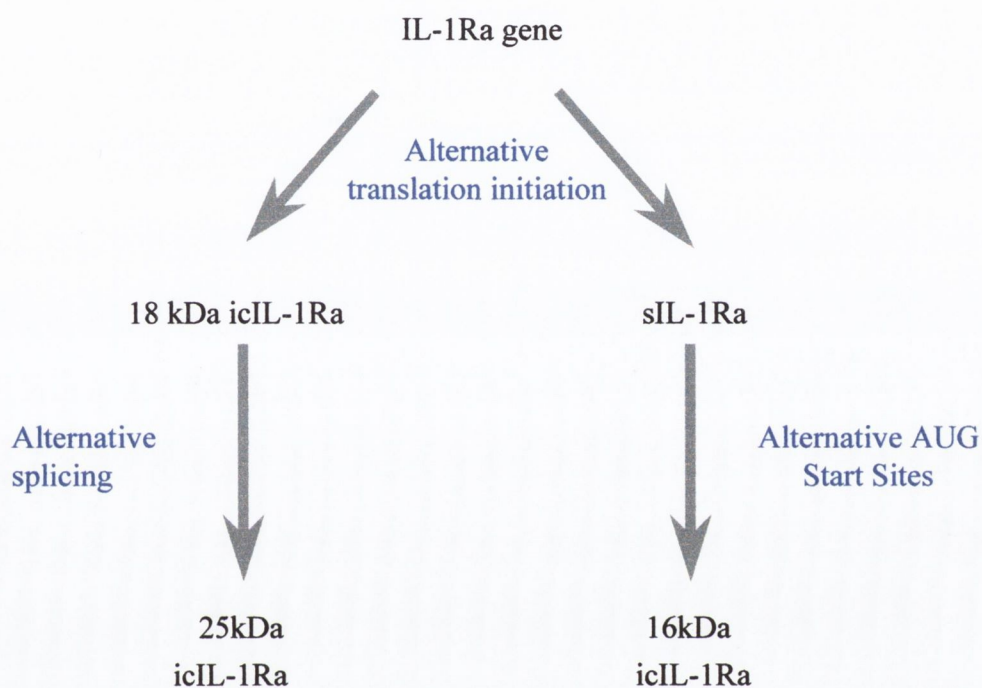


Figure 1.2.1 Alternative forms of IL-1Ra produced from the *IL-1RA* gene. The *IL-1RA* gene is known to produce four different isoforms; secreted and intracellular forms are produced by use of alternative first exons of *IL-1RA*, while use of alternative transcriptional start site within the first exon of sIL-1RA can lead to the production of a 16 kDa icIL-1Ra. Alternative splicing of icIL-1Ra produces a 25 kDa type II icIL-1Ra, which has a unique insertion in its N-terminal domain.

icIL-1Ra) is transcribed from an alternative first exon and the internal splice acceptor site of this isoform is located in the first exon of sIL-1Ra, near the 3' end of the leader sequence and thus it lacks a complete leader sequence and therefore is not secreted from the cell (Butcher et al., 1994; Haskill et al., 1991). A low molecular weight, 16 kDa icIL-1Ra has also been identified, which is thought to arise from the use of alternative translational initiation sites of sIL-1Ra (Malyak et al., 1998). Also, a 25kDa splice variant of icIL-1Ra has been identified and is known as type II icIL-1Ra (Muzio et al., 1995). This larger form of icIL-1Ra has a unique 21 amino acid insertion in its N-terminal domain that is encoded by a separate exon downstream of exon 1 (Muzio et al., 1999). sIL-1Ra can be produced from any cell type capable of synthesising IL-1 (Arend et al., 1998). It is produced in response to IL-1, IL-6, GM-CSF, IL-4 as well as acute phase proteins (APP) and lipopolysaccharide (LPS) (Table 1.2.2) (reviewed in Arend et al., 1998). Production of the 18 kDa icIL-1Ra, however, is more restricted compared with sIL-1Ra with constitutive expression in epithelial cells (Gabay et al., 1997) and delayed expression in LPS stimulated monocytes and macrophages (Malyak et al., 1998). Its expression has been shown to be unable to inhibit exogenous signalling by IL-1 β (Watson et al., 1995). It is likely that icIL-Ra acts as an internal reservoir of IL-1Ra and that its release upon cell death may serve to limit pro-inflammatory responses to cellular debris (Muzio et al., 1999). The ability of IL-1Ra to inhibit IL-1 activity is important to prevent the development of disease state in which IL-1 becomes chronically active. As such it has been tested in the clinical environment for the treatment of diseases such as arthritis and septic shock. The degree of success however, depends on the disease treated, with most efficacy achieved in the reduction of joint destruction in rheumatoid arthritis but little efficacy achieved against septic shock (Freeman and Buchman, 2001).

1.2.3 IL-18

Interleukin 18 (IL-18) was first identified by its ability to induce interferon γ (IFN γ) and was originally called interferon γ inducing factor (IGIF) (Okamura et al., 1995). It has low sequence identity to IL-1 α and IL-1 β (12% and 19% respectively) but its structure is predicted to be the same (Bazan et al., 1996). It is however the only member of the family

Cytokines	Other stimuli
GM-CSF	Adherent IgG
M-CSF	LPS
G-CSF	APP
IL-1	Products of human CMV early genes
IL-2	Soluble CD23 (with IL-1)
IL-3	IgA
IL-4	IgD
IL-6	Th2 cells (direct contact)
IL-10	Fibronectin
IL-13	β -glucan
TGF- β	
IFN- α	
IFN- γ	

Table 1.2.2 Induction of IL-1Ra production in human monocytes. Factors which have been shown to cause induction of sIL-1Ra are listed (After Arend et al., 1998).

not to map to chromosome 2 of the human genome (Nolan et al., 1998). Like IL-1, it is produced as pro-IL-18 and is activated by enzymatic cleavage by caspase-1, the same enzyme that activates IL-1 β (Pirhonen et al., 1999). IL-18 also shares similar signalling pathways to IL-1 (see section 1.5.7). The full range of activities of this molecule are probably still to be defined but it plays a major role in the development of T cells, specifically in the differentiation of T_h0 cells into T_h1 cells. It directs this development mainly through the production of IFN γ , which is a T_h1 specific cytokine; a role previously thought to be specific to IL-12. In fact, IL-18 is likely to act by increasing the production of IL-12 receptors on the surface of T_h1 cells (Ahn et al., 1997). IL-18 also has the ability to increase pro-inflammatory cytokine production required for the response to bacterial infection, thus classing it as a pro-inflammatory cytokine itself (Sugawara et al., 1999). Also, IL-18 has been shown to have effects on bone metabolism where it can inhibit the formation of osteoclasts (Udagawa et al., 1997). Interestingly this is an activity that is biologically antagonistic to that of IL-1 (Jimi et al., 1998).

1.3 Novel IL-1 Family Members

The past four years has seen the description of six novel proteins that belong to the IL-1 family (Figure 1.3.1) and the main features of each of the novel family members is summarised in Table 1.3.1. A nomenclature has been adopted for these proteins that will be used in this text and which names the new proteins IL-1F5 to IL-1F10 (Sims et al., 2001). The sequence identity in the family ranges from 57% at its highest to 13% at its lowest. Gene localisation places all the novel genes in the same locus as *IL-1A*, *IL-1B* and *IL-1RA* (Nicklin et al., 2002). Examination of the locus also indicates that there are no new members of the family left to be identified (Nicklin et al., 2002; Taylor et al., 2002).

The cloning of IL-1F5 has been described six times (human and murine IL-1L1 (Barton et al., 2000), human IL-1RP2 (Busfield et al., 2000), human IL-1 δ (Debets et al., 2001), murine IL-1H3 (Kumar et al., 2000), human IL-HY1 (Mulero et al., 1999), human FIL-1 δ (Smith et al., 2000)). The human and murine forms share 92% sequence identity at the

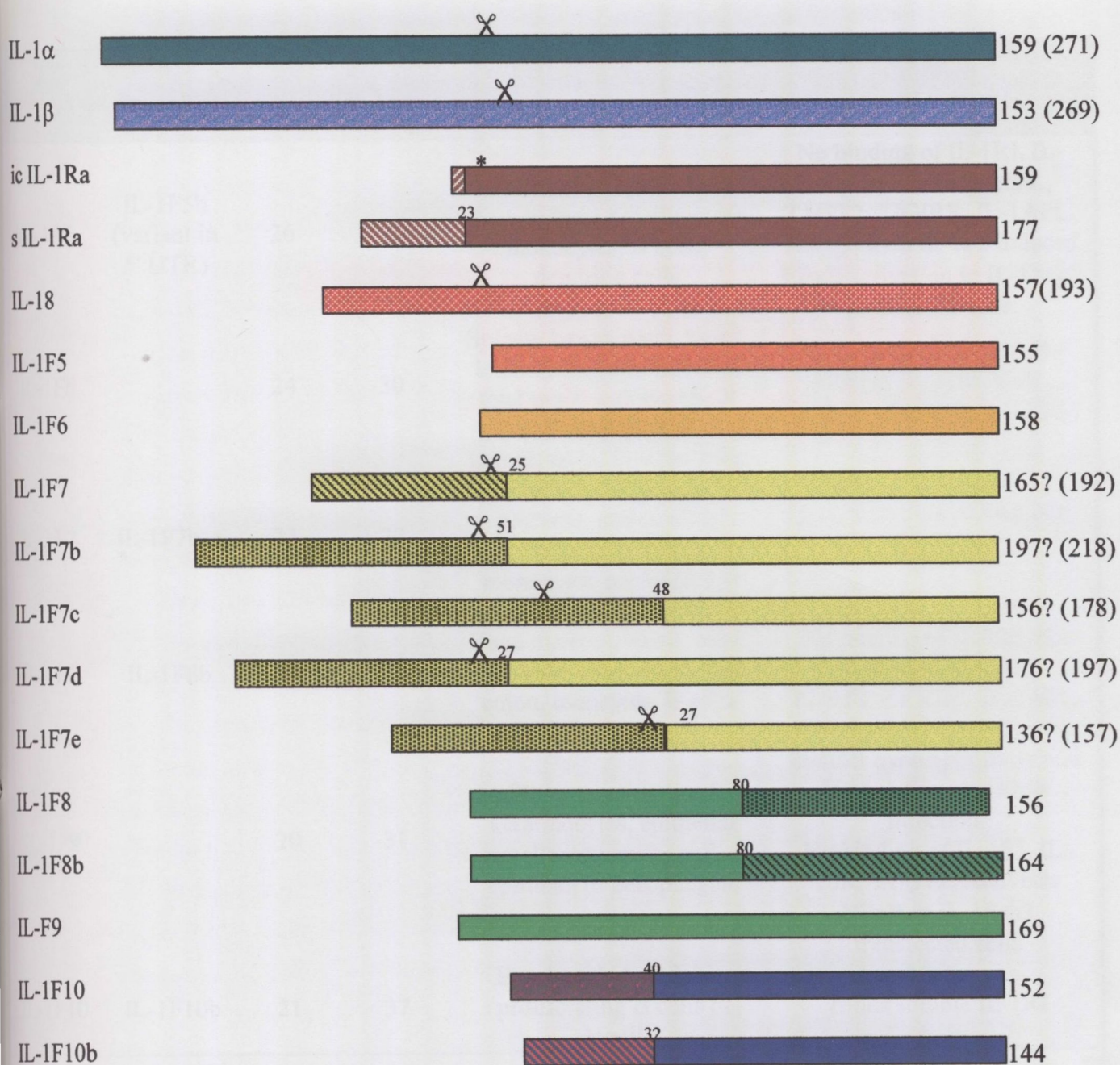


Figure 1.3.1 IL-1 Family Members. Each member of the IL-1 family is represented schematically with numbers to the right representing the length of each protein in terms of amino acids, and numbers in parentheses representing the length of the pro-form of the protein. The approximate positions of cleavage sites within the proteins is indicated by a scissors symbol. Where splice variants occur this is represented by alternatively shaded boxes with the residue number at which the sequence divergence occurs indicated. In the case of icIL-1Ra, the existence of a variant which results from the use of an alternative translation start site is indicated by an asterisk.

Name	Splice Variants	% Amino Acid Similarity		Expression	Features
		IL-1 β	IL-1ra		
IL-1F5	IL-1F5b (variant in 5' UTR)	26	52	Placenta, uterus, skin, brain, heart, kidney, keratinocytes, monocytes, B cells, dendritic cells	No binding of IL-1RI, IL-1RAcP, IL-18R, AcPL, T1/ST2, TIGIRR, IL-1APL. Antagonises IL-1F9 induced NF κ B activation in IL-1Rrp2 transfected cells.
IL-1F6		24	30	Spleen, lymph node, tonsil, leukocytes, bone marrow, fetal brain, monocytes, B cells and T cells	No binding of IL-1RI, IL-1RAcP, IL-18R, AcPL, T1/ST2, TIGIRR, IL-1APL.
IL-1F7	IL-1F7b-e	21	27	Lymph node, thymus, bone marrow, lung, testis, placenta, uterus, skin, colon, NK cells, monocytes, stimulated B cells, keratinocytes.	IL-1F7b binds IL-18R and IL-18BP
IL-1F8	IL-1F8b	27	31	Bone marrow, tonsil, heart, placenta, lung, testis, and colon, monocytes, B cells.	No binding of IL-1RI, IL-1RAcP, IL-18R, AcPL, T1/ST2, TIGIRR, IL-1APL. Up-regulated upon chronic contact hypersensitivity and Herpes-Simplex virus infection.
IL-1F9		20	31	Placenta, stimulated keratinocytes, epithelial cells, squamous cell epithelia of oesophagus.	No binding of IL-1RI, IL-18R, T1/ST2. Activates NF κ B in IL-1Rrp2 transfected cells
IL-1F10	IL-1F10b	21	37	Basal epithelia of skin, proliferating B cells of tonsil	Binds soluble IL-1RI

Table 1.3.1 Percentage amino acid similarity with IL-1 β and IL-1Ra, expression patterns and features of novel IL-1 family members.

amino acid level. IL-1F5 is most closely related to IL-1Ra and they share 44% identity on the amino acid level based on a structural alignment derived from crystallographic data. IL-1F5 has two mRNA isoforms which result from the use of alternative transcription initiation sites. These transcripts may have implications for the regulation of translation of IL-1F5 (Mulero et al., 2000, Barton et al., 2000). Expression of the protein is much more restricted than that of the classical members of the IL-1 family, with strong expression detected in fetal skin (Mulero et al., 1999) and adult skin, with keratinocytes being the main source of the expression (Barton et al., 2000; Busfield et al., 2000; Debets et al., 2001). It is also expressed in placenta (Barton et al., 2000; Busfield et al., 2000; Smith et al., 2000), tonsil (Smith et al., 2000) and thymus (Barton et al., 2000), as well as being abundant in psoriatic skin lesions (Debets et al., 2001). Expression is also detected in immune cells such as monocytes (Barton et al., 2000; Smith et al., 2000), B cells and dendritic cells (Smith et al., 2000), as well as LPS stimulated macrophages (Mulero et al., 1999). Experiments using Fc-fusion proteins of the extracellular domains of IL-1RI, IL-1 receptor accessory protein (IL-1RAcP), IL-18 receptor (IL-18R), IL-18 receptor accessory protein (IL-18RAcP), IL-1 receptor related protein 2 (IL-1Rrp2), T1/ST2 (Smith et al., 2000), three immunoglobulin-domain containing IL-1 receptor related (TIGIRR) or IL-1 receptor accessory protein-like (IL-1RAPL) (Born et al., 2000) indicate that IL-1F5 cannot bind any of these receptors. Using an IL-6 production assay the ability of IL-1F5 to either mimic or inhibit IL-1 activity was tested but no activity was found (Barton et al., 2000). The same result was obtained in an IFN- γ production assay when testing for the ability of IL-1F5 to mimic or inhibit IL-18 signals (Barton et al., 2000). However, transfection of Jurkat cells with a plasmid encoding IL-1Rrp2, indicates that IL-1F5 is able to inhibit the activation of an NF κ B-luciferase reporter plasmid by another novel IL-1; IL-1F9 (Debets et al., 2001). A polymorphism in the gene for IL-1F5 has also been linked to the occurrence of severe forms of alopecia areata, suggesting IL-1F5 may be involved in this disease (Tazi-Ahnini et al., 2002).

IL-1F6, cloned as human FIL-1E (Smith et al., 2000), is most similar to IL-1F9 based on amino acid comparison and the two have the highest sequence identity in a pairwise comparison of the whole IL-1 family (Lin et al., 2001). Expression is most abundant in

tonsil, but is also in fetal brain (Smith et al., 2000). At a cellular level IL-1F6 is found in LPS stimulated monocytes, as well as resting monocytes and B cells (Smith et al., 2000). Experiments using Fc-fusion proteins of the extracellular domains of IL-1RI, IL-1RAcP, IL-18R, IL-18RAcP, IL-1Rrp2, T1/ST2 (Smith et al., 2000), TIGIRR or IL-1RAPL (Born et al., 2000) indicate that IL-1F6 cannot bind any of these receptors.

Human IL-1F7 has been cloned independently by four groups as FIL-1 ζ (Smith et al., 2000), IL-1RP1 (Busfield et al., 2000), IL-1H4 (Kumar et al., 2000) and IL-1H (Pan et al., 2001), and it does not seem to have a murine orthologue (Taylor et al., 2002). It exists in five isoforms; IL-1F7 and IL-1F7b-e. IL-1F7b (Kumar et al., 2000; Pan et al., 2001) and IL-1F7d (Taylor et al., 2002) both have a variant N-terminal region. Both IL-1F7c and IL-1F7e are short isoforms of IL-1F7b, with amino acids 49 to 89 missing from IL-1F7c (Pan et al., 2001) and residues 29 to 89 missing from IL-1F7e (Taylor et al., 2002). Since these proteins would be lacking some of the β -strands required for the 12 stranded β -trefoil fold it is uncertain whether they form functional proteins. Expression of IL-1F7 has been detected in lymph node, bone marrow stroma, lung, testis and placenta (Smith et al., 2000). Expressed sequence tags (ESTs) for IL-1F7 have been detected in mixed fetal lung, colon, testes and B cell libraries (Kumar et al., 2000). Pan et al. (2001) also report abundant expression in testis, thymus and uterus, with expression being up-regulated in response to PMA in various cells lines including A431 epithelial cells, normal human dendritic cells, peripheral blood mononuclear cells, THP-1 macrophages and KG-1 bone marrow cells. IL-1F7 is the only member of the novel IL-1 family members that has a pro-domain like that of IL-1 α , IL-1 β and IL-18 (Smith et al., 2000). When expressed in CHO or HEK293 cells, a cleaved form of the protein is detected in cellular supernatants with a N-terminal sequence beginning at Val46 (Pan et al., 2001). However, caspase-1, the same protein that cleaves IL-1 and IL-18, has been shown to cause the cleavage of IL-1F7b *in vitro* at a different site, with cleavage occurring at Glu21 (Kumar et al., 2002). Both mature and cleaved IL-1F7b have been shown to bind the extracellular portion of IL-18R, but not IL-1RI (Kumar et al., 2002; Pan et al., 2001). However, this binding does not seem to induce signal transduction and IL-1F7b is reported to be unable to induce IFN- γ production in KG1 cells (Kumar et al., 2000; Pan et

al., 2001). Recently, IL-1F7b has been shown to inhibit IL-18 activity in the presence of IL-18 binding protein (IL-18BP) (see section 1.4.3) (Bufler et al., 2002). Binding of IL-1F7b to IL-18BP can be detected in a crosslinking assay and it is hypothesised that this complex can recruit IL-18RAcP. The trimeric complex would then be able to inhibit formation of the IL-18R/IL-18/IL-18RAcP active signalling complex and by this mechanism inhibit IL-18 activity. In addition to this, IL-1F7b is reported to be able to form dimers (Kumar et al., 2000). No other member of the IL-1 family is reported to act in a similar manner, except for a degradative product of IL-1Ra (Chang et al., 1996). Whether this IL-1F7 dimer is of physiological relevance is not known.

IL-1F8, originally named FIL-1 η (Smith et al., 2000), has a splice variant IL-1F8b, originally IL-1H2 (Kumar et al., 2000), which varies in the C-terminal region. It is most similar to IL-1F6 and IL-1F9 with 46% and 45% amino acid sequence identity respectively (Lin et al., 2001). It is found expressed in tonsil, bone marrow, placenta, lung, testis, and fetal brain, as well as in LPS stimulated monocytes and resting monocytes and B cells (Smith et al., 2000). An EST has also been detected in an osteoclastoma cell library (Kumar et al., 2000). Experiments using Fc-fusion proteins of the extracellular domains of IL-1RI, IL-1RAcP, IL-18R, IL-18RAcP, IL-1Rrp2, T1/ST2 (Smith et al., 2000), TIGIRR or IL-1RAPL (Born et al., 2000) indicate that this protein is unable to bind these receptors.

IL-1F9 has been cloned by three independent groups as IL-1RP2 (Busfield et al., 2000), IL-1 ϵ (Debets et al., 2001) and IL-1H1 (Kumar et al., 2000). The murine IL-1H1 described by Kumar et al (2000) is more likely to be the mouse orthologue of human IL-1F6 (Taylor et al., 2002). IL-1F9 is expressed in lung, TNF and IFN γ stimulated keratinocytes and epithelial cells, as well as oesophageal squamous mucosa and macrophages (Kumar et al., 2000). Expression in keratinocytes could be induced in a chronic inflammatory model, as well as in Herpes Simplex infected cells (Kumar et al., 2000). Debets et al (2001) also report expression in IL-1 β /TNF stimulated keratinocytes, with up-regulation detected in lesions of psoriatic skin. Furthermore, it has been shown

that IL-1F9 can induce NF κ B activation in IL-1Rp2 transfected Jurkat cells in a gene reporter assay (Debets et al., 2001).

IL-1F10, has been cloned by two groups as IL-1HY2 (Lin et al., 2001) and as IL-1F10 (Bensen et al., 2001), and is the final member of the IL-1 family to be described. It is most similar in sequence to IL-1F5 and IL-1Ra, sharing 41% and 37% amino acid identity respectively. Expression is detected in fetal skin and spleen cDNA libraries and immunohistochemistry detects expression in activated B-cells of the tonsil. It is secreted from cells, and has been shown to be able to bind a soluble form of IL-1RI, although its affinity for the receptor is significantly less than that of IL-1 β or IL-1Ra. The functional significance of this binding is not known.

1.4 The IL-1 Receptor Super-family

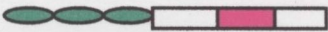
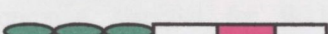
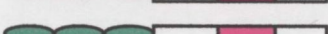
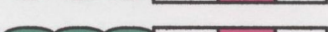
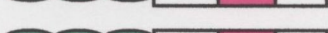
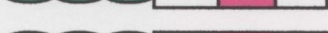
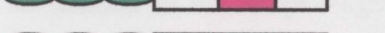
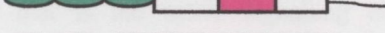
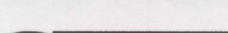
To date the IL-1 receptor family has ten members (O'Neill and Dinarello, 2000). These receptors share a common overall structure, with an intracellular Toll-IL-1 receptor (TIR) domain and/or three extracellular immunoglobulin (Ig)-like domains (Figure 1.4.1). The family members include the type I IL-1 receptor (IL-1RI), which directly binds IL-1 (Sims et al., 1988), the type II IL-1 receptor (IL-1RII) (McMahan et al., 1991), the IL-1 Receptor Accessory Protein (IL-1RAcP), which is required for IL-1 signalling (Greenfeder et al., 1995a), the IL-18 Receptor (formerly IL-1Rp1) (Thomassen et al., 1998) and its accessory protein, IL-18RAcP (formerly IL-1RAcPL) (Born et al., 1998), IL-1Rp2 (Lovenberg et al., 1996), which is reported to be responsive to IL-1F9 and IL-1F5 (Debets et al., 2001), and finally T1/ST2 (Klemenz et al., 1989; Tominaga et al., 1999), IL-1RAPL (Carrie et al., 1999) and TIGIRR/IL-1RAPL2/IL-1R9 (Born et al., 2000; Ferrante et al., 2001; Jin et al., 2000; Sana et al., 2000), which are all orphan receptors with unknown ligands. Single Ig-domain containing IL-1 receptor related (SIGIRR) is an IL-1R family member with only one extracellular immunoglobulin domain (Born et al., 2000), similar to IL-18 binding protein (Aizawa et al., 1999; Dinarello et al., 1998). Toll-like receptors (TLRs), which also have intracellular TIR

Name

Domains

Ligand

 TIR
  Ig
  LRR

Sub-group 1	IL-1RI		IL-1/IL-1Ra/IL-1F10
	IL-1RII		IL-1
	B15R		IL-1
	IL-1RAcP		—
	IL-18R		IL-18/IL-1F7b
	IL-18RAcP		—
	T1/ST2		?
	IL-1Rrp2		IL-1F9/IL-1F5?
	IL-1RAPL		?
	TIGIRR		?
	SIGIRR		?

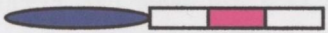
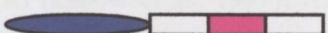
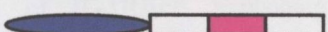
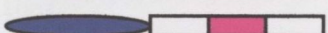
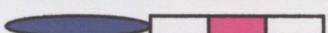
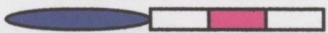
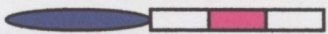
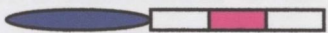
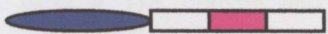
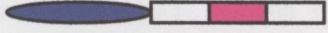
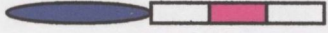
Sub-group 2	dToll 1-8		Spaetzle etc
	TLR1		Bacterial lipopeptides
	TLR2		Peptidoglycan, lipopeptides, etc.
	TLR3		d/s RNA
	TLR4		LPS, LTA, fibronectin, F protein
	TLR5		Flagellin
	TLR6		Mycobacterial lipopeptides
	TLR7		Imiquimod
	TLR8		Imiquimod
	TLR9		CpG DNA
	TLR10		?

Figure 1.4.1 IL-1 Receptor/TLR Superfamily. All the known members of the IL-1R family are shown, as well as the transmembrane members of the Toll receptor family, which are related to IL-1Rs on account of their TIR domains. TIR; Toll/IL-1 receptor, Ig; immunoglobulin, LRR; Leucine rich repeat.

domains, are classed with the IL-1 family to form the IL-1/TLR superfamily of receptors (O'Neill, 2000).

1.4.1 IL-1 Receptors

IL-1RI is an 80 kDa glycoprotein essential for IL-1 activity (Sims et al., 1993). Glycosylation of the receptor also seems to be required for optimal ligand binding and activity of the protein (Einstein et al., 1996; Mancilla et al., 1992). Binding of IL-1 to IL-1RI induces structural changes that allow dimerisation with the IL-1RAcP. Recruitment of the IL-1RAcP results in a stabilisation of the receptor–ligand complex (Wesche et al., 1998). It is this heterodimer that is the active signalling complex (Greenfeder et al., 1995a). IL-1RAcP deficient cells indicate that IL-1 cannot signal without the presence of IL-1RAcP (Cullinan et al., 1998), as was originally thought to be the case (Curtis et al., 1989). Meanwhile, IL-1RI deficient cells show that IL-1 cannot form a complex with IL-1RAcP in the absence of IL-1RI (Labow et al., 1997). IL-1Ra binding to IL-1RI however does not induce recruitment of IL-1RAcP, explaining why it cannot induce signal transduction (Greenfeder et al., 1995a).

IL-1RII is the 60 kDa product of a separate gene to that of IL-1RI (McMahan et al., 1991). Its extracellular domain is highly similar to that of IL-1RI but it possesses a truncated intracellular tail that lacks a TIR domain and, as a result, is not capable of transducing a signal upon ligation with IL-1 (Colotta et al., 1993; Sims et al., 1993; Stylianou et al., 1992). Thus, IL-1RII acts as a negative regulator of IL-1 action and can be termed a 'decoy receptor'. Human IL-1 α , IL-1 β and IL-1Ra have similar affinities for IL-1RI (Ruggiero et al., 1997; Stylianou et al., 1992), however IL-1RII only has a high affinity for IL-1 β and does not bind IL-1 α or IL-1Ra as effectively (Dripps et al., 1991b). This ensures that its role as a negative regulator is not compromised by needlessly binding IL-1Ra and reducing the effective concentration of the antagonist. In addition, ligated IL-1RII is also able to recruit IL-1RAcP, offering another means by which it can inhibit IL-1 signalling by causing formation of non-functioning complexes which limit the availability of the required accessory protein (Lang et al., 1998).

IL-1RII (Giri et al., 1990; Symons et al., 1991) and possibly IL-1RI (Svenson et al., 1993; Svenson et al., 1992) also exist naturally as 'soluble' receptors in the circulation. Soluble IL-1RII is formed when the extracellular domains of the receptors are cleaved from their membrane anchors by metalloproteases (Orlando et al., 1997). In the circulation these receptors can bind IL-1 β , as well as pro-IL-1 β , protecting the pro-form from proteolytic processing (Symons et al., 1991; Symons et al., 1995). Meanwhile it loses affinity for IL-1Ra (Symons et al., 1995). In this manner, these receptors can act as inhibitors of IL-1 activity. At lower concentrations, however, the binding of IL-1 by the soluble receptors may serve to prolong the action of the cytokine by increasing its half-life. An analogous situation, in which soluble receptor of TNF protects the ligand from proteolytic cleavage, causes the preservation of TNF signalling (Aderka et al., 1992). A splice variant of IL-1RAcP also exists giving rise to a soluble form in T cell lymphomas (Korherr et al., 1997), in rat brain (Gayle et al., 1997) and in liver where it may serve to inhibit IL-1 signalling (Jensen et al., 2000).

1.4.2 T1/ST2 Receptor

T1/ST2 is an orphan receptor of the IL-1 receptor family with no known ligand yet found (Yanagisawa et al., 1993). It has been shown to be incapable of binding IL-1, however two putative ligands have been partially purified, but not identified, using receptor precipitation with T1/ST2-Fc chimeric receptors (Gayle et al., 1996; Kumar et al., 1995). These ligands were also shown to activate p38 MAP kinase (Kumar et al., 1995). The T1/ST2 receptor is preferentially expressed in T_H2 cells and is therefore implicated in directing development of these cells and influencing their effector activities (Lohning et al., 1998). Furthermore, antibodies to T1/ST2 were effective in inhibiting the development of lung mucosal immune responses in a model of asthma, an event that is T_H2 dependent (Lohning et al., 1998). Studies show that, unlike other members of the IL-1 receptor family, T1/ST2 seems unable to activate NF κ B, but is able to activate JNK and p38 MAP kinase (Brint et al., 2002).

1.4.3 IL-18 Receptor

The IL-18 receptor (IL-18R) was originally cloned as IL-1Rrp1 (Thomassen et al., 1998). Like IL-1, IL-18 forms a trimeric complex with a receptor and accessory protein (Born et al., 1998). Furthermore, a natural inhibitor of IL-18 function has been identified as IL-18 binding protein (IL-18BP) (Aizawa et al., 1999; Novick et al., 1999). This molecule is a member of the immunoglobulin superfamily but, unlike the other receptor family members, has only one Ig domain and only has limited homology to IL-1RII (Novick et al., 1999). The protein is thought to act as a decoy receptor, in a manner similar to IL-1RII. Six isoforms of the protein have been identified in human and mouse, with varying affinities for IL-18 (Kim et al., 2000).

1.4.4 IL-1RAPL and TIGIRR

IL-1RAPL and TIGIRR are two closely related members of the IL-1R family (Jin et al., 2000). They have three Ig domains and an intracellular TIR domain as well as an additional 120 amino acid C-terminal tail, which does not have any significant similarity to other known proteins. IL-1RAPL expression is found in brain and heart, as well as other tissues (Born et al., 2000; Carrie et al., 1999), but TIGIRR expression seems to be specifically limited to the developing brain (Ferrante et al., 2001; Sana et al., 2000), although weak expression is also detected in skin (Born et al., 2000). Mutations in, and deletions of, the IL-1RAPL gene have been linked with the development of mental retardation (Carrie et al., 1999; Jin et al., 2000). The similarity between IL-1RAPL and TIGIRR has been used to suggest that this protein may also be involved in the development of mental retardation (Jin et al., 2000). The implicated involvement of these proteins in the development of the nervous system suggests that IL-1R family members, like *Drosophila* Tolls, may function in both immune and developmental processes.

1.4.5 Toll-like Receptors

The human toll-like receptors (TLRs) are a family of 10 related receptors that can be classed with the IL-1 receptors to form a superfamily. This superfamily is formed on the basis of the structure of the intracellular domains of TLRs, which are very similar to that of IL-1RI (O'Neill and Greene, 1998). The extracellular domain of these receptors does not however resemble the IL-1 receptor and their most striking feature is the presence of a leucine rich repeat (LRR) domain (O'Neill, 2000). TLRs are implicated in binding and responsiveness to bacterial, viral and fungal derived pathogen products that are important for the initiation of innate immune responses (reviewed in O'Neill, 2000). The TLRs have also been shown to signal in a manner similar to that of IL-1, utilising a similar array of messenger molecules to effect cellular function (see section 1.5), (reviewed in O'Neill, 2000).

1.5 IL-1 signalling

IL-1 exerts its effects on cells by altering the expression of target genes and the stability of target mRNAs. The signalling pathway leading from the IL-1 receptor complex to changes in transcription factor activity has been an area of intense research over many years. This has led to the identification of many different molecules that become activated after IL-1 stimulation. From these studies two major pathways have emerged as integral parts of IL-1 signalling, that is a) the pathway leading to nuclear factor κ B (NF κ B) activation, and b) the pathway leading to activation of the AP-1 class of transcription factors (Figure 1.5.1). These pathways are not however mutually exclusive and both may be involved in cross talk interactions.

1.5.1 NF κ B Activation

NF κ B is a transcription factor originally identified by its ability to bind the promoter of the κ light chain in B cells (Sen and Baltimore, 1986) but has since been shown to be present and functional in many other cell types (reviewed in Ghosh et al., 1998). At

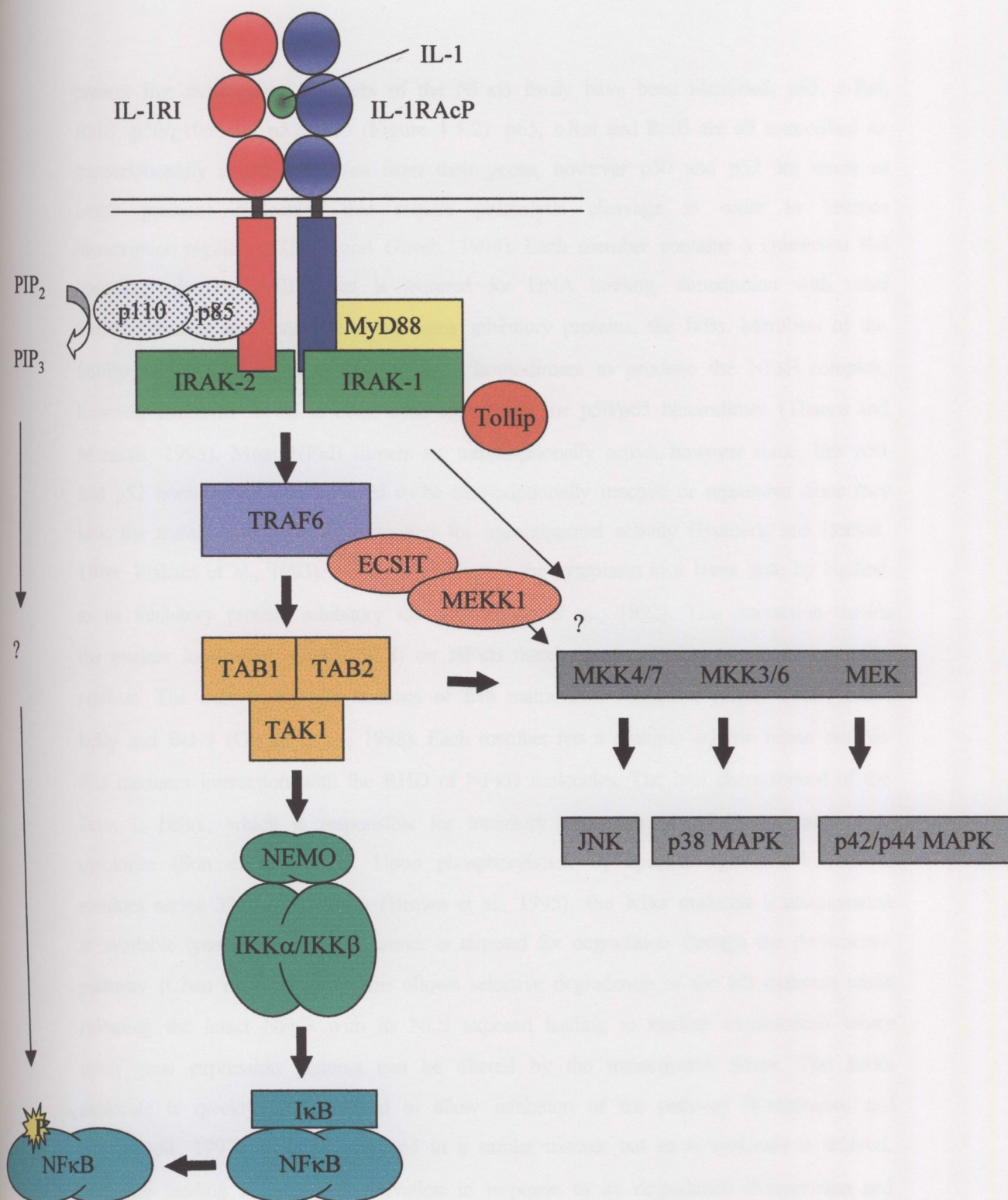


Figure 1.5.1 Signalling Pathways Activated by IL-1. See text for details.

present five mammalian members of the NF κ B family have been identified; p65, c-Rel, RelB, p50/p105 and p52/p100 (Figure 1.5.2). p65, c-Rel and RelB are all transcribed as transcriptionally active molecules from their genes, however p50 and p52 are made as larger precursor molecules that require proteolytic cleavage in order to become transcription regulators (May and Ghosh, 1998). Each member contains a conserved Rel homology domain (RHD) that is required for DNA binding, dimerisation with other family members and interactions with their inhibitory proteins, the I κ Bs. Members of the family are able to form both hetero- and homodimers to produce the NF κ B complex, however the term NF κ B is often used to refer to the p50/p65 heterodimer (Thanos and Maniatis, 1995). Most NF κ B dimers are transcriptionally active, however some, like p50 and p52 homodimers, are believed to be transcriptionally inactive or repressive since they lack the transactivation domain required for transcriptional activity (Baeuerle and Henkel, 1994; Plaksin et al., 1993). NF κ B is retained in the cytoplasm in a latent state by binding to its inhibitory protein, inhibitory κ B (I κ B) (Beg et al., 1992). This interaction shields the nuclear localisation signal (NLS) on NF κ B thereby preventing its translocation to the nucleus. The I κ B family also consists of five mammalian members; I κ B α , I κ B β , I κ B ϵ , I κ B γ and Bcl-3 (Ghosh et al., 1998). Each member has a multiple ankyrin repeat domain that mediates interaction with the RHD of NF κ B molecules. The best characterised of the I κ Bs is I κ B α , which is responsible for transitory activation of NF κ B in response to cytokines (Sun et al., 1993). Upon phosphorylation by specific upstream kinases at residues serine 32 and serine 36 (Brown et al., 1995), the I κ B α molecule is ubiquitinated at available lysines and in this manner is targeted for degradation through the proteasome pathway (Chen et al., 1995). This allows selective degradation of the I κ B molecule while releasing the intact NF κ B with its NLS exposed leading to nuclear translocation where upon gene expression patterns can be altered by the transcription factor. The I κ B α molecule is quickly re-synthesised to allow inhibition of the pathway (Krappmann and Scheidereit, 1997). I κ B β is regulated in a similar manner but its re-synthesis is delayed, therefore leading to persistent activation in response to its degradation (Krappmann and Scheidereit, 1997; Thompson et al., 1995)

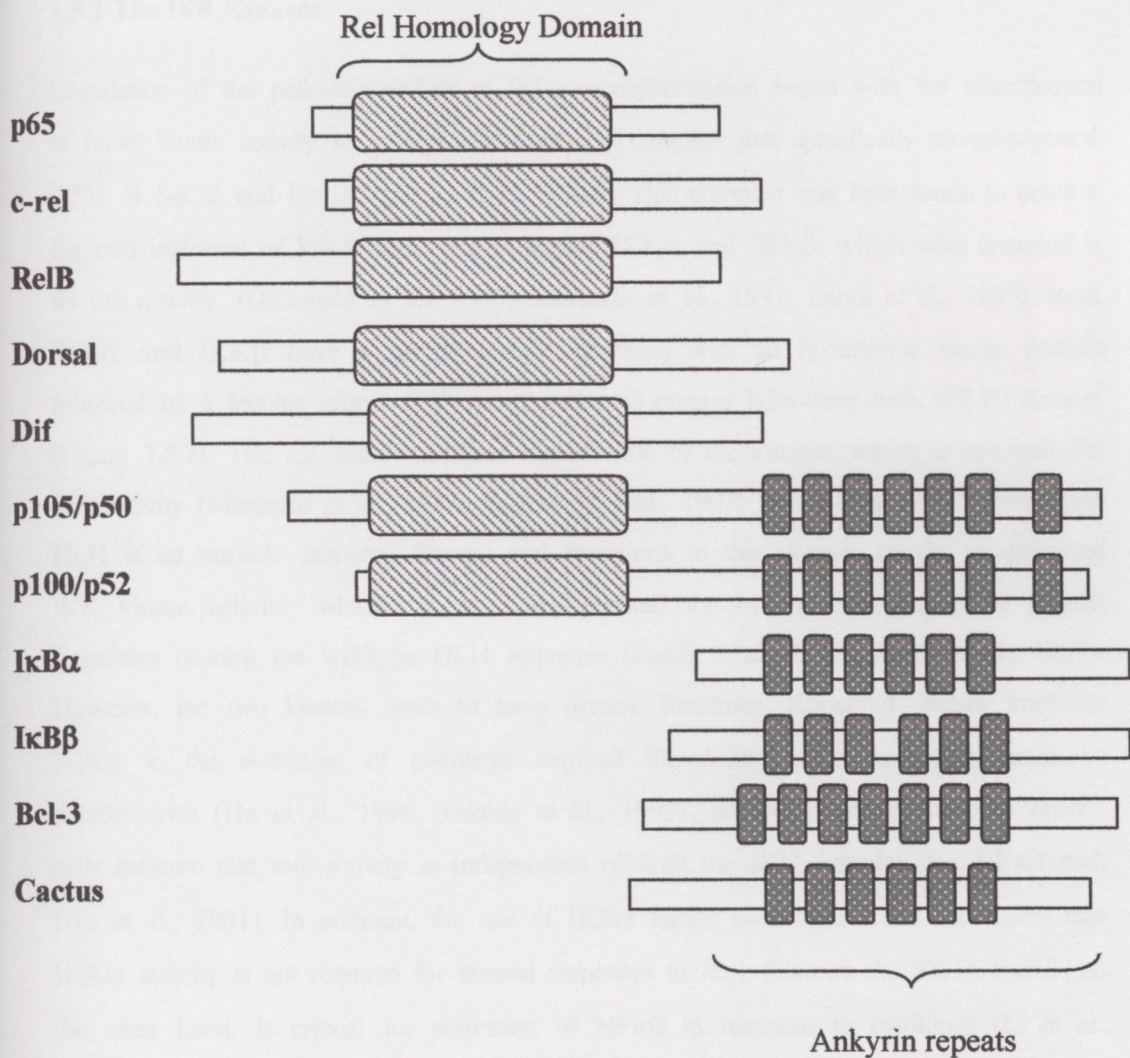


Figure 1.5.2 NFκB and IκB family of proteins. Members of the NFκB family of proteins are characterised by the Rel homology domain which mediates DNA binding and protein-protein interactions. The IκB family of proteins are characterised by multiple ankyrin repeats which mediate the interaction to the Rel Homology domain. Dif, dorsal and cactus are *Drosophila* proteins. (After Baldwin, 1996)

1.5.2 The I κ B Kinases

Elucidation of the pathway leading to I κ B α phosphorylation began with the identification of novel kinase activity in a large 700-900 kDa complex that specifically phosphorylated I κ B α at Ser32 and Ser 36 (Chen et al., 1996). This complex was later found to contain the two isoforms of I κ B kinase (IKK), namely IKK α and IKK β , which were responsible for this activity (DiDonato et al., 1997; Mercurio et al., 1997; Zandi et al., 1997). Both IKK α and IKK β have a similar overall structure, with an N-terminal kinase domain followed by a leucine zipper (LZ) motif and a C-terminal helix-loop-helix (HLH) domain (Figure 1.5.3). The LZ motif mediates dimerisation of the kinases, which is essential for their activity (Mercurio et al., 1997; Woronicz et al., 1997; Zandi et al., 1997), while the HLH is an intrinsic activator domain and mutations in this domain results in decreased IKK kinase activity, which can be complemented for by the introduction of peptide fragments bearing the wildtype HLH sequence (Zandi et al., 1998; Zandi et al., 1997). However, the two kinases seem to have diverse functions. Knockout studies implicate IKK α in the activation of pathways required for differentiation and proliferation of keratinocytes (Hu et al., 1999; Takeda et al., 1999), however experiments with *Ikk α ^{-/-}* cells indicate that this activity is independent of both the IKK complex and NF κ B itself (Hu et al., 2001). In addition, the use of IKK α kinase inactive knockin mice show that IKK α activity is not required for normal responses to IL-1 (Cao et al., 2001). IKK β , on the other hand, is critical for activation of NF κ B in response to cytokines (Li et al., 1999b) and this is supported by data which show that serine to alanine mutations in the activation loop abolish cytokine responsiveness when present in the IKK β isoform and not in IKK α (Delhase et al., 1999).

Another important component of the IKK complex is NF κ B essential modulator (NEMO)/IKK γ , which acts as an essential scaffold protein for the IKKs (Mercurio et al., 1999; Rothwarf et al., 1998; Yamaoka et al., 1998). Yamaoka et al. (1998) cloned NEMO by complementation of a mutant cell line, which, like NEMO^{-/-} cells (Rudolph et al., 2000), did not activate NF κ B in response to various stimuli including IL-1. NEMO interacts, via an N-terminal α -helical region, with the IKKs through a short interaction

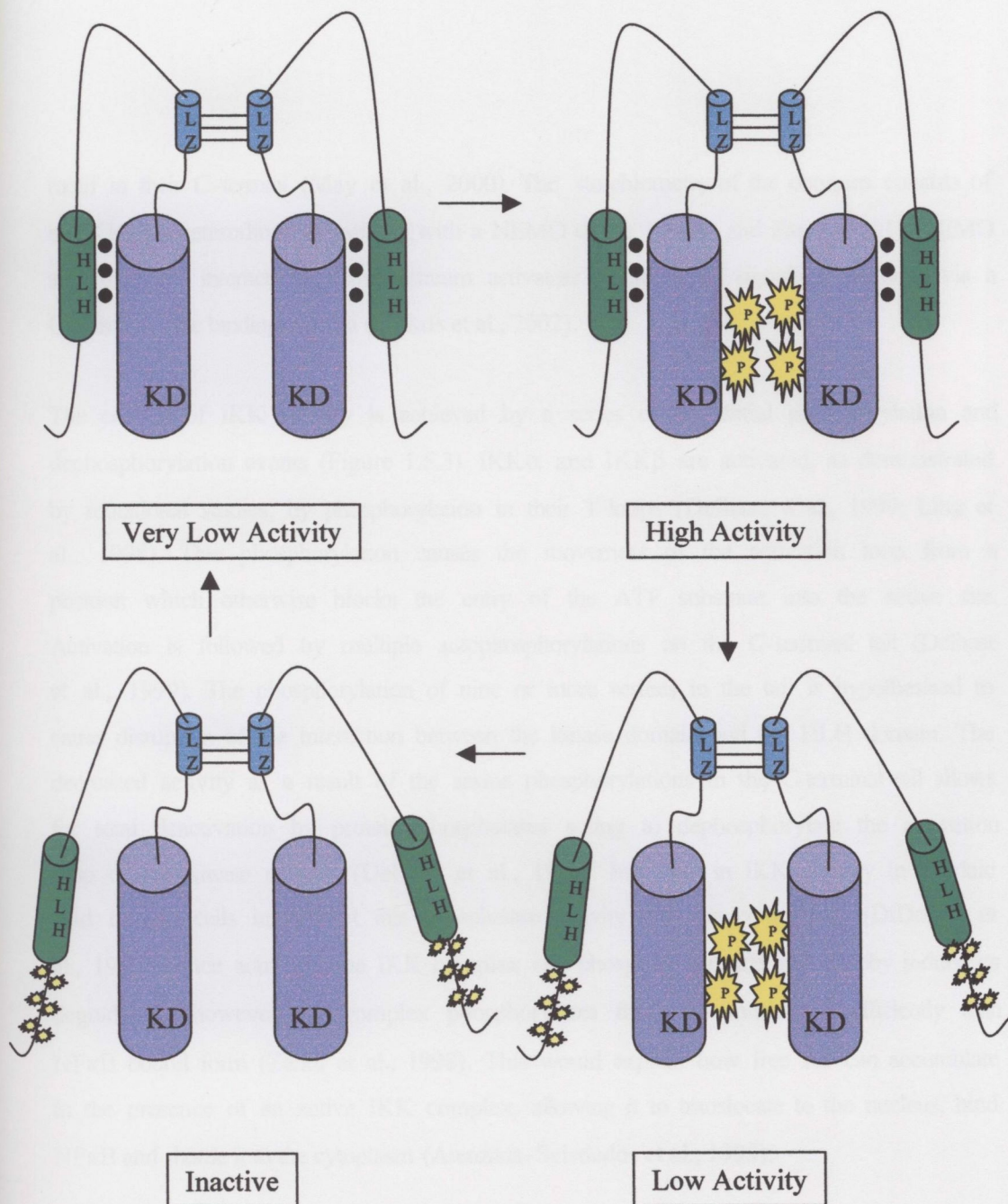


Figure 1.5.3 Regulation of the kinase activity of IKK by phosphorylation. IKK α and IKK β dimerise via their leucine zipper motifs (LZ), with the helix-loop-helix (HLH) and C-terminal domains interacting with the kinase domain (KD). Phosphorylation by upstream kinases in the activation loop induces the kinase activity in the dimer. This is followed by multiple autophosphorylation events in the C-terminal region which disrupts the interaction of the HLH and the KD, decreasing the intrinsic kinase activity. The decreased activity allows for inactivation of the IKKs by phosphatases. (After Delhase *et al.*, 1999).

motif in their C-termini (May et al., 2000). The stoichiometry of the complex consists of an IKK α/β heterodimer associated with a NEMO dimer (Miller and Zandi, 2001). NEMO also mediates interactions with upstream activators in the TNF signalling pathway via a C-terminal Zinc binding domain (Makris et al., 2002).

The control of IKK activity is achieved by a series of sequential phosphorylation and dephosphorylation events (Figure 1.5.3). IKK α and IKK β are activated, as demonstrated by mutational studies, by phosphorylation in their T-loops (Delhase et al., 1999; Ling et al., 1998). This phosphorylation causes the movement of the activation loop from a position which otherwise blocks the entry of the ATP substrate into the active site. Activation is followed by multiple autophosphorylations on the C-terminal tail (Delhase et al., 1999). The phosphorylation of nine or more serines in the tail is hypothesised to cause disruption of the interaction between the kinase domain and the HLH domain. The decreased activity as a result of the serine phosphorylations in the C-terminal tail allows for total deactivation by protein phosphatases acting to dephosphorylate the activation loop of the kinase domain (Delhase et al., 1999). Increases in IKK activity in okadaic acid treated cells imply that this phosphatase activity may be constitutive (DiDonato et al., 1997). Once activated the IKK complex can phosphorylate I κ B and thereby induce its degradation, however the complex phosphorylates free I κ B much less efficiently than NF κ B bound form (Zandi et al., 1998). This would explain how free I κ B can accumulate in the presence of an active IKK complex, allowing it to translocate to the nucleus, bind NF κ B and shuttle it to the cytoplasm (Arenzana-Seisdedos et al., 1995).

1.5.3 IKK Kinase

The kinase responsible for the activation of IKK is still a matter of debate. Initially this role was designated to NF κ B inducing kinase (NIK), the over-expression of which activated IKK (Regnier et al., 1997; Woronicz et al., 1997). Also, NIK was used in a yeast-two-hybrid screen to isolate IKK α (Regnier et al., 1997). However, the physiological relevance of these findings has been questioned. Although NIK has been

shown to interact with TRAF6, the up-stream activator of NF κ B in the IL-1 pathway, it has also been shown that NIK binds to most TRAF proteins indiscriminately (Song et al., 1997). Also, *in vitro* experiments have shown that NIK preferentially phosphorylates IKK α , the isoform of IKK that has been shown not to be important for NF κ B activation in response to IL-1 and TNF (Ling et al., 1998). This suggested that NIK may not be required for the phosphorylation of IKK in response to pro-inflammatory cytokines. NIK deficient cells confirmed this and they retain responsiveness to treatment with either IL-1 or TNF, showing that these pathways do not rely on NIK for activation (Yin et al., 2001). NIK is however required for activating NF κ B transcriptional activity in response to lymphotoxin β (Yin et al., 2001).

Another candidate for the IKK kinase is the TAK1/TAB complex. TGF β activated kinase 1 (TAK1) is a kinase that was originally identified as a mediator of transforming growth factor- β (TGF β) signalling, and is a member of the mitogen activated protein kinase kinase kinase (MAP3K) family of proteins (Yamaguchi et al., 1995). TAK1 activity is dependent on the associated protein TAB1 (Shibuya et al., 1996), and this complex has been shown to be activated in an IL-1 dependent manner, causing the activation of TAK1 kinase activity and association of the complex with TRAF6 (Ninomiya-Tsuji et al., 1999). This association is mediated by an adapter molecule TAB2 (Takaesu et al., 2000). TAK1 has also been implicated in the activation and phosphorylation of the IKK complex in a TRAF6 dependent manner (Wang et al., 2001). Uniquely the activation of TRAF6 is shown to be a ubiquitin controlled event, but it is not dependent on protease activity (Wang et al., 2001).

1.5.4 Receptor to IKK

Investigations into events further upstream of IKK phosphorylation have revealed the existence of transitory multi-protein complexes at the IL-1 receptor. Thus upon dimerisation, the IL-1RI/IL-1RAcP complex recruits the adapter molecule MyD88 (Burns et al., 1998; Muzio et al., 1997; Wesche et al., 1997). The importance of MyD88 to IL-1 signalling has been demonstrated in MyD88 deficient mice. These mice show

impaired responses to stimulation with IL-1 in terms of thymocyte proliferation and production of acute phase proteins and pro-inflammatory cytokines such as TNF α and IL-6 (Adachi et al., 1998). The protein contains two interaction domains, a Toll-IL-1 receptor (TIR) domain and a death domain (DD). The TIR domain indicates the relationship between MyD88 and the IL-1 and Toll-like receptors (Hultmark, 1994) and has been shown to mediate homotypic protein-protein interactions (Burns et al., 1998). The DD, which is present in many other molecules such as RIP, FADD and TRADD, is also well characterised in apoptosis pathways for its role in protein-protein interactions (Boldin et al., 1995). The presence of these two interaction domains therefore suits the function of MyD88 as an adapter molecule very well, allowing it to act as a link between different proteins. Indeed, immuno-precipitation assays have shown that MyD88 can interact with IL-1RAcP through its TIR domain (Muzio et al., 1997), while it interacts with under-phosphorylated IL-1 Receptor Associated Kinase (IRAK) through its DD (Wesche et al., 1997).

IRAK is a kinase which also has a DD and has been implicated in IL-1 signalling (Cao et al., 1996a; Croston et al., 1995). IRAK is recruited to the IL-1 receptor complex upon IL-1 stimulation in a process that is mediated by the IRAK binding protein Tollip (Burns et al., 2000). A dominant negative truncated form of IRAK has been shown to inhibit IL-1RI-IL-1RAcP induced NF κ B activation in gene reporter assays (Muzio et al., 1998). Interestingly, the kinase activity of this molecule is not required for signalling to NF κ B (Li et al., 1999a; Vig et al., 1999) and MyD88 has been shown to preferentially interact with underphosphorylated IRAK (Wesche et al., 1997). However, phosphorylation has been shown to be followed by IRAK degradation in a proteasome dependent manner and may therefore be a signal to limit IRAK activity (Yamin and Miller, 1997). The requirement for IRAK in IL-1 signalling has been demonstrated using IRAK deficient mice, since embryonic fibroblasts from these mice fail to activate NF κ B in response to treatment with IL-1, but not TNF. Also, the mice show attenuated, but not eradicated, production of IL-6 and TNF α in response to injection with IL-1 β (Thomas et al., 1999). This implies that functional redundancy occurs with other members of the IRAK family.

Four isoforms of the kinase exist, namely IRAK (IRAK-1), IRAK-2 (Muzio et al., 1997), IRAK-M (Wesche et al., 1999) and IRAK-4 (Li et al., 2002), as well as a splice variant of IRAK, IRAK-1b (Jensen and Whitehead, 2001). Studies have shown that IRAK-1 is preferentially recruited to IL-1RAcP (Huang et al., 1997; Muzio et al., 1997), while IRAK-2 is recruited to IL-1RI (Muzio et al., 1997). IRAK-M expression is mainly restricted to myeloid cells (Wesche et al., 1999) and may be involved in negative regulation of signalling (Kobayashi et al., 2002). IRAK-1b has been shown to have similar properties to IRAK, but it does not undergo phosphorylation and is more stable than IRAK-1 after IL-1 stimulation (Jensen and Whitehead, 2001). IRAK-4, unlike the other IRAKs, seems to require its kinase activity for signalling and this kinase activity is required for its interaction with MyD88 and TRAF6 (Li et al., 2002). Furthermore, IRAK-4 knockout cells are completely devoid of activation of NF κ B and cytokine production in response to treatment with IL-1 (Suzuki et al., 2002). Overexpression studies in knockout cells indicate that IRAK-4 signals upstream of TRAF6 and downstream of MyD88 (Suzuki et al., 2002). It may function to interact with and activate IRAK-1, in a process that is reliant on its kinase activity (Li et al., 2002).

The recruitment of IRAK to the receptor complex has been shown to lead to the recruitment of another protein called TNF Receptor Associated Factor 6 (TRAF6) (Cao et al., 1996b). TRAF6 has been shown to be required for activation of NF κ B and AP-1 in response to IL-1 (Baud et al., 1999; Cao et al., 1996b). Since IRAK stimulation of NF κ B activity is inhibited by dominant negative TRAF6, this places TRAF6 downstream of IRAK on the signalling pathway from IL-1 (Muzio et al., 1998). The structure of TRAF6 is shown in figure 1.5.4. It has an N-terminal RING finger and a Zinc finger domain, followed by a coiled coil region and a well conserved C-terminal TRAF domain (Ishida et al., 1996). Chimeric proteins that allow controlled oligomerisation of TRAF6 have shown that this is an important event in the activation of both NF κ B and AP-1 (Baud et al., 1999). These chimeras are truncated forms of TRAF6 and only possess the N-terminal RING finger and Zn finger domains. Therefore, these data suggest that the effector activities of TRAF6 are only achieved when oligomerisation occurs through its conserved C-terminal domain, which in turn is likely to lead to a stabilisation of the

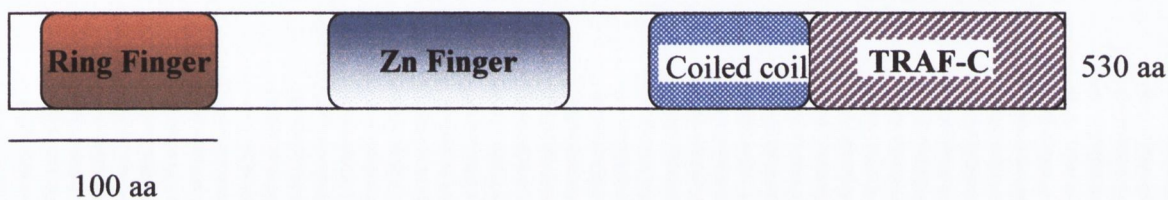


Figure 1.5.4 Structure of TRAF6. Relative positions of each domain is shown. Ring and Zinc fingers domains are important for NF κ B and AP-1 activation, while the C-terminal domain mediates protein-protein interactions. The bar represents a segment 100 amino acids long.

interaction with its effector molecules. The importance of TRAF6 in IL-1 signalling has been shown through the use of knockout cells, which are defective in NF κ B activation, as well as JNK activation (Lomaga et al., 1999). A TRAF6 binding protein has also been identified which binds TRAF6 in a manner that is mutually exclusive to that of IRAK-1, and does not involve signalling to NF κ B or JNK (Ling and Goeddel, 2000). A TRAF6 binding motif defined by the sequence Pro-X-Glu-X-X-Ar/Ac (aromatic/acidic) has been identified and is found to be present in IRAK-1, IRAK-2 and IRAK-M (Ye et al., 2002), but it is not present in IRAK-4. The interaction of TRAF6 with IRAK-1 through this motif then leads to the translocation of TRAF6 from the membrane to the cytosol where it then interacts with and activates the TAK1/TAB complex (Qian et al., 2001). As mentioned above this process is mediated by the adapter protein TAB2, which facilitates the interaction of TRAF6 with the TAK1/TAB complex (Takaesu et al., 2000; Takaesu et al., 2001). The TAK/TAB complex can then phosphorylate downstream targets such as IKK.

1.5.5 Mitogen Activated Protein Kinases

The mitogen activated protein kinases (MAP kinases) are the second major targets of IL-1 signalling. IL-1 has been shown to activate p38 MAP kinase and c-Jun N terminal kinase (JNK) (Derijard et al., 1995; Lin et al., 1995; Raingeaud et al., 1995) and in certain cell types p42/p44 MAP kinase (Bird, 1991; Guy et al., 1991). Furthermore, knockout studies with cells deficient in p38 α show that IL-1 loses its ability to induce IL-6 production in MEF cells (Allen et al., 2000), while cells deficient in JNK1 and JNK2 have only partial responses to IL-1 in terms of induced AP-1 activity and induction of collagenase-3 expression (Han et al., 2001).

The MAP kinases are activated by dual phosphorylation on the tripeptide Thr-X-Tyr motif by upstream kinases MAP kinase kinases (MKK). In general, MKK3, MKK4 and MKK6 are believed to phosphorylate p38, MKK4 and MKK7 are believed to phosphorylate JNK, and MKK1 and MKK2 are believed to phosphorylate p42/p44 (reviewed in Dong et al., 2002). MKK3 deficient cells however are still responsive to IL-

IL-1 induced p38 signalling but not TNF α , suggesting that MKK3 is not essential for p38 activation in response to IL-1 (Wysk et al., 1999). However, *Mkk4*^{-/-} cells are deficient in p38 activation in response to IL-1, implicating this kinase in phosphorylation of p38 (Ganiatsas et al., 1998). MKK4 is also able to phosphorylate JNK along with MKK7 (reviewed in Davis, 2000). Using knockout cells it has been shown that *Mkk7*^{-/-} cells are not responsive to IL-1 in terms of JNK activation, whereas as in *Mkk4*^{-/-} cells JNK activation is reduced but not abolished (Tournier et al., 2001). This suggests that MKK7 is essential for JNK activation in response to IL-1, whereas MKK4 is required for optimal activation. Upstream of p42/44, IL-1 has been shown to activate MEK1 (Saklatvala et al., 1993).

The proteins acting upstream of the MKKs are still unclear. MEKK1, which has been shown to be a potent activator of JNK activity (Minden et al., 1994), is also able to interact with ECSIT (evolutionarily conserved signalling intermediate in Toll pathways), a protein that seems to interact specifically with TRAF6 and can activate AP-1 activity as well as NF κ B (Kopp et al., 1999). However, knockout studies with *Mekk1*^{-/-} cells call into question its role in NF κ B and JNK activation. The *Mekk1*^{-/-} cells indicate that requirement for MEKK1 in IL-1 induced JNK activation seems to be cell type specific and that it is not required for NF κ B activation (Xia et al., 2000; Yujiri et al., 2000). It is also possible that the effects of MEKK1 may be masked by redundancy with other MAPKKs. TAK1 is also another up-stream activator of the MAP kinase pathway that is capable of activating JNK (Deng et al., 2000; Wang et al., 2001). The activation of MKK1/2 is less clear, although activation of p42/44 by IL-1 has been shown to be dependent on focal adhesion complexes (FAC), which may direct the activation of the MAP kinase pathway leading to p42/44 (MacGillivray et al., 2000). IL-1 has also been shown to activate Raf, a known activator of MKK1 (Huwiler et al., 1996). Evidence has also been presented for the involvement of other small Gproteins in the activation of the MAP kinase pathway in response to IL-1. Dominant negative Rac1 can inhibit IL-1 induced activation of p38 and JNK (Zhang et al., 1995) and has also been shown to be involved in regulation of IL-1 stimulated transactivation of p65 through p38 and p42/p44 (Jefferies and O'Neill, 2000). Also, lethal toxin, which inactivates Ras, Rac and Rap by

glucosylation, inhibits IL-1 induced activation of p38 (Palsson et al., 2000) and Ras has been shown to be involved in activation of p38 MAP kinase in response to IL-1 in a process that involves IRAK, IRAK-2, TRAF6 and TAK1 (McDermott and O'Neill, 2002).

1.5.6. Bifurcation of the Pathway

The TAK/TAB complex represents a possible point of divergence of the IL-1 pathway to the MAP kinases and NFκB. It has been shown to be activated by IL-1 in time dependent manner and to specifically phosphorylate IKKβ in a stimulus dependent manner (Wang et al., 2001). Previous studies have shown that TAK1 mutants can inhibit the activation of JNK by ceramide (Shirakabe et al., 1997), and that it is able to phosphorylate MKK6 and MKK3, both upstream activators of p38 (Moriguchi et al., 1996; Wang et al., 2001). Therefore, these data present evidence that TAK1 may activate both the pathways leading to NFκB activation and MAP kinase activation.

Other evidence using IRAK mutants suggests that it may be a point of divergence in the pathway. A deletion mutant on the unclassified N-terminal proximal region of IRAK was shown to be impaired in its ability to activate NFκB while it retained its ability to activate JNK (Li et al., 2001). This mutant was also impaired in its ability to bind TRAF6. Experiments using truncated forms of TRAF6 also indicate a point of divergence at TRAF6, since a C-terminal domain deleted mutant inhibits TLR induced NFκB activation but not JNK activation (Muzio et al., 1998). TRAF6 however, has been shown to be required for JNK activation (Lomaga et al., 1999).

1.5.7 Other IL-1 Induced Signals

IL-1RI has been shown to be capable of interacting with other signalling components such as phosphatidylinositol 3 (PI3) kinase (Reddy et al., 1997). PI3 kinase activity is induced by IL-1 and expression of the p110 catalytic subunit of PI3 kinase can drive both

NFκB and AP-1 activation (Reddy et al., 1997). The activation of NFκB transcriptional activity by PI3 kinase is independent of IκBα degradation and is via a transactivation pathway that leads to phosphorylation of the p65 subunit of NFκB (Sizemore et al., 1999). IL-1 induced PI3 kinase can also activate the anti apoptotic kinase, Akt (Chen et al., 2002; Madge and Pober, 2000).

The protein kinase C (PKC) family of proteins has also been implicated in IL-1 signalling, however their participation in IL-1 responses remains controversial. Although calphostin, an inhibitor of PKC, has been shown to inhibit IL-1 induced NFκB activation, this activity is cell type specific (Bonizzi et al., 1999).

1.5.8 IL-18 Signalling

The features of IL-18 signalling so far described are very similar to those for IL-1 and differences between their respective components have not yet been identified. Like IL-1, activation of cells by IL-18 leads to NFκB activation (Matsumoto et al., 1997; Robinson et al., 1997). This signalling has been shown to be dependent on MyD88 using *Myd88*^{-/-} cells, which also fail to activate AP-1 in response to IL-18 (Adachi et al., 1998). IRAK has also been shown to be phosphorylated in response to IL-18 (Robinson et al., 1997), and in IRAK null cells, IL-18 signalling, in terms of IFN-γ induction, is attenuated but not eradicated, as is the case with IL-1 (Thomas et al., 1999). This suggests that the other members of the IRAK family can compensate for the loss of IRAK. Activation of JNK in response to IL-18 is however abolished in IRAK null cells, while NFκB activation is only impaired (Kanakaraj et al., 1999). This suggests that IRAK may be a point of divergence for signalling to NFκB or JNK, as has been suggested for IL-1 (Li et al., 1999a). IL-18 has also been shown to induce the interaction of IRAK with TRAF 6 (Kojima et al., 1998). In addition, IL-18 can induce serine phosphorylation of TAB1, while a kinase dead mutant of TAK1 inhibits IL-18 mediated NFκB activation (Wald et al., 2001). This implicates the TAK/TAB complex in IL-18 signalling. Furthermore, it has been shown that mutations in NEMO associated with an X-linked immunodeficiency disease leads to

impaired IL-18 signalling (Doffinger et al., 2001). Finally, PI3 kinase inhibitors have been shown to inhibit IL-18 induced adhesion molecule expression, suggesting that IL-18 may also be able to activate the PI3 kinase pathway (Morel et al., 2001). However, despite having very similar signalling pathways, mutant cells lines that are unresponsive to IL-1 but are still capable of responding to IL-18 suggest that there are IL-18 signalling components that are unique from those used by IL-1 (Wald et al., 2001).

1.6 Structural Aspects of the IL-1 Family

1.6.1 The β -Trefoil Fold

Crystal and nuclear magnetic resonance (NMR) structures of IL-1 β (Clare et al., 1991; Finzel et al., 1989; Priestle et al., 1988b; Veerapandian et al., 1992), IL-1 α (Graves et al., 1990) and IL-1Ra (Schreuder et al., 1995; Vigers et al., 1994) have all been solved. These studies show that all three family members have a common β -trefoil fold. This fold groups the cytokines in a structural superfamily along with the Kunitz family of protease inhibitors (Sweet et al., 1974), ricin-like toxins (Lord et al., 1994), histactophilin (Habazetti et al., 1992) and fibroblast growth factors (Eriksson et al., 1991). The fold is defined by three repeating units of a trefoil (Y) structure each consisting of four β strands of two bonded pairs connected by three loops (Figure 1.6.1a). As a result, the structure has a pseudo three-fold symmetry. The third loop of the repeat is longer than the first two, allowing the last strand to fold back to associate with the first. The structure can thus be viewed as consisting of the repeating $\beta\beta\beta L\beta$ unit, where l is a short loop and L is a long loop (figure 1.6.1b). The complete structure therefore consists of 12 β -strands. Six strands, two from each trefoil unit, consisting of strands 1, 4, 5, 8, and 9, associate to form an anti-parallel β -barrel. The other six strands form three hairpins consisting of strands 2 and 3, 6 and 7 and 10 and 11, and form a cap at one end of the β -barrel in a triangular triplet hairpin structure. This is illustrated in figure 1.6.2 with the barrel structure represented by red strands and the cap structure represented by yellow strands.



Figure 1.6.2 Structure of IL-1 β . The six stranded anti-parallel barrel (red) topped by a three hairpin cap (yellow) are highlighted.

The members of this structural family, despite folding in a similar manner, do not share any great sequence identity and neither do they have functional homology; the Kunitz family bind tightly to and inhibit proteases, histactophilin binds and helps assemble actin and FGF mediates mitogenic signals via cell surface receptors. Furthermore, they act in both an extra- and intracellular context. Although they all act as ligands they do not all share similarities in the type of molecules that they bind to or the regions in their tertiary structure with which this binding is associated. Despite this Ponting and Russell do suggest that this fold may have arisen from a common ancestral protein in eukaryotic cells (Ponting and Russell, 2000). Although the sequences of the family members vary greatly they do exhibit conservation in terms of residue hydrophobicity and volume at sites that are important for allowing the structural determinants of the fold to develop (Murzin et al., 1992). These determinants include the geometry of the barrel and the cap, and the interface of the two. In the case of the barrel the diameter of the barrel, the angle between the axis of the barrel and the direction of the strands are important for establishing a stable structure. For a β -trefoil barrel with six β -strands the diameter of the barrel is about 16 Å and the angle between the strands is about 56° (Murzin et al., 1992). The coiling of the hairpins in the cap, as well as the interaction between its residues and those protruding from the barrel structure, are the key features that determine the cap's formation and stability.

1.6.2 The Barrel Structure

The barrel structure is made up of three layers of residues with each strand in the barrel contributing one residue to each layer. The bottom layer is exposed to the solvent and the residue side chains do not point to the interior of the barrel but out towards the solvent. The residues that make up this layer are therefore quite heterogeneous in nature. The middle and top layers are more conserved, with residues here being strongly hydrophobic. Where hydrophilic residues do occur, their sidechains protrude through the barrel to the surface. The middle layer is made up of tightly packed sidechains that fill the interior of the protein. These residues must be fairly bulky in nature, thereby allowing the residues to occupy the full interior of the barrel such that a diameter of 16 Å is achieved.

The mean volume of the residues in this layer are $170 \text{ \AA}^3$, this is compared to $140 \text{ \AA}^3$ for an eight stranded barrel, which has a smaller interior (Murzin et al., 1992). The top layer of the barrel has residues that are both large and hydrophobic, three of the residues reach into the barrel interior while the remaining three point in the opposite direction and interact with residues from the cap structure. Water molecules are also important in this structure. Ordered water molecules in the structure act as hydrogen bond bridges between strands in the barrel, in particular the residues Leu10 and Val40 in IL-1 β are linked by a water molecule in this way. This seems to be a very important bond since a Thr9Gly mutant in IL-1 β is lacking this water molecule and it has relatively unchanged receptor binding activity but 200 fold decreased biological activity (Simon et al., 1993).

1.6.3 The Hairpin Triplet

The strands of the hairpin triplet are coiled in such a way that the inner and outer strands can be defined. Inner strands hydrogen bond, not only to the other strand in the hairpin, but also form inter-hairpin hydrogen bonds. Outer strands however, only form hydrogen bonds with the inner strand of their hairpin (Figure 1.6.3). In IL-1 β only two of the inner strands form hydrogen bonds directly with the other, the third strands forms hydrogen bonds with the other through a water molecule. Like the barrel, the cap structure is made up of layers of amino acids. There are two layers in this structure with the top layer furthest away from the barrel. They are made up of hydrophobic residues and very often these positions are filled by leucine or valine residues. The second layer is proximal to the barrel and three residues, one from each of the outer strands, make up this layer along with three residues pointing downwards from the barrel. These interlocking residues therefore form the interface of the barrel and the cap.

1.6.4 Loop Structures

The loops of the β -trefoil structures vary greatly in length, amino acid sequence and in the confirmations they adopt. They act to cover most of the barrel and cap such that these

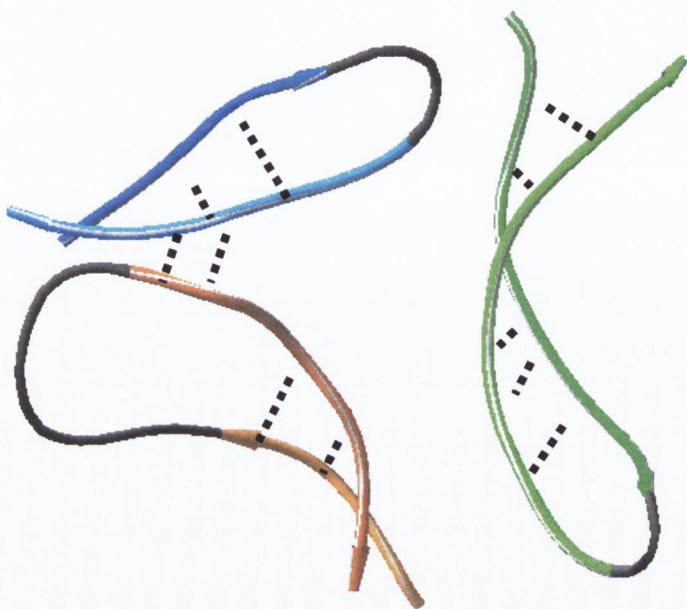


Figure 1.6.3 Schematic representation of the arrangement of β -strands in the triangular cap in IL-1 β . The strands of the cap are shown by green , blue or orange arrows, the black lines represent the approximate position of hydrogen bonds between the β -strands, based on the structure of IL-1 β .

become buried with the loop structures and make up about 70% of the exposed surface of the protein. This ability of the fold to accommodate such variations in loop structures means that it is ideally suited to being capable of adapting the fold to different functions. It also indicates that any conservation in the fold is likely to occur at sites that form the core layers of the barrel and cap structures, whereas evolution of functional divergence is likely to arise from variation in the sequence of the loops.

1.6.5 Structure of IL-1RI

The structure of the IL-1 receptor consists of three repeated domains with an immunoglobulin (Ig) like fold (Schreuder et al., 1997; Vigers et al., 1997). Each consists of two β -sheets packed against each other in a β -sandwich structure. In a generalised format each domain consists of seven β -strands, named A to G from the N to the C terminus. Four β -strands contribute to one sheet in the sandwich and three to the other. Each domain is linked by a flexible linker region, which is longer between domains 1 and 2 than between domains 2 and 3. All three domains have a conserved pair of cysteines, as found in other Ig domains, and also have a conserved tryptophan about 14 residues after the first cysteine. Domains 1 and 2 are held together by a disulphide bond between Cys104 on the linker of the two domains and Cys147, on the loop between strand C2 and D2 (Figure 1.6.4). In the ligand bound form domains 1 and 2 form a groove into which the ligand can bind, and in this way the domains form a cap over half of the hairpin structure and one side of the β -barrel (Figure 1.6.5). Also, the linker between domain 2 and 3 is almost completely stretched out in such a way that domain 3 lies below and almost at right angles to the cap structure of domains 1 and 2, and gives the receptor a very open structure allowing accommodation of the ligand. However, it is not known whether this is the conformation of the unbound receptor. The crystal structure of a 21mer peptide inhibitor bound form of the receptor has also been solved (Vigers et al., 2000). This structure shows a much more compact receptor with domain 3 rotated by about 170° relative to its position in the ligand bound form and also with the ligand binding region twisted such that it points away from the centre of the structure (Figure



Figure 1.6.4 Structure of IL-1RI domains 1 and 2 linked by a disulphide bond. Domains 1 and 2 of the IL-1 β bound form of IL-1RI are shown with the N terminus of domain 1 highlighted in green and the C terminus of domain 2 highlighted in red. The position of disulphide bonds in the domains is depicted in stick representation with the residues Cys104 and Cys147 labelled to highlight the bond linking domains 1 and 2.

1.6.6). This shows the degree of flexibility of this linker region and that the three Ig domains are not at all restrained in the conformations that they can adopt.

1.6.6 Structural Determinants of IL-1 β and IL-1Ra Receptor Binding

The structures of IL-1 β (Vigers et al., 1997) and IL-1Ra (Schreuder et al., 1997) in complex with IL-1RI have both been solved. The solving of these structures revealed a new mode of cytokine binding, which is seen as similar but distinct from the binding mode of fibroblast growth factor, which binds a receptor with two immunoglobulin-like domains but in a manner different to that of IL-1 (Plotnikov et al., 2001). The overall binding of IL-1 resembles that of the ligand being surrounded by a question mark (Figure 1.6.5). This is more pronounced in the structure of IL-1 β bound to the receptor, where the membrane proximal domain (domain 3) is rotated by approximately 20° relative to that of the IL-1Ra bound receptor. This is due in part to the loop between strands 7 and 8 of IL-1 β . This loop is highly solvent accessible in the unbound form and has a patch of positively charged lysines at its centre. The side chains of these residues point out into the solvent and are thereby able to interact with an oppositely charged patch of aspartate and glutamate residues on domain 3 of the receptor. This patch of residues is located on a loop between strands C and D of the third Ig domain β -strands which are positioned in such a way that they point up towards the ligand (Figure 1.6.5). This rotation, to a more closed structure in the IL-1 β complex, is made possible by the flexible linker region between domains 2 and 3 of the receptor. IL-1Ra on the other hand does not have a similarly charged positive patch on its 7-8 loop and therefore is unable to interact well with the distant domain 3. The loop between strands 4 and 5 of IL-1 β also interacts with an area on domain 3, nearer the linker region than that which interacts with the charged patch of loop 7-8. The residues in this loop bind to residues on and between β -strands F and G of domain 3 using a combination of hydrophilic and hydrophobic interactions. IL-1Ra also interacts with the receptor in this region close to the top of domain 3, even though the loop in the ligand is much shorter in this region than in IL-1 β . However, mutagenesis studies have shown that insertion of the loop from IL-1 β can increase the

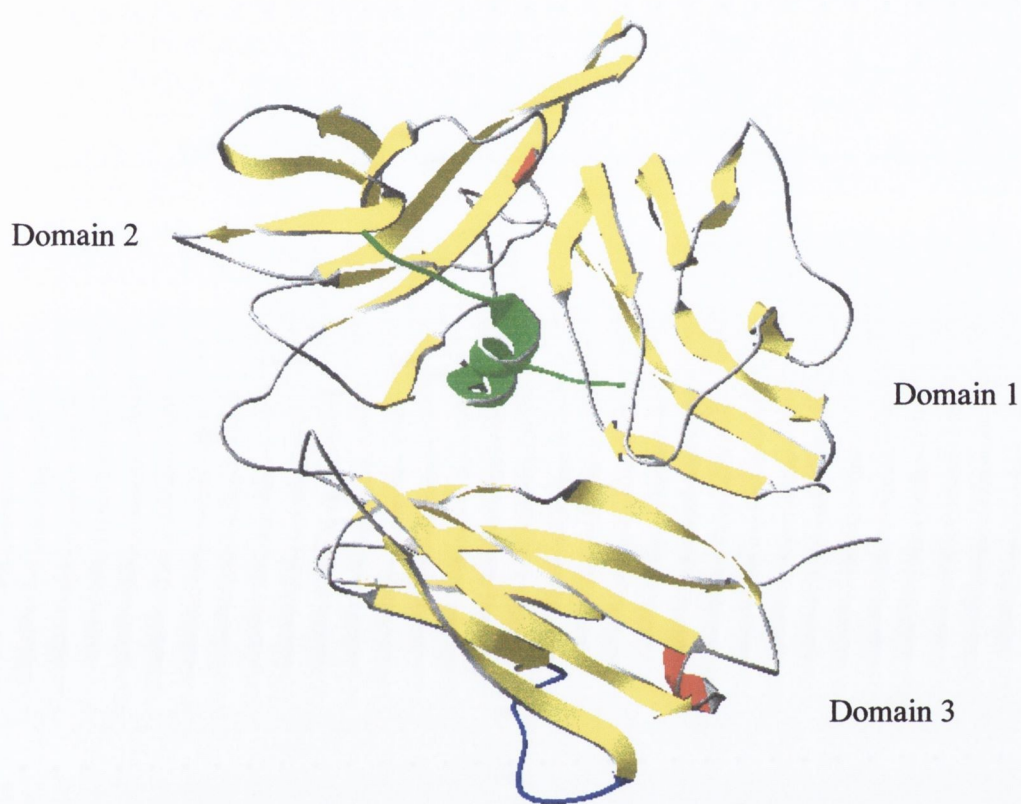


Figure 1.6.6 Structure of IL-1RI with a bound peptide inhibitor. IL-1RI is shown in yellow, with the 21mer peptide (ETPFTWEESNAYYWQPYALPL) shown in green. The position of the loop which interacts with loop 7-8 of the bound IL-1 β ligand is shown in blue, highlighting that domain 3 is twisted by 170° relative to the ligand bound structure.

agonist properties of the IL-1Ra K145D mutant (Greenfeder et al., 1995b, see section 1.6.7). Furthermore, partial deletion of the loop from IL-1 β reduces receptor binding 10 fold and biological activity 1,000 fold (Simoncsits et al., 1994), while insertion of the short loop from IL-1Ra into IL-1 β also reduces the biological activity and receptor binding affinity of IL-1 β 1,000 fold (Boraschi et al., 1995) clearly suggesting that the length of this loop is important for receptor binding and biological activity.

The binding at domains 1 and 2 is more conserved between IL-1 β and IL-1Ra, and contacts are made to strands A1, loop B1-C1, A2, B2, and loop B2-C2. The residues involved in this binding overlap in both IL-1 β and IL-1Ra and consist of residues from β -strands 1, 2, 3, 10, 11 and 12, and the loops 1-2, 2-3 and 3-4. The largest of these loops, the loop between strands 3 and 4, fits in the groove between domains 1 and 2. In most cases the interactions in this region are hydrophilic in nature. There are more residues involved in IL-1Ra binding to domains 1 and 2 than for that of IL-1 β , which may compensate for its lack of strong binding through domain 3 and therefore still allow tight binding. Indeed, a truncated form of the receptor consisting solely of domains 1 and 2 binds IL-1Ra with high affinity (28nM) but only binds IL-1 β with low affinity (7 μ M) (Schreuder et al., 1997). In the case of IL-1Ra, binding results in about 20% of the total surface area being covered which made indicate why such tight binding is observed between receptor and ligand.

1.6.7 Uncoupling of Ligand Binding and Biological Activity in IL-1 β

1 Asp145

Many site directed mutagenesis studies have identified residues which are important for the receptor binding activity of IL-1 β , IL-1 α and IL-1Ra, and many of the conclusions drawn from those studies were confirmed when the crystal structure of the IL-1 β and IL-1Ra in complex with IL-1RI were solved. However, there were also some mutations that affected IL-1 biological activity but did not affect its receptor binding capacity. Such mutations can be understood as altering in some way the ligand's ability to interact with

IL-1RAcP. Asp145 is the most significant of these residues. Ju et al. (1991) showed that conversion of the aspartate to the oppositely charged lysine, as found in IL-1Ra, converted IL-1 β to an antagonist. Meanwhile, in the reverse situation, IL-1Ra can be converted to a partial agonist when its lysine is mutated to an aspartate, although more than 1000-fold concentration of the IL-1Ra mutant is required to elicit a similar response to that of IL-1 β . Therefore, the residue at this position in the structure seems to be essential in determining the ability of the ligand to recruit IL-1RAcP and activate signal transduction. The same phenotype is seen in the equivalent IL-1 α mutant (Kawashima et al., 1992). Size, as well as charge, seems to be an important determinant of this activity also, as large residues such as tyrosine also compromise the ability of IL-1 α to activate signal transduction, whereas other acidic or small amino acid do not (Kawashima et al., 1992). Importantly, the bioactivity of the K145D mutant of IL-1Ra is inhibited by an antibody against IL-1RAcP, which suggests that this mutation is indeed sufficient to allow recruitment of IL-1RAcP to the receptor complex (Greenfeder et al., 1995b). It is not known whether the residue interacts directly with IL-RAcP, or if the mutant causes local changes in the structure that allow the recruitment to occur. However, since the crystal structure of the IL-1Ra K145D mutant is virtually identical to the wildtype, this latter scenario seems unlikely (Labriola-Tompkins et al., 1991).

2 Arg11

Mutation of Arg11 of IL-1 β to glycine results in a 100-fold decrease in biological activity, as measured by H³ thymidine incorporation into T-cells, but receptor binding affinity is reduced only by 25% (Gehrke et al., 1990). It has been argued that this mutation has the secondary effect of altering the position of the Asp145 in IL-1 β ; by removing the interaction of Arg11 with Lys16, the mutant protein allows strands 1 and 12 to collapse into an hydrated pocket located between strands 1, 2 and 4 (Auron et al., 1992). Other mutants in which larger amino acids than glycine replace the arginine do not have the same loss of function phenotype, since they can pack against Lys16 and prevent movement of strands 1 and 12 (Auron et al., 1992). However, this mutant still retains the ability to induce a limited amount of gene expression, specifically of genes that do not require *de novo* synthesis of protein such as *c-fos* and *c-jun*, *jun-B*, IL-1 β and IL-6.

However, expression of genes that are induced at a later time course and do require *de novo* protein synthesis, such as procollagenase, is abolished in cells treated with the Arg11Gly mutant (Conca et al., 1991). This effect was shown not to be due to the rapid inactivation or degradation of the mutant. Since IL-1RAcP is required for both early and late phase gene induction (Cullinan et al., 1998), this suggests that the Arg11Gly mutant is able to recruit the IL-1RAcP but in a manner where a stable complex is not formed to allow the full range of signal transduction to occur.

3 Thr9

Similar to the Arg11Gly mutation, mutation of Thr9 to glycine also results in loss of biological activity, while receptor binding capacity is completely retained (Simon et al., 1993). The structure of this mutant has been solved which shows only subtle changes in conformation, with the position of Asp145 unaltered in the mutant (Camacho et al., 1993). The most dramatic changes in structure that are seen in the mutant structure are attributed to crystal packing. However, since the crystal packing is altered from the wildtype protein this is an indication in itself that the mutant alters protein-protein interaction properties of IL-1 β . Specifically, alterations occur in the region of loop 3-4, which corresponds closely to changes also seen in the 2D NMR spectrum of the mutant at residues 28 and 29 (Simon et al., 1993). Whether these differences account for the changes in biological activity seen in the mutant is uncertain.

1.6.8 Overview of Ligand Binding Mechanism

In an overall view therefore, the binding mechanism for IL-1 ligands to the receptor can be described as below. Both IL-1 β and IL-1Ra bind through domains 1 and 2 but IL-1Ra does so more strongly (Schreuder et al., 1997). IL-1 β also interacts with domain 3 of the receptor and results in a rotation of this domain by about 20° relative to the IL-1Ra bound form. This difference in rotation must in some way allow recruitment of IL-1RAcP to the complex, which then forms the fully active signalling complex. This is likely to involve the key residue of Asp145 in IL-1 β . Asp 145 of IL-1 β is quite solvent accessible on the face of the ligand after receptor binding and would therefore be available to interact with IL-1RAcP (Figure 1.6.7

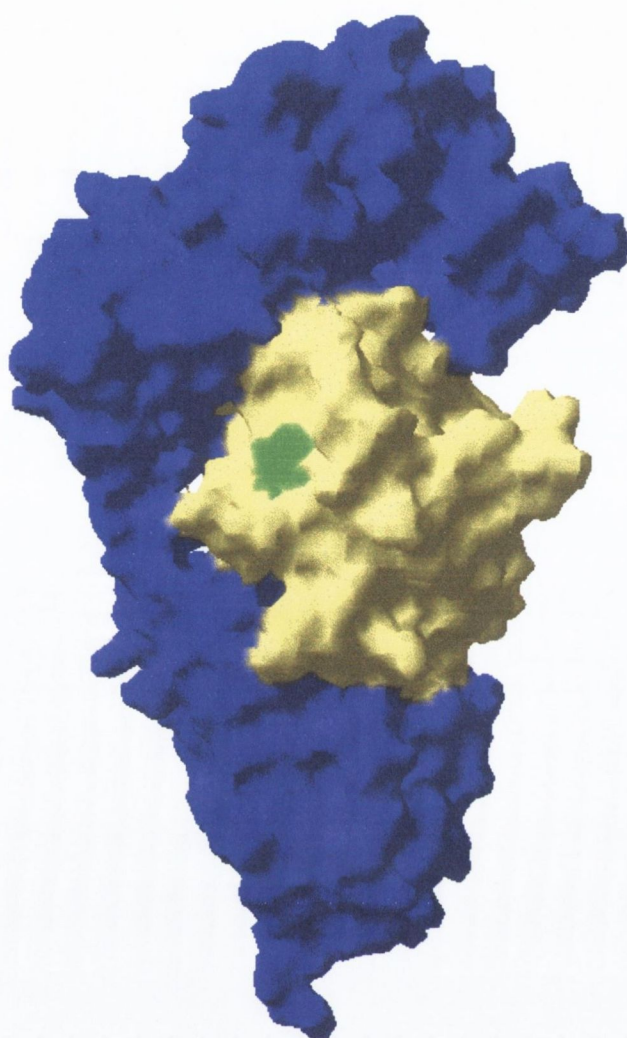


Figure 1.6.7 Molecular surface display of IL-1 β in complex with IL-1RI. The residue Asp145 is highlighted in green exposed on the surface free for interaction with IL-1RAcP.

). Indeed, the fact that the activity of the IL-1Ra K145D mutant is inhibited by antibodies to IL-1RAcP suggests that this residue allows for productive interaction of the mutant and the IL-1RAcP (Greenfeder et al., 1995b). The conformation adopted by IL-1RAcP is not known but it may not necessarily adopt one similar to that of the ligand bound IL-1RI. The structure of the peptide inhibitor shows that the three Ig domains are free to adopt very different conformations (Vigers et al., 2000). Modelling of the trimeric complex suggests that IL-1RAcP binds at the 'back' of the IL-1RI/ligand complex (Casadio et al., 2001). A binding model at the 'front' of the complex involving loop 6-7 is ruled out on the basis that antibodies to this region are unable to inhibit IL-1 signalling. However, the manner of this binding awaits crystal structures of the trimeric complex. There are indications that the initial recruitment and interaction is only dependent on the extracellular portion of the receptors since mutant receptors with no intracellular tails (Huang et al., 1997) and IL-1RII can both recruit IL-1RAcP (Lang et al., 1998; Malinowsky et al., 1998). Furthermore, the orientation of the membrane proximal domain seems an important determinant since chimeric proteins of IL-1RII with a stalk region from the EGF receptor losses its ability to interact with IL-1RAcP (Neumann et al., 2000). An understanding of this structure would lead to more insights into how the IL-1 system is activated and could lead to improvements in the design of inhibitor drugs targeting the receptor complex. Since the site of action would be at the head of the signal transduction pathway such drugs would be powerful inhibitors of IL-1 activity.

1.7 Aims of Study

At the outset of this study the only known information on the novel IL-1 family members was their DNA and amino acid sequences, which were used to class the new proteins as members of the IL-1 family. This study therefore aimed to:

1. Use sequence information to predict a function for IL-1F5
2. To express and purify IL-1F5 for use in functional and structural studies
3. Carry out functional assays to test the activity of both IL-1F5 and IL-1F6
4. Use crystallographic methods to gain insights into the structure and function of IL-1F5.

Chapter 2. Materials and Methods

2.1 Materials

Human IL-1 α was obtained from the National Institute for Biological Standards and Control (Hertfordshire, UK). Murine IL-1Ra was obtained from R&D systems (Oxon, UK) and the National Institute for Biological Standards and Control (Hertfordshire, UK). Murine IL-1F5 and IL-1F6 were initially obtained from Millennium Pharmaceuticals (MA, USA).

The NF κ B consensus probe was obtained from Promega and is a 22 sequence shown below, with consensus sequence underlined;

5'-AGTTGAGGGACTTTCCCCAGGC-3'

The STAT3 probe was obtained from Santa Cruz Biotechnology (Santa Cruz, California, USA) and has a 24 base pair sequence shown below with consensus sequence underlined;

5'-GATCCTTCTGGGAATTCCTAGATC-3'

Mutant probe from the same source is mutated in the three underlined base pairs;

5'-GATCTTTCTGGGCCGTCCTAGATC-3'

γ^{32} P- ATP, 9.25 Ci/mmol was obtained from Amersham Life Science Products (Uppsala, Sweden) and T4 nucleotide kinase was obtained from Promega (Madison, Wisconsin, USA).

Maxi prep, endotoxin free plasmid purification kits were obtained from Promega (Madison, Wisconsin, USA). The NF κ B-luciferase was a kind gift of Dr. R. Hofmeister (University of Regensburg, Germany) and contains five κ B sites upstream of the luciferase gene. The E-selectin-luciferase plasmid was obtained from Dr. M. Muzio (University of Michigan, Medical School, Department of Pathology, Ann Arbor, MI, USA). Gene juice was obtained from Novagen (Madison, Wisconsin, USA).

M2 anti-FLAG antibody was obtained from Millennium Pharmaceuticals (Cambridge, MA, USA), anti-JNK and anti-p38 antibodies were obtained from Promega (Madison,

Wisconsin, USA). FITC labelled anti mouse IgG₁ was purchased from Pharmigen (Becton Dickinson, San Diego, CA, USA). Mouse serum was obtained from Sigma (Poole, Dorset, UK).

For PCR of murine IL-1F5 primers were designed to the coding region using Primer 3. Output, the primers, giving a 424 bp product, had the following sequence:

Forward primer: 5'-TTC CGA ATG AAG GAT TCA GC-3'

Reverse primer: 5'-AGA AGT CTG TGA TGG GAG CA-3'

Primers to murine aldolase 1, giving a 442 bp product were:

Forward primer: 5' TTC CAC GAG ACA CTG TAC CAG AAG 3'

Reverse primer: 5' ACC ATG TTG GGC TTC AGC AAT G 3'

Primers to human β -actin, giving a 441 bp product were:

Forward primer: 5' GAGAAGATGACCCAGATCATGT 3'

Reverse primer: 5' ACTCCATGCCAGGAAGGAAGG 3'

Primers to murine β -actin, giving a 493bp product were:

Forward primer: 5' CAGATCATGTTTGAGACCTTC 3'

Reverse primer: 5' ACTTCATGATGGAATTGAATG 3'

Primers to murine GAPDH, giving a 239 bp product were:

Forward primer: 5' TGA ATG ACA TCA AGA AGG TGG TGG AG 3'

Reverse primer: 5' TCC TTG GAG GCC ATG TAG GCC AT 3'

RTase was obtained from Sigma (Poole, Dorset, UK) and Taq polymerase was obtained from Promega (Madison, Wisconsin, USA).

Both the G75-sephadex and the MonoQ ion exchange column were obtained from Pharmacia (Piscataway, New Jersey, USA).

2.2 Bio-informatic Sequence Analysis

Sequence alignments were compiled using CLUSTALW 1.82, edited by hand using SEAVIEW and formatted using ESPript (http://prodes.toulouse.inra.fr/ESPript/cgi-bin/nph-ESPript_exe.cgi). This alignment was used to generate a dendrogram in Jalview

calculated using percentage identities. Secondary structure prediction was carried out using nine different prediction programs: Protein Predict (<http://cubic.bioc.columbia.edu/predictprotein/>), PSI pred (<http://insulin.brunel.ac.uk/psipred>) Hierarchical Neural Network, SOPMA, GOR (<http://pbil.ibcp.fr>) Nnpredict (www.cmpharm.ucsf.edu), Discrimination of Protein Secondary Structure Class (DSC) (www.bmm.icnet.uk/~prof/dsc.html), Bioinbgu (www.cs.bgu.ac.il/~bioinbgu), 3D-PSSM (www.bmm.icnet.uk/~3dpssm). Tertiary structure prediction was carried out using Swiss-Model with the Swiss-Prot PDB-viewer interface (www.expasy.ch/spdv/mainpage.html). Individual sequences were submitted to the protein data bank to determine suitable templates onto which to model the sequences. The structures of IL-1 β and IL-1Ra chosen for this purpose were; 1I1B, human IL-1 β at 2 Å, 7I1B, human IL-1 β solved by NMR, 8I1B, murine IL-1 β at 2.4 Å, 1ILR, human IL-1Ra at 2.1 Å and 1IRAX, the ligand chain of IL-1RI/IL-1Ra complex at 2.7 Å. Hydropathy plots were generated using ProtScale from Expasy (<http://ca.expasy.org/>) with Kyte and Doolittle parameters.

2.3 Subcloning

2.3.1 Polymerase Chain Reaction

Primers were designed to the first and last 15 bases of the murine IL-1F5 gene with the forward primer incorporating an EcoRI restriction site and the reverse primer incorporating an NdeI restriction site. The EcoRI site was protected by three bases (marked in bold) and the NdeI site was protected by seven bases (marked in italics). The sequence of the primers is given below:

Forward 5' TTA ATT **TCA** *TAT* GGT CCT GAG TGG GGC GCT G 3'

Reverse 5' AAA **GAA** **TTC** CTA GTC ACA CTG CTG GAA GTA 3'

PCR reactions were set up with 1X buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 1.5 unit Taq polymerase, 200 μ M of each dNTP, 100 ng plasmid DNA, 1.5 mM MgCl₂ (unless otherwise indicated) and 400 nM of each primer. Two to three drops of mineral

oil was added on top of each reaction mix. Amplification was carried out using the conditions given below:

95°C for 5 minutes

35 cycles of 95°C for 60 seconds

58°C for 60 seconds

72°C for 60 seconds

Final extension 72°C for 10 minutes.

2.3.2 Gel Cleaning of PCR products

PCR products were resolved on 1% agarose gels in 1X TAE buffer (4.84 g/L Tris base, 1.142 ml/L glacial acetic acid, 0.744g/L EDTA, pH 8.5) at 90 volts for 30 minutes. The gel was stained with ethidium bromide and visualised under UV radiation. The products were then excised from the gel using sterile razor blades and placed in pre-weighed microfuge tubes. The tubes were re-weighed and the weight of the slice calculated. Using this figure 3 volumes of 6 M NaI was added to the microfuge and then incubated at 52°C until all the agarose was melted. 15 µl of a silica suspension containing 100 mg/ml silica in 3 M NaI was added. About 10 µl of the suspension is sufficient to bind ~3 µg of DNA. The mixture was then incubated at room temperature for 10 minutes with intermittent shaking to ensure the silica remained in suspension. The silica was then pelleted by centrifugation at 1,300 x g for 30 seconds. The supernatant was removed and the pellet was washed three times with wash buffer containing 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 2.5 mM EDTA and 50% (v/v) ethanol. The pellet was then allowed to air dry for 5 minutes and the DNA was dissolved in 20 µl T/E buffer pH 7.4.

2.3.3 Digestion of PCR Products and Plasmid

Approximately 1 µg DNA to be digested was incubated with 1 unit of the appropriate restriction enzyme and 1X buffer as recommended by the manufacturers in a final volume

of 20 μ l. If required BSA was also added to the mixture at a final concentration of 100 μ g/ml. The reaction mix was then incubated at 37°C for 1 hour for analysis purposes or overnight if complete digestion was required.

2.3.4 Spin Column Cleaning of Digested Products

250 μ l binding buffer was added to the digested products and mixed well before applying to a spin column filter. The filter was inserted into a 1.5 ml microfuge tube, which was spun at maximum speed for 30 seconds. The flowthrough was discarded and 250 μ l of wash buffer was applied to the column. The column was again spun at maximum speed for 30 seconds and the flowthrough was discarded. Another 100 μ l of wash buffer was added and the column spun at maximum speed for 1 minute and the flowthrough discarded. Finally 50 μ l of elution buffer was added to the column and spun at maximum speed for 1 minute. The flowthrough containing the DNA used immediately or stored at – 20°C until required.

2.3.5 Ligation Reaction

Ligations were carried out using the Rapid DNA Ligation Kit (Boehringer Mannheim). 50 ng of insert DNA was reacted with varying ratios of insert DNA ranging from 0 to 6 molar excess concentrations. DNA was diluted with dilution buffer to a final concentration of 1X and then DNA ligation buffer was added at a 1 in 2 dilution factor. Finally, 0.5 units of T4 ligase was added to the reaction mix. The reaction was allowed to proceed for 30 minutes at room temperature.

2.3.6 Preparation of Competent Cells

A single colony of BL-21(DE3) or DH5 α *E.coli* was inoculated into a 50 ml LB overnight culture. The culture was diluted 1 in 40 in a final volume of 500 ml and grown to a density with an A_{600} of 0.45. Cells were pelleted by centrifugation at 2,500 xg for 20

minutes at 4°C. The cells were then resuspended in 20 ml of ice-cold trituration buffer (100 mM CaCl₂, 70 mM MgCl₂, 40 mM sodium acetate, pH 7.5). Once resuspended the cells were diluted with trituration buffer to a final volume of 500 ml and incubated on ice for a further 45 minutes. Cells were then pelleted at 2,000 x g for 15 minutes and again resuspended gently in 20 ml trituration buffer. DMSO was then added to a final concentration of 7% (v/v), the cells were aliquoted and snap frozen in liquid N₂ and stored at -80°C for later use.

2.3.7 Transformation of Ligation Reaction

Ligation reactions were incubated with 100 µl of ultra competent bacteria on ice for 10 minutes. The mixture was then plated out on pre-warmed LB-ampicillin agar plates, allowed to air dry and incubated at 37°C overnight. The colonies that resulted from the transformation were streaked onto an agar plate with numbered grids and grown overnight at 37°C and thereafter stored at 4°C, glycerol stocks of each colony were also prepared and stored at -70°C.

2.3.8 PCR Analysis of Ligation Colonies

Colonies resulting from the ligation reaction were toothpicked into a PCR reaction mixture and the PCR carried as described in section 2.3.1 with the exception that the initial heating step at 95°C was extended to 10 minutes.

2.3.9 Plasmid Mini-Prep from Ligation Colonies

Colonies from the ligation reaction were toothpicked into a 10 ml LB-ampicillin culture and grown overnight at 37°C at 200 rpm. 1.5 ml of the culture was pelleted by centrifugation at 10,000 x g for 5 minutes. Plasmids were isolated using the Wizard® Plus SV Minipreps DNA purification System from Promega. Supernatant was discarded and the pellet resuspended in 250 µl of resuspension buffer, followed by 250 µl of lysis

buffer. Mixing was achieved by inverting 4-6 times and the mixture let stand for 5 minutes at room temperature. 350 ml of neutralization buffer was added and mixed immediately by inversion. The resulting lysate was cleared by centrifugation at 13,000 x g for 10 minutes. The cleared lysate was removed by decanting into a spin column, which was then spun at maximum speed for 1 minute. The column was washed with 95% ethanol. DNA was eluted with 100 µl of sterile T/E buffer pH 7.4.

2.3.10 Digestion Analysis of Plasmids from Ligation Colonies

As for section 2.3.3, with digestions analysed for digestion products by separation on 1% agarose gels stained with ethidium bromide and visualised under UV light.

2.4 Expression and Purification of IL-1F5

2.4.1 Induction of Expression

10 ml of an overnight culture of BL21 transformed with pET21a-IL1-F5 were incubated with 0.4 mM and 1 mM IPTG for 1 hour and 3 hours at 37°C. Cells were grown in M9 minimal medium containing 1 mM MgSO₄, 0.1 mM CaCl₂, 1 mM Thiamine-HCl, 2% glucose, 20 mg Proline and 1X M9 salts (6 g/L Na₂HPO₄, 3 g/L KH₂PO₄, 1 g/L NH₄Cl and 0.5 g/L NaCl, pH 7.4) supplemented with 5 g/L yeast extract. Salts were prepared as a 10X stock and autoclaved before use, all other components were filter sterilized with a 0.2 µm filter and stored at 4°C before use. Samples of the bacterial cultures were diluted back to the OD₆₀₀ of the culture at the time of induction and 1 ml was pelleted by centrifugation at 1,300 x g for 2 minutes. The pellet was resuspended in 25 µl 5X SDS-sample buffer (125 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 0.0002 % (w/v) bromophenol blue, 1% (v/v) β-mercaptoethanol) and boiled for 5 minutes at 100°C. The whole cell lysates were then examined by SDS-PAGE analysis (see section 2.9.2) for the expression of IL-1F5.

2.4.2 Preparation of Crude Cell Lysates

Cultures were pelleted by centrifugation at 10,000 x g for 10 minutes and the supernatant removed. Cells were then lysed in 30 ml per litre original culture of ice cold NETN lysis buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl pH 8, 0.5% (v/v) NP-40). 20 µg/ml lysozyme was added and the lysate incubated at room temperature for 10 minutes with continuous rolling. This was followed by sonication on ice with 5 second pulses followed by 5 second pauses to prevent over heating of the preparation. The suspension was cleared of insoluble material by centrifugation 38,000 x g for 1 hour.

2.4.3 Ammonium Sulphate Precipitation

The soluble fraction of the bacterial lysate was then precipitated with a 50% saturated ammonium sulphate solution (1.31 g ammonium sulphate per ml of lysate). The ammonium sulphate was added slowly with continuous stirring on ice. Once all the ammonium sulphate was added, the preparation was left stirring at 4°C for a minimum of 2 hours. Precipitated protein was pelleted by centrifugation at 12,000 x g for 2 hours. The supernatant was discarded and the pellet stored at 4°C until use.

2.4.4 Packing of Sephadex G-75 Column

53 g of Sephadex G-75 powder (Pharmacia) was allowed to swell in 1.5 L of 50 mM ammonium acetate for 24 hours. Enough buffer was added to the settled gel to give approximately a 75% slurry before it was de-gassed under a vacuum for 2 hours. Air was excluded from the outlet of the column to be used by injection of water into the outlet tubing, which was then immediately closed. The gel was gently mixed to give an even consistency and then poured in one operation into the column with an extension attached to accept the full volume of the gel. The slurry was poured slowly and the column was tilted at an angle to minimised introduction of air bubbles. Once the gel was poured the outlet was opened to allow quick and even settling of the gel. As soon as the extension

was not required, it was removed and flow of buffer into the column was continued from the reservoir. The column was equilibrated with at least 5 bed volumes of buffer.

The column was calibrated using dextran blue to estimate the void volume and IL-1Ra to estimate the expected elution volume of IL-1F5. 10 mg IL-1Ra and 30 mg dextran blue were mixed in 10 ml of 50 mM ammonium acetate and loaded on the column. They were allowed to run into the column and eluted with 50 mM ammonium acetate. The column ran at a rate of 1.4 ml/min and 5 minute fractions were collected. The elution profile of dextran blue was followed by absorbance readings at 630 nm, while that of IL-1Ra was followed by the absorbance of fractions at 280 nm.

2.4.5 Gel Filtration on Sephadex G75 Column

Precipitated protein was resuspended in 50 mM ammonium acetate and cleared of any insoluble material by centrifugation before loading on the column. Buffer was removed from the top of the column until the level reached the frit at the top of the settled bed. Samples were then gently loaded onto the column and allowed to run into the column before the column was replenished with fresh buffer and connected to the reservoir. The void volume was allowed to run through and then 5 ml fractions were collected until a total volume of 550 ml had run through the column. Samples from every second fraction were then analysed by SDS-PAGE for the presence of IL-1F5. Fractions containing IL-1F5 were shell frozen in a round bottomed flask in an ethanol ice bath and dried in a vacuum freeze drier.

2.4.6 Anion Exchange

2.4.6.1 DE-trisacryl

A 1:1 (w:v) slurry of DE-trisacryl in 0.01 M phosphate buffer pH 7.2, was prepared and washed in 0.01 M phosphate buffer pH 7.2. Protein from the bacterial lysate soluble fraction was dilute 1 in 10 with 0.01 M phosphate buffer pH 7.2 and 10 ml was incubated

with the 100 µl DE-trisacryl slurry for 15 minutes at room temperature with gentle agitation. The DE-trisacryl was pelleted by centrifugation at 1,000 x g for 5 minutes and washed in 500 µl wash buffer by rolling for 5 minutes at room temperature. Wash buffers of various stringencies were used with 25 mM phosphate buffer pH 7.2 and from 25 mM NaCl to 200 mM NaCl. Protein was then eluted with 25 mM phosphate buffer pH 7.2, 1 M NaCl and fractions analysed on Commassie blue stained SDS-PAGE gels.

2.4.6.2 Mono Q

After freeze drying samples were reconstituted in 25 mM Tris-HCl pH 8 using 1 ml per litre of original culture. The protein was then passed through a MonoQ column (Pharmacia), equilibrated with 25 mM Tris-HCl, pH 8, using the HPLC. The column as run at 4 ml/min and the salt concentration was increased from 0 M to 0.2 M NaCl from 5 minutes to 38 minutes and then from 0.2 M to 1 M NaCl from 38 minutes to 40 minutes. 2 ml fractions were collected and analysed for IL-1F5 by SDS-PAGE.

2.4.7 Circular Dichroism Analysis

Material for circular dichroism (CD) analysis was dialysed overnight into water before use in order to aid the reduction of noise in the spectra. Measurements were taken in a Jasco 800 instrument which had been pre-flushed for 30 minutes with N₂ gas prior to use. Ellipicity was measured from 250 nm to 180 nm, with a response time of 1 second, a scan speed of 20 nm/min and a bandwidth of 1 nm. Measurements were taken in a 3 ml quartz cuvette with protein at a concentration of 37.5 µg/ml. Six cumulative scans were taken in each case and baseline measurements were subtracted from the spectra.

2.4.8 Fluorescence Studies

20 µg/ml of protein was dialysed into water and analysed in a Perkin Elmer LS 50B fluorimeter. Emission spectra from 300 nm to 400 nm were taken at an excitation

wavelength of 280 nm unless otherwise stated. A bandwidth of 5 nm and a scan speed of 240 nm/min was used. 50 mM glycine-HCl was used as the buffer for pH solutions at pH 3.5 and 50 mM glycine-H₂SO₄ for pH 8.5 to 9, 40 mM sodium acetate-HCl was used for solutions of pH 4 to 6, 50 mM MOPS was used for pH 6.5 and 50 mM HEPES was used for solutions of pH 7 to 8. All guanidinium hydrochloride samples were made from a common stock of a 10 M solution and all solution used were filtered in a 0.2 µm filter and equilibrated to room temperature before measurements were made.

2.5 Antibody Production

A stock solution of IL-1F5 in 0.025 M Tris-HCl, pH 8, 100 mM NaCl at a concentration of 0.8 mg/ml was diluted 1 in 2 with complete Freund's adjuvant in a final volume of 2 ml. The mixture was then shaken vigorously for 1 hour at room temperature. 1 hour prior to injection of IL-1F5 the rabbits were injected with sterile filtered 2% (w/v) Evan's Blue dye in 0.9% (w/v) NaCl. This dye binds strongly to serum albumin and leaves the circulation only very slowly. Injection of the dye thus allows for enhanced visualisation of the popliteal lymph nodes. Rabbits were then anaesthetised using halothane and the hair on the inner thigh was removed and the skin cleaned and covered in antiseptic. An incision was made through the skin and the popliteal node identified. The node was injected with 0.1 ml (40 µg of protein). The procedure was repeated for both popliteal nodes. 14 days after injection the rabbit was given a booster of multiple subcutaneous injections of IL-1F5 prepared as described above except that incomplete Freund's adjuvant was used. This was repeated again on day 27 of the injection schedule. 9 days later a test bleed of about 10 ml was taken and analysed for antibody titre (described below). The titre was found to be sufficient and exsanguination was carried out.

Blood collected from the rabbits was ringed with a small spatula to prevent clot formation on the side of vessels. The blood was then allowed to clot at 4°C overnight. The blood was then centrifuged at 2,500 x g for 10 minutes at 4°C and the serum removed by decantation. The serum was aliquoted and stored at -80°C until required.

The serum was then analysed using the dot blot method. Nitrocellulose strips were cut and the purified IL-1F5 protein was dot blotted on to the nitrocellulose in concentrations ranging from 4 µg to 125 ng for high range analysis and 25 ng to 0.064 pg for low range analysis. As a control the strips were also dotted with PBS, and equivalent or highest doses of bovine serum albumin and lysozyme. The strips were then allowed to air dry for 1 hour, were washed twice in TBS-tween and then placed in blocking buffer of 5% non-fat dried milk in TBS-tween. The strips were then washed and incubated in the test serum at dilutions of 1 in 500, 1 in 1000 and 1 in 2000 in 5% non-fat dried milk TBS-tween, for 1 hour at room temperature. The strips were again washed and incubated with anti-rabbit HRP-antibody for 1 hour at room temperature. Blots were then developed using the enhanced chemiluminescence reagents.

2.6 Cell Culture

EL-4.NOB.1, murine thymoma cells and Jurkat, T-lymphocytoma cells were grown in RPMI medium (GibcoBRL, Life Technologies, Paisley, UK), T24, human bladder carcinoma cells were grown in medium 199 (Sigma-Aldrich Inc., St. Louis, MO, USA) and HeLa, human cervical carcinoma cells, Saos-2 (kind gift of Dr. S. Marmioli, Institute of Cytomorphology, CNR Chieti, Bologna, Italy), human osteoblast-like carcinoma cells, neuro2a cells (European Collection of Animal Cell Culture), 1321N1 glioblastoma and U87MG astrocytoma were grown in Dulbecco's modified Eagle's medium (DMEM). All media were supplemented with 10% (v/v) fetal calf serum (FCS), 1% (v/v) L-glutamine and 10ng/ml gentamycin (GibcoBRL, Life Technologies, Paisley, UK). Cells were seeded at 1×10^5 cells/ml and sub-cultured every 3-4 days. Cell viability was determined using trypan blue staining.

2.7 Electrophoretic Mobility Shift Assay (EMSA)

2.7.1 Preparation of Cells

Monolayer cells were seeded at 1×10^5 cell/ml in 6 well plates and grown for 48 hours before treatment. Suspension cells were seeded in 1 ml at 2.5×10^6 cell/ml and allowed to equilibrate at 37°C for 15 minutes before treatment.

2.7.2 Nuclear Extract Preparation

Nuclear extracts were prepared according to the protocol of Osborn et al (1989); the reaction was stopped by diluting suspension cells in 5 ml of ice-cold phosphate buffered saline (PBS) or by placing adherent cells on ice, aspirating off medium and washing twice with ice-cold PBS. Suspension cells were pelleted by centrifugation at $2,000 \times g$ for 10 minutes, washed in 1 ml Buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl) and pelleted again by centrifugation at $13,000 \times g$ for 10 minutes at 4°C. Adherent cells were scraped from wells in 800 μ l Buffer A and pelleted by centrifugation at $13,000 \times g$ for 10 minutes. Cells were incubated on ice in 20 μ l Buffer A plus 0.1 % (v/v) Nonidet P-40 (Sigma) for 15 minutes to cause lysis. The nuclei were pelleted by centrifugation at $13,000 \times g$ for 10 minutes at 4°C. The pellet was resuspended in 15 μ l Buffer C (20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% (v/v) glycerol) and incubated on ice for 15 minutes to cause nuclear lysis. The samples were again pelleted by centrifugation at $13,000 \times g$ for 10 minutes at 4°C and the supernatant transferred to 50 μ l Buffer D (10 mM HEPES pH7.9, 50 mM KCl, 0.2 mM EDTA, 5% (v/v) glycerol) for storage. All of the above buffers were supplemented with 5 μ l/ml 100 mM Phenylmethylsulfonyl Fluoride (PMSF) and 0.5 μ l/ml 1 M Dithiothreitol (DTT) prior to use to reduce protein degradation and oxidation.

2.7.3 Protein Concentration Determination

Determination of protein concentration was carried out by the use of the Bradford assay (Bradford, 1970). 5 µl samples were diluted to 20 µl final volume with Buffer D. The samples were then incubated for 5 minutes at room temperature with 200 µl Bradford reagent (0.01% (w/v) Commassie Brilliant Blue G-250, 4.7% (v/v) ethanol and 8.5% (v/v) orthophosphoric acid). The absorbance of the samples was measured at 570 nm. Protein concentration was calculated using a standard curve of Bovine Serum Albumin (BSA) concentrations from 0-20 µg/20 µl. All determinations were carried out in duplicate.

2.7.4 Electrophoretic Analysis

Nuclear extracts were analysed by the method of Sen and Baltimore (1986). 4 µg of sample was diluted with buffer D to a total volume of 15 µl and incubated at room temperature for 30 minutes with 2 µl poly (dIdC) (Pharmacia), 2 µl binding buffer (40% (v/v) glycerol, 10 mM EDTA, 100 mM Tris-HCl pH 7.5, 1 M NaCl, 1 mg/ml BSA, 50 mM DTT for NFκB binding, and 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% (v/v) glycerol for STAT3 binding) and 1 µl of 10,000-30,000 cpm ³²P labelled DNA consensus sequence probe. The reaction was stopped by the addition of 2 µl of 10X Final Sample Buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 10% (v/v) glycerol). The samples were then loaded on a 5% polyacrylamide gel in 0.5 X Tris Borate EDTA (TBE) (5.4 g/L Tris, 2.75 g/L Boric acid, 2ml/L 0.5 M EDTA, pH 8) buffer and allowed to pass through the gel at a constant voltage of 140 V for 90 minutes. Gels were then dried onto Whatmann paper and autoradiographed using Kodak X-Omat diagnostic film.

2.8 Luciferase gene reporter assay

2.8.1 Plasmid Preparation

2.8.1.1 Preparation of Competent Cells

1 ml of an overnight culture of *E.coli* DH5 α was added to 50 ml L-Broth (10 g/L Bacto-peptone, 5 g/L dried yeast, 10 g/L NaCl) and grown at 37°C in an orbital incubator at 200 rpm until the absorbance at 600 nm reached a reading of 0.6. Following centrifugation at 6,000 x g for 10 minutes at 4°C cells were resuspended in 500 μ l ice-cold 100 mM CaCl₂ and left on ice for 20 minutes. Cells were aliquoted, snap frozen in liquid N₂ and then stored at -70°C until required.

2.8.1.2 Transformation of Bacterial Cells

200 μ l of competent cells were incubated on ice for 30 minutes with 500 ng of DNA. The cells were then heat shocked by incubation at 43°C for 2 minutes and then placed on ice for 2-3 minutes. Next, 1 ml of pre-warmed L-Broth was added to the cells and they were incubated for 1 hour at 37°C. 100 μ l of this was plated on agar plates supplemented with ampicillin (10 μ g/ml) and allowed to grow for 16 hours at 37°C. A single colony from this plate was purified and grown under the same conditions. A scraping from this purification was then added to 100 ml L-broth supplemented with 100 μ l ampicillin (100 μ g/ μ l). The culture was allowed to grow for 16 hours in a shaking incubator at 37°C.

2.8.1.3 Purification of Plasmid DNA

Plasmids were purified using Wizard® PureFection Plasmid DNA Purification System from Promega. Bacterial cultures were pelleted by centrifugation at 10,000 x g for 10 minutes at 4°C. Cells were resuspended in 6.25 ml cell resuspension buffer (50 mM Tris-HCl pH 7.5, 10 mM EDTA, 100 μ g/ml RNase A). 8.25 ml cell lysis buffer (0.2M NaOH, 1% (w/v) SDS) was then added and allowed to incubate for 5 minutes at room

temperature. The reaction was stopped by the addition of 8.75 ml Neutralisation Buffer (1.32 M potassium acetate, pH 4.8) and mixed thoroughly. Cellular debris was removed by centrifugation at 10,000 x g for 40 minutes. Supernatant was retained and purified of endotoxin using Endotoxin Removal Resin. Plasmid DNA was then mixed with 5 ml of 5 M guanidine thiocyanate and purified using MagneSil™ paramagnetic particles. In order to purify the DNA further, it was washed with 5 ml 4/40 Wash Solution (4.2 M guanidine-HCl/40% (v/v) isopropanol) and multiple times with 80% (v/v) ethanol. Plasmid DNA was then dissolved in 6 ml water and precipitated using 3 ml 7.5 M sodium acetate and 22.5 ml 95% ethanol. DNA was pelleted by centrifugation at 14,000 x g for 15 minutes, washed in 70% ethanol and pelleted again by centrifugation at 14,000 x g for 5 minutes before dissolving in 500 µl TE buffer pH 7.4. Plasmid DNA quality and concentration was determined by measuring the absorbance at 260 nm and 280 nm and visualised on a 0.8% (w/v) agarose gel stained with ethidium bromide.

2.8.2 Transfection by Electroporation

Adherent cells were seeded at 1×10^5 cells/ml and allowed to grow to ~80% confluence before trypsinisation with trypsin-EDTA. Trypsinisation was halted by addition of 20 ml medium and cells were then pelleted by centrifugation at 1,000 x g for 5 minutes. The pellet was resuspended in 20 ml PBS and pelleted by centrifugation at 1,000 x g for 5 minutes. The pellet was resuspended in 750 µl PBS. 250 µl of the cells were incubated on ice for 10 minutes with the required amount of DNA in an electroporation cuvette. The cells were electroporated using an Invitrogen electroporator set at 250 Volts, 25 Watts and 25 mAmps. The cells were mixed with 1 ml pre-warmed medium and any dead cells, which formed a layer at the top of the medium, were removed by pipetting before diluting the cells to a volume of 9 ml with medium. Cells were plated out using 1 ml of cells per well in a final volume of 3 ml in 6 well plates or 100 µl per well of a 6 well plate. After 24 hours the medium was aspirated from the cells and they were washed with PBS and then feed with fresh medium. The cells were allowed to rest for a further 24 hours before stimulation

2.8.3 Gene Juice Transfection

The liposomal based transfection agent from Novagen was used for transfection of Neuro2a cells. Neuro2a cells were seeded at a density of 1×10^5 cells/ml and plated with 0.4 ml per well in 24 well plates or 200 μ l per well in 96 well plates and allowed to grow for 16 hours. For 24 well transfections DNA with a final mass of 7 μ g consisting of appropriate amounts of each required plasmid was incubated with a Gene juice:Serum free medium mix (1.5:18.5) for 15 minutes at room temperature to allow DNA:Gene juice complexes to form. 20 μ l of this mixture was added to cells. After 24 hours the cells were stimulated. For 96 well plates DNA with a total mass of 400 ng was incubated consisting of appropriate amounts of each required plasmid was incubated with a Gene juice:Serum free medium mix (0.8:9.2) for 15 minutes at room temperature to allow DNA:Gene juice complexes to form. 10 μ l of this mixture was added to cells. After 24 hours the cells were stimulated.

2.8.4 Preparation Cellular Lysates

Medium was aspirated off cells and they were washed with 1X PBS. The cells were lysed for 15 minutes at room temperature in 250 μ l 1X Passive Lysis Buffer (Promega, Madison, Wisconsin, USA) for 6 well plate samples, and 50 μ l for 24 or 96 well plate samples. Cells were scraped from 6 well plates and pelleted at $13,000 \times g$ for 10 minutes and the supernatant removed. Samples in 24 or 96 well plates were centrifuged at $2,000 \times g$ for 7 minutes and supernatants were taken as required.

2.8.5 Measurement of Luciferase Activity

20 μ l of sample was incubated with 20 μ l Luciferase Assay Mix (20 mM Tricine, 1.07 mM $(\text{MgCO}_3)_4\text{Mg}(\text{OH})_2 \cdot 5\text{H}_2\text{O}$, 2.67 mM MgSO_4 , 0.1mM EDTA, 33.3 mM DTT, 270 mM co-enzyme A, 470 mM luciferin, 530 mM ATP) to measure firefly luciferase and the luminescence was immediately measured for a total of 5 seconds using a luminometer.

The same procedure as used to measure renilla luciferase but colenterazine substrate, diluted to 1 in 500 in PBS, was used in place of luciferase assay mix.

2.8.6 Protein Concentration Determination

Determined as per section 2.7.3

2.8.7 β -Galactosidase Activity Measurement

20 μ l of sample was incubated at 37°C for 1 hour with 40 μ l ortho-nitro-phenyl galactose (ONPG) substrate (4 mg/ml) and 140 μ l PM-2 buffer pH7.3 (23 mM NaH₂PO₄, 77 mM Na₂HPO₄, 0.1 mM MnCl₂, 2 mM MgSO₄, 40 mM β -mercaptoethanol) and the absorbance was measured at 630 nm.

2.8.8 Transactivation Assay

To assay transactivation of p65, a variation on the classical gene reporter assay was used. In this assay, cells are transfected with a plasmid containing GAL4 binding sites upstream of the reporter luciferase gene, as well as a plasmid encoding for a p65-GAL DNA binding domain chimera protein. Upon transfection the chimeric protein is constitutively expressed and binds to the consensus binding sequences in the reporter plasmid. Transactivation of p65 drives expression of the luciferase gene. This allows the reporter system to respond solely to the transactivation status of p65 and is not dependent on its DNA binding activity. Cells were transfected by electroporation (see section 2.8.2) with 30 μ g GAL4-luciferase and 2.5 μ g p65-GAL-DNA binding domain, after 24 hours the cells were washed and given fresh medium. Following a further 24 hours cells were treated as required and luciferase expression measured as normal.

2.9 Western Blot Analysis

2.9.1 Preparation of Whole Cell Lysates using p38 Sample Buffer

Cells were set up as per section 2.7.1. After appropriate treatment cells were washed with 1X PBS and lysed in 150 µl sample buffer (62.5 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 50 mM DTT, 10% (v/v) glycerol, 0.1% (w/v) bromophenol blue) and scraped into minifuge tubes. Samples were then sonicated for 10 seconds at 80% strength and boiled at 100°C for 5 minutes.

2.9.2 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples were resolved on sodium dodecylsulphate (SDS) polyacrylamide gels in 1X running buffer (25 mM Tris, pH 8.3, 0.1% (w/v) SDS and 0.192 M glycine) using a constant current of 25 mA per gel. Samples were first run through a stacking gel (1 ml 30% acrylamide: bisacrylamide (37.5:1) mix, 0.75 ml 1 M Tris-HCl pH 6.8, 60 µl 10% (w/v) ammonium persulphate and 6 µl TEMED made up to 6 ml with H₂O) to condense protein and then resolved according to size using 10-15% polyacrylamide gels (required volume of 30% acrylamide: bisacrylamide (37.5:1) mix, 3.75 ml 1.5 mM Tris-HCl pH 8.8, 150 µl 10% (w/v) ammonium persulphate, 6 µl TEMED made up to 15 ml with H₂O). Samples were run with pre-stained protein markers (New England Biolabs) used as molecular weight standards.

2.9.3 Electrophoretic Transfer

Resolved proteins were transferred to nitrocellulose or polyvinylidenedifluoride (PVDF) membranes. All materials were soaked in transfer buffer before use. The PDVF membrane was activated by soaking in methanol for 30 seconds and then washing in water for 2 minutes. The polyacrylamide gel was placed on top of filter paper and the paper cut to the size of the gel. The PDVF membrane was laid on top with another piece of filter paper laid above this. Again membrane and filter paper were cut to size. Thus

arranged the gel and membrane were placed between sponges and fitted in the transfer cassette and placed in a tank filled with transfer buffer pH 8.5 (25 mM Tris, 0.2 M glycine, 20%(v/v) methanol). The protein was transferred to the membrane by passing a constant current of 150 mA through the gel for 2 hours or overnight at 30 mA.

2.9.4 Antibody Blotting

Membranes were blocked for non-specific binding by incubation in Blocking Buffer (5% (w/v) non-fat dried milk in 1%(v/v) Tris Buffered Saline (TBS)-Tween), for 1 hour at room temperature or at 4°C overnight. The membrane was then washed for 5 minutes in 1%(v/v) TBS-Tween three times. The membrane was then incubated for 1 hour with the antibody of interest (for anti phospho and total p42/p44, anti phospho and total p38; 1 in 1,000 dilution, for anti phospho and total JNK; 1 in 1,000 dilution, for anti-M2-flag; 1 in 5,000 dilution in 5% fatty acid free BSA, for anti-p50; 1 in 1,000 in 5% non-fat dried milk). Next the membrane was washed three times and incubated for 1 hour with the appropriate secondary horseradish peroxidase linked enzyme antibody diluted 1 in 2,000 in 5% non-fat dried milk.

2.9.5 Chemiluminescence

Membrane was covered in 1X peroxide and buffer mix (New England Biolabs) for 1 minute and placed between acetate sheets before exposure to X-Omatic film (Kodax).

2.10 Coomassie and Silver Gel Staining

Resolved gels were soaked in Coomassie blue stain (50% (v/v) methanol, 10% (v/v) glacial acetic acid, 2.5 g/L Coomassie blue dye) for at least 1 hour and the washed several times with destain until clear bands appeared.

For silver staining, resolved gels were fixed by incubation in fixation buffer (50% (v/v) methanol, 5% (v/v) glacial acetic acid, 45% (v/v) H₂O) for 30 minutes. The gel was washed in water and then left in water for 1 hour with shaking. The gel was then incubated in sensitizing buffer (0.02% (w/v) sodium thiosulphate) for 2 minutes and then rinsed with two changes of water. The gel was then placed in cold 0.1% (w/v) silver nitrate for 30 minutes at 4°C followed by washing with two changes of water. The gel was developed by incubation in 0.04% (v/v) formaldehyde in 2% (w/v) sodium carbonate. The stained gel was then washed and stored in 1% (v/v) acetic acid.

2.11 Reverse Transcriptase-Polymerase Chain Reaction

2.11.1 Preparation of RNA

Balb/c mice 15 days old were killed by exsanguination and perfused with saline for 10 minutes before extraction of organs. Tissues were weighed and 1ml of Trizol per 50-100 mg was added to tissue before homogenisation in a power homogeniser until the preparation was consistent.

For cells grown in culture, 1 ml of Trizol reagent was added per 5×10^6 cells. All samples were then incubated for 5 minutes at room temperature. 200 μ l of chloroform was added per 1 ml Trizol and the mixture was shaken vigorously for 15 seconds before being allowed to settle for 3 minutes at room temperature. Samples were then centrifuged at 12,000 x g for 20 minutes at 4°C. The top aqueous layer was pipetted off and added to 500 μ l isopropanol for every 1 ml of Trizol used. This mixture was incubated overnight at -20°C. Precipitated RNA was collected by centrifugation at 12,000 x g for 15 minutes at 4°C. The supernatant was decanted off and the pellet was washed in 100% ethanol. The pellet was again centrifuged at 7,500 x g for 10 minutes at 4°C. The supernatant was removed and the pellet allowed to air dry for 1 hour before being dissolved in 100 μ l sterile distilled water.

2.11.2 Quantitation of RNA

A 1 in 20 dilution of purified RNA was prepared and the absorbance read at 260nm and 280nm with water acting a blank. 1 OD unit at 260 nm was taken to be equivalent to approximately 50 µg RNA. An A260/A280 ratio of ≥ 1.8 was taken as an indication that the preparation of RNA was pure.

2.11.3 DNase Treatment of RNA

Contaminating DNA was removed by treatment with the enzyme DNase (Ambion). A reaction mixture of 1X buffer, 2 units of RNase inhibitor, 1 unit of DNase, 100 µl of prepared RNA solution and sufficient water to bring the final volume to 120 µl was prepared and incubated at 37°C for 30 minutes. Enzymes were then denatured by incubation at 70°C for 30 minutes.

2.11.4 Reverse Transcription of RNA

1 µg of RNA was added to 5µM of random hexanucleotide primers in a final volume of 10 µl and this mixture was incubated at 65°C for 10 minutes to remove any RNA secondary structure. The RNA was transcribed to cDNA using Reverse Transcriptase in a reaction containing 1X buffer, 20 mM DTT, 0.5 mM of each dNTP and 10 unit/µl M-MLV RTase (Gibco BRL) in a final volume of 20 µl. This reaction mixture was incubated at 37°C for 1 hour and then the enzyme was denatured by heating at 90°C for 5 minutes.

2.11.5 Polymerase Chain Reaction (PCR)

PCRs were set up using 3 µl of prepared cDNA along with 1 µl of each appropriate 20 mM primer stock, 20 µl sterile water and 25 µl Sigma Ready Mix containing 20 mM Tris-HCl, 100 mM KCl, 3 mM $MgCl_2$, 0.002% gelatin, 0.4 mM dNTP, 60 units Taq

polymerase/ml. 2 drops of sterile mineral oil was dropped on top of the reaction mixture.

The following reaction conditions were used for the amplification of IL-1F5:

95°C for 5 minutes

2 cycles of 95°C for 30 seconds

64°C for 30 seconds

72°C for 45 seconds

2 cycles of 95°C for 30 seconds

62°C for 30 seconds

72°C for 45 seconds

2 cycles of 95°C for 30 seconds

60°C for 30 seconds

72°C for 45 seconds

35 cycles of 95°C for 1 minute

58°C for 1 minute

72°C for 1 minute

Final extension at 72°C for 5 minutes.

The primers used to amplify murine IL-1F5 were:

Forward 5' TTC CGA ATG AAG GAT TCA GC 3'

Reverse 5' AGA AGT CTG TGA TGG GAG CA 3'

Primers to murine aldolase were:

Forward primer: 5' TTC CAC GAG ACA CTG TAC CAG AAG 3'

Reverse primer: 5' ACC ATG TTG GGC TTC AGC AAT G 3'

Primers to human β -actin were:

Forward primer: 5' GAG AAG ATG ACC CAG ATC ATG T 3'

Reverse primer: 5' ACT CCA TGV CCA GGA AGG AAG G 3'

Products were diluted with 6X sucrose loading buffer, separated on 1% agarose gels stained with ethidium bromide and visualised under UV light.

2.12 Crystallisation of IL-1F5

2.12.1 Preparation of Protein

Protein was dialysed into water and concentrated using centricon® spin columns (Millipore) to a concentration of 5 mg/ml, as determined using the Bradford assay (see section 2.7.3).

2.12.2 Preparation of Selenomethionine Derivative Protein

Bacterial cultures were prepared as described in section 2.4.1 with the exceptions that the M9 medium was not supplemented with yeast extract and when the OD₆₀₀ of the starting culture reached 0.3 the medium was supplemented with 100 mg/l L-lysine, 100 mg/l L-phenylalanine, 100 mg/l L-threonine, 50 mg/l L-isoleucine, 50 mg/l L-leucine and 50 mg/l L-Valine and 50 mg/ml L-selenomethionine. 15 minutes after the amino acids were added, and methionine synthesis was inhibited, the culture was induced with 1 mM IPTG for 15 hours. Protein was purified as for the native protein with the exception that all buffers were degassed under a vacuum and supplemented with 10 mM DTT.

2.12.3 Crystallisation Trials

Initial trials were set up using the Hampton Research Crystal Screen 1 and 2 kits (see section 6.2.1). The protein was crystallised by the hanging drop method. 1 ml of the precipitant was put in the reservoir well. 2 µl of this buffer was mixed with 2 µl of 5 mg/ml IL-1F5 on a siliconised coverslip, also included was a control drop with 2 µl of water instead of 2 µl of protein. The coverslip was then placed over the well and it was sealed with vacuum wax. Plates were then incubated at either room temperature or at 4°C in a place with little vibration. Drops were inspected for the development of crystals and scored as to whether they were clear, had precipitate, had quasi crystals or had crystals.

2.12.4 X-Ray Diffraction, Phase Determination and Structure Refinement

X-ray diffraction data of single IL-1F5 crystal was collected at 1.5 Å in beam line ID14 EH2, Grenoble, France at wavelength of 0.934 Å, through 120° at 1 minute per degree. Data was processed at 1.58 Å in MOSFLM (Leslie, 1986). Data was sorted, scaled and truncated using CCP4 package (CCP4, 1994).

Initial phase of the IL-1F5 structure was obtained by molecular replacement using AMoRe (Navaza, 2001). Homology searching models of IL-1F5 derived from murine IL-1β (PDB code, 2mib), human IL-1Ra (PDB code, 1irp), ligand chain of human IL-1β complexed to IL-1RI (PDB code, 1itb), ligand chain of human IL-1Ra complexed to human IL-1RI (PDB code, 1ira), *Bos Taurus* acidic fibroblast growth factor (PDB code, 1afc), human acidic fibroblast growth factor (FGF) (PDB code, 1afg), and human basic FGF (PDB code, 2fgf) were used for the rotation function search. Murine interleukin-1β (2mib) with 24.5% sequence identity yielded the highest rotation solution at correlation of 45.7% and the lowest R-factor of 57.5%. 26 top rotation solutions were sequentially applied to the translation function search. It yielded correlation of 50.2% and a R-factor of 56.2%. An initial model of IL-1F5 with a correlation of 60.0% and an R-factor of 53.6% were obtained by following a rigid body and B-overall refinement in AMoRe (Navaza, 2001).

Crystal structure of murine IL-1F5 was refined using reflection data to 1.6 Å. 5% of randomly selected reflections were used as the free R-factor for cross validation (Brünger, 1992). Maximum likelihood refinement, anisotropic b-factor refinement and bulk solvent correction (solvent density is 0.36 e/Å³) were employed to minimise the difference between the model of IL-1F5 and X-ray data using CNS. Following a few cycles of refinement with CNS and manual model rebuilding using O (Jones et al., 1991), the model was improved to R-factor of 29.2% and free R-factor of 30.1%. Then, water molecules were searched at peaks where sigma was above 3. Sigma-weighted $2F_c - F_o$ and $F_c - F_o$ electron density map were used to guide the manual model rebuilding (Read, 1990). The secondary structure of IL-1F5 was assigned using DSSP (Kabsch and Sander, 1983).

Solvent accessibility of IL-1F5 and interface between IL-1F5 and potential docked receptors were calculated in CNS using the methods of Lee and Richards (Lee and Richards, 1971) using a 1.4 Å radius probe. Contact and hydrogen bonds between IL-1F5 and potential receptors are assigned in CNS by picking all intermolecular non-hydrogen atoms separated by 3.5 Å or less.

The atomic coordinates have been deposited in the Brookhaven Protein Data Bank with accession code 1md6.

Chapter 3 Sequence and Structural analysis of IL-1 Family Members with Specific Attention to IL-1F5.

3.1 Introduction.

IL-1 belongs to a structural family of proteins, which includes the Kunitz-type soyabean trypsin inhibitors, ricin-like toxins, hisactophilin and fibroblast growth factor (Ponting and Russell, 2000; Vigers et al., 1994). The proteins all share a common β -trefoil fold consisting of 12 β -strands joined by 11 loop regions. Crystal and NMR structure of IL-1 α , IL-1 β and IL-1Ra show that these three family members are very similar in their respective β -strand regions, but their structures diverge in the loop regions. This suggests that functional diversity is achieved by the divergence of sequence in these regions. In addition, the structures of both IL-1 β and IL-1Ra have been solved in a complex with the extracellular domain of the type I IL-1 receptor (Schreuder et al., 1997; Vigers et al., 1997). These studies give good insights into which regions and residues of the proteins are important for interacting with and activating the receptor. In the absence of structural information about the novel members of the family, sequence based analysis is important for determining whether the proteins are validly placed in the family and whether they are likely to have similar secondary and tertiary structures to the classical IL-1 family members. This would confirm that these proteins have an amino acid composition that is likely to allow them to adopt the same β -trefoil fold as IL-1. Such information would also imply that they are likewise able to bind IL-1RI-like receptors in manner similar to IL-1 itself. Furthermore, close examination of the sequence can also lead to insights into the possible function of the proteins. In particular, the features known to determine the agonistic and antagonistic properties of IL-1 β and IL-1Ra respectively can be used to direct predictions as to the functions of the novel IL-1 family members. These techniques are applied with specific attention to IL-1F5.

3.2 Results and Discussion.

3.2.1 Alignment of IL-1 Family Members

The protein sequences of all the members of the IL-1 family were aligned using ClustalW 1.82. This alignment was subsequently edited by hand using the alignment editor Seaview and formatted using ESPript (Figure 3.2.1). The alignment includes all the identified splice variants of the novel family members. IL-1F7 has 5 splice variants; IL-1F7, IL-1F7b and IL-1F7d have divergent N-terminals, while IL-1F7c, like IL-1F7e, is a shorter version of IL-1F7b. Since these two shorter versions do not possess the 12 β -strands of the other family members it is possible they do not form functional proteins. IL-1F8b has a variant C-terminal sequence, while IL-1F10b has a variant N-terminal sequence. All the novel members lack a pro or signal sequence with the exception of IL-1F7, which has a predicted cleavage site at Asn27 (Smith et al., 2000) and IL-1F7b which is reported to have a caspase 1 cleavage site at Glu21 (Kumar et al., 2002; Kumar et al., 2000), with another cleavage site at Val46 also reported (Pan et al., 2001). IL-1 α , IL-1 β and IL-18 are all aligned in their mature, processed forms. All sequences in the alignment are human in origin; sequence identities to murine orthologues are given in Table 3.2.1 (Taylor et al., 2002).

Regions of high sequence identity between the proteins are marked with boxes and conserved residues are highlighted in red to allow for quick visualisation of areas of conservation. I have identified five regions of high sequence identity and defined these as boxes 1-5. The existence of these boxes across the family confirms that the proteins are indeed all related. Box1 occurs at a position equivalent to β -strand 5 in IL-1 β and IL-1Ra. It is characterised by hydrophobic residues at positions 1, 3 and 5, interspersed by a variable residue at position 2 and a well conserved glycine at position 4, which occurs in all but IL-1 α and IL-18. Box 2 occurs in a region corresponding to β strand 6 of IL-1 β . It consists of a strongly conserved cysteine, which occurs in all of the novel family members as well as IL-1Ra, followed by a non-polar residue, conserved as leucine in all

Figure 3.2.1 Alignment of IL-1 Family. Alignment was compiled using ClustalW 1.8, edited by hand using Seaview and formatted using ESPrit. Only human forms of the proteins are aligned, using mature sequences for the classical members, but complete open reading frames for the novel members. Sequences are coloured according to percentage amino acid identity, blue dashed lines indicate positions of defined boxes of conservation 1-5, arrows represent position of β -strands in IL-1 β and IL-1Ra, while coils represent helical structures. Sequences were taken from GeneBank™ IL-1 α accession number X03833, IL-1 β accession number X04500, IL-1Ra accession number NM000577, IL-18 accession number E17138, IL-1F5 accession number AF201830, IL-1F6 accession number AF201831, IL-1F7 accession number AF201832, IL-1F7b accession number AF200496, IL-1F7c accession number AF251120, IL-1F7d accession number AY071840, IL-1F7e accession number AY071841, IL-1F8 accession number AF201833, IL-1F8b accession number AF200494, IL-1F9 accession number AF200492, IL-1F10 accession number AF334755.

IL-1betaAPVRSLNCTIRDSQQKSL
IL-1alphaSAPFSFSLSNVKYNFMRIIKYEFILNDALNQSILIKYADQKA
IL-1F10MCSLPMARYYIIRDSRQMVV
IL-1F10bMSSSFLPEPLP
IL-1F9MRGTPGDADGGGRAVYQSMCKPITGTINDLNQQVW
IL-1F8MNPQREAAPKSYAIRDSRQMVV
IL-1F8bMNPQREAAPKSYAIRDSRQMVV
IL-1F7MSGCDRRETETKGNKSPFKRLRGPKVKNLNPCKFSLHDQDHKVL
IL-1F7b MSFVGENSGVKMGSEDEWEKDEPQCCELDPAVSPLEPGPSLPTMNFVHTSPKVKNLNPCKFSLHDQDHKVL
IL-1F7c MSFVGENSGVKMGSEDEWEKDEPQCCELDPAVSPLEPGPSLPTMNFVHTSPKVKNLNPCKFSLHDQDHKVL
IL-1F7d MSFVGENSGVKMGSEDEWEKDEPQCCELDPAVSPLEPGPSLPTMNFVHTSPKVKNLNPCKFSLHDQDHKVL
IL-1F7e MSFVGENSGVKMGSEDEWEKDEPQCCELDPAVSPLEPGPSLPTMNFVHTSPKVKNLNPCKFSLHDQDHKVL
IL-1F6MEKALKIDTPQQGSLQDINHRVW
IL-1F5MVLSGALCFRMKDSALKVL
IL-18YFGKLESKLSVIRNLNDQVLFILWVNVQKTF
IL-1RaMALETICRPSGRKSSKMQAFRIWVNVQKTF

IL-1beta VMSGPYELKALHLQGDMEQVVFSSFSVQ.GEESNDKIPVALGLK.EKNLYISCVLKDDK.PTLQLLES
IL-1alpha IRAN.DQYLTAALHN.LDEAVKFDMGAYKSSKDDAKITVILRIS.KTQLYVTAQDEDO.PVLLKEM
IL-1F10 LYTR.DGQLLVGDPVA..DNCCAETICTLPNRLDRTKVPVIFLGIQ.GGSRCLACVETEEL.PSLQLED
IL-1F10b AKSL.QHGVPLSLDSS..LSSLLEKICILPNRGLARTKVPVIFLGIQ.GGSRCLACVETEEL.PSLQLED
IL-1F9 TLQG.QNLVAVPRSDSVTPVTVAVITCKYPEALEQGRGDPVIFLGIQ.NPEMCLYCEKVGQ.PTLQLKE
IL-1F8 VLSG.NSLIAAPLSRSIKPVTLHLIAACRDTEFSDKEKGNMVYLGIIK.GKDLCLYCAEIQ.K.PTLQLKE
IL-1F8b VLSG.NSLIAAPLSRSIKPVTLHLIAACRDTEFSDKEKGNMVYLGIIK.GKDLCLYCAEIQ.K.PTLQLKE
IL-1F7 VLDG.GNLIAVPDKNYIRPEIFFALASSL.SSASAEKGSPIILGV.S.KGEFCLYCDKDKGQSHPSLQLKK
IL-1F7b VLDG.GNLIAVPDKNYIRPEIFFALASSL.SSASAEKGSPIILGV.S.KGEFCLYCDKDKGQSHPSLQLKK
IL-1F7c VLDG.GNLIAVPDKNYIRPEIFFALASSL.SSASAEKGSPIILGV.S.KGEFCLYCDKDKGQSHPSLQLKK
IL-1F7d VLDG.GNLIAVPDKNYIRPEIFFALASSL.SSASAEKGSPIILGV.S.KGEFCLYCDKDKGQSHPSLQLKK
IL-1F7e VLDG.GNLIAVPDKNYIRPEIFFALASSL.SSASAEKGSPIILGV.S.KGEFCLYCDKDKGQSHPSLQLKK
IL-1F6 VLQD.QTLIAVPRKDRMSPVTIALISCRHVETLEKDRGNPIYLGILN.GLNLCLMAKVGDQ.PTLQLKE
IL-1F5 YLHN.NQLLAGGLHAGKVIKGEIISVVPNRWLDAS..LSPVILGVQ.GGSQCLSCG.VGQE.PTLTLEP
IL-18 DQGN.RPLLEDMTSDSDCRDNAPRTIFIRMYKDSQPRGMATISVKCEKISTLSCENKIIISFKEMNPPDN
IL-1Ra YLRN.NQLVAGYLGQPNVNLEEKIDVPIEP.....HALFLGIH.GGKMLSCVKSQDE.TRLQLLEA

IL-1beta VDP...KNYPKKKMEKRFVFNKIEINN.KLEFESAQFPNWIISTSAENMPVFLGSGTKGGQ...DITDST
IL-1alpha PEIP...KITGSETNLFFWETHGT.KNYFTSVAHPNLFIAATKQDYWVCLAGGPP...SITDST
IL-1F10 VNIEEL.YKGGEAATRTFFQSSSSGS.AFRLEAAAWPGWFLCGPAEPQQPVOLTKESEPS...ARTKY
IL-1F10b VNIEEL.YKGGEAATRTFFQSSSSGS.AFRLEAAAWPGWFLCGPAEPQQPVOLTKESEPS...ARTKY
IL-1F9 QKIMDL.YGQPEPVKPFLLFYRAKTGR.TSTLESVAFPDWFIASSKRD.QPIILTSELGKS...YNTAE
IL-1F8 KNIMDL.YVEKKAQKPFLLFYHNKESGS.TSVFQSVSYPGWFIATSTTSGQPIFLTKERGIT...NNTNY
IL-1F8b QGSDQNIKDTCTCKWLVGIHTCINLDV.RESCFMGTLDQWGI.GVGRKKWKSSF..QHHHLRK...KDKDS
IL-1F7 EKLMLKLAQKESARRPFIFYRAQVGS.WNMLESAHPGWFICTSCNCNEPVGVTDKFENR...KHIES
IL-1F7b EKLMLKLAQKESARRPFIFYRAQVGS.WNMLESAHPGWFICTSCNCNEPVGVTDKFENR...KHIES
IL-1F7c EKLMLKLAQKESARRPFIFYRAQVGS.WNMLESAHPGWFICTSCNCNEPVGVTDKFENR...KHIES
IL-1F7d EKLMLKLAQKESARRPFIFYRAQVGS.WNMLESAHPGWFICTSCNCNEPVGVTDKFENR...KHIES
IL-1F7e EKLMLKLAQKESARRPFIFYRAQVGS.WNMLESAHPGWFICTSCNCNEPVGVTDKFENR...KHIES
IL-1F6 KDIMDL.YNQPEPVKSFLLFYHSQSGR.NSTFESVAFPGWFIASVSEGGCLILTQELGKA...NTTDS
IL-1F5 VNIMEEL.YLGAKESKSTFFYRRDMGL.TSSFESAAYPGWFLCTVPEADQPVRLTQLPENGWGNAPITDY
IL-18 I.....KDTKSDIIFQFQSVPGHDNKMQFESSSYEGYFLACEKERDLFKLILKKEDDEL.DRSIMT
IL-1Ra VNIITDLSNRKQDKR.FAFIRSDSGP.TTSFESAAYPGWFLCTAMEADQPVSLTNMPDEG...VMVTKY

IL-1beta MQFVSS.....
IL-1alpha ILENQA.....
IL-1F10 FEQSW.....
IL-1F10b FEQSW.....
IL-1F9 LNIND.....
IL-1F8 LDSVE.....
IL-1F8b SMRTNIGMPGRM....
IL-1F7 FQPVCKAEMSPSEVSD
IL-1F7b FQPVCKAEMSPSEVSD
IL-1F7c FQPVCKAEMSPSEVSD
IL-1F7d FQPVCKAEMSPSEVSD
IL-1F7e FQPVCKAEMSPSEVSD
IL-1F6 LTMLF.....
IL-1F5 FQOCD.....
IL-18 VQSED.....
IL-1Ra FQEDE.....

Protein	% Amino Acid Identity
IL-1 α	55
IL-1 β	78
IL-1Ra	77
IL-18	65
IL-1F5	91
IL-1F6	54
IL-1F7	-
IL-1F8	62
IL-1F9	56
IL-1F10	82

Table 3.2.1 Percentage amino acid identity between mature forms of human and murine IL-1 Family members.

but IL-1 α which has a threonine. This is followed by a more variable residue which is predominantly polar, in turn followed by another highly conserved cysteine. Box 3 occurs in the region equivalent to β strand 7 of IL-1 β , and like the previous two boxes, a pattern of polar or small residues followed by non-polar residues is seen in this box. Box 4 is the region of strongest conservation between all family members. It contains the sequence already identified as a motif that can act as a signature of IL-1 family members (Prosite PDOC00226). This motif is also present in all the novel family members with some variations and it is least well conserved in IL-1F8b. For example, in IL-1F5 this motif runs from amino acid 111 to 131 with the sequence FESAAYPGWFLCTVPEADQPV which is consistent with the motif consensus of {FL}-x-S-{ASLV}-x(2)-P-x(2)-{FYLV}-{LI}-{SCA}-T-x(7)-{LIVM}.

Also highlighted is a further box 5, which is a region of both conservation and functional importance. This box contains a well conserved threonine and a completely conserved phenylalanine, which both flank the site of the functionally important residue equivalent to aspartate 145 in IL-1 β . The phenylalanine is of interest since it is the only completely conserved residue across the family. This residue is located in the top layer of the six-stranded β -barrel of the β -trefoil structure. It points away from the barrel core and instead interacts with residues from the hairpin cap structure. Saturation mutagenesis was used to identify this residue as a contributor to binding and biological activity in IL-1 α (Gayle et al., 1993). However, it has not been identified in IL-1 β or IL-1Ra as a residue sensitive to mutagenesis. Nonetheless its position at the barrel and cap interface may mean it has an important role in the stabilisation of the β -trefoil structure as a whole (Auron et al., 1992). It may also support the functionally important residue next to it in an active conformation (see below).

The residue directly preceding this phenylalanine has been shown to be important for the agonistic properties of IL-1 β and IL-1 α , and the antagonistic properties of IL-1Ra. Mutagenesis of this lysine to aspartate converts IL-1Ra to a partial-agonist (Ju et al., 1991), while in the reverse IL-1 β or IL-1 α aspartate to lysine mutant, agonist activity is lost without affects on binding capacity (Ju et al., 1991; Kawashima et al., 1992). This

demonstrates the importance of this residue in determining the biological activity of the cytokine. Size and charge seem to be the important properties involved in determining activity, with large and/or positive residues conferring antagonistic properties (Kawashima et al., 1992). In the structure of IL-1 β complexed with the receptor the Asp145 residue is still exposed to the solvent on the side of the molecule (Figure 1.6.7). Therefore, it seems possible that this residue is directly involved in binding IL-1RAcP, which is required to complex with IL-1RI in order to form the active signalling complex. The formation of an active signalling complex can be understood to involve two steps; 1) the binding of the ligand to IL-1RI and 2) the recruitment of the IL-1RAcP. There are only a few mutations that have been identified in IL-1 β which alter its biological activity but not its binding capacity to IL-1RI (see section 1.6.7), these include mutations at Asp145 (Ju et al., 1991), Arg11 (Gehrke et al., 1990) and also Thr9 (Simon et al., 1993). Asp145 itself lies in a cleft or depression on the surface of IL-1 β , which is hydrated and hydrophilic in nature (Figure 3.2.2). The cleft is held in position partly by an hydrated pocket which is formed by residues from β -strands 1, 2 and 4 as well as water molecules. These residues include Arg11, which makes a bond with Lys16, and it has been argued that this bond prevents β -strands 1 and 12 from collapsing into the pocket (Auron et al., 1992). Movement of strand 12, which has Asp145 at its N-terminal, would therefore result in a displacement of Asp145 from the centre of the cleft on the surface of the protein. Indeed, when Arg11 is mutated to a small amino acid IL-1 β loses its biological activity but equivalent sized amino acids, on the other hand, have little affect on activity (Auron et al., 1992). Therefore, the Arg11 mutant indicates that the structural integrity of this cleft is essential for a functional molecule. Furthermore, the conserved Phe mentioned above packs against the base of this cleft and may therefore be important for both maintaining its structure and positioning the preceding residue in such a way that it remains solvent accessible at the centre of the cleft. The equivalent residues in the novel family members are also likely to be at the centre of a similar cleft seen in IL-1 β , since they all have the phenylalanine at the same position. In addition, they all, with the exception of IL-1F10b, have large amino acids at the position equivalent to Arg11 of IL-1 β . Therefore, it is likely that these residues are functionally similar across the family.

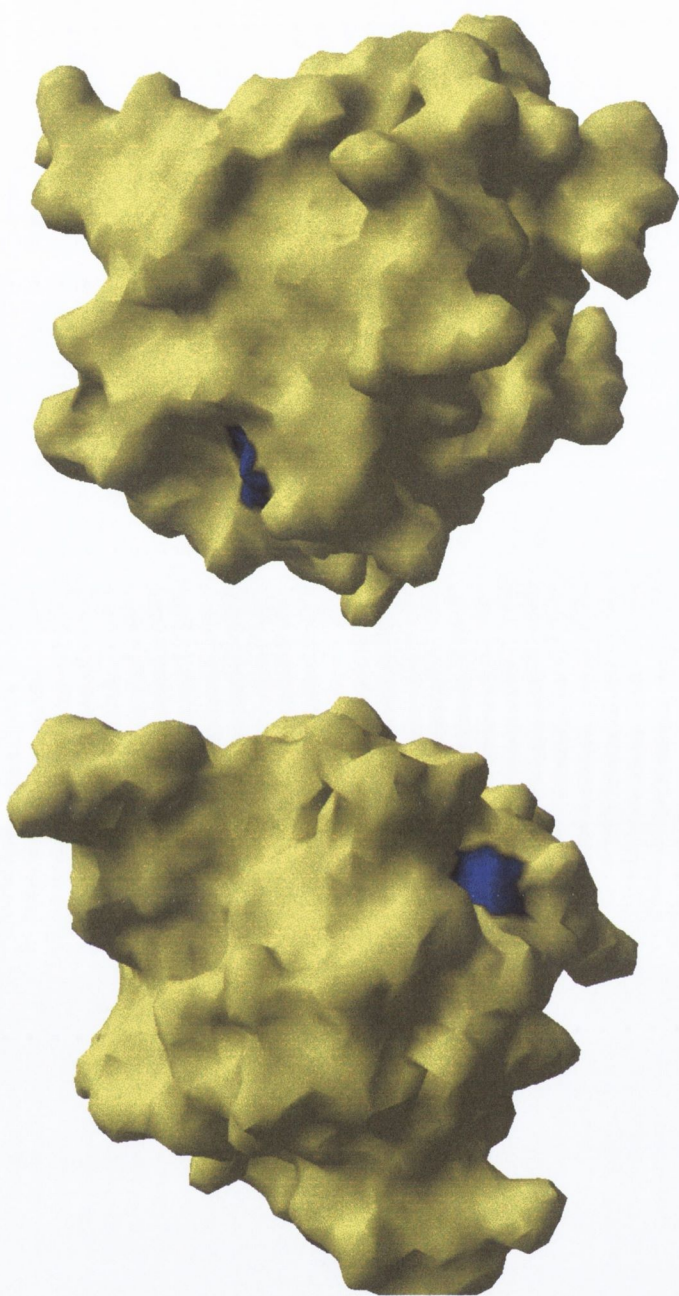


Figure 3.2.2 Position of Asp145 in IL-1 β . Two alternative views of the molecular surface of IL-1 β highlighting the position of Asp145 (blue) at the centre of a cleft on the surface of the protein.

Examination of the aligned residues shows that IL-1F5 also has an aspartate in box 5, which suggests it too may be an agonist and be able to recruit an accessory protein. The same is true of IL-1F6 and IL-1F8b. IL-1F9 has a neutral and small alanine at this position. It has been shown that mutation of Asp151 in IL-1 α to an alanine does not greatly affect its activity (Kawashima et al., 1992), therefore it is possible that IL-1F9 could also recruit an accessory protein. Indeed, this protein has been shown to induce NF κ B activation in IL-1Rrp2 transfected cells (Debets et al., 2001). IL-1F7 has a negative glutamate, which suggests an agonist role for this protein. Indeed, recently it has been postulated that this protein is able to recruit the IL-18RAcP in a complex with IL-18BP, but not in complex with IL-18R (Bufler et al., 2002). IL-1F10 and IL-1F10b both have a lysine like IL-1Ra in this position and IL-1F8 has a large asparagine, which may suggest antagonistic roles for these members.

Another area of interest in the alignment is the region equivalent to the loop joining β -strands 4 and 5 in IL-1 β . In IL-1Ra this loop is significantly shorter and this is reflected in the sequence alignment of the family. Although the shortened loop of IL-1Ra is still able to interact with domain 3 of the receptor, it seems to alter its ability to interact with the receptor in such a way as to allow activation to occur. This is borne out by the fact that insertion of the loop into the K145D IL-1Ra mutant increases the mutant's agonist activity (Greenfeder et al., 1995b). Furthermore, when the short loop of IL-1Ra is substituted for the longer loop in IL-1 β , then IL-1 β loses both receptor binding activity and biological activity (Boraschi et al., 1995). This indicates that the length of the loop does indeed affect receptor binding even in the presence of the binding patch on loop 7-8. IL-1 β must require both binding sites to domain 3 in order to bind with full affinity to the receptor. No other member of the family has an equivalently short loop in this region.

The alignment indicates the regions of the sequences of both IL-1 β and IL-1Ra that contribute to each of the twelve β -strands of the proteins; these are indicated by black arrows above and below the alignment. This clearly highlights that most conservation between the structures occurs in regions that make up the β -strands while regions that form loops are less strongly conserved. Furthermore, it has been postulated that it is the

residues which make up the core layers of the β -barrel and the layers of the cap structure of the β -trefoil fold that are conserved in terms of their size and hydrophobicity. This conservation even holds across the whole family of β -trefoil structures, which are not strongly homologous at a sequence or functional level (Murzin et al., 1992). It is these residues that are important for establishing the correct geometry of the β -trefoil fold. New members of the family would therefore be expected to have similar if not identical residues at these important sites, especially if they are all likely to have evolved from a common ancestor. On isolating these residues from the alignment it can be seen that they are well conserved in terms of their hydrophobicity and size across the whole family (Figure 3.2.3). This is another indication that the novel family members are likely to adopt the same fold.

3.2.2 Dendrogram of IL-1 Family

Based on the sequence alignment of these proteins a dendrogram of the family was drawn using the nearest neighbour scores from percentage identities (Figure 3.2.4). This tree shows the close relationship between the five novel members of the family. IL-1F6 and IL1F9 are very closely related and share 56% sequence identity in a pairwise comparison. The two next most closely related proteins are IL-1F5 and IL-1Ra, which share 50% amino acid identity based on a sequence alignment. This similarity between IL-1Ra and IL-1F5 suggests the possibility that they may share a similar function, and indeed some groups have termed it a novel IL-1Ra (Mulero et al., 2000; Mulero et al., 1999). The classical members of the family are more distantly related in terms of sequence identity; IL-1 α and IL-1 β for instance, share only 24% identity. This highlights however that lack of sequence identity does not automatically mean differences in function. A pairwise comparison of each of the family members based on percentage amino acid similarity is given in table 3.2.2 and also shows the relationships between the different family members.



Figure 3.2.3 Alignment of residues from the novel IL-1s with those that contribute to the protein core of IL-1 β . The 24 residues, known from crystal structure analysis to contribute to the core of IL-1 β , were aligned against the sequences of the novel IL-1 family members and coloured according to hydrophobicity, from red for hydrophobic residues to blue for non-hydrophobic residues. Residue numbers corresponding to IL-1 β residues are indicated above the alignment.

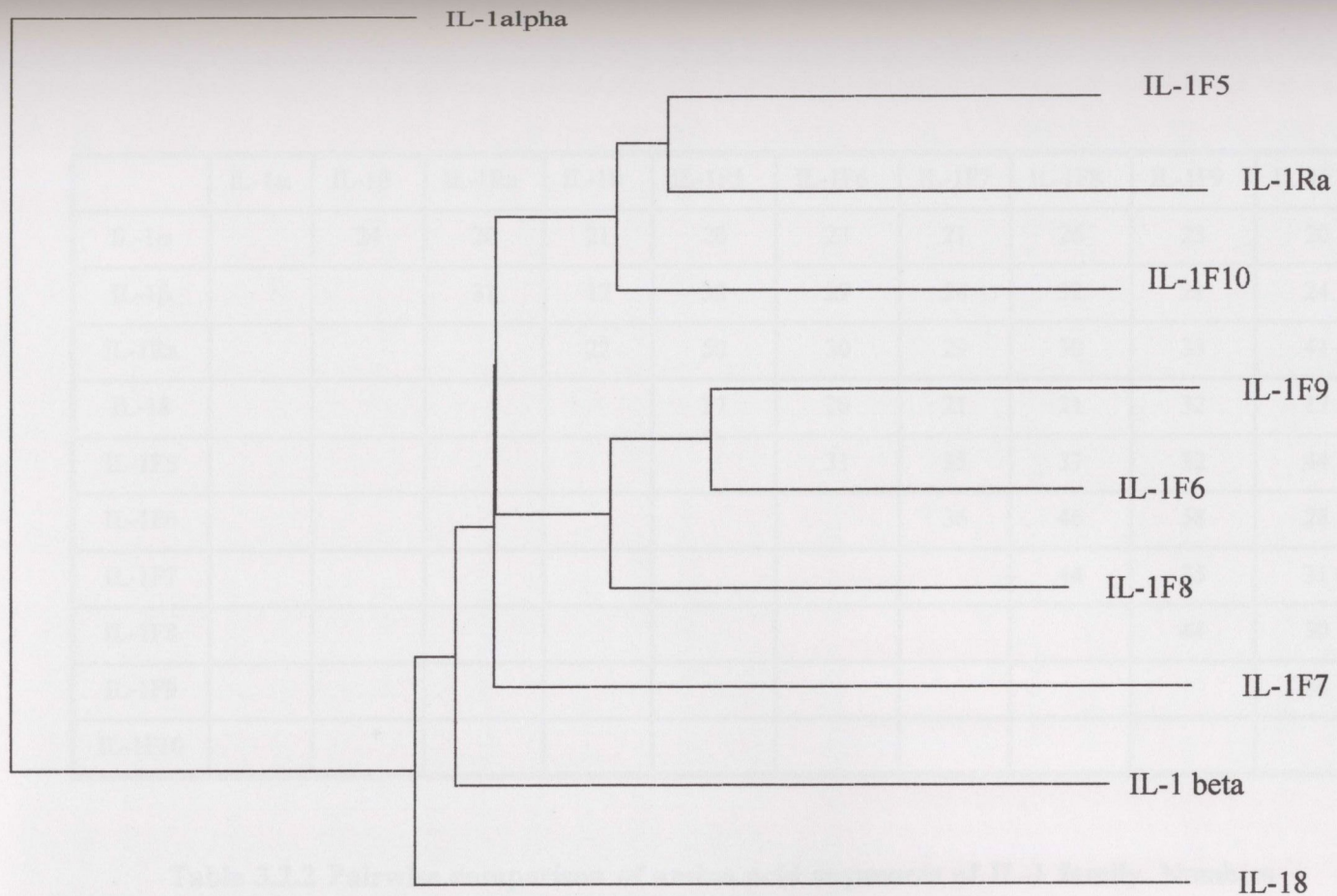


Figure 3.2.4 Dendrogram of IL-1 Family. The dendrogram was compiled using the edited alignment from ClustalW 1.8 and generated by the neighbour joining method using percentage identities.

	IL-1 α	IL-1 β	IL-1Ra	IL-18	IL-1F5	IL-1F6	IL-1F7	IL-1F8	IL-1F9	IL-1F10
IL-1 α		24	20	21	20	23	21	26	23	20
IL-1 β			31	17	32	27	24	32	21	24
IL-1Ra				22	50	30	29	30	33	41
IL-18					27	20	21	21	32	17
IL-1F5						31	35	37	32	44
IL-1F6							36	46	58	28
IL-1F7								44	35	31
IL-1F8									44	30
IL-1F9										34
IL-1F10										

Table 3.2.2 Pairwise comparison of amino acid sequences of IL-1 family. Numbers presents percentage amino acid identity between each member of the family. IL-1 α , IL-1 β , IL-1Ra and IL-18 sequences were taken in their mature processed forms, IL-1F7a was used for comparison using residues 27-192.

3.2.3 Secondary Structure Prediction of Novel IL-1 Family Members.

Secondary structure prediction for the novel family members was carried out using web-based software. A consensus conclusion from the data compiled from these programs is consistent with the presumption that novel family members are likely to have a similar secondary structure to that of the previously characterised members. The data collected with regard to IL-1F5 indeed suggest that a) IL-1F5 has predominantly β -strands in its secondary structure and b) that these strands occur in approximately the same location as in IL-1 β (Figure 3.2.5). The programs strongly predict β strands in similar regions to IL-1 β at the C-terminal section of the sequence, while N-terminal β strands are less strongly predicted by the majority of programs. However, these prediction programs do not give a 100% accurate prediction; feeding of IL-1 β into the programs for instance, correctly predicts the general position but not length of 11 out of its 12 β -strands, while for IL-1 α the position of only seven of the 12 are correctly predicted. However, the predictions represent a best guess of secondary structure in the absence of any experimentally derived information.

On the basis of these results it is legitimate to use the β strands of IL-1 β as a reference point with which to infer the position of β -strands in IL-1F5 and other novel IL-1 family members. Indeed, in the alignment of the family it can be seen that the structurally determined β -strand positions of both IL-1 β and IL-1Ra correlate very well, lending further support to the use of the presumed conservation of β -strand positions across the family. Therefore, using the positions of the β -strands of IL-1 β and IL-1Ra as reference, it can be seen from the alignment of the family that the areas of conservation occur mostly in regions that β -strands occur. This indicates that these regions are likely to be regions that are important contributors to the folding and stability of the protein and is another indication that the members of the family are likely to fold in a similar manner.

It is known from the structures of IL-1 β complexed to IL-RI that the majority of residues of functional importance are within the loop structures of the protein. Of the 19 residues that are within 3.5 Å of the receptor, 14 of these are in the loop regions of IL-1 β , which

		1	2	3	4	5	6	7	8	9	10			1	2	3	4	5	6	7	8	9	10	
1	M											52	A											
2	V			b				b				53	S											
3	L			b		a						54	L											
4	S					a	b					55	S					b				b		
5	G					a	a				b	56	P	b				b	b	b	b	b		
6	A	b	b			a	a	b		b	b	b	57	V	b	b	b	b	b	b	b	b	b	
7	L	b	b			a	a	b		b	b	b	58	I	b	b	b	b	b	b	b	b	b	
8	C	b	b			a	b	b	b	b	b	b	59	L	b	b	b	b	b	b	b	b	b	
9	F	b	b			a	b	b		b	b	b	60	G	b	b	b	b	b	b	b	b	b	
10	R	b	b			a	b	b	b	b	b	b	61	V	b	b	b	b	b	b	b	b	b	
11	M	b	b			a		b		b	b	b	62	Q		b						b		
12	K	b				a				b	b	b	63	G										
13	D	b				a			a				64	G										
14	S					a			a				65	S	b					b	b	b		
15	A					a			a				66	Q	b			b		b	b	b	b	
16	L	b			b	a		b	a				67	C	b			b	b	b	b	b	b	
17	K	b			b	a	b	b	a	b	b		68	L	b			b	b	b	b	b	b	
18	V	b			b	a	b	b	a	b	b	b	69	S	b			b	b	b	b	b	b	
19	L	b			b	a	b	b	a	b	b	b	70	C	b			b		b		b	b	
20	Y	b			b	a	b	b	a	b	b	b	71	G	b			b	b		b		b	
21	L					a	b	b	a	b	b	b	72	V	b									
22	H					a	b		a		b	b	73	G										
23	N								a				74	Q										
24	N	b				a			a				75	E										
25	Q	b				a	a		a		b	b	76	P										
26	L	b				a	a	b	a	a	b	b	77	T	b			b		b	b	b	b	
27	L				b	a	a	b	a	a	b	b	78	L	b			b		b	b	b	b	
28	A						a	b	a	a	b	b	79	T	b			b		b	b	b	b	
29	G						a		b	a	b	b	80	L	b			b		b	b	b	b	
30	G				b			a		b	a	b	81	E	b			b		b	b	b	b	
31	L				b			b		b	a	b	82	P				b		b	b	b	b	
32	H				b			b				b	83	V				b	a		b			
33	A				b				b				84	N				b	a		b	b		
34	G				b	b			b				85	I				a		b				
35	K					b		b	b				86	M				a		b				
36	V					b		b	b				87	E				a		b				
37	I					b		b		b			88	L				b		b				
38	K								b				89	Y				b						
39	G									b		b	90	L										
40	E	b	a					b		b	b	b	91	G										
41	E	b	a				b		b		b	b	92	A										
42	I	b	a				b		b		b	b	93	K										
43	S	b	a				b		b		b	b	94	E										
44	V	b	a				b				b	b	95	S						b	a			
45	V	b	a								b	b	96	K					b					
46	P	b									b		97	S					b	b	a	b		
47	N					a				b			98	F	b	b	b	b	b		b	b	b	
48	R					a			b				99	T	b	b	b	b	b		b	b	b	
49	W					a							100	F	b	b	b	b	b		b	b	b	
50	L					a	b						101	Y	b	b	b	b	b		b	b	b	
51	D						a						102	R	b	b	b	b	b		b	b	b	
													103	R	b	b	b	b	b		b	b	b	

Figure 3.2.5 Secondary Structure Prediction for IL-1F5. Results of nine different prediction programs are given. They are, according to column number: 1) Protein Predict, 2)PSI pred, 3) Hierarchical Neural Network, 4)SOPMA, 5)GOR, 6)Nnpredict, 7)Bioinbgu 8)Discrimination of Protein Secondary Structure Class (DSC) 9)3D-PSSM. ‘b’ represents a β-strand, ‘a’ an α-helix. The position of the structurally determined β-strands of IL-1β are shown in red in column 10.

represents 74% of the contacting residues, while for IL-1Ra, out of the 21 residues making contact with the receptor at 3.5 Å, 13 of these are in loop regions, which represents 62% of the contact residues. When a comparison of sequence identity is carried out taking secondary structure into account then it is seen that IL-1F5 and IL-1Ra are only ~24% identical in predicted loop regions, while in β -strand regions this identity increases to ~54%. Therefore, most of the conservation occurs in the β strands. As noted above, this may reflect a requirement to maintain tertiary structure stability. Meanwhile, the functionally important loops are more divergent, and this may suggest that the two proteins do not share a common function despite their high sequence identity.

In addition to this, hydropathy plots of both IL-1Ra and IL-1F5 were also calculated. This analysis uses two important characteristics of each amino acid to assign a numerical value to the degree of their hydrophobicity: a) the water-vapour transfer free energy, which is an indicator of the tendency of an amino acid to be partially or completely buried and b) the distribution of the amino acids on the exterior and interior of 12 known globular proteins (Kyte and Doolittle, 1982). A positive score indicates a hydrophobic residue, while a negative score indicates a hydrophilic residue. These values help to give an insight into the portions of a protein sequence that would form either the buried or accessible regions of the protein. Using a pairwise alignment of both IL-1Ra and IL-1F5 and superimposing their respective hydropathy plots it can be seen that especially towards the middle and C-terminal regions of the proteins there is good agreement of the hydropathy scores (Figure 3.2.6). This again indicates that the two proteins are likely to adopt a similar fold with similar regions of the proteins exposed to solvent. The hydropathy plots correlate well with the idea that the β -strands of the proteins, where most conservation of sequence occurs, are also hydrophobic regions of the proteins and which make up part of the structural interior of the protein. In some regions of the plot a zig-zag effect is clear with alternating hydrophobic and hydrophilic residues. This arises from the fact that successive residues along the β -strands of the protein point alternatively toward and away from the core of the protein. On the other hand loop regions of the protein are hydrophilic and are exposed to solvent.

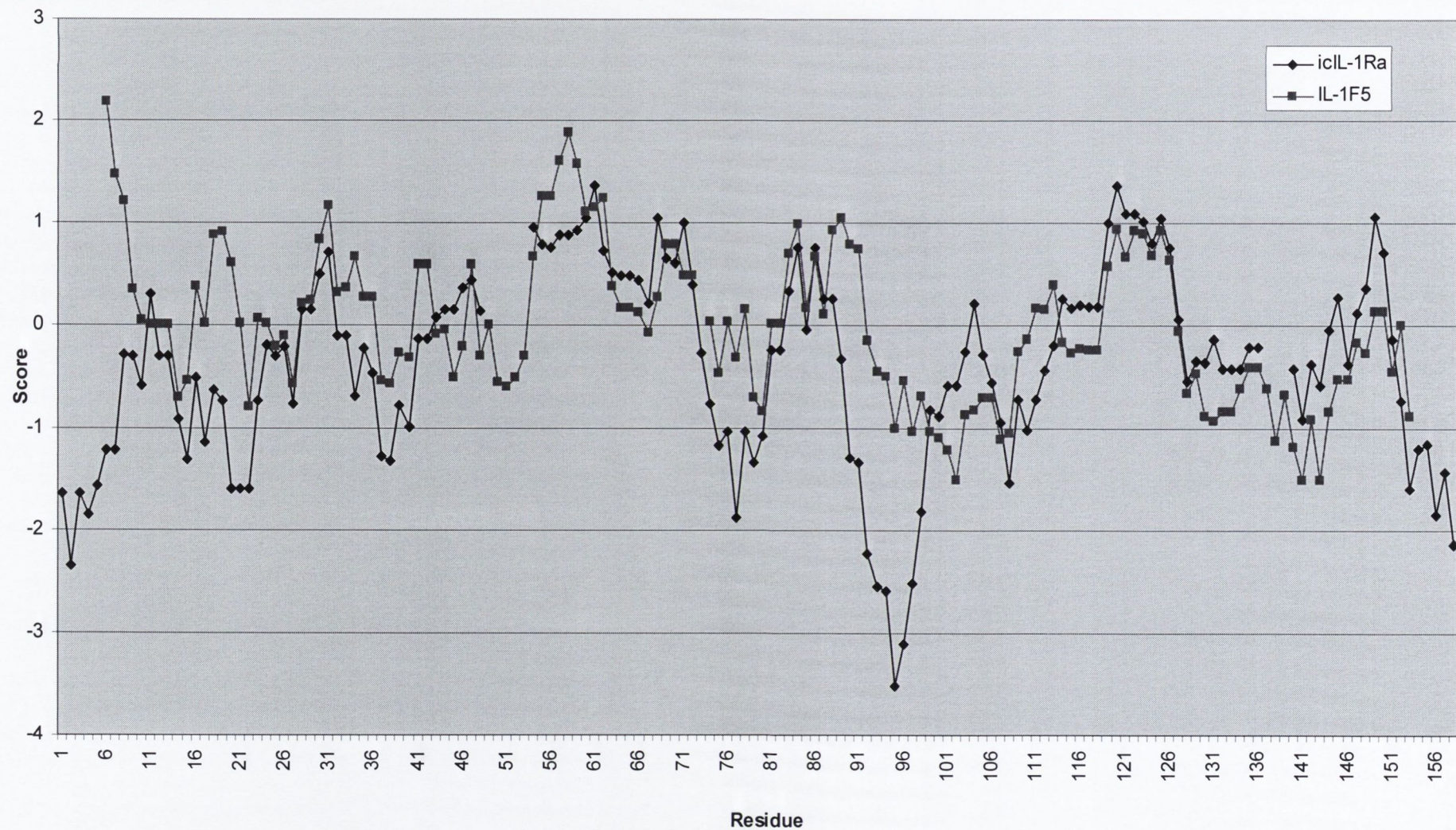


Figure 3.2.6 Hydrophobicity plot of IL-1F5 and IL-1Ra. Hydrophobicity plots of IL-1F5 (squares) and IL-1Ra (diamonds) were determined using the Kyte and Doolittle (1982) parameters. The resulting plots were superimposed using their pairwise alignment generated from ClustalW.

3.2.4 Tertiary Structure Prediction of IL-1 Family Members

Secondary structure prediction was supported further with tertiary structure prediction using SwissModel with the Swiss-Prot PDB Viewer. Threading of IL-1F5 on the superimposed structures of five different crystal or NMR structures of IL-1 β and IL-1Ra (three for IL-1 β and two for IL-1Ra) results in the prediction of a fold that is very similar to the known structures. The prediction clearly indicates that the protein is likely to adopt the β -trefoil fold. Loop regions are areas of less reliability in these predictions (Bajorath et al., 1993). The predicted structure more closely resembles IL-1 β in the loop 4-5 region, but mimics IL-1Ra in the loop 7-8 region (Figure 3.2.7). Again, although these predictions are only giving a 'best guess' result, they do yield information in that the β -trefoil structure is likely to be used by novel family members and that subtle differences in structure, most probably in loop regions, are likely to lead to differences in biologically activities of the various members.

3.2.5 Predicting a Function for IL-1F5

Initial predictions suggested that IL-1F5 would be an antagonist of the IL-1 system based on the amino acid homology with IL-1Ra. However, there are several lines of evidence described here that together suggest that this is not the case and in fact it is possible that IL-1F5 is even an agonist. In particular, the functionally important residue of box 5, Asp145 in IL-1 β , which is likely to be important for IL-1RAcP binding is conserved as an aspartate in IL-1F5. Also, unlike IL-1Ra, IL-1F5 does not have a similar short loop between β -strands 4 and 5. In IL-1 β this loop is seen to interact with domain 3 of IL-1RI and also to affect ligand binding and biological activity in mutagenesis studies. When the short loop of IL-1Ra is inserted into IL-1 β it loses agonist properties (Boraschi et al., 1995), and if the loop is shortened, IL-1 β likewise loses biological activity (Simoncsits et al., 1994). Meanwhile, if the long loop of IL-1 β is inserted into the K145D mutant of IL-1Ra it gains increased agonist activity (Greenfeder et al., 1995b). The alignment indicates that IL-1F5 has a loop of similar length to that in IL-1 β , suggesting it may be an agonist. The alignment also shows that IL-1F5 and IL-1Ra, although highly conserved in

predicted β -strand regions, diverge significantly in loop regions, where they share only about 24% sequence identity. Since these loop regions are likely to determine the functionality of the protein, this may indicate that the functions of IL-1Ra and IL-1F5 have diverged. Thus, the hypothesis from global alignment information alone that IL-1F5 might act as a novel IL-1Ra is not strongly supported by closer examination of the sequence data. However, the report by Debets et al. (2001) indicates that IL-1F5 may be an antagonist to IL-1F9 through IL-1Rrp2, although direct binding of the ligand to the receptor remains to be shown in order to confirm this finding. From the sequence analysis carried out here I suggest that IL-1F5 may be capable of acting in an agonistic manner.

Chapter 4 Cloning, Expression and Purification of IL-1F5

4.1 Introduction

Large quantities of IL-1F5 were desired not only for extensive *in vitro* work but also for crystallisation studies, which might lead to insights into the structure and function of IL-1F5. This required a high expression system to produce milligram amounts of protein. The expression system chosen for this purpose was a bacterial system using the pET vectors from Novagen (Figure 4.1.1). These systems have the advantage of producing high yields in a tightly controlled expression system under the control of the T7 polymerase. T7 polymerase is only induced upon the addition of isopropyl- β -D-thiogalactopyranoside (IPTG), which relieves the inhibition on the *lac* promotor by the *lac* repressor, thereby allowing expression of the T7 polymerase, the T7 polymerase in turn induces expression of the target gene, which is under the control of the T7*lac* promotor and is similarly inhibited by the *lac* repressor.

In order to achieve the most relevant material possible it was important not to introduce any foreign amino acids into the protein which might effect either function or structure, therefore no tags were added in the cloning process. This is especially important for IL-1 family members where N and C terminal residues are likely to be important for establishing and determining the fold and activity of the protein (see section 1.6). Expressed protein was purified to >90% homogeneity using a three step procedure involving ammonium sulphate precipitation, size exclusion chromatography and anion exchange.

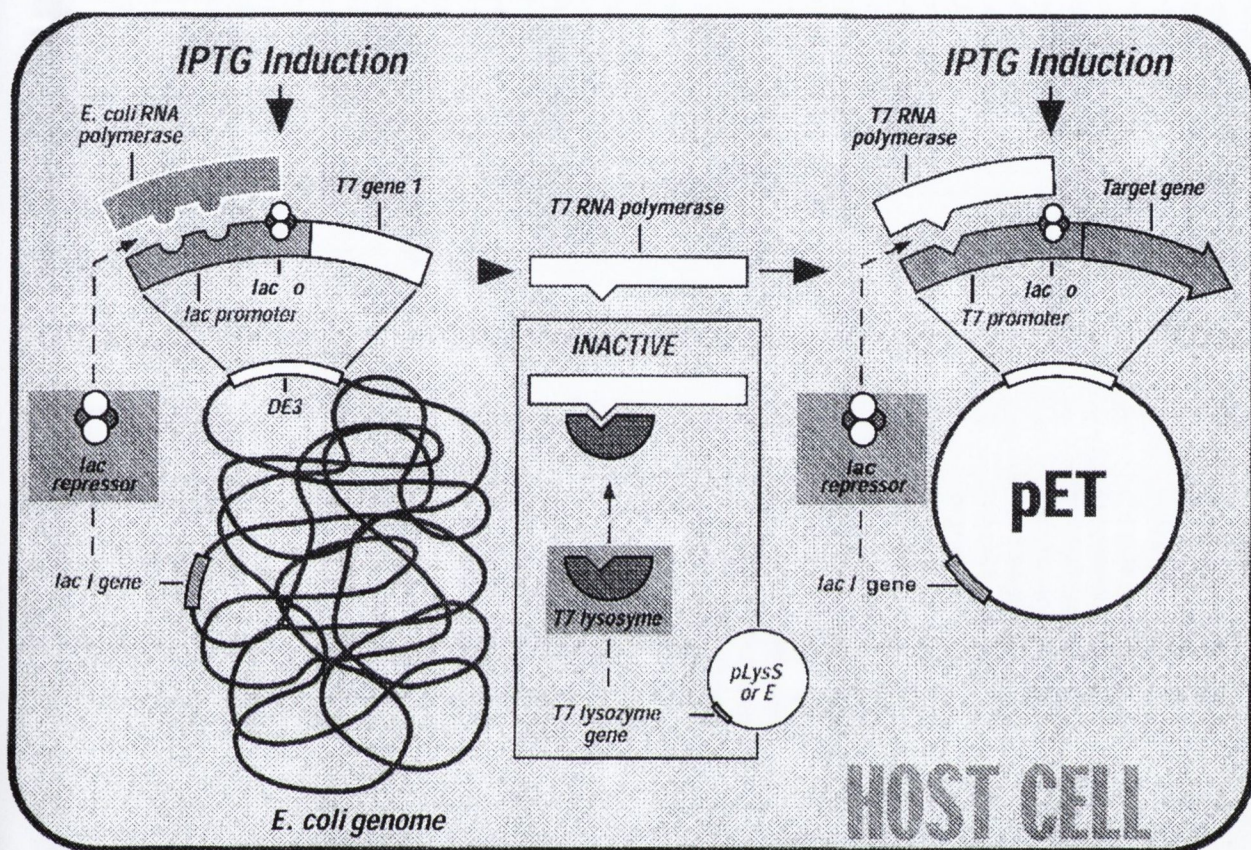


Figure 4.1.1 Schematic of the pET expression system. The host genome (λ DE3 lysogen), containing the T7 polymerase gene, expresses T7 polymerase upon induction with IPTG, which relieves the inhibition resulting from the *lac* repressor. The T7 polymerase then acts to induce expression of the target gene on the introduced vector, which is also under negative control of the *lac* repressor, which is relieved by IPTG induction.

4.2 Results

4.2.1 Sub-cloning of IL-1F5

The IL-1F5 gene was obtained in a yeast expression vector and the first step was to sub-clone it into the high expression pET vector. The IL-1F5 gene was therefore amplified from the yeast expression vector using the primers that were designed using the published cDNA sequence (Barton et al., 2000) (Figure 4.2.1). These include a forward primer with a well protected NdeI restriction site as this restriction enzyme requires at least seven base pairs either side of the cleavage site in order to cut efficiently. The reverse primer also included the translation termination codon so that no tag was introduced to the protein and it would be expressed in its native state. The primers were used at an annealing temperature of 58°C and the IL-1F5 gene was well amplified over a range of Mg^{2+} concentrations (Figure 4.2.2a). The resulting PCR product was digested with SacI, which cuts the PCR product at position 273. This digest yielded two bands corresponding to fragments of size 273 and 214, the expected products from a SacI digest of IL-1F5. In this experiment the restriction enzymes BglII and SmaI acted as negative controls (Figure 4.2.2b).

A Mg^{2+} concentration of 2 mM was chosen for scaled up PCR reactions, which could yield enough DNA for sub-cloning purposes. The resulting product was cleaned using the agarose gel cleaning method which involved the melting of the agarose gel and subsequent binding of the DNA to silica, washing and elution in Tris/EDTA buffer. This method insures proper separation of plasmid and insert, giving a clean product ready for the restriction enzyme digest. The vector, pET21a (Figure 4.2.3), and insert were both digested with NdeI and EcoRI overnight to insure complete digestion. The digested products were cleaned using the quicker method of spin column purification, which also results in less loss of product during the cleaning process but allows for adequate separation of digested product from the small cut away fragments.

Products were quantified by measuring absorbance at 260 nm and 50 ng of plasmid was incubated in the presence of T4 DNA ligase with various amounts of IL-1F5 insert that

Primer	Direction	Sequence	Length	GC content	Tm
pETIL-1F5F	Forward	TTA ATT TCA <u>TAT</u> GGT CCT GAG TGG GGC GCT G NdeI	31	48.4%	68.2 °C
pETIL-1F5R	Reverse	AAA GAA TTC <u>CTA</u> GTC ACA CTG CTG GAA GTA EcoRI	30	40%	64.0 °C

Figure 4.2.1 Sequence of primers used to amplify IL-1F5 coding DNA by PCR. Sequence of forward and reverse primers are given, with GC content and predicted transition melting point indicated. The primers were used at an annealing temperature of 58°C. The initiation ATG codon in the forward primer and the antisense CTA termination codon in the reverse primer are indicated with a horizontal bar.

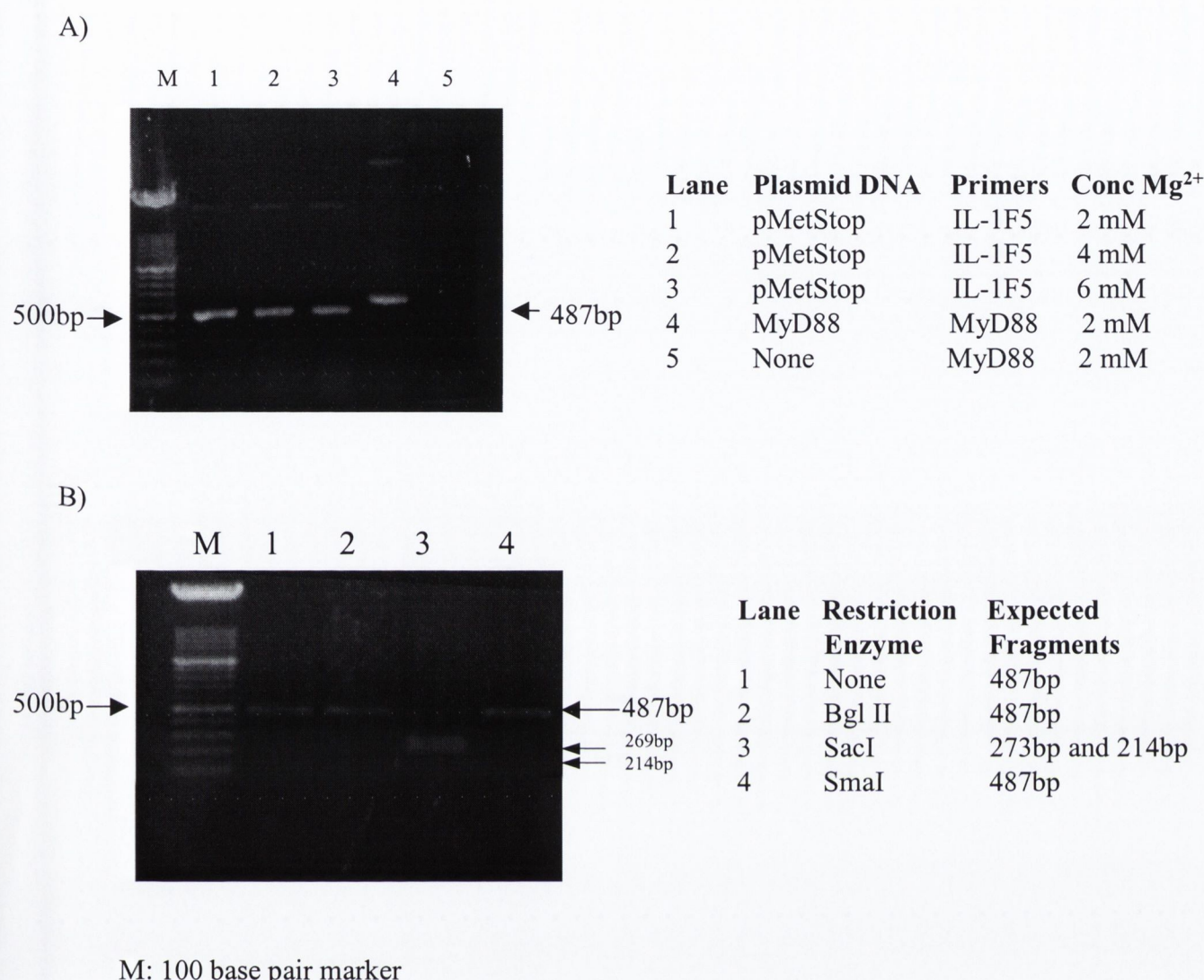


Figure 4.2.2 PCR amplification of IL-1F5 from pMetStop plasmid. A) PCRs were set up using primers designed against the coding region of IL-1F5 and using varying concentrations of Mg^{2+} from 2 mM to 6 mM. A plasmid encoding MyD88 was used as a positive control using MyD88 directed primers. Reactions were run for 35 cycles of amplification using an annealing temperature of 58°C. B) Restriction digest analysis of the PCR product from pMetStop plasmid. The PCR product was cleaned using a GenElute™ spin column and incubated with restriction enzymes for 1 hour at 37°C. DNA was separated on a 1% agarose gel, stained with ethidium bromide and visualised under UV light.

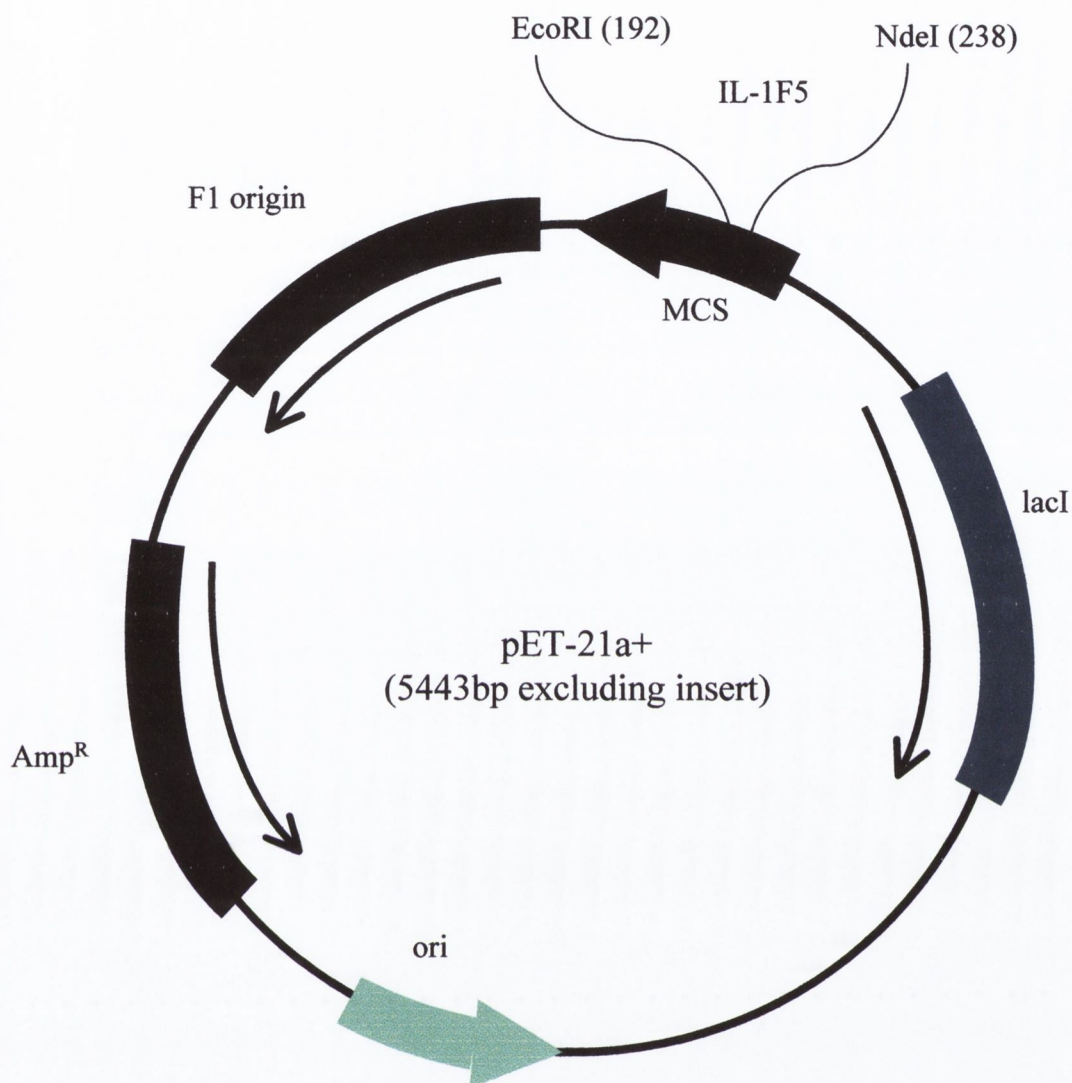


Figure 4.2.3 Map of the pET-21a-IL-1F5 construct. The 5.5 kb pET21a plasmid contains genes encoding for ampicillin resistance and lacI, the inhibitor of transcription at the T7 promoter. The gene encoding IL-1F5 was cloned into the multiple cloning site (MCS) between the EcoRI and NdeI restriction sites. The positions of each restriction site on the vector is represented by lines and their exact position on the parent plasmid given by the number in parentheses following their names.

were equivalent to 2, 4 and 6 times the molar equivalent of the plasmid. This helped ensure that there was an excess of insert in the ligation reaction and thereby minimised self-religation of the phsmid. As a control reaction, ligation with vector alone was also carried out. The reaction mixtures were then transformed directly into highly competent DH5 α *E.coli* and incubated on ice for 5 minutes before plating out directly onto pre-warmed ampicillin containing LB agar plates. The plates were incubated overnight at 37°C and then examined for any colonies. The ratio of colonies on the vector alone plate should always be less than that for the plates with both vector and insert as an indication that the colonies on the vector plus insert plate are not a result of self religation reaction of the vector. This was the case and the number of colonies that grew from the transformation is given in figure 4.2.4.

Colonies were analysed by two methods to determine if they contained the IL-1F5 gene in the pET21a vector. Firstly colonies were analysed by PCR using the cloning primers. A toothpick scraping of each purified colony was added to a complete PCR reaction mixture and heated at 95°C for 10 minutes. This is sufficient to lyse bacterial cells and release DNA for amplification. The results of this analysis are given in figure 4.2.5a which show that colonies number 3, 7, 9, 11, 12 and 13 were positive for the presence of the IL-1F5 gene by this method of detection. In addition these colonies were also analysed by restriction digest analysis. Small cultures of each colony were grown overnight and rapid mini-prep plasmid DNA was prepared as described in section 2.3.9. The purified plasmids were digested with NdeI and EcoRI restriction enzymes and analysed for a ~500 bp digest product corresponding to the IL-1F5 gene. Figure 4.2.5b shows that colonies number 3, 7, 9 and 12 were positive for the IL-1F5 gene. On the basis of this analysis colonies number 7 and 9 were chosen for trial induction experiments.

Plate (insert:vector)	No. Colonies
0:1	0
1:0	1
2:1	7
4:1	20
6:1	30

Figure 4.2.4 Colony numbers resulting from the ligation transformation. Analysis of transformations resulting from ligation reactions of varying insert to vector molar ratios. Ligation reactions were carried out and transformed into DH5 α *E. coli*, plated out on LB agar plates and incubated at 37°C overnight. The number of colonies on each plate was then counted.

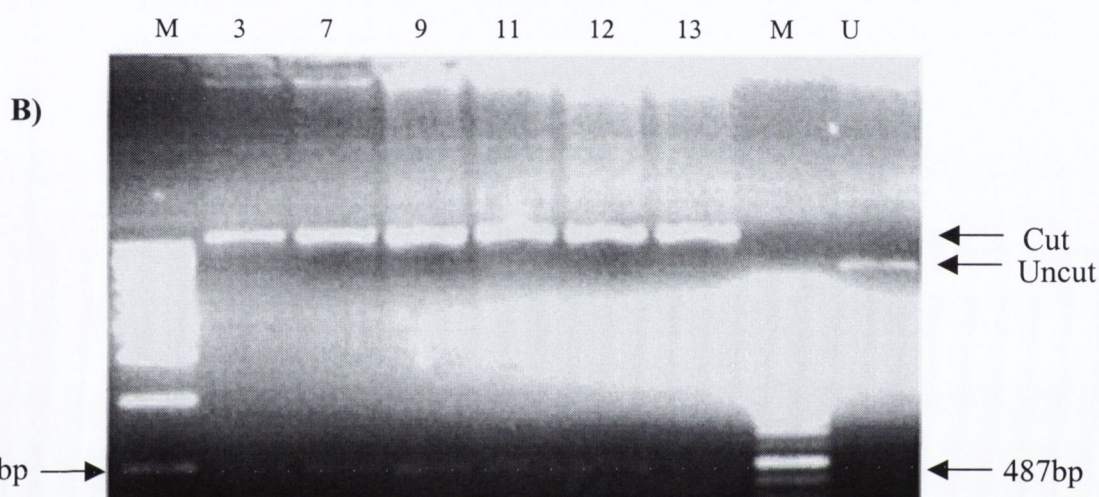
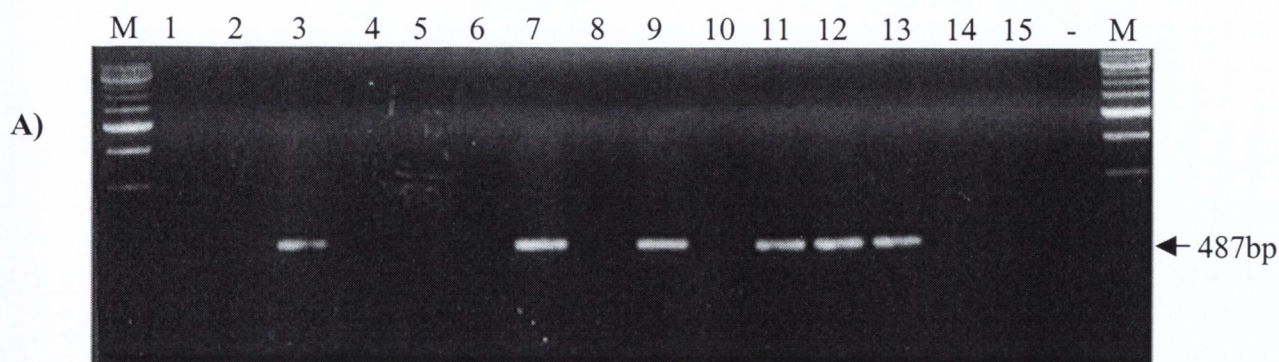
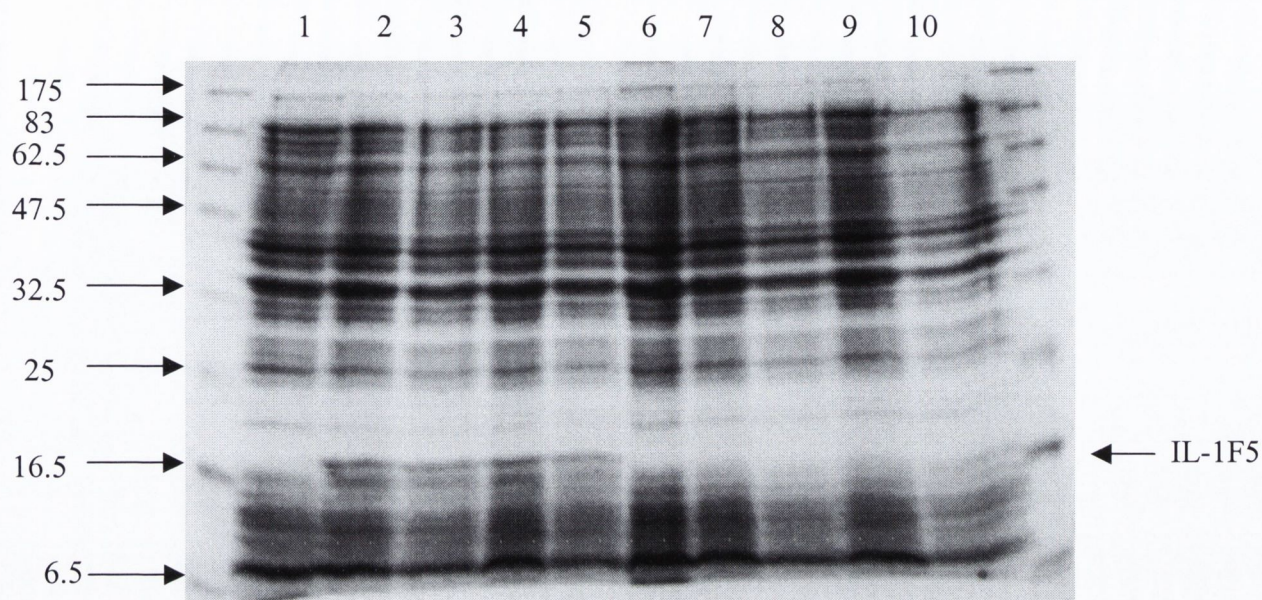


Figure 4.2.5 Analysis of ligation transformants. A) PCR of ligation colonies using IL-1F5 primers. Colonies resulting from the ligation transformation were streaked onto an agar plate with numbered grids and the colonies allowed to grow overnight. A sample of each colony was then introduced into a PCR reaction mixture containing primers to IL-1F5 using a toothpick. The PCR was then run with an initial heating step of 95°C for 10 minutes, followed by 30 amplification cycles using an annealing temperature of 58°C. DNA was separated on a 1% agarose gel, stained with ethidium bromide and visualised under UV light. Numbers above the lanes refer the colony number, M is a 100bp marker, '-' represent a negative control with no bacterial colony added. B) Restriction digest of plasmids isolated from ligation colonies. Plasmids were isolated from colony numbers 3, 7, 9, 11, 12 and 13 and were digested with EcoRI and NdeI at 37°C for 1 hour. DNA was separated on a 1% agarose gel stained, with ethidium bromide and visualised under UV light. The 487 bp fragment is the expected size for the IL-1F5 insert. U denotes uncut plasmid.

4.2.2 Expression of IL-1F5

4.2.2.1 Induction of Expression

Plasmid preparations of colonies number 7 and 9 were prepared and transformed into competent BL21(DE3) *E.coli*. Single colony purifications of transformed cells were prepared and used to inoculate small overnight cultures in supplemented M9 medium which minimised leaky expression from the pET21a vector. 10 ml cultures were allowed to grow to an optical density of 0.6 and then induced with either 0.4 mM or 1 mM IPTG for 2 or 4 hours. As shown in figure 4.2.6, a band running just above the 16.5 kDa molecular weight was induced from colony number 7 in all conditions examined. However, the level of expression of IL-1F5 was quite low and therefore other conditions of induction were examined to try and increase the level of expression of the induced protein. The plasmid isolated from colony 7 was also transformed into competent CodonPlus BL21 *E.coli*. If a mammalian gene has codon usage that varies significantly from that of bacterial cells, this can inhibit the expression of the recombinant protein due to insufficient transfer RNAs (tRNAs) in the bacterial pool. The CodonPlus cells have tRNAs which correspond in amounts to those typically found in mammalian cells and can therefore overcome this problem. However expression of the induced protein was not increased in these cells when transformed with plasmid from colony 7 (Figure 4.2.7a). Furthermore, the plasmid was also transformed into BL21 pLysS *E.coli*. These cells contain the gene for the protein lysozyme S. This protein binds to and inhibits T7 polymerase thereby controlling its activity in uninduced cells. However only very faint expression of induced protein was detected in these cells (Figure 4.2.7a). Other conditions of induction were also examined in which inoculated cultures were induced with IPTG from the start of the culture and allowed to grow for 5 hours in total. However, this procedure also failed to increase the levels of expression of the induced protein (Figure 4.2.7b). Thus, although the level of expression detected was not to a very high level, it was sufficient to allow for purification and isolation of IL-1F5.

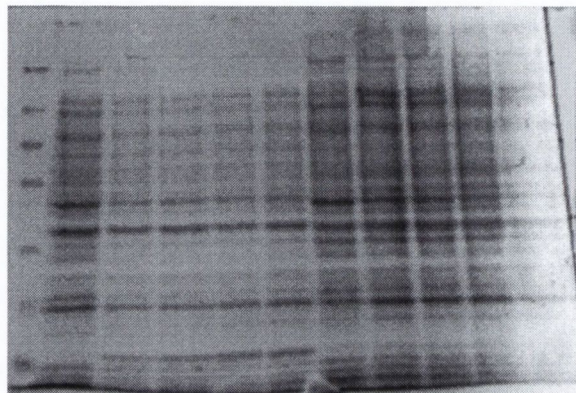


Lane	Colony	IPTG (mM)	Time (hours)
1	7	0	0
2	7	0.4	2
3	7	0.4	4
4	7	1	2
5	7	1	4
6	9	0	0
7	9	0.4	2
8	9	0.4	4
9	9	1	2
10	9	1	4

Figure 4.2.6 Trial induction of IL-1F5 Expression. Cultures of BL21(DE3) *E. coli* bacteria known to contain plasmids encoding IL-1F5 were grown overnight, diluted back 1 in 40 and grown to an OD₆₀₀ of 0.6 before incubation with either 0.4 mM or 1 mM IPTG for 2 or 4 hours. 1 ml of bacteria, diluted to an OD₆₀₀ of 0.6 was taken, pelleted and lysed in 25 µl SDS-sample buffer. The whole cell lysates were then boiled and analysed for protein content on a 13% SDS-polyacrylamide gel stained with Coomassie blue.

A)

M 1 2 3 4 5 6 7 8 9 10



16.5 →

← IL-1F5

B)

M 1



16.5 →

← IL-1F5

A)

Lane	Strain	Conc IPTG	Time
1	Codon Plus	0	0
2	Codon Plus	0.5 mM	2 h
3	Codon Plus	1 mM	2 h
4	Codon Plus	0.5 mM	4 h
5	Codon Plus	1 mM	4 h
6	pLysS Star	0	0
7	pLysS Star	0.5 mM	2 h
8	pLysS Star	1 mM	2 h
9	pLysS Star	0.5 mM	4 h
10	pLysS Star	1 mM	4 h

B)

Lane	Strain	Conc IPTG	Time
1	BL21	1 mM	5 hr (from start of culture)

Figure 4.2.7 Optimisation of IL-1F5 expression. A) The IL-1F5 expression plasmid was transformed into codon plus BL21 and pLysS Star BL21 *E. coli*, grown to an OD₆₀₀ of 0.6 and induced with IPTG at either 0.5 mM or 1 mM, for 2 or 4 hours. B) BL21 cells transformed with IL-1F5 expression plasmid were induced from the start of the culture with 1 mM IPTG for a total induction time of 5 hours. 1 ml of bacteria, diluted such that it had an OD reading equivalent to that of the OD at the time of induction was taken, pelleted and then lysed in 25 µl SDS-sample buffer. Samples were boiled for 5 minutes at 100°C and analysed on a 13% SDS-polyacrylamide gel stained with Coomassie blue.

4.2.2.2 Sequence Analysis of cDNA Insert

A preparation of the plasmid from colony number 7 was purified. 10 µg of the purified plasmid was precipitated and dried and sent for sequencing to MWG Biotech (Germany). Primers to IL-1F5 were supplied for this process. The results are shown in Figure 4.2.8. The DNA sequence shows three differences from the published sequence by Barton et al. (2000) (accession number AJ250429). However, when translated into the amino acid sequence, no differences from the published sequence are present (Figure 4.2.9).

4.2.3 Purification of IL-1F5

4.2.3.1 IL-1F5 Solubility

Next a procedure for the purification of IL-1F5 was investigated. The first thing to be determined was whether the IL-1F5 was expressed into the soluble or insoluble fraction of the bacterial lysate. A 1 L culture was grown overnight, pelleted and lysed according to the method described in section 2.4.2. Analysis of both the soluble and insoluble fractions showed that IL-1F5 appeared in the soluble fraction (Figure 4.2.10). Some IL-1F5 protein could also be detected in the insoluble fraction. This is likely to be due to some of the soluble fraction binding to insoluble matter and being brought down in the pellet with the insoluble fraction. Knowing that IL-1F5 could be extracted from the soluble fraction, this fraction was thus collected and put through further purification steps.

4.2.3.2 Anion Exchange

At first an anion exchange step was tested using DEAE-trisacryl based on the predicted pI of IL-1F5 of 5.1. However, figure 4.2.11 shows that no purification of IL-1F5 was achieved using this method. This may have been due to the presence of large amounts of DNA in the preparation, which also would have a negative charge and is likely to bind the DEAE-trisacryl.


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1  MVLSGALCFRMKDSALKVLYLHNNQLLAGGLHAEKVIKGEEISVVPNRAL  50
   ||||||||||||||||||||||||||||||||||||||||||||||||
2  MVLSGALCFRMKDSALKVLYLHNNQLLAGGLHAEKVIKGEEISVVPNRAL  51

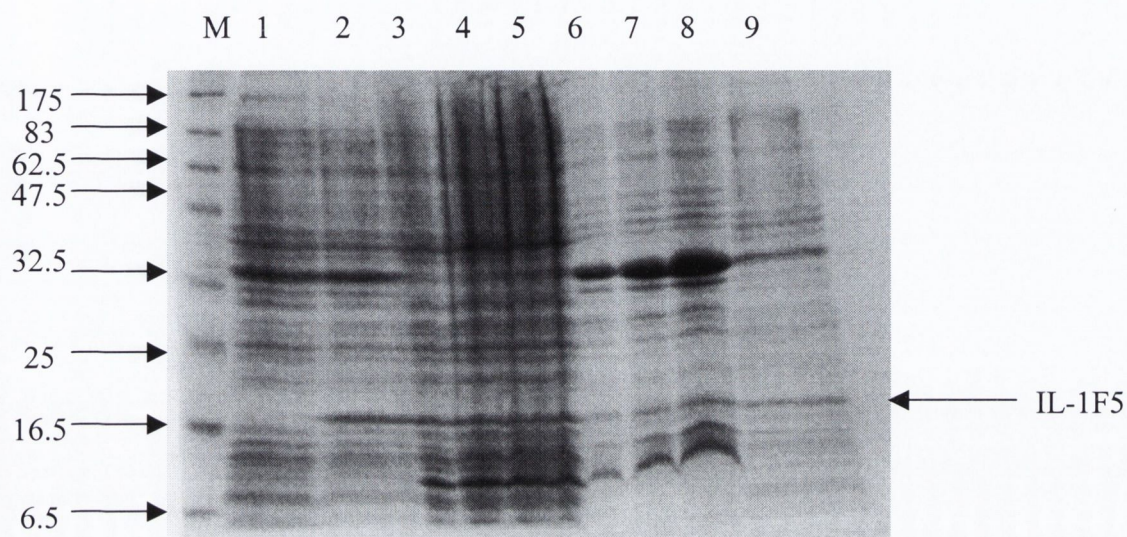
51 DASLSPVILGVQGSQCLSCGTEKGPILEPVNIMELYLGAKESKSFTF 100
   ||||||||||||||||||||||||||||||||||||||||||||||||
52 DASLSPVILGVQGSQCLSCGTEKGPILEPVNIMELYLGAKESKSFTF 101

101 YRRDMGLTSSFESAAYPGWFLCTSPEADQPVRLTQIPEDPAWDAPITDFY 150
    ||||||||||||||||||||||||||||||||||||||||||||||||
102 YRRDMGLTSSFESAAYPGWFLCTSPEADQPVRLTQIPEDPAWDAPITDFY 151

151 FQQCD 155
    |||||
152 FQQCD 156

```

Figure 4.2.9 Predicted amino acid sequence of IL-1F5 gene in pET21a. Comparison of the expected amino acid sequence of the IL-1F5 gene in the pET21a-IL-1F5 plasmid and the published sequence (Barton et al., 2000). No amino acid changes are present.



Lane	Sample
1	Uninduced whole cell lysate
2	Induced whole cell lysate
3	5µl soluble fraction
4	10µl soluble fraction
5	20µl soluble fraction
6	5µl insoluble fraction
7	10µl insoluble fraction
8	20µl insoluble fraction
9	Induced whole cell lysate

Figure 4.2.10 Distribution of IL-1F5 between soluble and insoluble fractions of bacterial cell lysate. Bacterial cultures induced to express IL-1F5 were collected and lysed. Soluble and insoluble fractions were separated by centrifugation and analysed for the presence of IL-1F5 by separation on a 15% SDS-polyacrylamide gel stained with Coomassie blue.

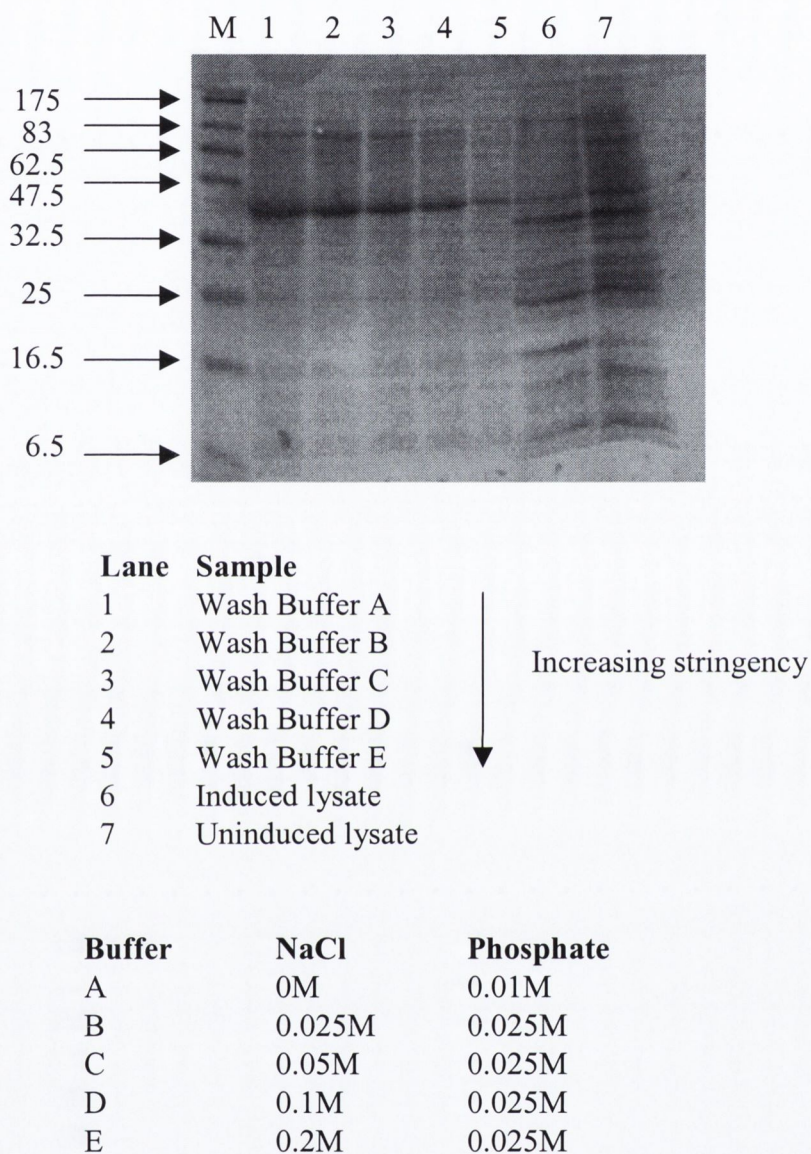
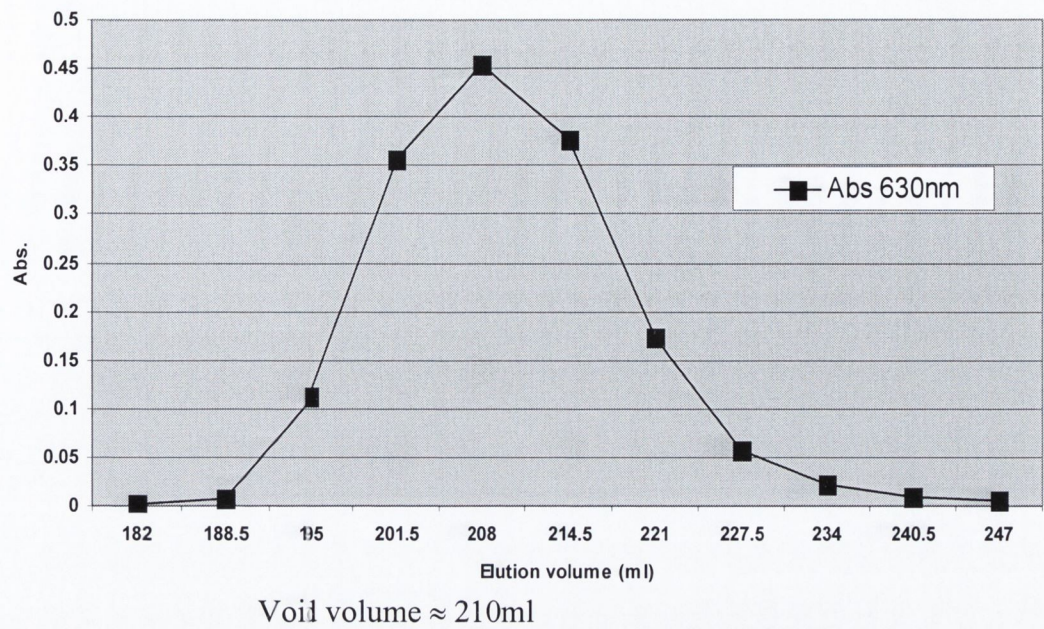


Figure 4.2.11 Trial anion exchange purification. A bacterial culture induced to express IL-1F5 was lysed and the soluble fraction collected. The soluble fraction was incubated with DEAE-trisacryl for 15 minutes at room temperature with gentle agitation. Elution was carried out under varying salt concentrations. After elution samples were analysed by separation on a 15% SDS-polyacrylamide gel stained with Coomassie blue.

4.2.3.3 Size Exclusion Chromatography and Ammonium Sulphate Precipitation

Next size exclusion chromatography was tried as a first step in the purification. A G-75 sephadex column was prepared as per section 2.4.4. The height of the column was measured at 84 cm and the diameter was 3.5 cm, which gives a calculated bed volume of 808 cm³. The column was calibrated for void volume using dextran blue dye and for the elution volume of IL-1Ra, which should behave in a manner similar to IL-1F5. This 800 ml column had a void volume of 210 ml and elution volume for IL-1Ra of 390 ml (Figure 4.2.12). In order for gel filtration to have a good resolution it is important for the load volume of the sample to be reasonably concentrated. For sephadex G-75 the recommended load volume is 0.5%-5% of the total bed volume. Therefore, a maximum of about 40 ml could be loaded on the column. The soluble fraction from the bacterial lysate would typically give a volume of about 150 ml to 200 ml from a 5 L culture, and therefore had to be concentrated to a smaller volume. This was achieved by ammonium sulphate precipitation. Figure 4.2.13 shows a trial ammonium sulphate precipitation experiment. Proteins were precipitated by increasing percentages of saturated ammonium sulphate solutions from 45% to 65% saturation. Precipitated protein was collected by centrifugation and any unprecipitated protein was recovered from the supernatant. Both fractions were analysed by SDS-PAGE. This analysis showed that a 50% saturated ammonium sulphate solution allows for complete precipitation of IL-1F5 (Figure 4.2.13). It also had the advantage of removing some higher molecular weight proteins, which did not precipitate well at this percentage of ammonium sulphate saturation. Precipitated protein was then collected by centrifugation and resuspended in 20 ml of 50 mM ammonium acetate per 5 L of culture. The resuspension was cleared of any insoluble material by centrifugation at 23,000 x *g* for 10 minutes. This ensured that the sephadex did not get clogged and thus maintained a good flow rate in the column. The sample was also checked for viscosity since increases in viscosity lead to deterioration of separation due to irregularity in the flow rate pattern and instability of the zone or region of the mobile phase containing the protein of interest. Viscosity was determined to be

A)



B)

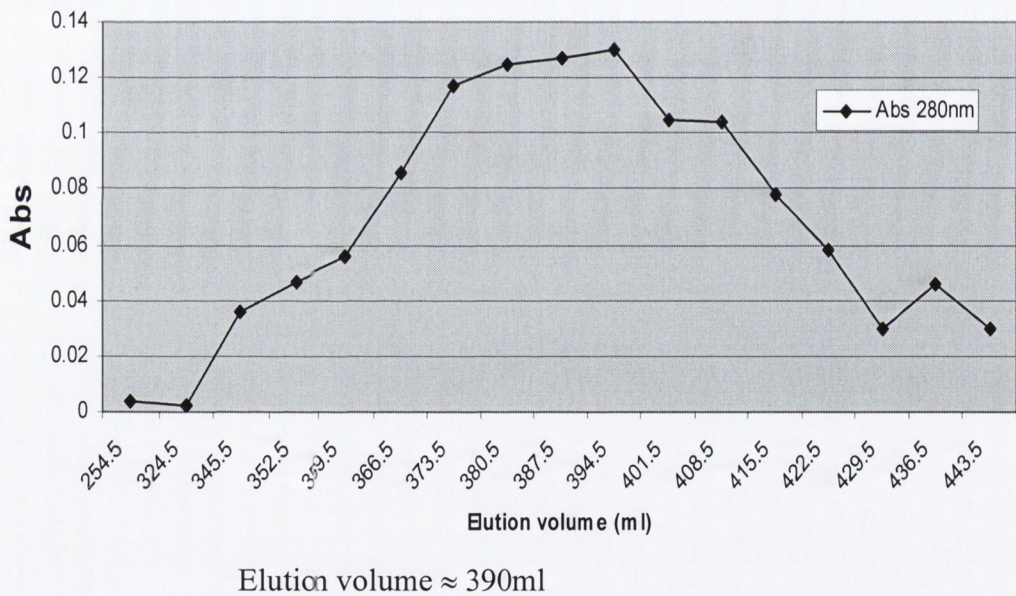


Figure 4.2.12 Calibration of G75 Sephadex column by determination of void volume and elution volume of IL-1Ra. 10 mg IL-1Ra and 30 mg dextran blue were mixed in a volume of 10 ml and loaded on the column. 5 minute fractions were collected and analysed for the presence of dextran blue and IL-1Ra. A) The void volume was calculated using the elution profile of dextran blue by measuring the absorbance of the fractions at 630 nm. B) the elution profile of IL-1Ra was measured by monitoring the absorbance of fractions at 280 nm.

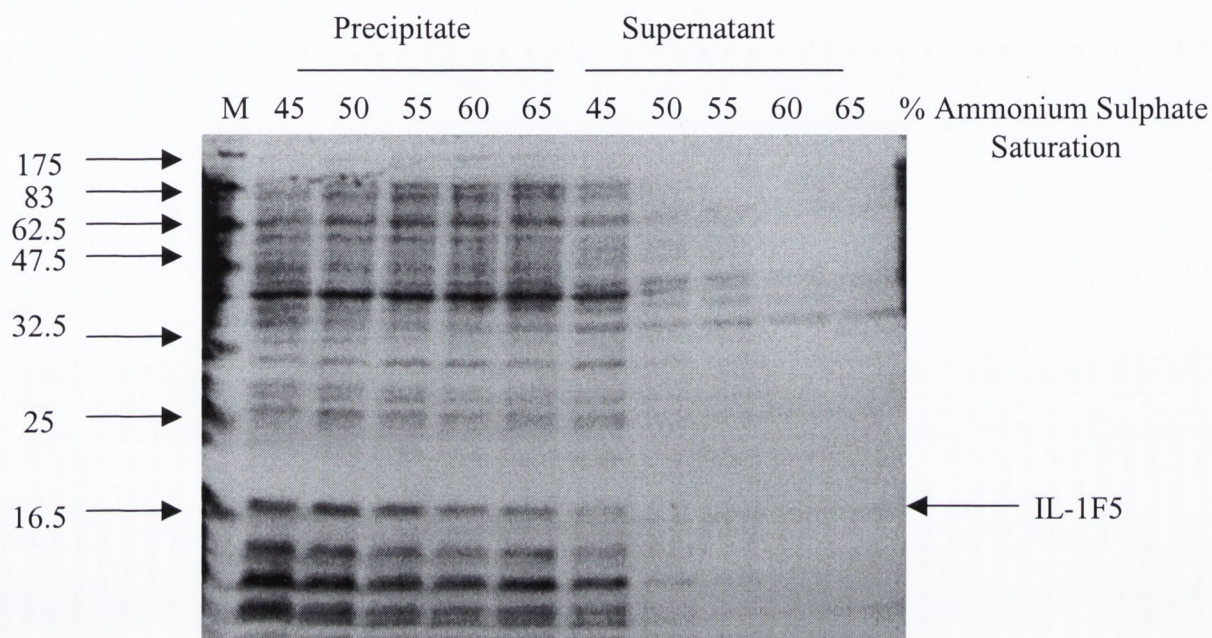


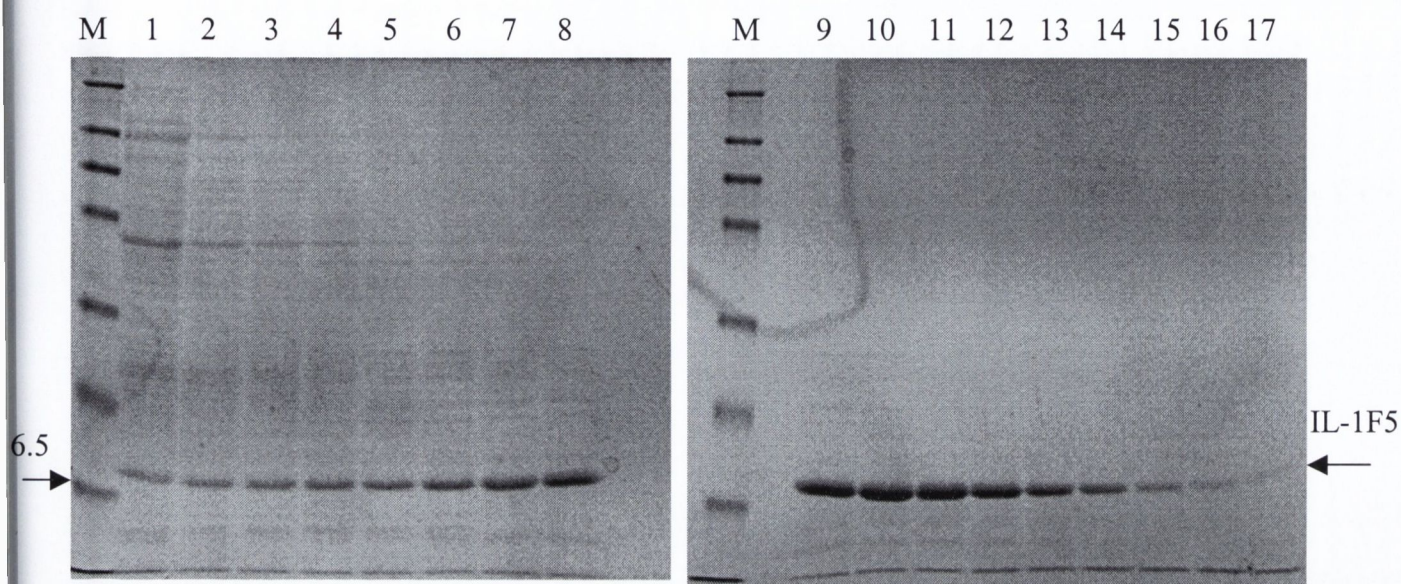
Figure 4.2.13 Trial ammonium sulphate precipitation. A bacterial culture, induced to express IL-1F5, was lysed and the soluble fraction collected and divided. Proteins in the fraction were precipitated with varying amounts of ammonium sulphate, added to give a final percentage saturation from 45% to 65%. Precipitated protein was collected by centrifugation and both the precipitate and the supernatant were analysed for protein content on a 15% SDS-polyacrylamide gel stained with Coomassie blue.

approximately equal to that of the ammonium sulphate buffer as measured by comparing emptying times from a pipette.

The resuspended protein was then loaded onto the column. The void volume was allowed to run through the column before 5 minute fractions were collected up to 500 ml elution volume. Fractions were then analysed for the presence of IL-1F5 by SDS-PAGE. A typical elution profile obtained for IL-1F5 is shown in figure 4.2.14. Fractions that contained the most concentrated and purified IL-1F5 (generally corresponding to an elution volume of 350 ml-450 ml, which correlates well with the elution volume of IL-1Ra which elutes at 390 ml) were collected. The conductivity of the collected samples was measured to ensure that the ammonium sulphate, which might interfere with subsequent purification steps, had indeed been separated from the protein; the conductivity of 50 mM ammonium acetate was measured as 4.8 mS whereas the protein solution had a measured conductivity of 5.4 mS. The protein was then shell frozen in a round bottomed flask using an ethanol bath and dried in a vacuum freeze drier. This procedure allows for concentration and buffer exchange ready for the next step in the purification procedure.

4.2.3.4 Mono-Q Anion Exchange

For the next step in the purification an anion exchange system was used. A 10 ml MonoQ anion exchange column was used linked to a high performance liquid chromatography (HPLC) machine. This allowed for controlled increases of salt concentrations to be applied to the column which yielded high quality and rapid separation and purification of IL-1F5. The preparation from the size exclusion chromatography was resuspended in 5 ml per 5 L of culture in 25 mM Tris-HCl pH 8 and loaded on the column. With the column running at a rate of 4 ml/min any protein not binding was allowed to elute off the column in the first 5 minutes. The concentration of salt in the running buffer was then increased slowly from 0% to 20% of 1 M NaCl over 5 to 35 minutes. Thereafter the running buffer was switched quickly to 100% 1 M NaCl to elute off any remaining protein. During this process the absorbance of the eluant was measured at 276 nm. A



Lane	Elution volume (ml)
1	323.5
2	332.25
3	341.0
4	349.75
5	358.75
6	367.75
7	376.75
8	385.75
9	394.75
10	403.75
11	412.75
12	421.75
13	430.75
14	439.75
15	448.75
16	457.75
17	466.75

Figure 4.2.14 Elution profile of IL-1F5 from size exclusion chromatography on G75 sephadex column. Protein precipitated with 50% ammonium sulphate saturation from a bacterial lysate containing IL-1F5 was resuspended in 50 mM ammonium acetate and loaded on a G-75 sephadex column. 10 minute fractions were collected and analysed for protein on a 13% SDS-polyacrylamide gel stained with Coomassie blue. Fractions corresponding to lanes 5-14 were pooled for further purification.

typical elution profile for this procedure is shown in figure 4.2.15. 2 ml fractions collected from the column were analysed by SDS-PAGE for the presence of IL-1F5. This showed that the peak coming off the column at about 23 minutes or 100 mM NaCl corresponded to purified IL-1F5. Purity was determined to be >90% as judged by SDS-PAGE analysis by Commassie Blue and silver staining (Figure 4.2.16). An absorption spectrum of the purified protein gives the expected peak at around 280 nm with a shoulder at 290 nm due to phenylalanine also present (Figure 4.2.17).

4.2.3.5 Mass Spectroscopic Analysis of Recombinant IL-1F5

The material from the anion exchange step was further analysed by mass spectroscopy to confirm that it was IL-1F5. The material was dialysed into 20% acetonitrile/0.025% trifluoroacetic acid and analysed in a mass spectrometer. This showed that the peak at 16871.38 daltons corresponds to the molecular weight of IL-1F5 in which the first methionine has been deleted, plus the existence of one disulphide bond which reduces the molecular weight by 2 daltons (Figure 4.2.18). The removal of the first methionine is something that is common in BL21 *E. coli* cells.

4.2.4 Structural Studies

4.2.4.1. Circular Dichroism

Purified IL-1F5 was analysed by circular dichroism (CD) to determine if it had a fold similar to that of IL-1Ra and as an indication that the protein had not been denatured or altered significantly during the purification procedure. CD measurements were made in a Jasco 800 instrument which was pre-flushed with N₂ gas in order to displace O₂ which absorbs light at wavelengths around 200 nm and below. Left and right circularly polarised light at wavelengths from 250 nm to 180 nm was passed through a sample of IL-1F5 at a concentration of 37.5 µg/ml in a 3 ml quartz cuvette, which had been dialysed into H₂O. Using water as a solvent helped reduce noise in the spectra, which is apparent especially at lower wavelengths where many buffers with chiral centres are optically

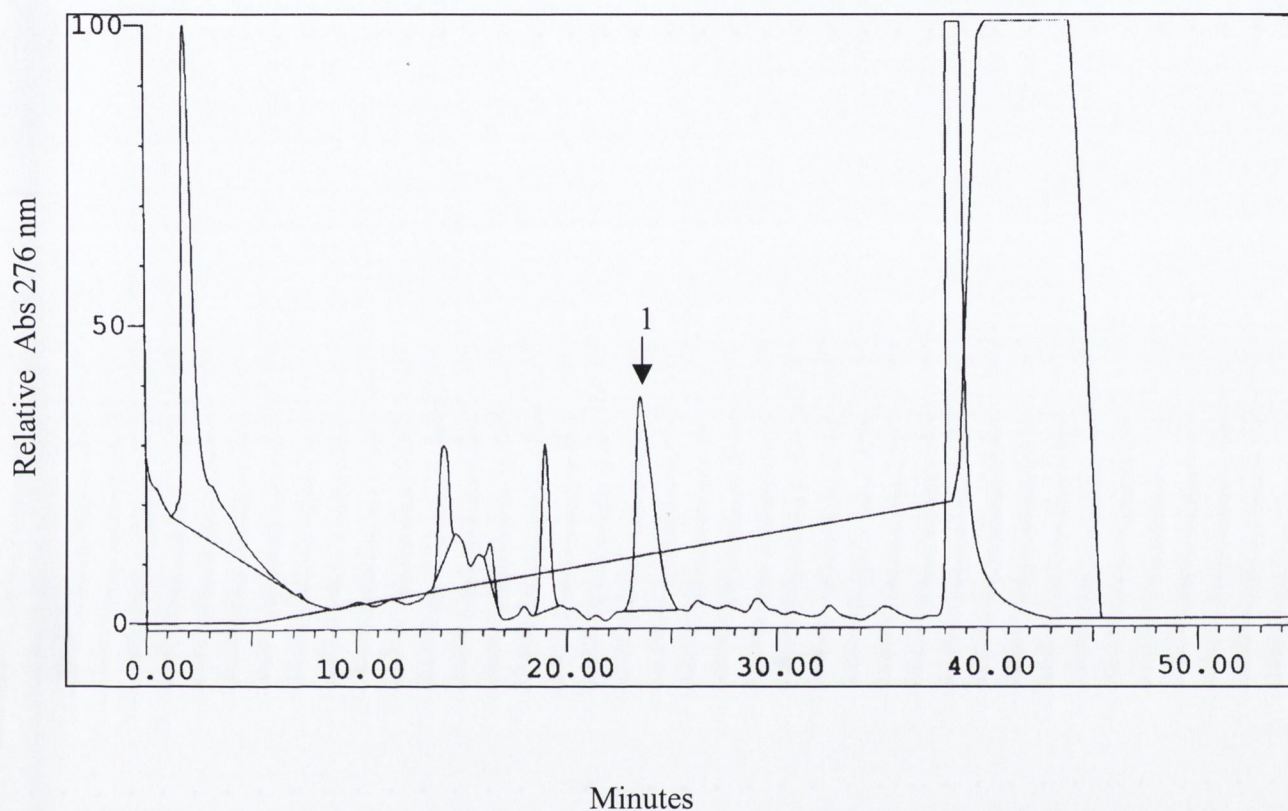


Figure 4.2.15 Elution profile from MonoQ anion exchange column. The column was equilibrated and run with 0.025 M Tris HCl. IL-1F5, obtained from size exclusion chromatography purification, was loaded on the column and eluted with increasing concentrations of NaCl. Numbered peak corresponds to IL-1F5 eluting after 23 minutes at a concentration of 0.1 M NaCl.

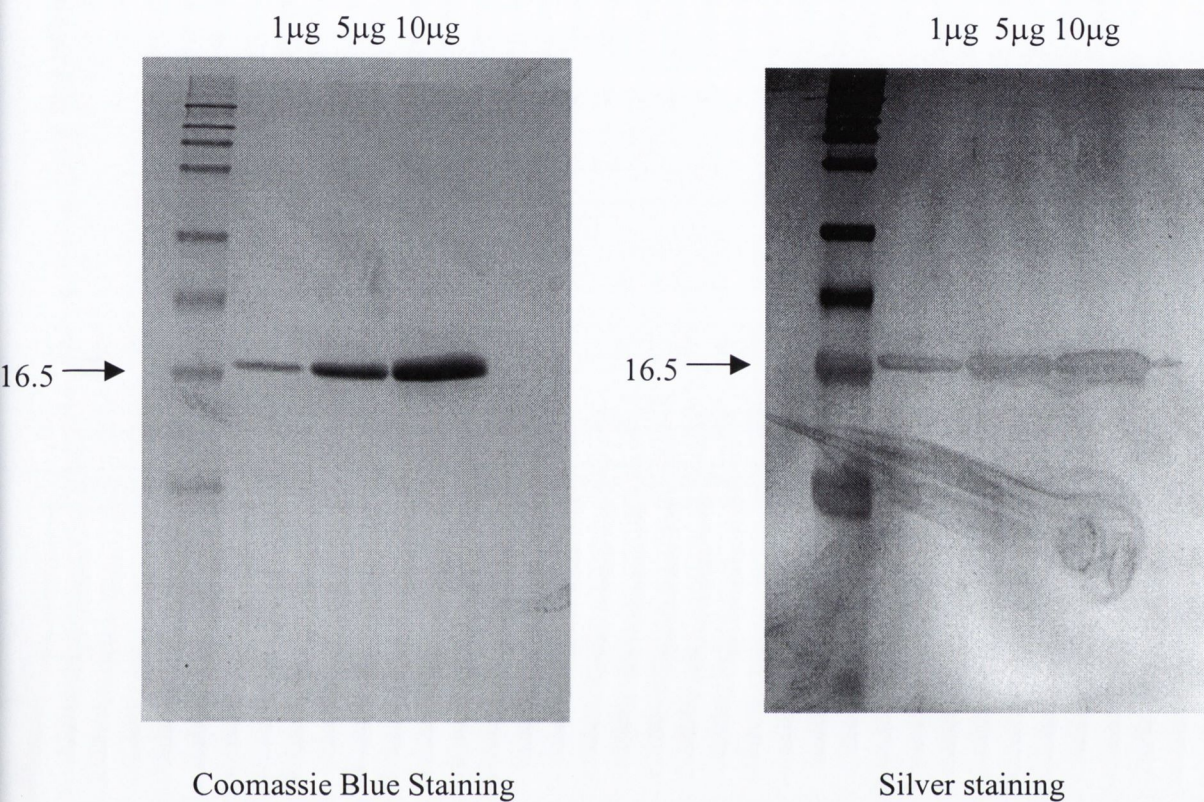


Figure 4.2.16 Analysis of purified IL-1F5 by Coomassie and silver staining. 1, 5 and 10 μg of purified IL-1F5 was run on a 20% SDS-polyacrylamide gel and stained with either Coomassie blue or silver nitrate.

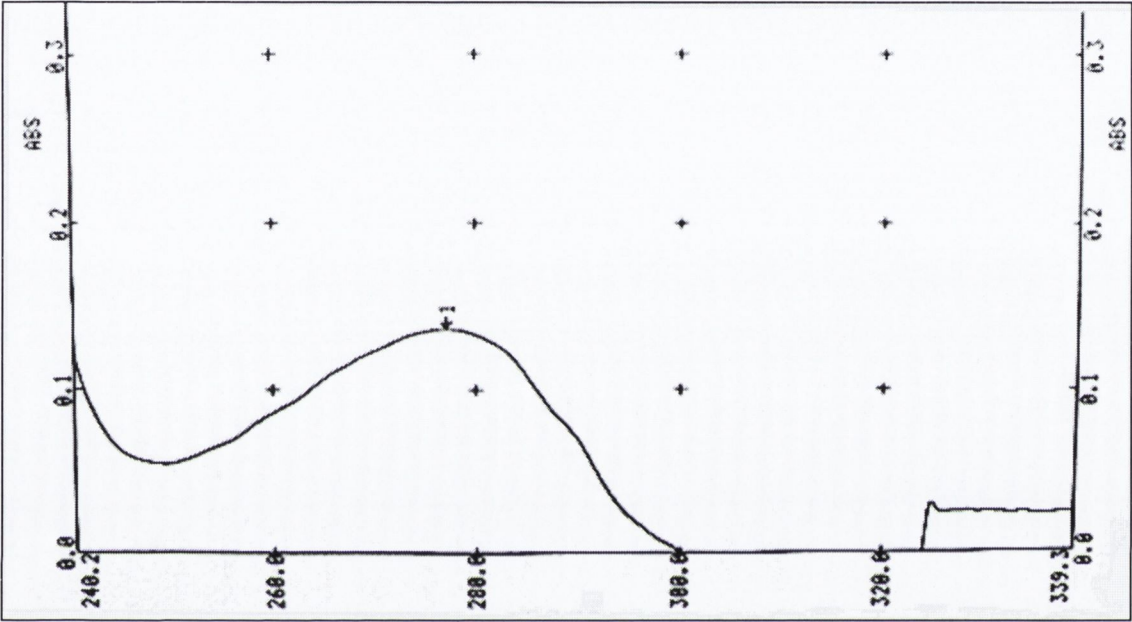


Figure 4.2.17 Absorption spectrum of IL-1F5. The absorption of purified IL-1F5 was measured between 240 nm and 340 nm. The instrument was zeroed on air before measurements were taken. The maximum absorption peak occurs at 277nm.

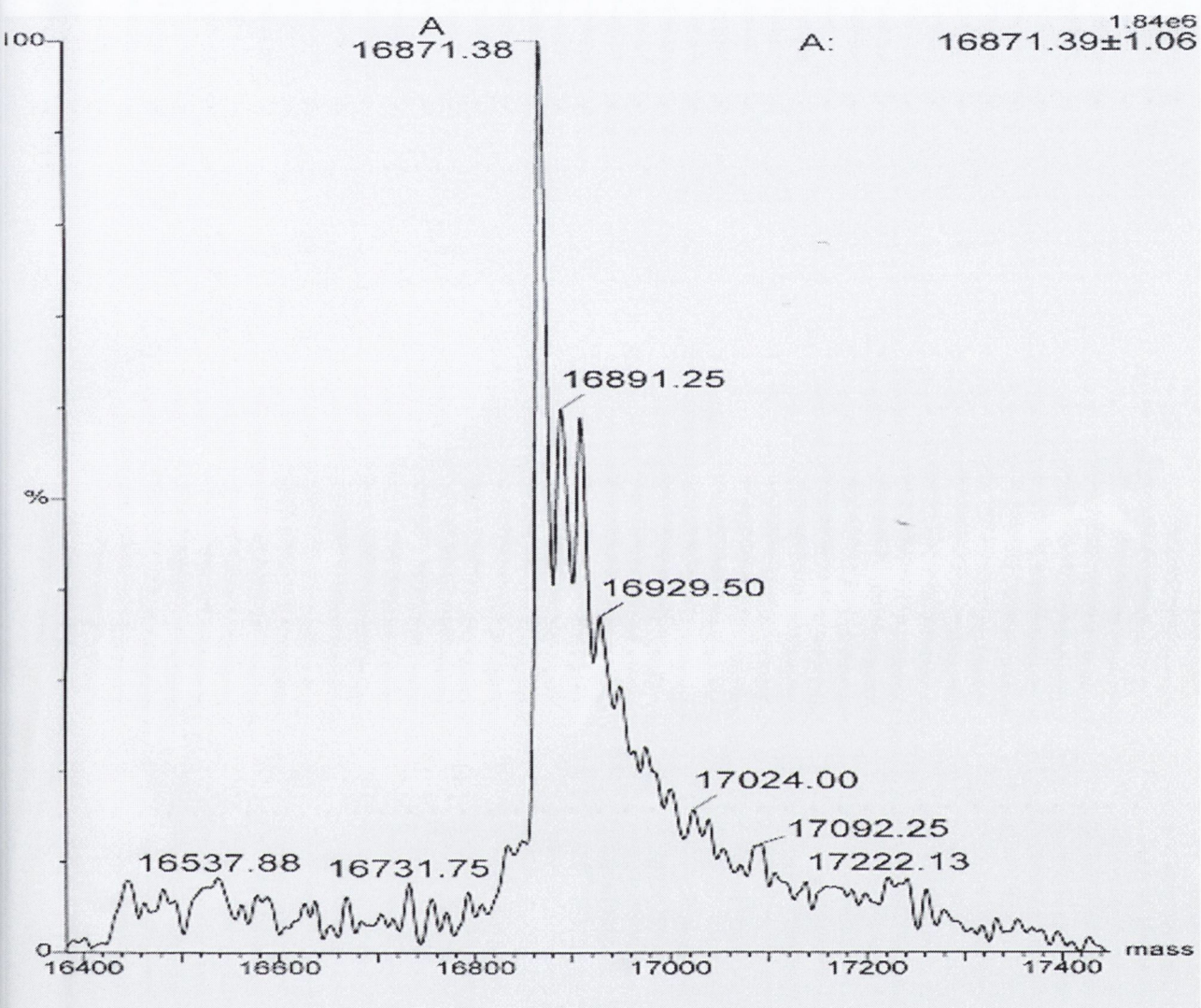


Figure 4.2.18 Mass spectroscopic analysis of purified of IL-1F5. The peak at 16871.38 daltons corresponds to the molecular weight of IL-1F5 in which the first methionine has been deleted, plus the existence of one disulphide bond, which reduces the molecular weight by 2 daltons. IL-1F5 was dialysed against 20% acetonitrile / 0.025% trifluoroacetic acid before analysis.

active. The UV/visible absorption spectrum of the sample was taken before measurement in the CD to ensure that absorbance did not exceed a total absorbance of 1.0, which would otherwise also lead to the introduction of noise into the spectrum. Measurements were taken at a scan speed of 20 nm/min, with a bandwidth of 1 nm, according to the equation:

$$\tau \times s < b/2$$

where τ = response time

s = scan speed

b= bandwidth

Scan speed was set at a value such that the response time would be as large as possible and therefore produce a better signal to noise ratio. A total of 6 cumulative scans were taken in each case. The same procedure was used to measure the CD spectrum of IL-1Ra, as well as a boiled sample of IL-1F5 and IL-1Ra. The spectrum of the buffer solution, in this case water was also measured and subtracted from the protein spectrum to remove any contribution to the spectrum of the buffer. Figure 4.2.19 shows that both IL-1F5 and IL-1Ra have negative ellipticity at around 200 nm which is indicative of β -strand structures and is similar to reported CD spectra for both IL-1Ra (Chang et al., 1996) and IL-1 β (Meyers et al., 1987). Furthermore, when the proteins were boiled for 10 minutes at 100°C, the negative ellipticity was abolished from the protein indicating an unfolded state has a very different CD spectrum to that of the native state (Figure 4.2.19).

4.2.4.2 Fluorescent Analysis

The effect of varying conditions of pH, salt concentration and the denaturing agent guanidine HCl on the structure and stability of IL-1F5 was also examined using fluorescence emission spectroscopy. IL-1F5 has two tryptophans, which absorb well at 280nm and emit strongly at 340-350 nm. This is shown in figure 4.2.20 where different excitation wavelengths were used to excite a solution of 20 μ g/ml IL-1F5 and the

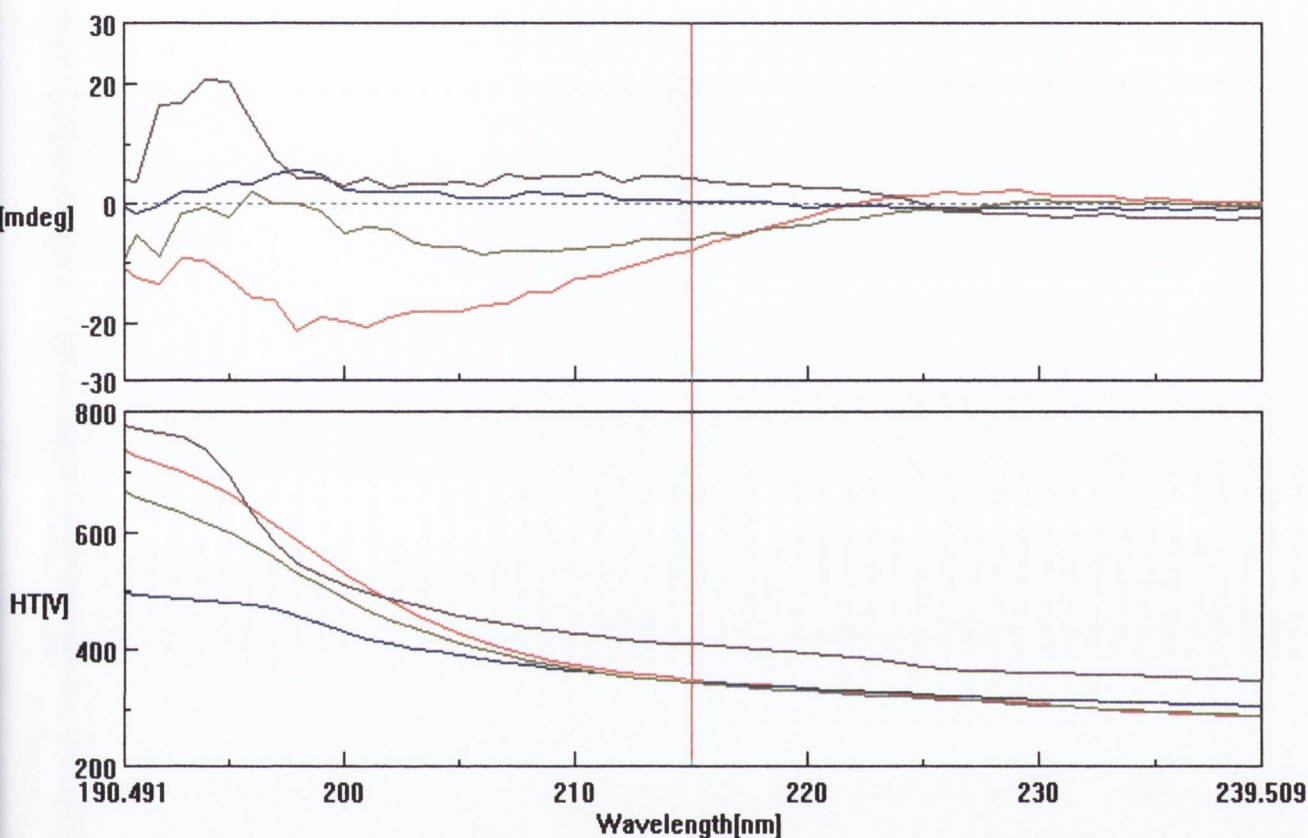


Figure 4.2.19 CD analysis of IL-1F5 and IL-1Ra. Spectra for the native (red) and denatured (blue) forms of IL-1F5 and also the native (green) and denatured (brown) forms of IL-1Ra are shown. Protein was dialysed into water overnight and diluted to 37.5 $\mu\text{g/ml}$. The CD spectrum taken from 190 nm to 240 nm, with a response time of 1 second, a scan speed of 20 nm/min and a bandwidth of 1 nm. Six cumulative scans were taken in each case and baseline measurements were subtracted from the spectra.

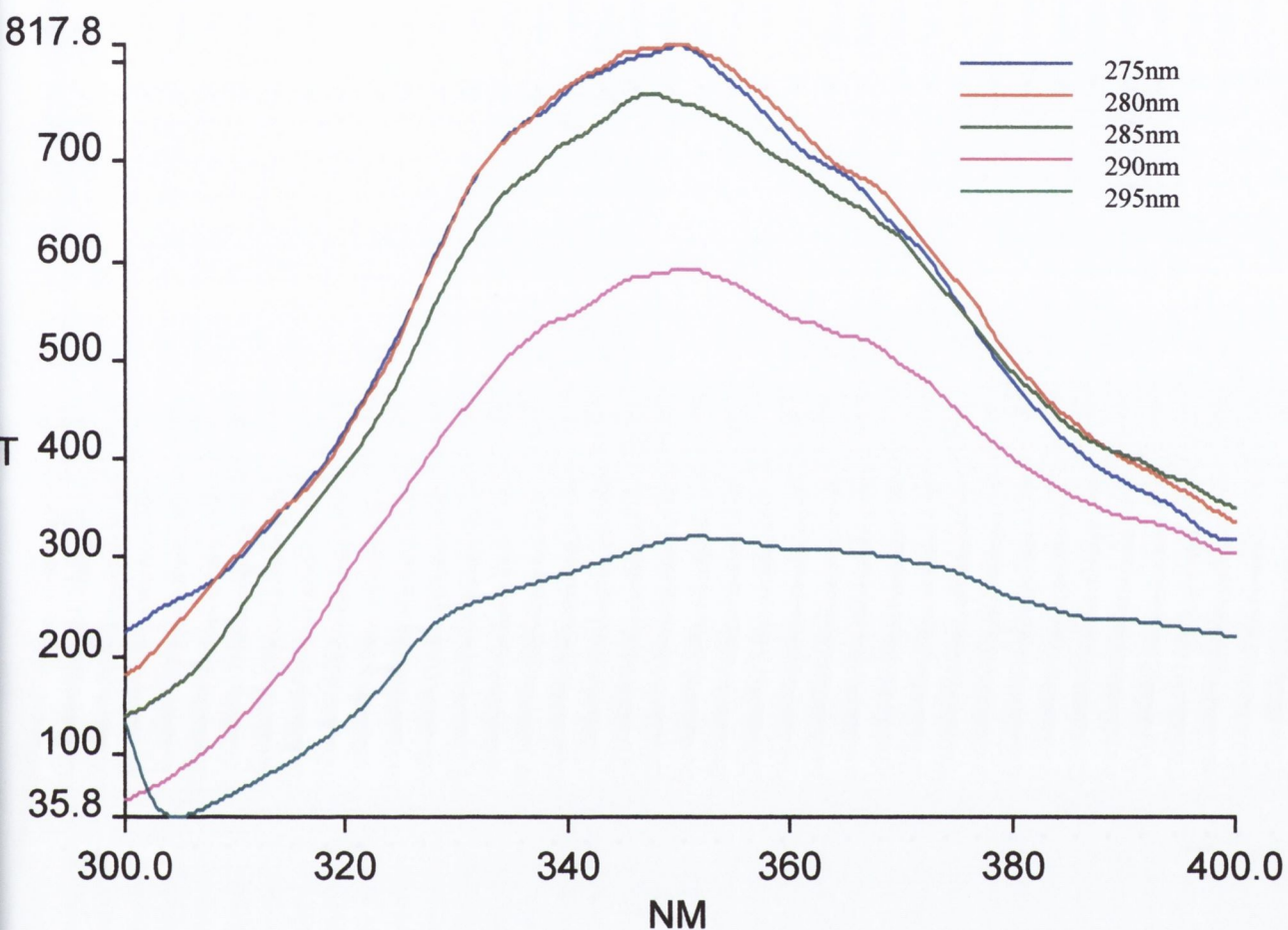


Figure 4.2.20 Fluorescence studies of IL-1F5. 20 $\mu\text{g/ml}$ IL-1F5 was excited at various wavelengths from 275 nm to 295 nm and the emission spectrum from 300 to 400 nm was measured. The slit wavelength was 5 nm and a scan speed of 240 nm/min was used.

emission spectrum from 300 nm to 400 nm was measured at a scan speed of 4 nm/sec and at a bandwidth of 5 nm. As can be seen, the emission is strongest when an excitation wavelength of 280 nm is used. This is due to the two tryptophans in the protein. IL-1F5 boiled for ten minutes was also measured for its emission spectrum, as can be seen from figure 4.2.21, the emission of the boiled sample is greatly reduced and is an indication that the protein is denatured. Samples of IL-1F5 were then buffer exchanged into different pH, salt and guanidine-HCl concentrations using PD-10 columns to give a final concentration of IL-1F5 of 20 µg/ml. The protein was then analysed in the fluorimeter using the parameters described above. Figure 4.2.22 shows the effects of pH on the stability of IL-1F5. The protein is stable over a range of pHs from pH 9 to pH 6. Below pH 6 the fluorescence decreases to pH 3.5 with a significant decrease between pH 4.5 and pH 4. Likewise increasing amounts of salt cause unfolding of the protein but this seems to occur in a more linear fashion (Figure 4.2.23). Unfolding seems to occur in a linear fashion with increasing amounts of guanidine-HCl and no transition state was obviously detectable in the unfolding process either when analysed immediately upon addition of guanidine-HCl or when analysed after incubation overnight at 22°C (Figure 4.2.24). This is compared to the unfolding of IL-1Ra in guanidine-HCl, which unfolds with a two-state mechanism with a transition midpoint of 1.8 M guanidine-HCl (Figure 4.2.25).

4.2.5 Antibody Production and Analysis

Recombinant IL-1F5 was injected into two healthy rabbits (A and B) via the popliteal lymph nodes. Each node was injected with 40 µg of protein. Two booster injections were given after 14 and 27 days. A test bleed was taken nine days after the second booster when IgG levels are maximal. Using the dot blot method (see section 2.5) the titre was found to be sufficient to detect as low as 5 ng of recombinant protein without cross reactivity to either BSA or Hen Egg White Lysozyme (Figure 4.2.26a and b). Based on this, and also considering the decreasing health state of the rabbits, it was decided to carry out exsanguinations. The sera of the two rabbits were isolated and further tested for cross reactivity to IL-1F5 that was denatured by boiling for 10 minutes either in PBS or SDS sample buffer (Figure 4.2.27). The antisera were able to detect both forms of IL-1F5 to a

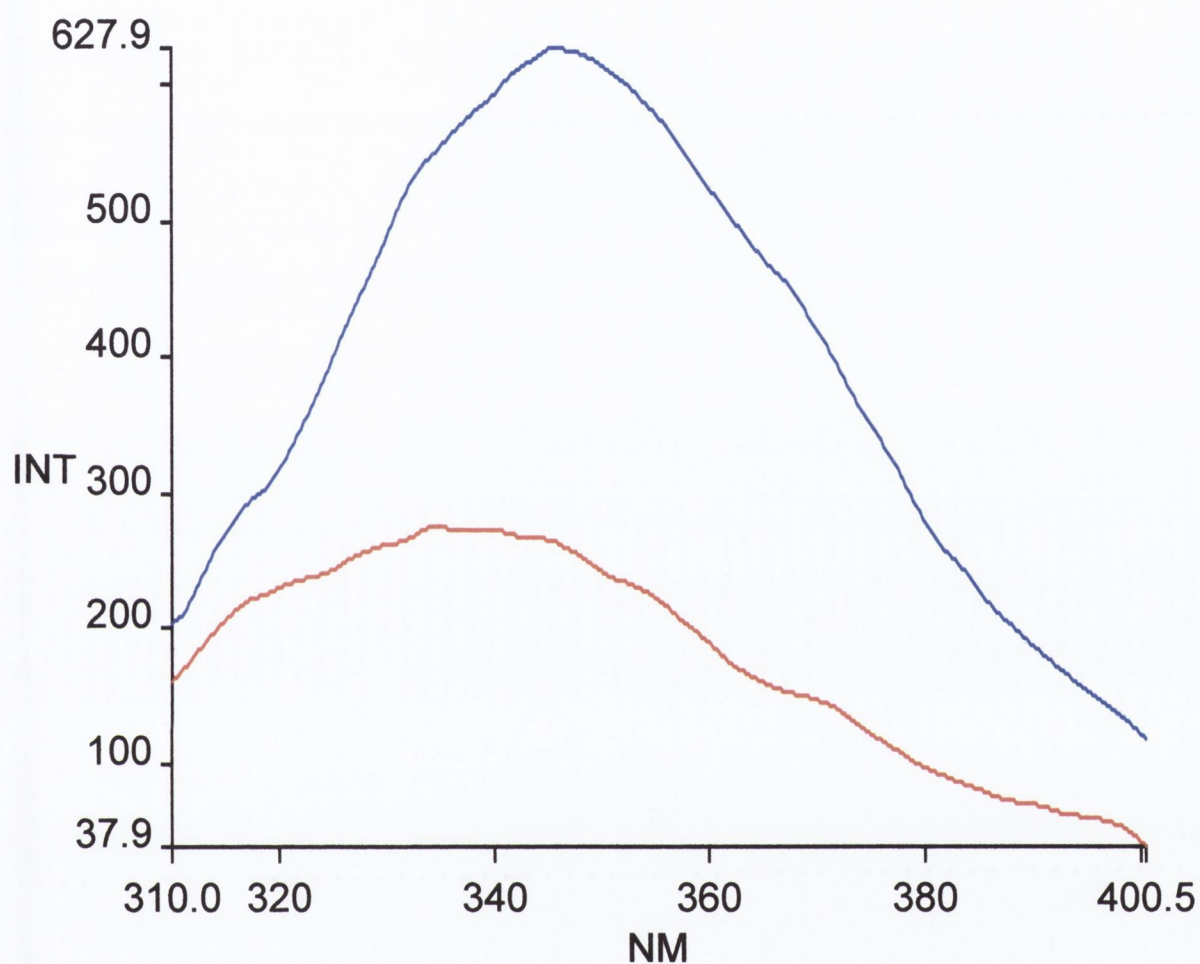
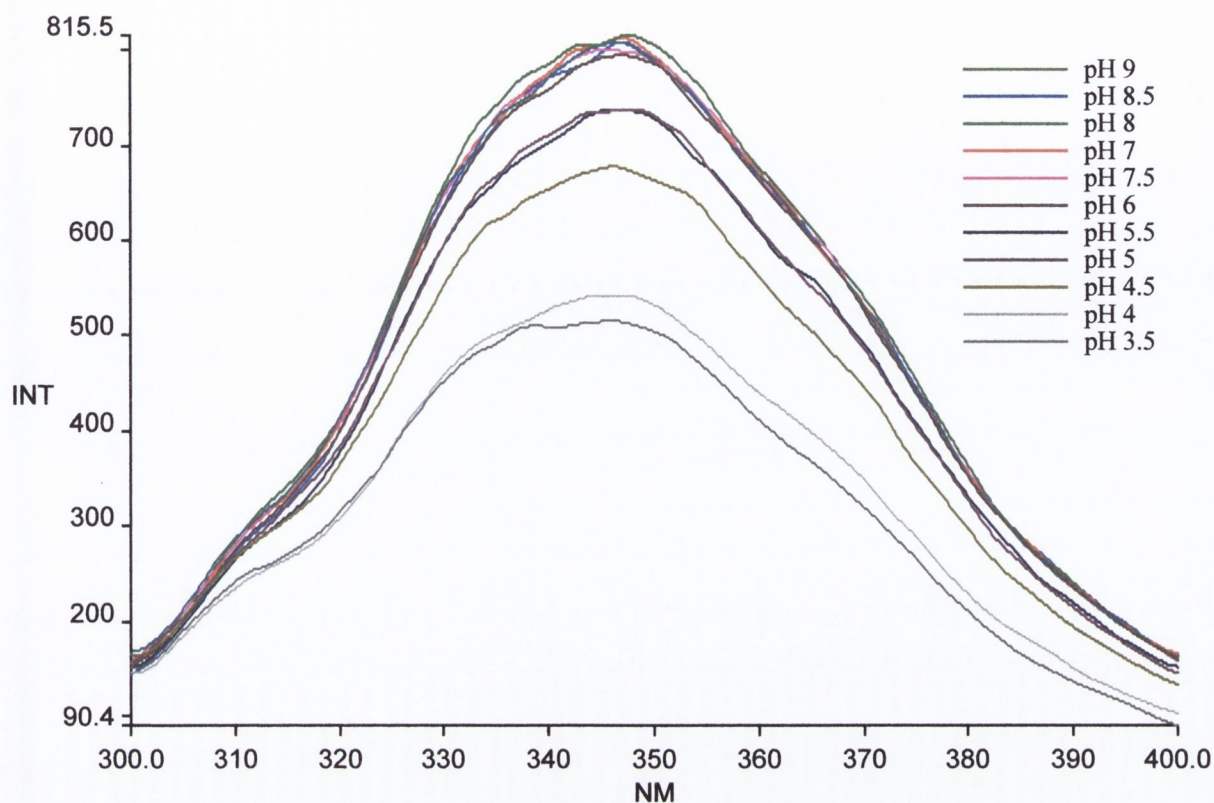


Figure 4.2.21 Effects of denaturation on fluorescence of IL-1F5. The emission spectrum of 20 $\mu\text{g/ml}$ IL-1F5 was measured from 300 nm to 400 nm using an excitation wavelength of 280 nm in its native state (blue) or after boiling at 100°C for 10 minutes (red). The slit wavelength was 5 nm and a scan speed of 240 nm/min was used.

A)



B)

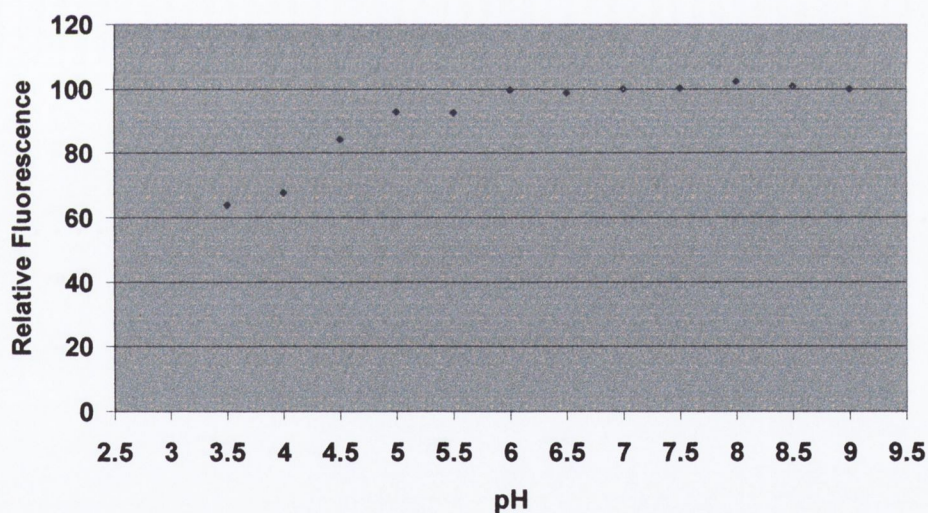
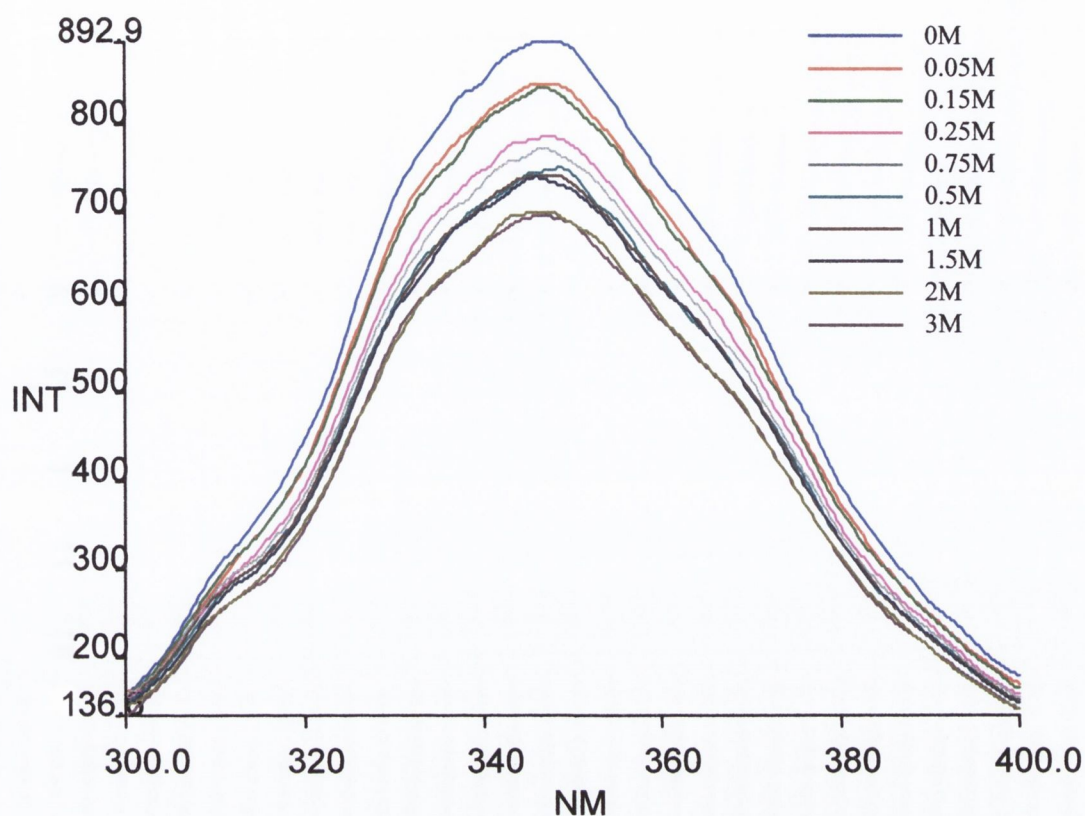


Figure 4.2.22 Effects of pH on fluorescence of IL-1F5. A) The emission spectrum of 20 $\mu\text{g/ml}$ IL-1F5 was measured from 300 nm to 400 nm using an excitation wavelength of 280 nm over a range of pH conditions from pH 3.5 to pH 9. The slit wavelength was 5 nm and a scan speed of 240 nm/min was used. B) Plot of the fluorescence of IL-1F5 in each pH condition relative to that of the fluorescence in pH 7 at an emission wavelength of 346 nm.

A)



B)

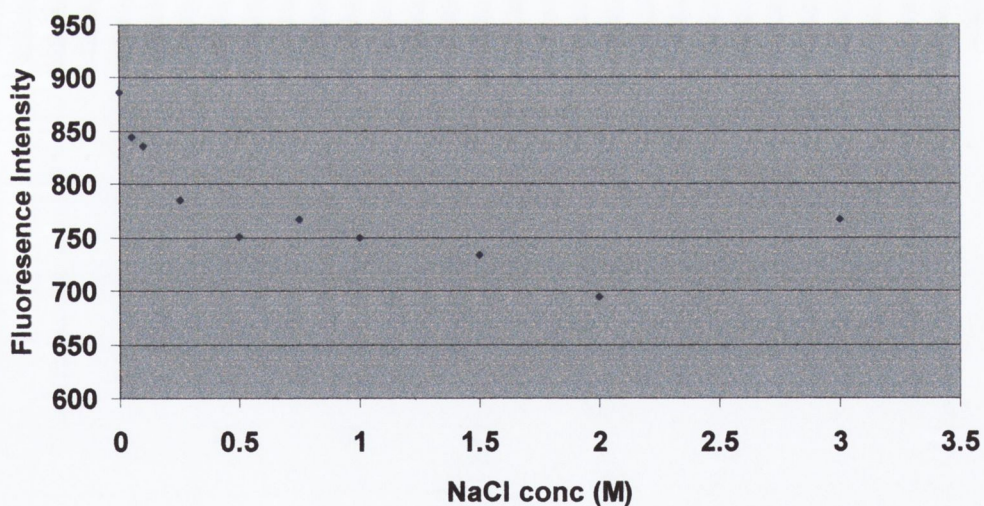


Figure 4.2.23 Effects of salt on fluorescence of IL-1F5. A) The emission spectrum of 20 $\mu\text{g/ml}$ IL-1F5 in 0.01 M Na phosphate buffer pH7.2 was measured from 300 nm to 400 nm using an excitation wavelength of 280 nm over a range of salt conditions from 0 M to 3 M NaCl. The slit wavelength was 5 nm and a scan speed of 240 nm/min was used. B) Plot of the fluorescence intensity of IL-1F5 in each salt concentration at an emission wavelength of 346 nm.

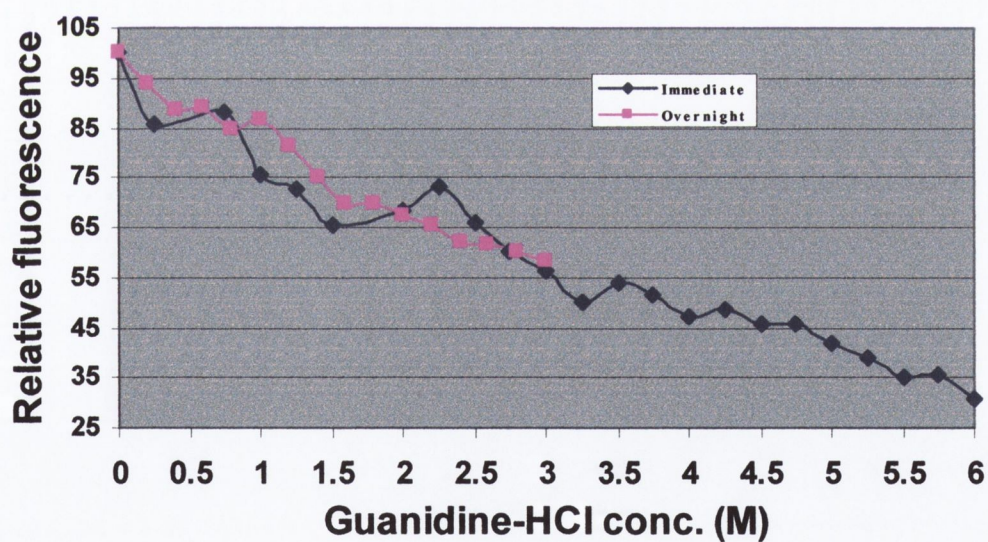
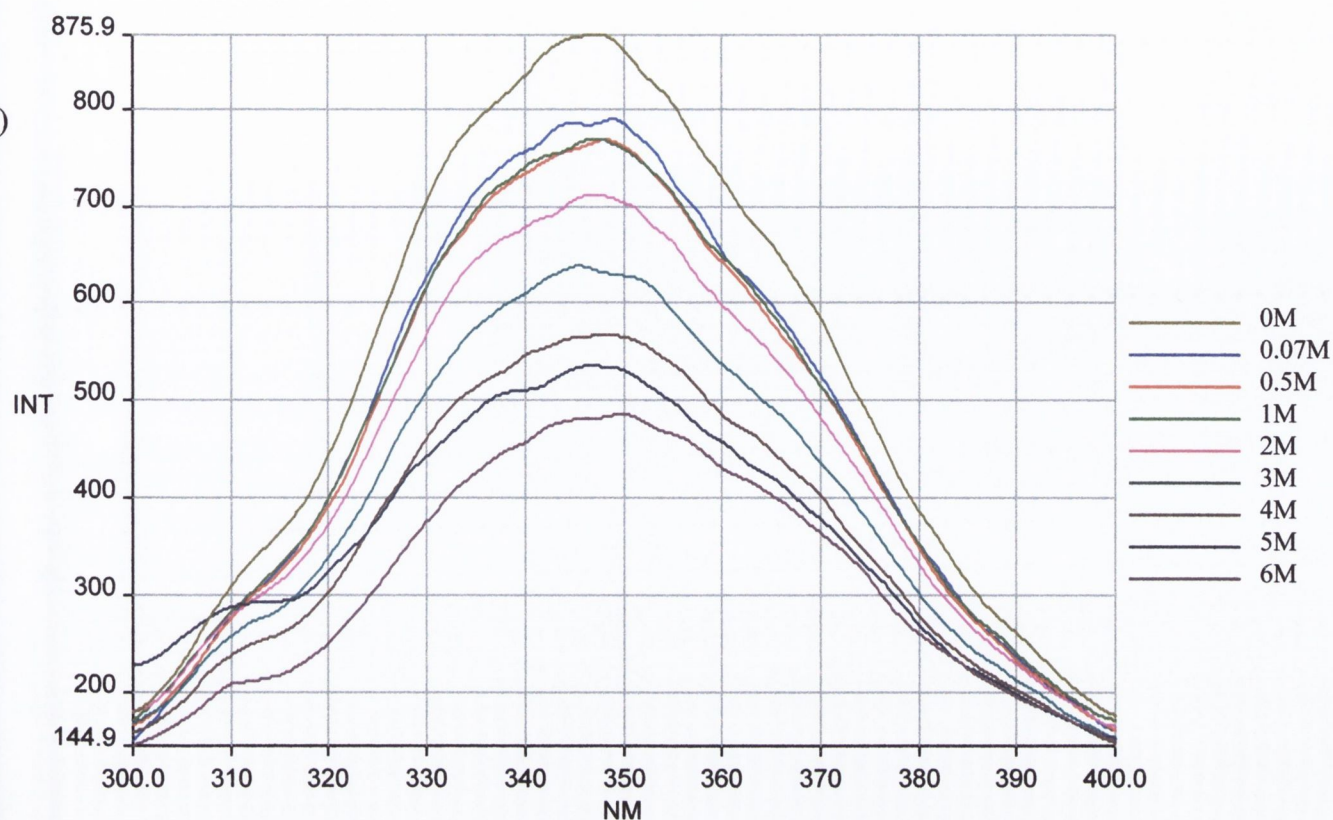


Figure 4.2.24 Effects of guanidinium-HCl on fluorescence of IL-1F5. A) The emission spectrum of 20 $\mu\text{g/ml}$ IL-1F5 was measured from 300 nm to 400 nm using an excitation wavelength of 280 nm over a range of guanidinium-HCl concentrations from 0 M to 6 M. The slit wavelength was 5 nm and a scan speed of 240 nm/min was used. B) Plot of the fluorescence of IL-1F5 in each guanidinium-HCl concentration relative to that of the fluorescence in 0 M guanidinium-HCl at an emission wavelength of 346 nm.

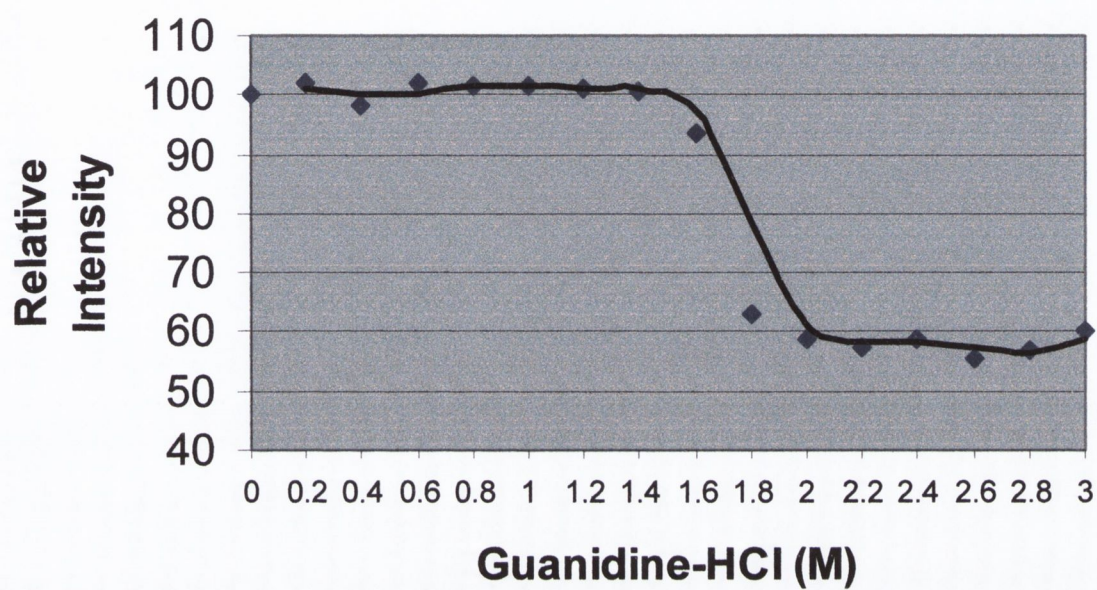
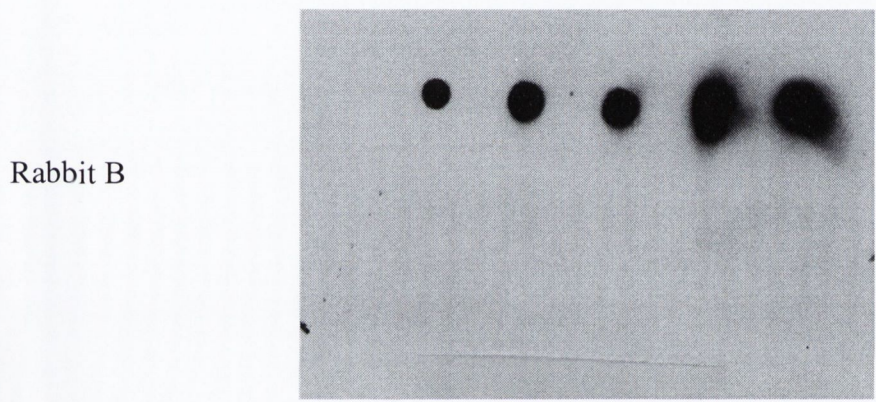
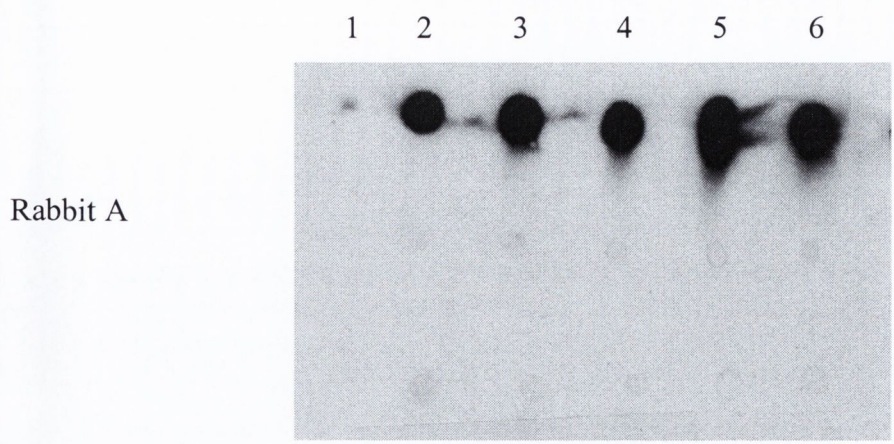
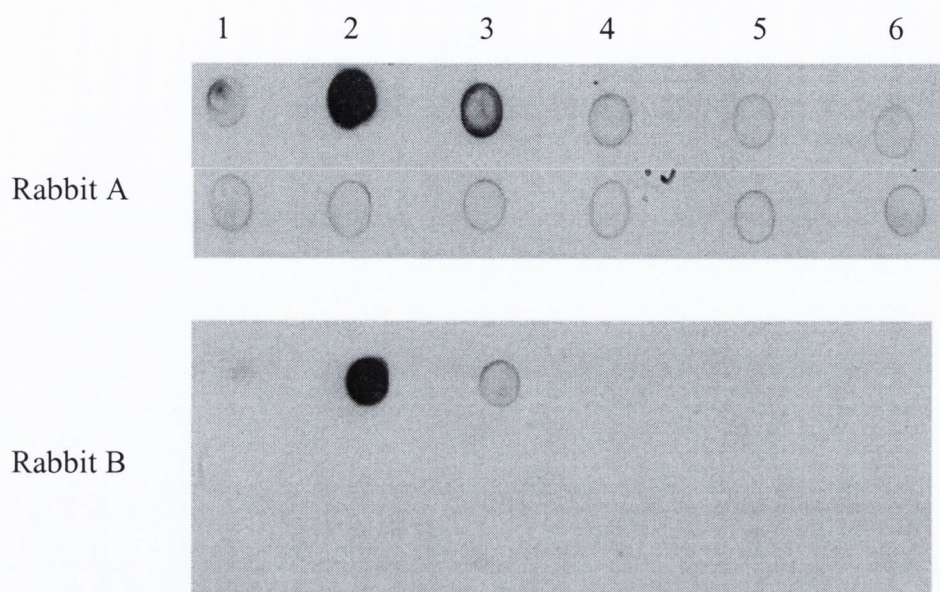


Figure 4.2.25 Effects of guanidinium-HCl on fluorescence of IL-1Ra. Plot of the fluorescence of IL-1Ra in varying guanidinium-HCl concentrations from 0 to 3 M relative to that of the fluorescence in 0 M guanidinium-HCl at an emission wavelength of 351 nm.



1	2	3	4	5	6
PBS	4 μ g IL-1F5	2 μ g IL-1F5	1 μ g IL-1F5	0.5 μ g IL-1F5	0.125 μ g IL-1F5
	4 μ g BSA	2 μ g BSA	1 μ g BSA	0.5 μ g BSA	0.125 μ g BSA
	4 μ g lysozyme	2 μ g lysozyme	1 μ g lysozyme	0.5 μ g lysozyme	0.125 μ g lysozyme

Figure 4.2.26a Dot-blot analysis of antisera raised against IL-1F5. Purified IL-1F5, BSA or egg white lysozyme was dotted on nitrocellulose in amounts indicated in table, from 125 ng to 4 μ g. The nitrocellulose was allowed to dry before washing with TBS-tween and was then blocked in 10% non-fat dried milk for 1 hour before probing with sera from rabbit A and B at concentrations of 1 in 2000 for 1 hour at room temperature. The nitrocellulose was then washed and probed with secondary HRP-linked anti-rabbit antibody for 1 hour at room temperature, washed and developed with ECL reagent.



1	2	3	4	5	6
PBS	25 ng IL-1F5	5 ng IL-1F5	1 ng IL-1F5	200 pg IL-1F5	40 pg IL-1F5
8pg IL-1F5	1.6 pg IL-1F5	0.32 pg IL-1F5	0.064 pg IL-1F5	25ng BSA	25ng Lys

Figure 4.2.26b. Dot-blot analysis of antisera raised against IL-1F5 detecting IL-1F5 in the nanogram range. Purified IL-1F5, BSA or egg white lysozyme was dotted on nitrocellulose in amounts indicated in table, from 0.064 ng to 25 ng. The nitrocellulose was allowed to dry before washing with TBS-tween and was then blocked in 10% non-fat dried milk for 1 hour before probing with sera from rabbit A and B at concentrations of 1 in 2000 for 1 hour at room temperature. The nitrocellulose was then washed and probed with secondary HRP-linked anti-rabbit antibody for 1 hour at room temperature, washed and developed with ECL reagent.

sensitivity of 5 ng with the signal from the membrane blotted with serum from rabbit B giving a stronger signal. Cross reactivity to IL-1Ra was also tested (Figure 4.2.28). This indicated that the antiserum from rabbit B cross reacted with IL-1Ra in the μg range when either in its native state or when boiled with SDS sample buffer. This indicates that although the antiserum from rabbit B is more sensitive it is also cross reactive. This may indicate that the antiserum contains a larger population of anti-IL-1F5 antibodies some of which recognise epitopes also present or similar to ones found in IL-1Ra. Furthermore, the antibody was also used to detect recombinant IL-1F5 run on SDS-PAGE (Figure 4.2.29). This led to the detection of an upper band equivalent to approximately 35 kDa. This might represent a dimer of the IL-1F5. To investigate this a native gel was run of the recombinant IL-1F5 (Figure 4.2.30). This shows a laddering effect of the IL-1F5, which may be indicative of either differently charged species or multimers within the preparation.

4.3 Discussion.

Murine IL-1F5 was successfully sub-cloned into a bacterial expression vector and a purification protocol designed and carried out to purify the protein to >90% homogeneity without the need to add any foreign tags to the protein.

DNA sequencing confirmed that the correct gene was encoded in the expression vector. The differences in the DNA sequence could be the result of natural sense polymorphisms in the gene between that cloned by Barton et al. (2000) and the one used in these procedures. The differences do not lead to any variation in sequence at the amino acid level.

The pET system of expression was chosen because it has been shown to offer high yields of expression under a very tightly controlled promoter system. Therefore any potential toxicity from the introduced foreign gene is minimised. This control is achieved as a result of the use of two inducible promoters. The target gene is under the control of the bacteriophage T7 promoter which is not recognised by any bacterial RNA polymerases

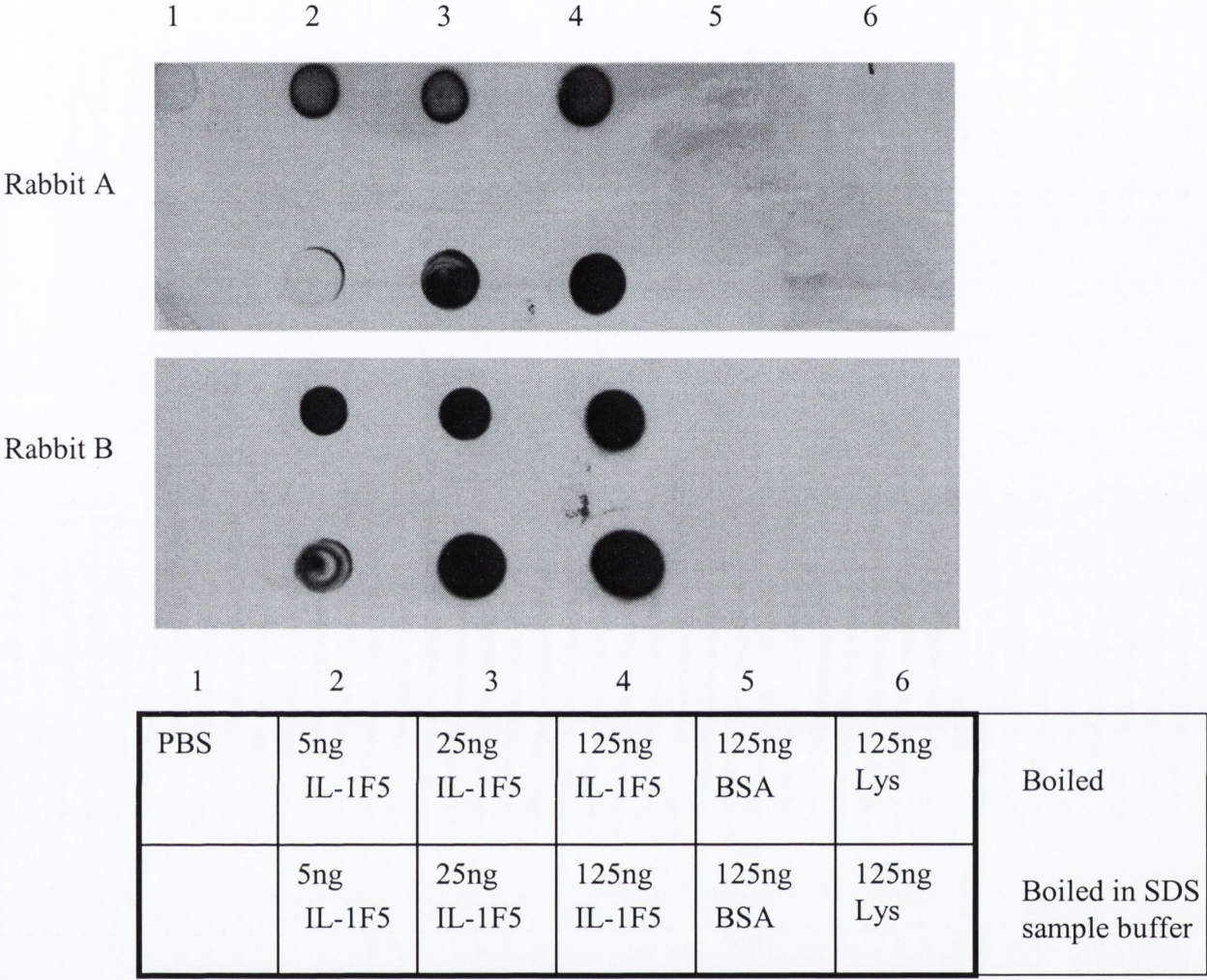
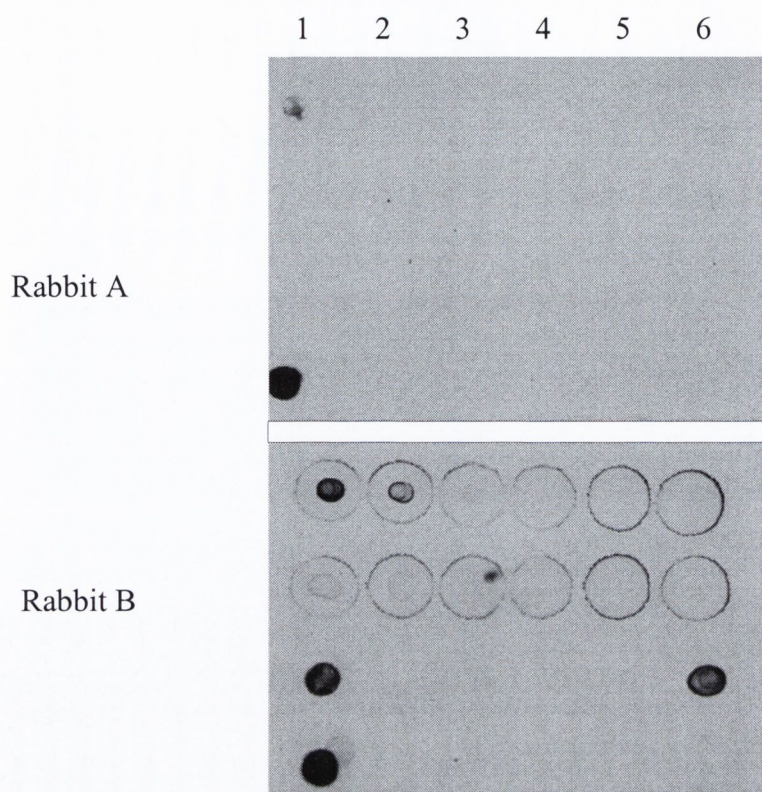


Figure 4.2.27 Dot-blot analysis of antisera raised against IL-1F5 detecting IL-1F5 in the nanogram range using boiled and SDS treated protein. Purified IL-1F5, BSA or egg white lysozyme was boiled for 4 minutes in the presence of buffer alone or 1X SDS sample and then dotted on nitrocellulose in amounts indicated in table. The nitrocellulose was allowed to dry before washing with TBS-tween and was then blocked in 10% non-fat dried milk for 1 hour before probing with sera from rabbit A and B at concentrations of 1 in 2000 for 1 hour at room temperature. The nitrocellulose was then washed and probed with secondary HRP-linked anti-rabbit antibody for 1 hour at room temperature, washed and developed with ECL reagent.



1	2	3	4	5	6	
1000ng IL-1Ra	500ng IL-1Ra	125ng IL-1Ra	62.5ng IL-1Ra	25ng IL-1Ra	5ng IL-1Ra	Native
1000ng IL-1Ra	500ng IL-1Ra	125ng IL-1Ra	62.5ng IL-1Ra	25ng IL-1Ra	5ng IL-1Ra	Boiled
1000ng IL-1Ra	500ng IL-1Ra	125ng IL-1Ra	62.5ng IL-1Ra	25ng IL-1Ra	5ng IL-1Ra	Boiled in SDS sample buffer
25ng IL-1F5	PBS					

Figure 4.2.28 Dot-blot analysis of antisera raised against IL-1F5 testing for cross reactivity with IL-1Ra. Purified IL-1Ra or IL-1F5 was boiled for 4 minutes in the presence of buffer alone or 1X SDS sample and then dotted on nitrocellulose in amounts indicated in table. The nitrocellulose was allowed to dry before washing with TBS-tween and was then blocked in 10% non-fat dried milk for 1 hour before probing with sera from rabbit A and B at concentrations of 1 in 2000 for 1 hour at room temperature. The nitrocellulose was then washed and probed with secondary HRP-linked anti-rabbit antibody for 1 hour at room temperature, washed and developed with ECL reagent.

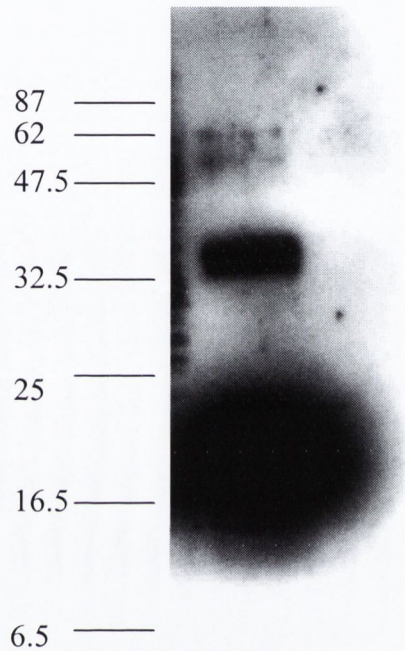


Figure 4.2.29 Western blot analysis of purified recombinant IL-1F5. 25 ng of IL-1F5 was run on a 15% SDS-polyacrylamide gel and transferred to nitrocellulose. The nitrocellulose was blocked in 10% non-fat dried milk for 1 hour before probing with sera from rabbit A at a concentration of 1 in 2000 for 1 hour at room temperature. The nitrocellulose was then washed and probed with secondary HRP-linked anti-rabbit antibody for 1 hour at room temperature, washed and developed with ECL reagent and left to expose to film for 10 minutes.



Figure 4.2.30 Native Gel Electrophoresis of IL-1F5. 500 ng of protein was run on a 15% polyacrylamide gel without the presence of SDS and stained with Coomassie blue, lane 1; IL-1F5, lane 2; BSA.

and there is therefore virtually no transcription from the gene in the absence of T7 polymerase. The T7 polymerase is induced with IPTG which is a stable lactose homolog which relieves the inhibition by the *lac* repressor. Furthermore, the starting culture was grown in minimal M9 medium in the presence of 0.2% glucose which ensures that no leakiness of expression from the *lac* and T7*lac* promoters occurred. Induction was carried out in BL21(DE3) *E. coli* cells which not only have the necessary T7 polymerase gene but are also deficient in both *lon* and *omp T* proteases which help minimise protein degradation during lysis and purification. Various expression conditions were tested to investigate if increased yields could be achieved. These included the use of IPTG induction from the beginning of the culture rather than allowing the culture to first reach log phase and also the use of different host strains including pLysS BL21(DE3), which have a vector encoding for T7 lysozyme which inhibits T7 polymerase function in uninduced cells, and Codon Plus cells which express tRNAs which are rare in bacterial cells and allow for efficient use of mammalian codons. None of these conditions increased the level of induced expression detected.

The purification of IL-1F5 was achieved with a three step process including ammonium sulphate precipitation, size exclusion chromatography and anion exchange chromatography. The precipitation yielded very concentrated protein ideal for use on the size exclusion chromatography which, due to its large size (84 cm long), achieved excellent purification of the small protein. This was further aided by the fact the whole cell lysates indicated that the bacterial whole cell lysate was particularly deficient in proteins around the mass of 20 kDa (*e.g.* see figure 4.2.6). Anion exchange was used as a last step in the purification to clean up the yield from the size exclusion. Using the high capacity, high resolution, strong anion exchanger MonoQ column ($-\text{CH}_2-\text{N}^+(\text{CH}_3)_3$) good resolution of the purified IL-1F5 protein could be achieved with 100 mM NaCl as the eluent. Using this procedure a yield of up to 4 mg/L culture was achieved.

Mass spectrometric analysis of the recombinant protein product was carried out which confirmed the identity of the protein. A mass of 16871.38 Da was determined which is consistent with the mass of IL-1F5 with the first methionine removed. This is a process

that occurs commonly in recombinantly produced proteins. The mass is also indicative of the presence of a disulphide bond. In addition to this, N-terminal sequencing of the protein also indicated that the product was indeed IL-1F5.

The purified IL-1F5 was then used to generate anti-sera in rabbits to the protein. The anti-sera generated were capable of detecting as little as 5 ng of purified protein in native, boiled and SDS-denatured forms. It did not cross react with either BSA or lysozyme. While the anti-serum from rabbit A did not cross react with IL-1Ra, that from rabbit B did in the μg range. This antiserum was also more sensitive than that from rabbit A suggesting it has a larger repertoire of antibodies, some of which recognise epitopes also present in IL-1Ra. This is not surprising given the level of sequence identity between the two proteins. The anti-serum from rabbit A was further used to detect IL-1F5 that had been run on a polyacrylamide gel. This indicated the possible existence of a dimer of IL-1F5 and possibly even higher order aggregates. IL-1F7b is the only member of the IL-1 family that is implicated in forming homodimers (Kumar et al., 2002), while IL-1Ra has been reported to form homodimers as a degradative, but still active product (Chang et al., 1996). Although the result suggests that IL-1F5 can form dimers, this may be a degradative product as for IL-1Ra since the elution profile of IL-1F5 on the gel filtration column is very similar to that of undegraded IL-1Ra and the structure of IL-1F5 suggests also that the protein is a monomer (see Chapter 6).

Circular dichroism (CD) was used to examine secondary structure composition of the purified protein. CD is a measure of the difference in the absorbance between left and right circularly polarised light, which occurs in any sample that has chiral centres. It is often used as a crude but effective way of determining the folding elements of biopolymers such as protein and DNA. Studies with IL-1F5 show that, like IL-1Ra, the protein has a high negative ellipticity at about 200 nm, which is indicative of high β -strand content of the protein. Also the lack of positive signal at 195 nm indicates the lack of a strong α -helical content of the protein. Boiling of the protein for ten minutes caused a disappearance of the strong negative signal at 200 nm suggesting that denaturation causes loss of the β -strand structure.

Fluorescent studies of the protein show that IL-1F5 is quite stable and there is little change in fluorescence over the pH range from 9 to 6. Salt also did not have a strong effect on the fluorescence read out of the protein, up to 3 M salt the protein still gave a strong fluorescent read out when compared to that of boiled denatured protein. This suggested that the increasing salt concentration does not greatly effect the environment of the fluorescing tryptophan residues. Furthermore, denaturation of the protein using guanidine-HCl shows that fluorescence decreases continually up to a concentration of 6 M guanidine-HCl with no plateau reached either at low or high concentrations of guanidine. The same curve is obtained whether the fluorescence was measured immediately upon addition of protein to the guanidine-HCl or when the protein-guanidine mixture was allowed to come to complete equilibrium by incubation at 22°C overnight. The denaturation curve obtained suggests that the pathway by which IL-1F5 unfolds is by a complex multi-state process (Saito and Wada, 1983). The sharpest decrease in fluorescence occurs at about 1.4 M guanidine-HCl, which perhaps indicates a transition region to a different folding state at this concentration. This value would correlate with a transition midpoint for IL-1Ra and IL-1β. Denaturing curves for IL-1Ra using the very same guanidine-HCl preparations show that IL-1Ra unfolds by a two-state mechanism, similar to IL-1β, with a transition mid point of about 1.8 M guanidine-HCl (Figure 4.2.25), while IL-1β that has a report transition midpoint of 1.35 M and 1.26 M guanidine-HCl (Chrnyk and Wetzel, 1993; Craig et al., 1987).

Chapter 5. Functional Analysis of IL-1F5 and IL-1F6.

5.1 Introduction.

IL-1 is a well-known mediator of inflammatory and immune responses. It regulates these responses through the activation of signal transduction pathways which in turn lead to the activation of the transcription factor NF κ B, as well as those transcription factors regulated by the MAP kinases (O'Neill, 2000). IL-18, the other well-characterised member of the family also activates similar, if not identical signal transduction pathways, which also lead to regulation of immune responses (Dinarello et al., 1998). Therefore it seems likely that the novel members of the IL-1 family will also activate signal transduction pathways which would lead to activation of NF κ B and the MAP kinases, and that they will be involved in inflammatory or immune responses also. With the availability of protein encoding both IL-1F5 and IL-1F6, these hypotheses were tested. Indeed, IL-1F5 mRNA expression is increased in lesions of psoriatic skin, suggesting it may be involved in this auto-immune disease (Debets et al., 2001). Also, given their similarities to IL-1Ra at the amino acid level (IL-1F5 52% identical and IL-1F6 30% identical) it seemed a possibility that one or both of these proteins might act as novel receptor antagonists, however as discussed in Chapter 3 it is also a strong possibility that they could also act as novel agonists. Therefore, the two proteins were tested in functional assays to examine their ability to mimic or antagonise IL-1 signalling pathways.

5.2 Results.

5.2.1 Detection of IL-1F5 and IL-1F6.

The first part of this study involved the analysis of the IL-1Ra-like activities of IL-1F5 and IL-1F6. IL-1F5 and IL-1F6 expressed as FLAG tagged proteins in HEK293 cell expression systems can be purified from the cellular supernatant using anti-FLAG

antibodies. These proteins, obtained from Millennium Pharmaceuticals (MA, USA), appeared as a single band on Coomassie stained polyacrylamide gels. Figure 5.2.1a shows that detection of the proteins in this manner indicated that they have a molecular weights of 20 kDa for IL-1F5 and 19.5 kDa for IL-1F6. The proteins were also detected by Western Blot techniques using anti-FLAG antibodies. As shown in figures 5.2.1b the proteins again displayed a molecular weight of 20 kDa and 19.5 kDa for IL-1F5 and IL-1F6 respectively. These proteins have a predicted molecular weight of 17 kDa and 18 kDa in the untagged state. The discrepancy between the predicted and observed molecular weight can be explained for IL-1F6 due the presence of the FLAG tag which has a molecular weight of 1 kDa, however the same consideration still leaves IL-1F5 with 2 kDa unaccounted for and this may be due to glycosylation of the protein. Indeed, although IL-1F5 and IL-1F6 do not have the Asn-X-Ser/Thr motif for N-linked glycosylation, IL-1F5 does have a strongly predicted O- linked GlnAc (N-acetylgalactosamine) site at Thr134, while IL-1F6 does not (Figure 5.2.2a and 5.2.2b) (Hansen et al., 1995). Later experiments in neuronal cells lines used IL-1F5 purified as described in Chapter 4.

5.2.2 Characterisation of IL-1 Induced Nuclear Translocation and DNA Binding Activity of NF κ B in Adherent and Suspension Cell Lines using EMSA.

The first system in which IL-1F5 and IL-F6 were studied was the effect on IL-1 induced activation of NF κ B as detected using the electrophoretic mobility shift assay (EMSA). Figure 5.2.3 shows that using this assay IL-1 induced a dose responsive activation of NF κ B in HeLa cells. In this, and all following experiments the term IL-1 refers to IL-1 α , which was used as the activating protein. Activation was detected with a dose as low as 1 ng/ml. Similar results were obtained using the suspension cell line EL4-NOB.1 (Figure 5.2.4). Figure 5.2.5 shows that using a dose of 10 ng/ml, NF κ B activation in HeLa cells was detected as soon as 15 minutes after treatment of cells with IL-1. This activation was transitory and decreased 1 hour after treatment. However, using time matched controls activation was again detected after a 24 hour treatment with IL-1. This biphasic activation

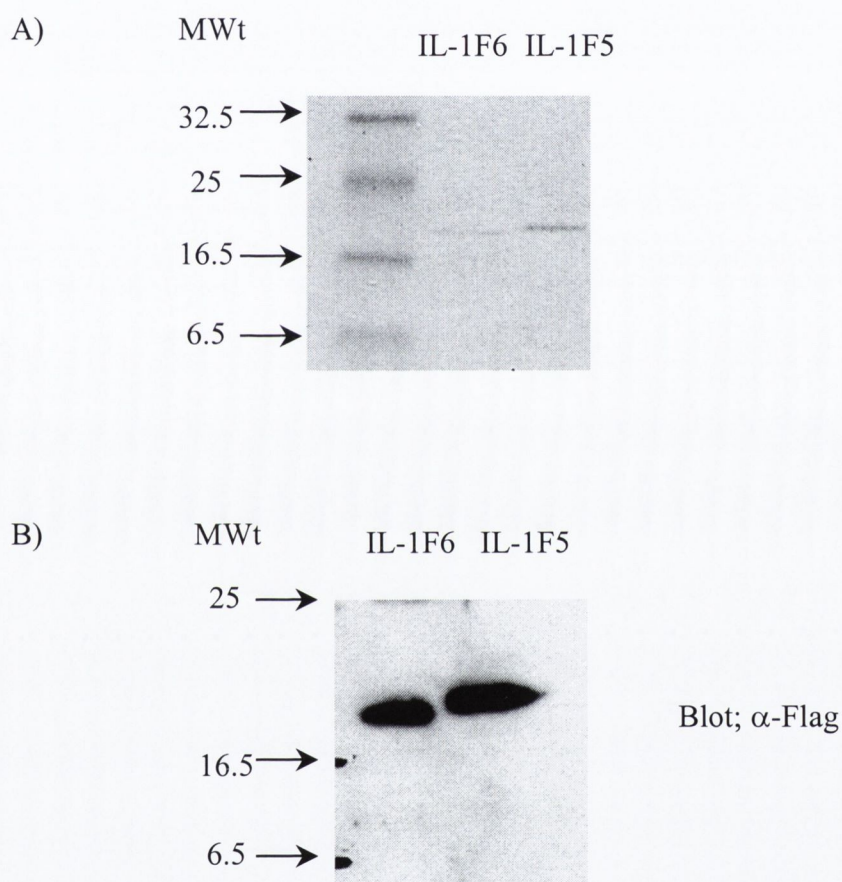


Figure 5.2.1 Coomassie and western blot analysis of the IL-1F5 and IL-1F6.

A) Coomassie stain of a 10% SDS polyacrylamide gel loaded with 500 ng IL-1F6 and 500 ng IL-1F5 (as calculated from information supplied by Millennium Pharmaceuticals) B) Western blot analysis of IL-1F5 and IL-1F6. 100 ng of each protein was resolved on a 10% SDS polyacrylamide gel, transferred to a PVDF membrane and detected with anti-FLAG antibody. Molecular weight markers are shown.

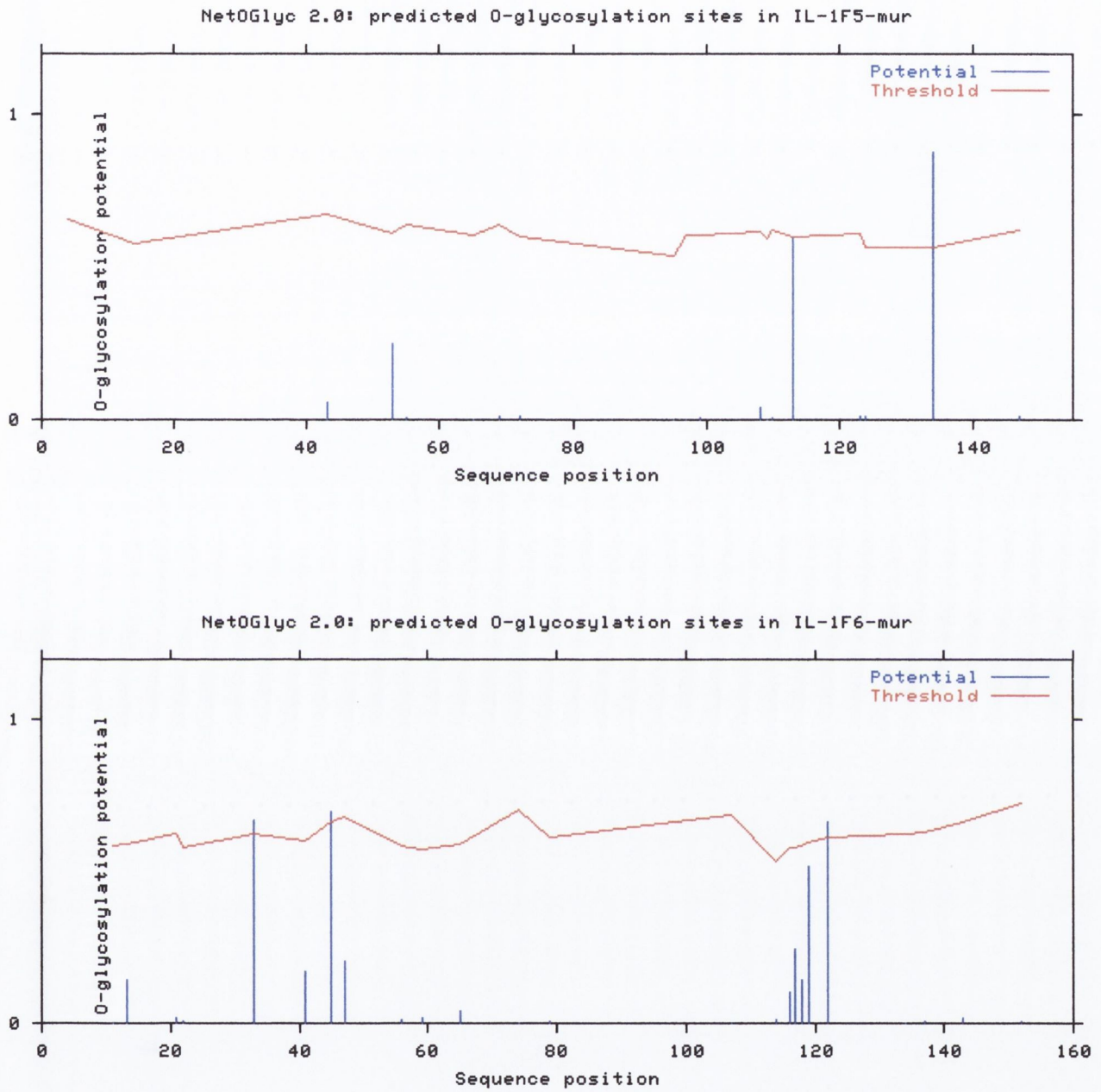


Figure 5.2.2 Predicted glycosylation sites in IL-1F5 and IL-1F6. The program NetOGly was used to predict GalNAc O-glycosylation sites in both murine IL-1F5 and IL-1F6. IL-1F5 is predicted to have a glycosylation site at Thr133.

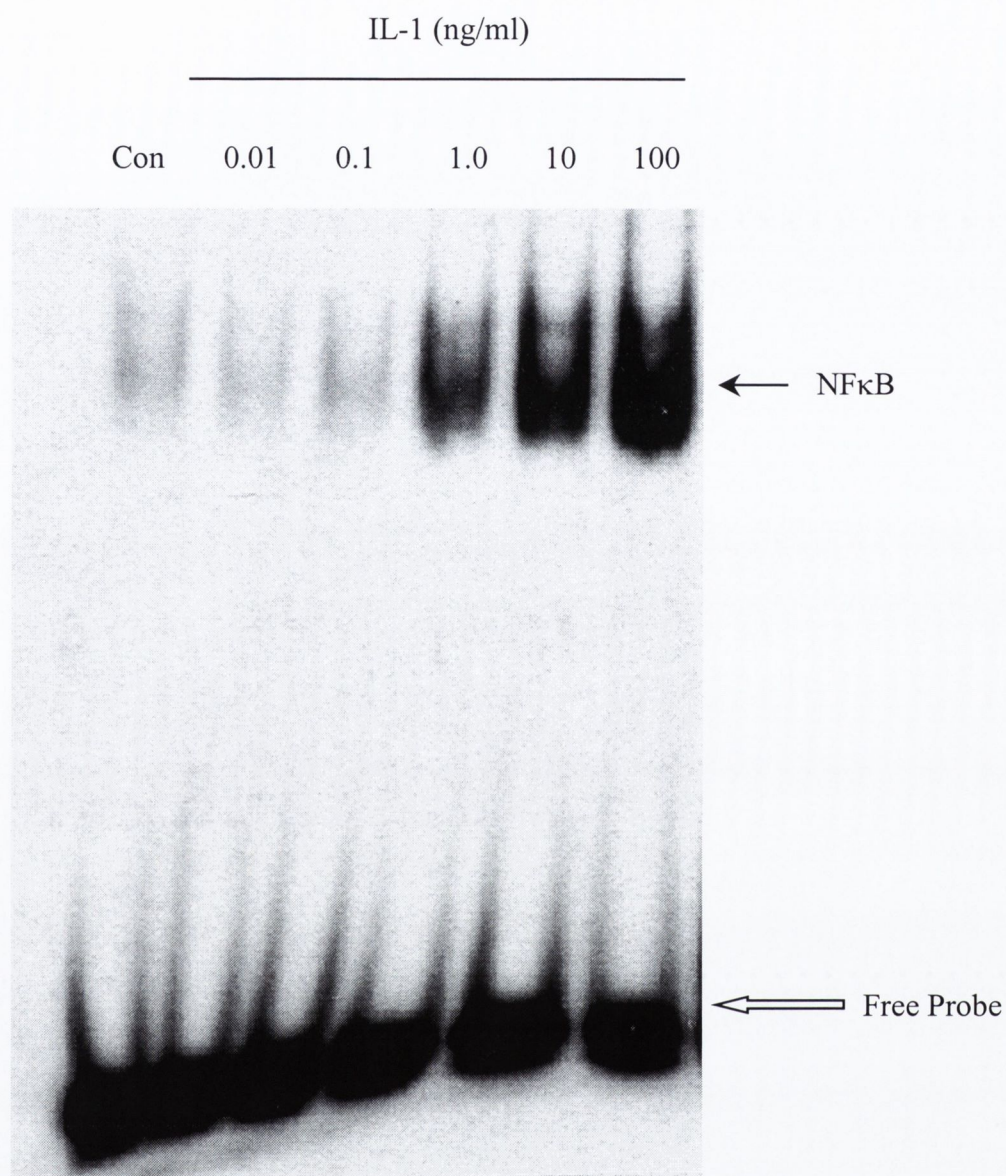


Figure 5.2.3 Dose response of IL-1 activation of NFκB in adherent HeLa cell line. Cells seeded at 1×10^5 cells/ml were grown in 6 well plates for 48 hours and treated with indicated doses of IL-1 for 30 minutes. Nuclear extracts were prepared and equal amounts of total protein were examined for NFκB activation. NFκB-DNA complex is marked by a filled arrow, free probe is marked by an open arrow. Result is representative of two independent experiments.

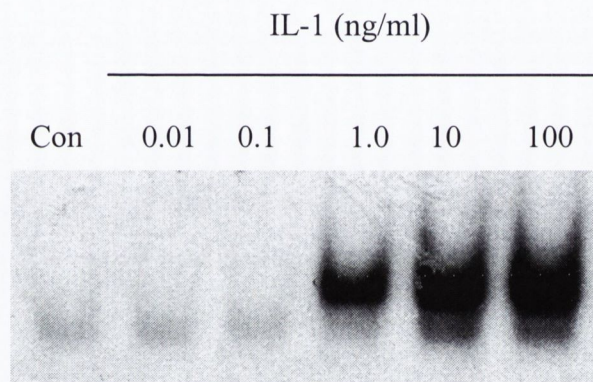


Figure 5.2.4 Dose response of IL-1 activation of NFκB in suspension cell line, EL4-NOB.1. Cells at a density of 2×10^6 cells/ml were treated with indicated doses of IL-1 for 30 minutes. Nuclear extracts were prepared and equal amounts of total protein were examined for NFκB activation. Bands represent NFκB-DNA complex. Result is representative of two independent experiments.

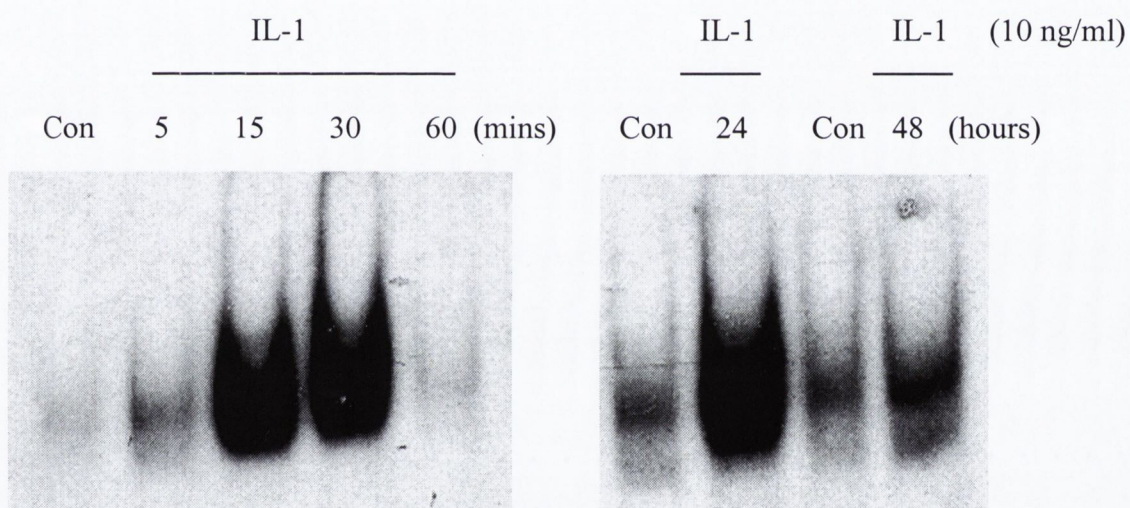


Figure 5.2.5 Time course of IL-1 activation of NFκB in HeLa cell line. Cells seeded at 1×10^5 cells/ml were grown in 6 well plates for 48 hours and treated for indicated times with 10 ng/ml of IL-1. One untreated time zero control was used for early time points, while time matched controls were used for the later time points. Nuclear extracts were prepared and equal amounts of total protein were examined for NFκB activation. Bands represent NFκB-DNA complex. Result is representative of two independent experiments.

may be due to an autocrine effect. EL4-NOB.1 cells showed similar rapid activation but longer time points were not examined (Figure 5.2.6).

5.2.3 Effect of IL-1F5 and IL-1F6 on IL-1 Induced Nuclear Translocation and DNA Binding Activity of NF κ B in Various Cell Lines.

Given their homology to IL-1Ra, IL-1F5 and IL-1F6 were assayed for inhibitory activity in response to IL-1 induced NF κ B activation. Cells were pre-treated with either IL-1F5 or IL-1F6 for 1 hour prior to stimulation with IL-1, and EMSA was used to detect the level of NF κ B activation. Figure 5.2.7 shows that in HeLa cells there was no inhibitory effect evident at concentrations up to 1000 fold that of IL-1. In these experiments IL-1Ra acted as a positive control for inhibition of IL-1 activity. IL-1Ra inhibited IL-1 induced NF κ B activation at a dose that was 100 fold that of IL-1. The T_H0 cell line EL4-NOB.1, which expresses high levels of the IL-1RI, also showed a similar lack of effect on IL-1 induced NF κ B activation in response to pre-treatment with either IL-1F5 or IL-1F6 (Figure 5.2.8). T24 bladder carcinoma cells, which have endothelial cell properties, were also used to assay for inhibitory effects of IL-1F5 and IL-1F6 on IL-1 induced activity. Figure 5.2.9 shows that concentrations up to 1000 fold that of IL-1 did not produce any inhibitory effects on IL-1 induced NF κ B activation. Finally, figure 5.2.10 shows the effect of pre-treatment of Saos-2 osteosarcoma cells with either IL-1F5 or IL-1F6 prior to stimulation with IL-1. Concentrations up to a 100 fold that of IL-1 did not produce any inhibitory effects in a response to 10 ng/ml IL-1. Thus in four different cell lines responsive to IL-1 no inhibitory effect of IL-1F5 or IL-1F6 was evident.

5.2.4 Effects of IL-1F5 and IL-1F6 on Nuclear Translocation and DNA Binding Activity of NF κ B.

Although very similar to IL-1Ra in sequence, the possibility that IL-1F5 or IL-1F6 might activate NF κ B themselves, in an IL-1 like activity, is also a real possibility as discussed in Chapter 3. Therefore it was also investigated whether treatment of cells with either IL-1F5 or IL-1F6 could activate such a signal. Figure 5.2.11 shows that in the HeLa cell line,

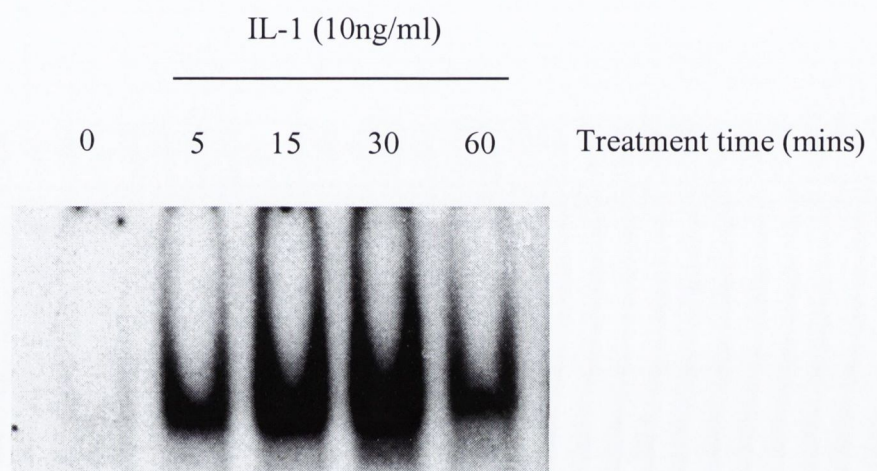


Figure 5.2.6 Time course of IL-1 activation of NFκB in suspension cell line, EL4-NOB.1. Cells at a density of 2×10^6 cells/ml were treated for indicated times with 10 ng/ml IL-1. One untreated time zero control was used for all time points. Nuclear extracts were prepared and equal amounts of total protein were examined for NFκB activation. Bands represent NFκB-DNA complex. Result is representative of two independent experiments.

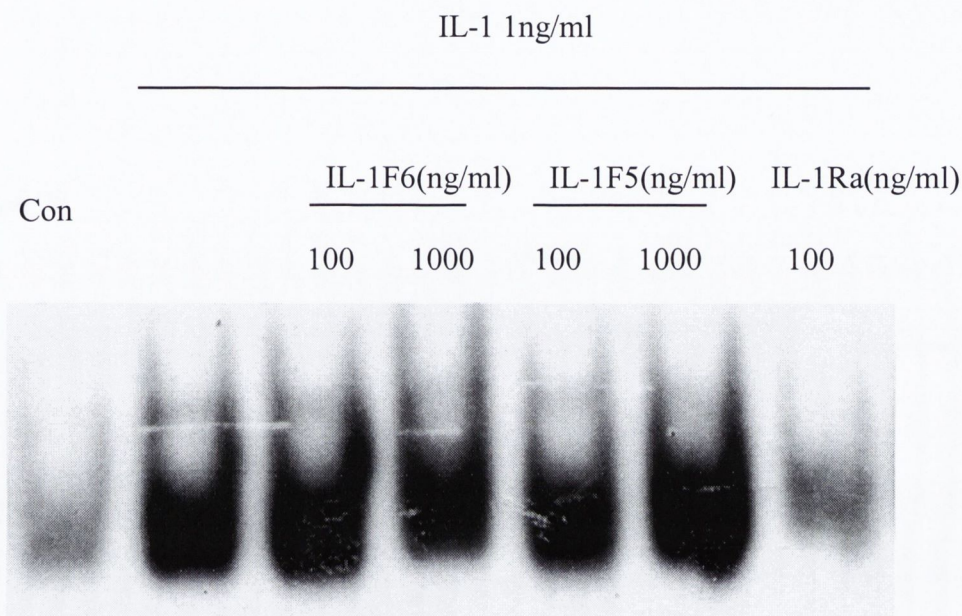


Figure 5.2.7 The effect of IL-1F5 and IL-1F6 on the IL-1 induced activation of NFκB in HeLa cells. Cells seeded at 1×10^5 cells/ml were grown in 6 well plates for 48 hours and pre-treated for 1 hour with indicated doses of IL-1F5, IL-1F6 or IL-1Ra. Cells were then treated with 1 ng/ml of IL-1 for 30 minutes. Nuclear extracts were prepared and equal amounts of total protein were examined for NFκB activation. NFκB-DNA complexes are shown. Result is representative of three independent experiments.

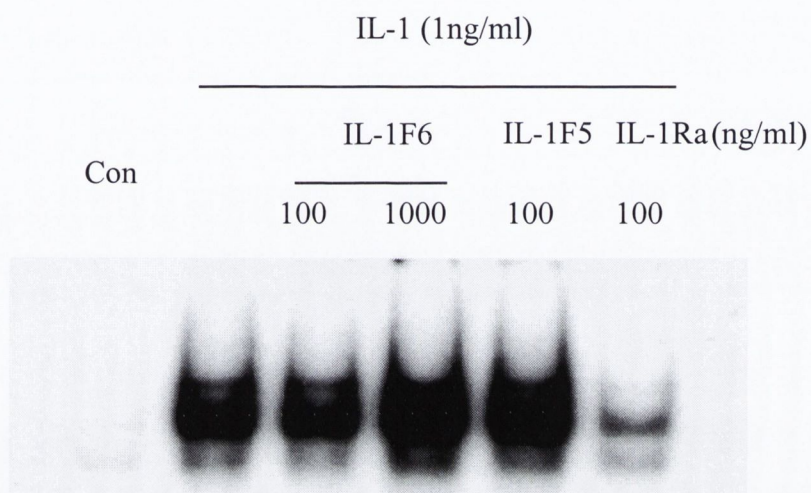


Figure 5.2.8 The effect of IL-1F5 and IL-1F6 on IL-1 induced activation of NFκB in EL4-NOB.1 cells. Cells at a density of 2×10^6 cells/ml were pre-treated for 1 hour with indicated doses of IL-1F5, IL-1F6 or IL-1Ra. Cells were then treated with 1 ng/ml of IL-1 for 30 minutes. Nuclear extracts were prepared and equal amounts of total protein were examined for NFκB activation. NFκB-DNA complexes are shown. Result is representative of three independent experiments.

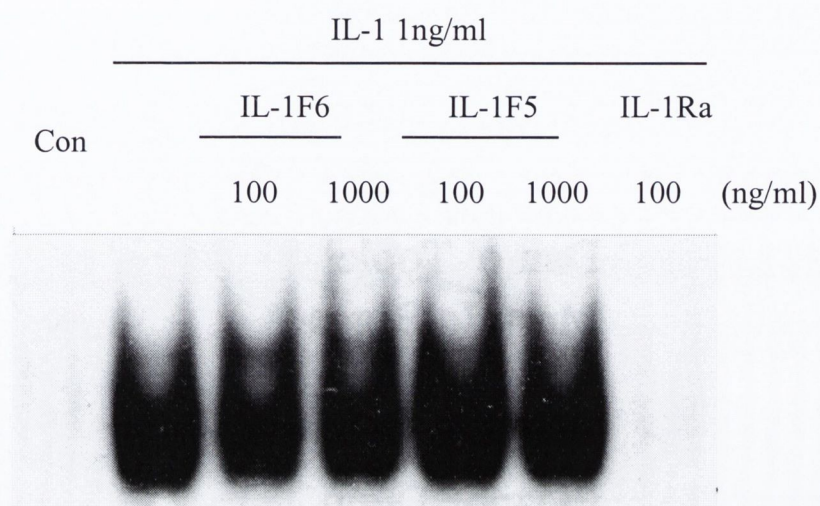


Figure 5.2.9 The effect of IL-1F5 and IL-1F6 on IL-1 induced activation of NFκB in T24 cells. Cells seeded at 1×10^5 cells/ml were grown for 48 hours and pre-treated for 1 hour with indicated doses of IL-1F5, IL-1F6 or IL-1Ra. Cells were then treated with 1 ng/ml of IL-1 for 30 minutes. Nuclear extracts were prepared and equal amounts of total protein were examined for NFκB activation. NFκB-DNA complexes are shown. Result is representative of two independent experiments.

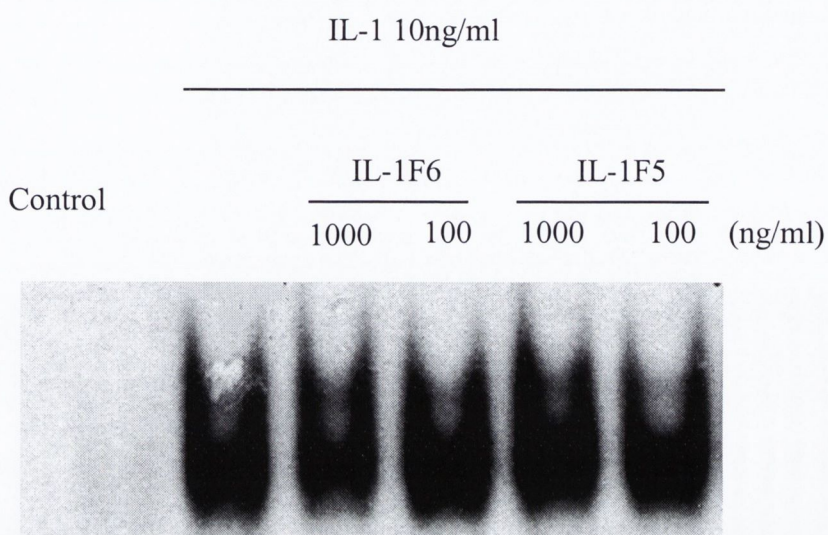


Figure 5.2.10. Gel mobility shift analysis of the effect of IL-1F5 and IL-1F6 on the activation NFκB activation in response to IL-1 in Saos-2 osteosarcoma cell line. Cells seeded at 1×10^5 cells/ml were grown in 6 well plates for 48 hours and pre-treated for 1 hour with indicated doses of IL-1F5, IL-1F6 or IL-1Ra. Cells were then treated with 10 ng/ml of IL-1 for 30 minutes. Nuclear extracts were prepared and equal amounts of total protein were examined for NFκB activation. NFκB-DNA complexes are shown. Result is representative of two independent experiments.

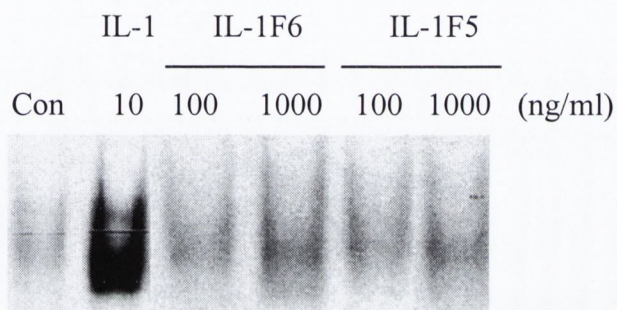


Figure 5.2.11 Effect of IL-1F5 and IL-1F6 on activation of NFκB in HeLa cells. Cells seeded at 1×10^5 cells/ml were grown in 6 well plates for 48 hours and treated for 30 minutes with indicated doses of IL-1F5, IL-1F6 or IL-1. Nuclear extracts were prepared and equal amounts of total protein were examined for NFκB activation. NFκB-DNA complexes are shown. Result is representative of five independent experiments.

treatment with either IL-1F5 or IL-1F6 for 30 minutes at concentrations up to 1 µg/ml did not produce any detectable activation of NFκB. In these experiments IL-1 acted as a positive control for NFκB activation. Similar results were obtained when EL4-NOB.1 cells were treated with up to 1 µg/ml of either IL-1F5 or IL-1F6 (Figure 5.2.12). In addition, IL-1F5 and IL-1F6 did not show any stimulatory effects at concentrations up to 1 µg/ml for IL-1F6 or 100 ng/ml for IL-1F5 in T24 cell line (Figure 5.2.13). Similar results were obtained when up to 400 ng/ml of either IL-1F5 or IL-1F6 was used to treat the osteosarcoma cell line, Saos-2. However, these cells were responsive to as little as 10 ng/ml of IL-1 (Figure 5.2.14).

5.2.5 Characterisation of the Activity of IL-18 in Various Cell Lines.

The possibility that IL-1F5 and IL-1F6 might inhibit IL-18 signalling was next investigated. The activity of IL-18 in various cell lines was thus examined in order to find conditions suitable for such investigations. Cells were treated with IL-18 at concentrations of 10 ng/ml and 100 ng/ml for 30 minutes and 1 hour. As shown in figure 5.2.15a, EL4.NOB-1 cells were shown to be responsive to IL-18, with strong activation detectable after 30 minutes of treatment with 100 ng/ml IL-18. Neither HeLa nor T24 cell lines were observed to be responsive to IL-18, in these cases IL-1 acted as a positive control for NFκB activation (Figure 5.15b and c).

5.2.6 Effect of IL-1F5 and IL-1F6 on IL-18 Induced Nuclear Translocation and DNA Binding Activity of NFκB in EL4-NOB.1 Cell Line.

To investigate the effect of IL-1F5 and IL-1F6 on IL-18 signalling EL4-NOB.1 cells were pre-treated for 1 hour with 1 µg/ml of either IL-1F5 or IL-1F6 prior to treatment with 100 ng/ml IL-18. As with studies looking at inhibition of IL-1 induced NFκB activation, neither IL-1F5 nor IL-1F6 showed any inhibitory activity on IL-18 induced activation of NFκB (Figure 5.2.16 a and b). As a negative control IL-1Ra was shown to be unable to inhibit the activation of NFκB by IL-18 (Figure 5.2.16 b).

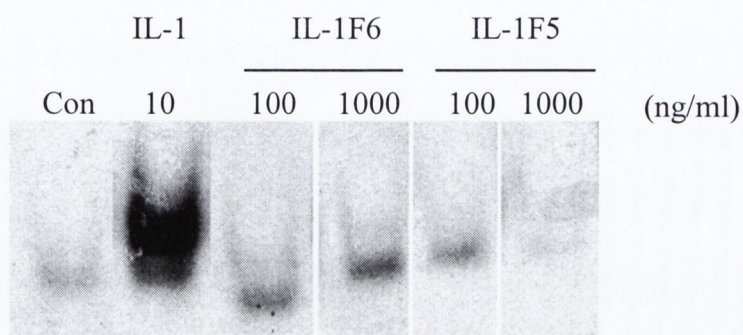


Figure 5.2.12. Effect of IL-1F5 and IL-1F6 on activation of NFκB in EL4-NOB.1 cells. Cells at a density of 2×10^6 cells/ml were treated for 30 minutes with indicated doses of IL-1F5, IL-1F6 or IL-1. Nuclear extracts were prepared and equal amounts of total protein were examined for NFκB activation. NFκB-DNA complexes are shown. Result is representative of two independent experiments.

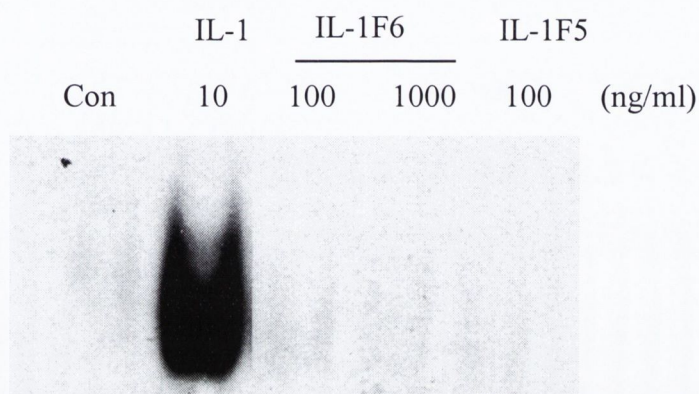


Figure 5.2.13. Effect of IL-1F5 and IL-1F6 on activation of NFκB in T24 cells. Cells seeded at 1×10^5 cells/ml were grown in 6 well plates for 48 hours and treated for 30 minutes with indicated doses of IL-1F5, IL-1F6 or IL-1. Nuclear extracts were prepared and equal amounts of total protein were examined for NFκB activation. NFκB-DNA complexes are shown. Result is representative of two independent experiments.

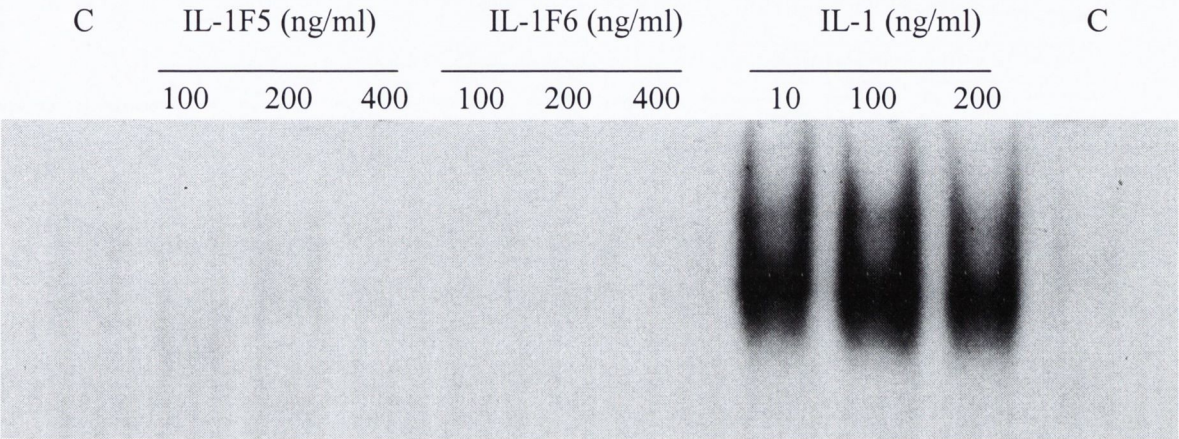


Figure 5.2.14. Effect of IL-1F5 and IL-1F6 on activation of NFκB in Saos2 cells. Cells seeded at 1×10^5 cells/ml were grown in 6 well plates for 48 hours and treated for 15 minutes with indicated doses of IL-1F5, IL-1F6 or IL-1. Nuclear extracts were prepared and equal amounts of total protein were examined for NFκB activation. NFκB-DNA complexes are shown. Result is representative of two independent experiments.

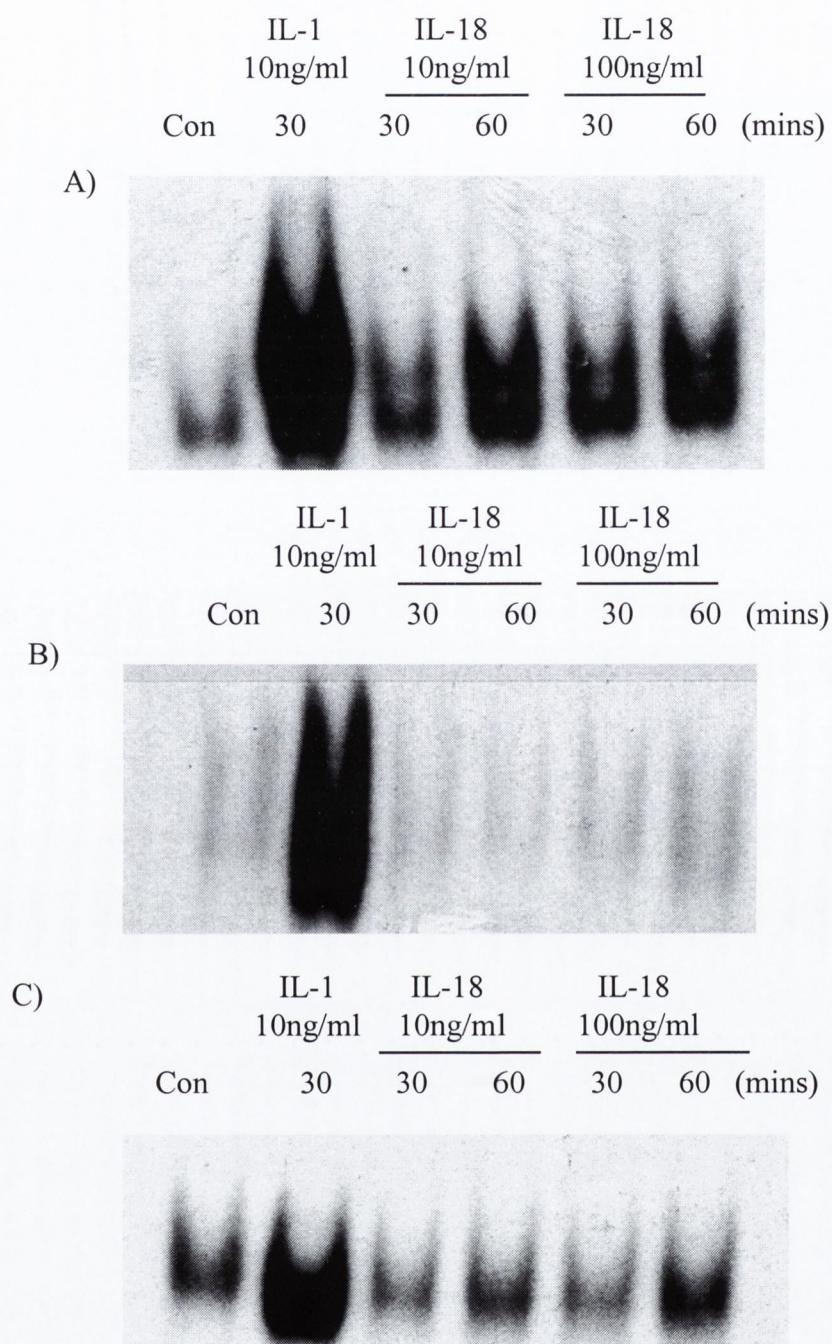


Figure 5.2.15 Gel mobility shift analysis of the ability of IL-18 to activate NFκB in A) EL4-NOB.1 cells B) T24 cells and C) HeLa cells. In the case of HeLa and T24 cell lines cells seeded at 1×10^5 cells/ml were grown in 6 well plates for 48 hours and treated for indicated times and with indicated doses of IL-18 and IL-1, while EL4-NOB.1 cells at a density of 2×10^6 cells/ml were treated for indicated times and with indicated doses of IL-18 and IL-1. Nuclear extracts were prepared and equal amounts of total protein were examined for NFκB activation. NFκB-DNA complexes are shown. Result is representative of two independent experiments.

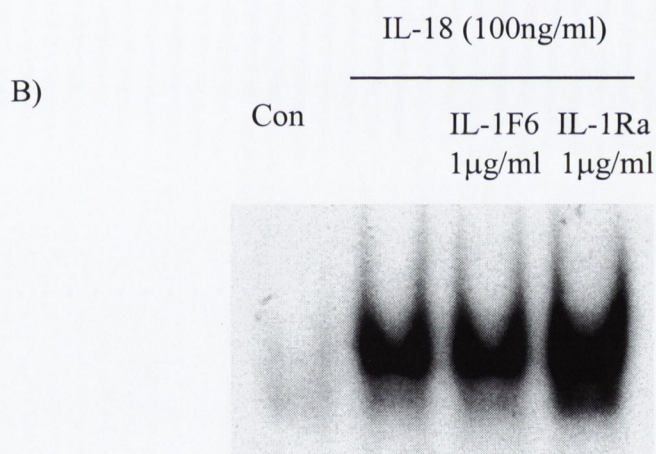
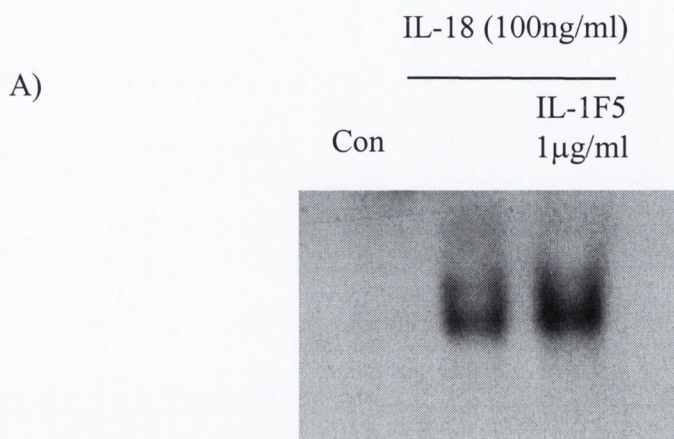


Figure 5.2.16 Effect of IL-1F5 and IL-1F6 on activation of NFκB in response to IL-18 in EL4-NOB.1. Cells at a density of 2×10^6 cells/ml were pre-treated for 1 hour with either A) 1 μg/ml IL-1F5 or B) 1 μg/ml IL-1F6 or IL-1Ra prior to treatment with IL-18 for 30 minutes at 100 ng/ml. Nuclear extracts were prepared and equal amounts of total protein were examined for NFκB activation. NFκB-DNA complexes are shown. Result is representative of two independent experiments.

5.2.7 Effects of IL-1F5 and IL-1F6 on Induction of Nuclear Translocation and DNA Binding Activity of NFκB in T1/ST2 Expressing Cells.

To investigate whether IL-1F5 or IL-1F6 might be ligands for T1/ST2, EMSA was used to detect possible activation of NFκB in response to treatment with IL-1F5 or IL-1F6 in cells known to express T1/ST2. As shown in figure 5.2.17, a concentration of 100 ng/ml of either IL-1F5 or IL-1F6 for 1 or 4 hours failed to produce any detectable activation of NFκB in HL-60 cells, which express T1/ST2 (Yanagisawa et al., 1997). In these experiments, TNF acted as a positive control for NFκB activation while IL-1 acts as a negative control. Similar results were obtained for experiments in K562 cells, which also express T1/ST2 (Yanagisawa et al., 1997) (Figure 5.2.17). This brings to six the number of cell lines which were tested but failed to show any activation of NFκB in response to treatment with either IL-1F5 or IL-1F6.

5.2.8 Effect of IL-1F5 and IL-1F6 on Induction of NFκB, c-Jun and ATF2 Transcriptional Activity using the Gene Reporter Assay in T24 Cell Line in the Presence and Absence of IL-1.

Transcriptional activation of transcription factors such as NFκB can be examined using the gene reporter assay. Cells transfected by electroporation with 10 µg E-selectin promoter-luciferase plasmid produce luciferase protein in response to treatment with IL-1. The Eselectin promoter binds the transcription factors NFκB, c-Jun and ATF2 (Nawa et al., 2000) and the luciferase linked promoter can therefore act as read out for the ability of these transcription factors to activate gene expression. The effect of a 1 hour pre-treatment with either IL-1F5 or IL-1F6 prior to stimulation with 10 ng/ml IL-1 for 6 hours produced a blunting of the IL-1 induced reporter gene expression, while it was completely inhibited by a 10-fold concentration of IL-1Ra (Figure 5.2.18). Furthermore, treatment of T24 transfected cells with either IL-1F5 or IL-1F6 alone did not result in any detectable stimulatory activity (Figure 5.2.19). This result was however not seen consistently but was seen in three out of five experiments completed.

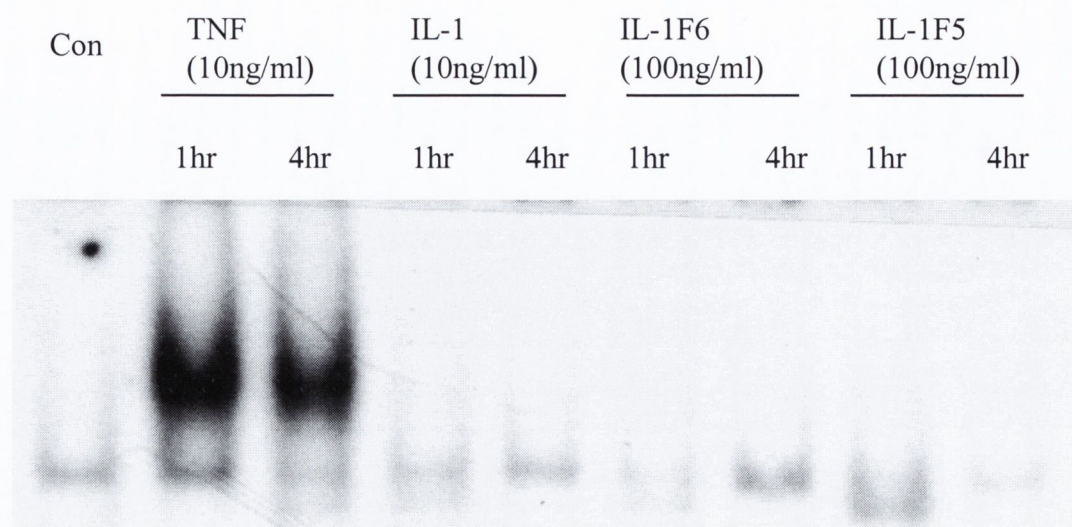


Figure 5.2.17a. Gel mobility shift analysis of the effect of IL-1F5 and IL-1F6 in HL-60 cell line. Cells grown in 0.5% (v/v) Fetal Calf Serum for 16 hours were seeded at 1×10^6 cells/ml 15 minutes prior to treatment with indicated doses and for indicated times with TNF, IL-1, IL-1F5 or IL-1F6. Nuclear extracts were prepared and equal amounts of total protein were analysed for NFκB activation. IL-1 acts as a negative control for these cells. Result is representative of two independent experiments.

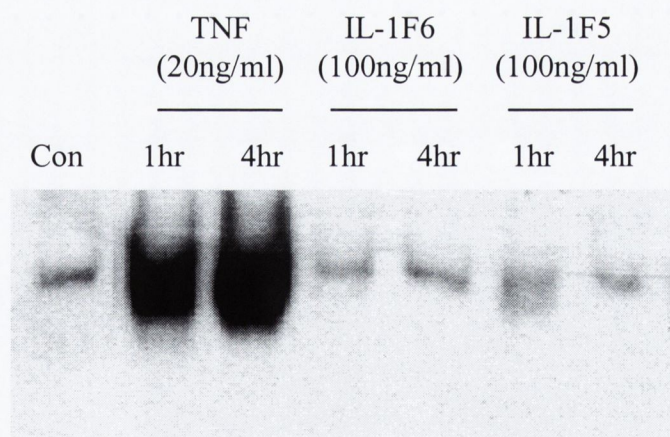


Figure 5.2.17b The effect of IL-1F5 and IL-1F6 on activation of NFκB in K562 cells. Cells were seeded at 1×10^6 cells/ml 15 minutes prior to treatment with indicated times and with indicated doses of IL-1F5, IL-1F6 or TNF. Nuclear extracts were prepared and equal amounts of total protein were analysed for NFκB activation.

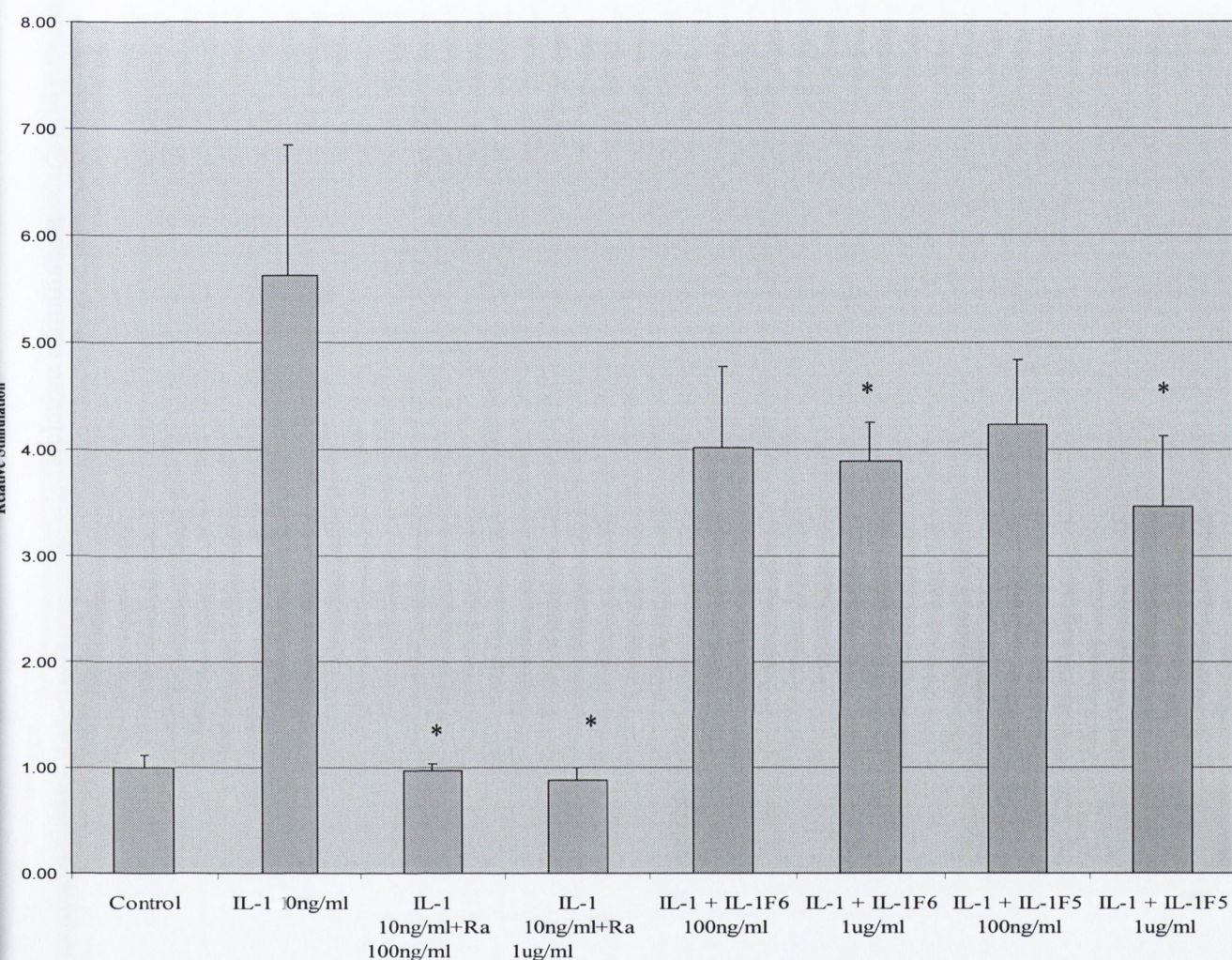


Figure 5.2.18. Effect of IL-1F5 and IL-1F6 on IL-1 induced activation of E-selectin promoter dependent luciferase expression in T24 cells. Cells seeded at 1×10^5 cells/ml were grown for 48 hours and transfected by electroporation with 10 μ g E-selectin promoter-luciferase plasmid. After 24 hours the cells were washed and given fresh medium. After a further 24 hours the cells were pre-treated for 1 hour with indicated doses of IL-1F5, IL-1F6 or IL-1Ra followed by a 6 hour treatment with 10 ng/ml IL-1. Cellular extracts were prepared and assayed for luciferase activity. Result represents mean values of quadruplicate samples $\pm$ standard deviation and standardised for protein levels. * indicates p -value < 0.05 in Student's t -test relative to IL-1 stimulated sample. Result is representative of three independent experiments of five experiments in total.

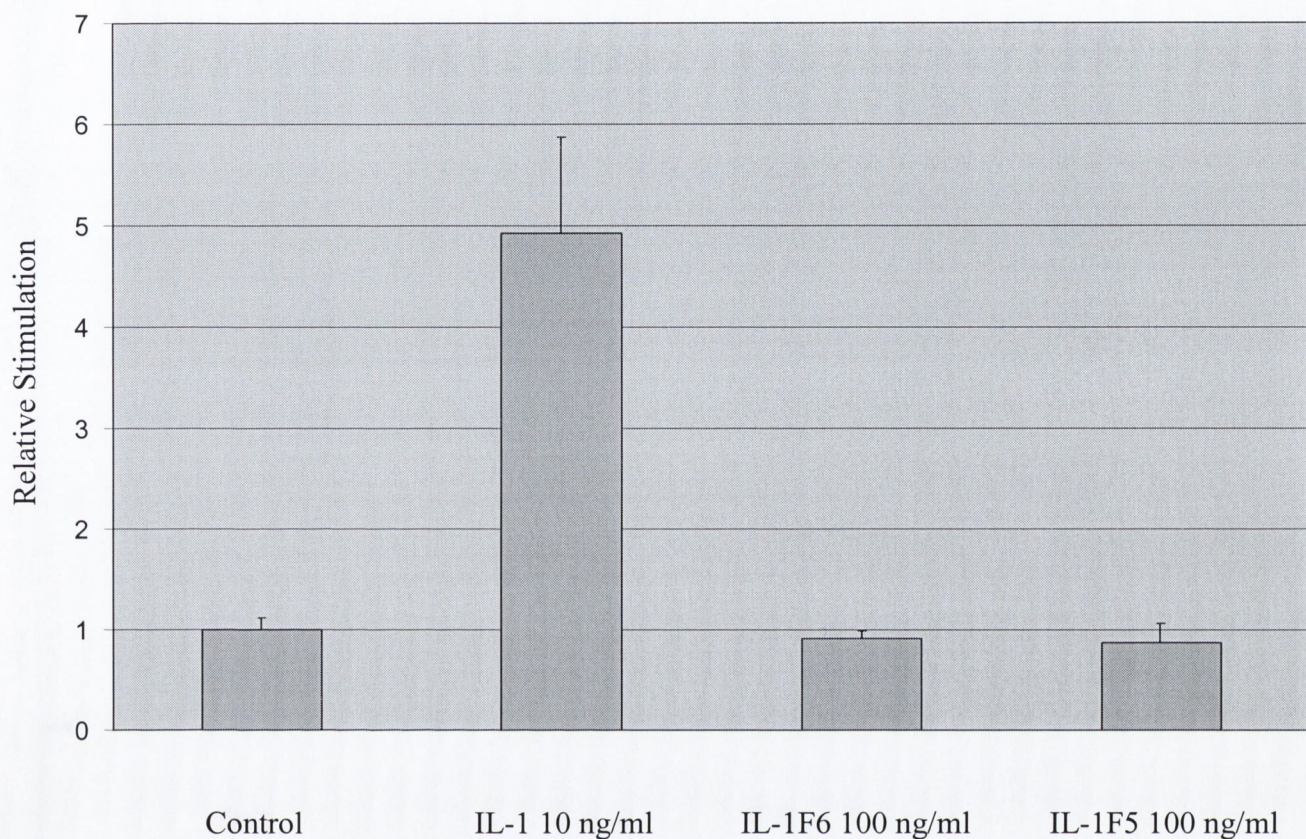


Figure 5.2.19. Effect of IL-1F5 and IL-1F6 on activation of E-selectin promoter dependent luciferase expression in T24 cells. Cells seeded at 1×10^5 cells/ml were grown for 48 hours and transfected by electroporation with 10 μ g E-selectin-luciferase plasmid. After 24 hours the cells were washed and given fresh medium. After a further 24 hours the cells were treated for 6 hours with indicated doses of IL-1F5 and IL-1F6 and IL-1. Cellular extracts were prepared and assayed for luciferase activity. Results are presented as mean values of triplicate samples standardised for protein levels $\pm$ standard deviation and is representative of three independent experiments.

5.2.9 Effect of IL-1F5 and IL-1F6 on IL-1 Induced Transactivation of Gene Expression by NFκB using Gene Reporter Assay in T24 Cell Line.

Because of the partial effect in the E-selectin promoter-luciferase reporter assay and the lack of effect on DNA binding it was possible that IL-1F5 and IL-1F6 were inhibiting transactivation of gene expression by NFκB. As shown in figure 5.2.20, IL-1 induced approximately a three-fold increase in gene expression in the p65 transactivation assay, which measures the ability of p65 to transactivate gene expression (see methods section 2.8.8). This effect on transactivation was not inhibited by pre-treatment for 1 hour with up to 1 μg/ml of either IL-1F5 or IL-1F6. However, IL-1Ra was capable of inhibiting this effect at concentrations 100 fold that of IL-1.

5.2.10 Effect of IL-1F5 and IL-1F6 on Activation of MAP Kinases in T24 and Saos-2 Cell Lines.

In addition to the activation of NFκB, treatment of responsive cells with IL-1 results in the activation of the MAP kinase pathways. The major downstream targets of these pathways are the MAP kinases p42/p44, c-Jun N Terminal Kinase (JNK) and p38. These MAP kinases, in particular JNK and p38 are also responsible for the activation of transcription factors such as c-Jun and ATF2, which are required for full activation of the E-selectin promoter (Tamaru and Narumi, 1999) and therefore regulation of these proteins may be responsible for the blunting effect seen in the E-selectin promoter gene reporter assays. As shown in figure 5.2.21 a and b, treatment of T24 cells for 30 minutes with up to 200 ng/ml of either IL-1F5 or IL-1F6 protein did not produce any detectable activation of either JNK or p38. However, 10 ng/ml IL-1 for the same incubation time produces a clear phosphorylation of both JNK and p38. Neither was pre-treatment for 1 hour with either IL-1F5 or IL-1F6 capable of inhibiting the activation of p38 in response to treatment with IL-1 (Figure 5.2.21 c). In another cell line, the Saos-2 osteosarcoma cell, similar results were obtained. In these cells a dose of 200 ng/ml of either IL-1F5 or IL-1F6 failed to induce detectable phosphorylation of either JNK or p38 at time points of 5, 15 or 30 minutes (Figure 5.2.22). Furthermore, pre-treatment of these cells for 1 hour

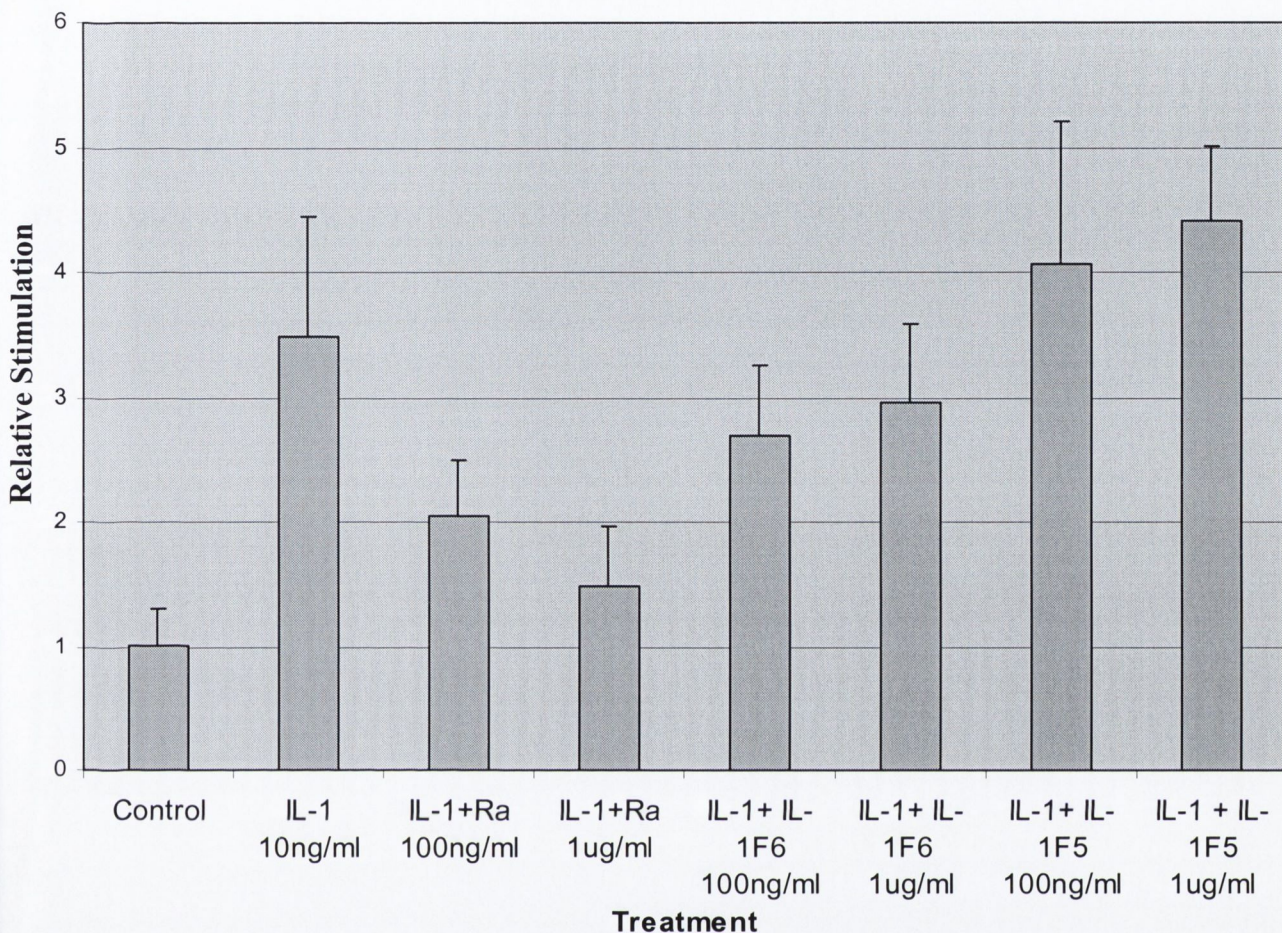


Figure 5.2.20 Effects of IL-1F5 and IL-1F6 on transactivation activity by p65 in T24 cells in response to IL-1. Cells seeded at 1×10^5 cells/ml were grown for 48 hours and transfected with 30 μ g GAL4-luciferase and 2.5 μ g p65-GAL DNA binding domain plasmid. After 24 hours the cells were washed and given fresh medium. After a further 24 hours the cells were pre-treated for 1 hour with indicated doses of IL-1F5, IL-1F6 or IL-1Ra followed by a 6 hour treatment with 10 ng/ml IL-1. Cellular extracts were prepared and assayed for luciferase activity. Results are presented as mean values of triplicate samples $\pm$ standard deviation and standardised for protein levels. Result is representative of three experiments.

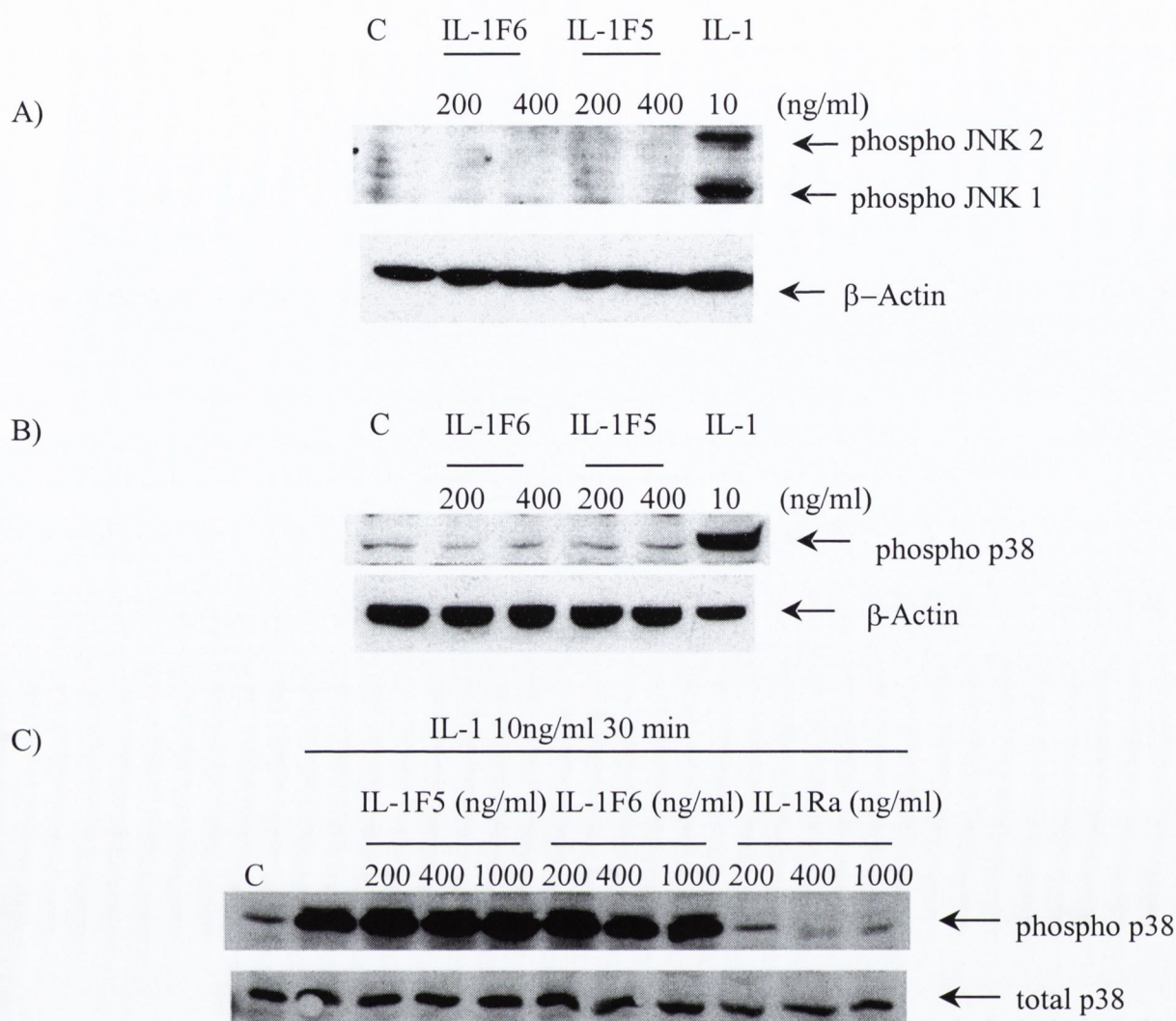
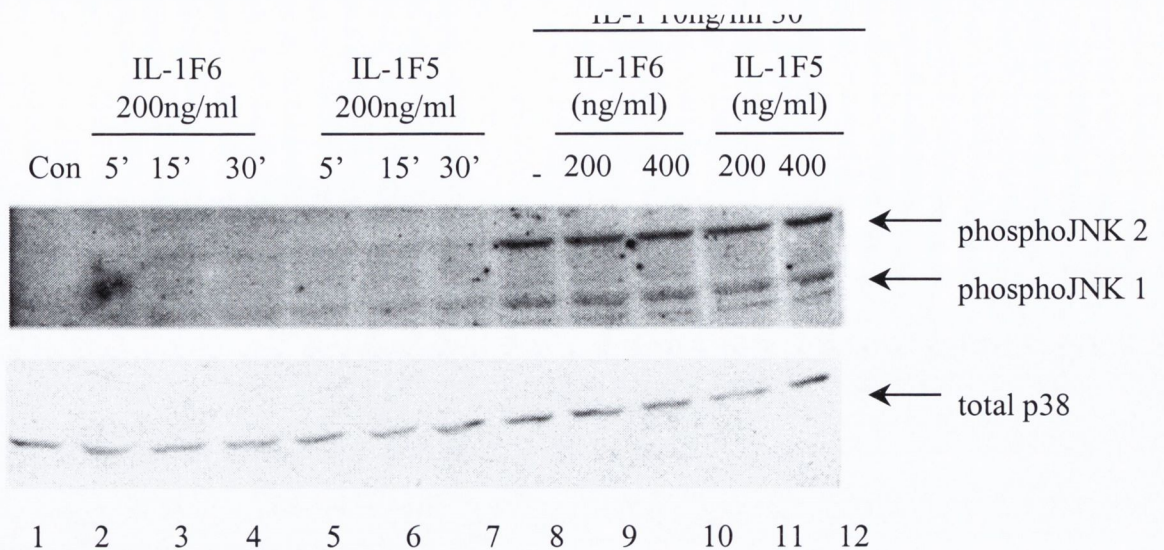


Figure 5.2.21 Effect of IL-1F5 and IL-1F6 on activation of MAP kinases in T24 cell line. Cells seeded at 1×10^5 cells/ml were grown in 6 well plates for 48 hours and treated with IL-1F5, IL-1F6 or IL-1 for 30 minutes at indicated doses, or pre-treated for 1 hour with IL-1F5, IL-1F6 or IL-1Ra before stimulation with IL-1 at 10 ng/ml for 30 minutes. Total cell lysates were prepared and DNA sheared with a 21 gauge needle. Proteins were separated on a 12% polyacrylamide gel, transferred to nitrocellulose and detected by western blot analysis using appropriate antibodies. A) Effect of IL-1F5, IL-1F6 and IL-1 on activation of c-Jun N-terminal kinase (JNK). β -actin acts as a control for equal protein loading between lanes. B) Effect of IL-1F5, IL-1F6 and IL-1 on activation of p38 MAP kinase. β -actin acts as a control for equal protein loading between lanes. C) Effect of IL-1F5, IL1F6 and IL-1Ra on IL-1induced p38 phosphorylation. Total p38 acts as a loading control. Result is representative of two independent experiments.

A)



B)

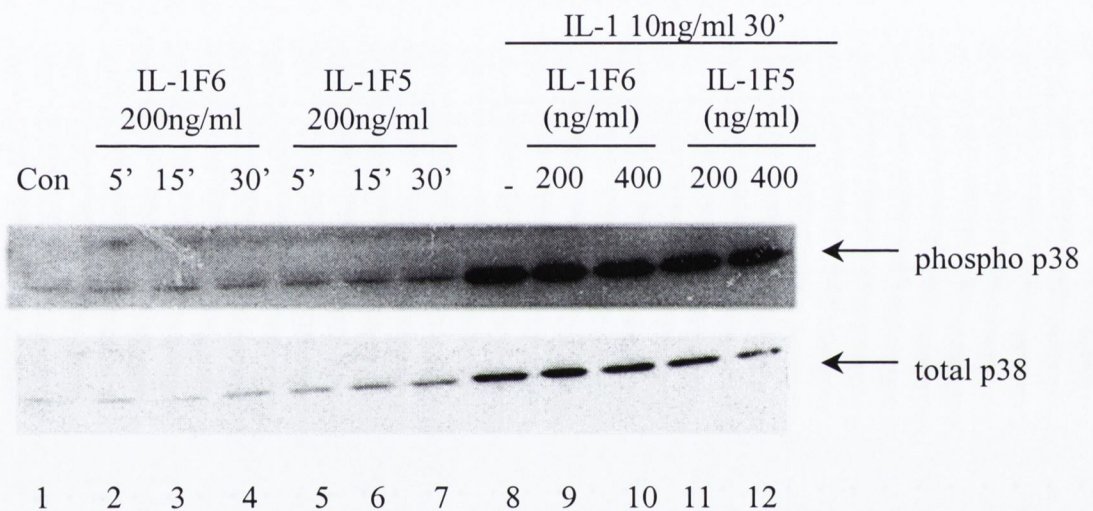


Figure 5.2.22 The effect of IL-1F5 and IL-1F6 on the activation of MAP kinase in Saos-2 cell line. Cells seeded at 1×10^5 cells/ml were grown in 6 well plates for 48 hours and either pre-treated for 1 hour with IL-1F5 or IL-1F6 at indicated doses followed by a 30 minute treatment with 10 ng/ml IL-1, or treated with IL-1F5 or IL-1F6 alone for indicated times and doses. Total cell lysates were prepared and DNA sheared with a 21 gauge needle. Proteins were separated on a 12% polyacrylamide gel, transferred to nitrocellulose and detected by western blot analysis using appropriate antibodies. A) Effect of IL-1F5 and IL-1F6 on activation of JNK (lanes 1-7) and the effect of IL-1F5 and IL-1F6 on IL-1 induced activation of JNK (lanes 1, 8-12). B) Effect of IL-1F5 and IL-1F6 on activation of p38 (lanes 1-7), and the effect of IL-1F5 and IL-1F6 on IL-1 induced activation of p38 (lanes 1, 8-12). Total p38 acts as a loading control. Result is representative of two independent experiments.

with up to 400 ng/ml of either IL-1F5 or IL-1F6 failed to show any inhibition of MAP kinase phosphorylation in response to 10 ng/ml IL-1 (Figure 5.2.22).

5.2.11 The Effect of IL-1F5 and IL-1F6 on Nuclear Translocation and DNA Binding Activity of STAT3 in T24 and Saos-2 Cell Lines.

At this point no evidence had been obtained for activity of either IL-1F5 or IL-1F6. Communications with other laboratories studying IL-1F5 in particular indicated that they too were having problems showing any effects. However, reports from Millennium Pharmaceuticals indicated that IL-1F5 and IL-1F6 might be able to induce proliferation of the IL-6 dependent cell line B9. This suggested that the proteins could signal in a manner similar to IL-6. IL-6 is a cytokine that utilises the gp130 signalling receptor and one of the major signals activated by this receptor is the activation of the transcription factor STAT3 (Taga and Kishimoto, 1997). Therefore, it was investigated whether IL-1F5 and IL-1F6 could activate STAT3 in various cell lines. As shown in figure 5.2.23 treatment of Saos-2 and T24 cells with up to 800 ng/ml of IL-1F5 or IL-1F6 did not show any detectable activation of STAT3 as assayed by EMSA using a probe with a STAT3 consensus sequence. However, a positive control for STAT3 activation is not included in these data, a stimulant such as IL-6 could be used for this purpose. It was subsequently learnt that the reported effects in B9 cells were due to contamination with LPS.

5.2.12 Expression Patterns of IL-1F5.

The expression patterns of IL-1F5 have been published in several reports. These patterns were, in general based on commercially available cDNA from various tissues and probed for expression using PCR. However, these results are not consistent between reports and little was carried out to localise expression to more defined areas of tissues, with the exception of hematopoietic cells (Smith et al., 2000). Three reports looked for expression of IL-1F5 in the brain and two of these returned positive results for expression. Therefore, it was decided to examine more closely the expression of IL-1F5 in the mouse brain. Using primers designed to the coding region of IL-1F5 (Figure 5.2.24) a band

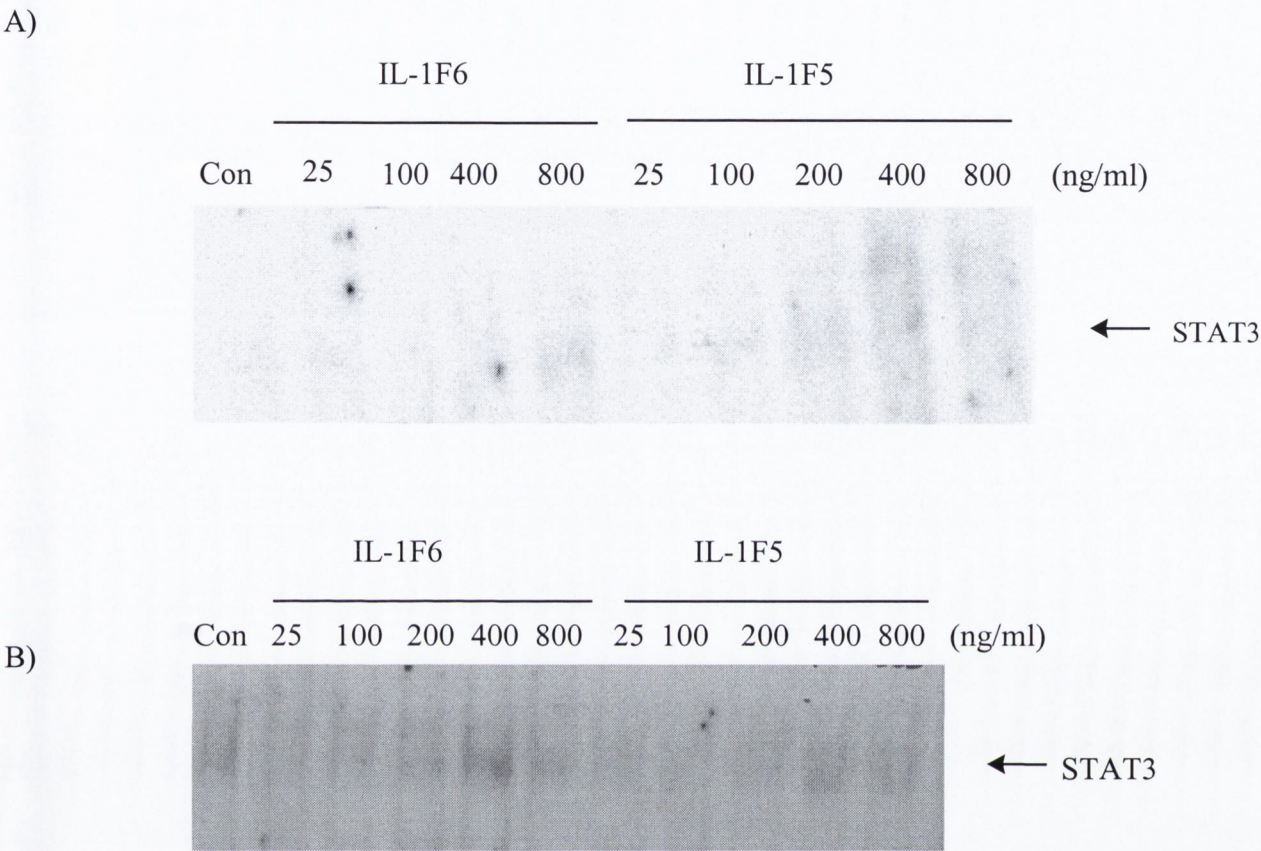


Figure 5.2.23 Gel mobility shift analysis of the effect of IL-1F5 and IL-1F6 activation STAT3 at various concentrations in a) Saos-2 and b) T24 cell line. Cells seeded at 1×10^5 cells/ml were grown in 6 well plates for 48 hour and treated with indicated doses of IL-1F5 or IL-1F6 for 15 minutes. Nuclear extracts were prepared and equal amounts of total protein were analysed by EMSA using a STAT3 consensus sequence binding probe. STAT3 DNA complexes are shown. Result is representative of two independent experiments.

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1    ATGGTTCTGA GTGGGGCACT ATGCTTCCGA ATGAAGGATT CAGCCTTGAA
51   GGTACTGTAT CTGCACAATA ACCAGCTGCT GGCTGGAGGA CTGCACGCAG
101  AGAAGGTCAT TAAAGGTGAG GAGATCAGTG TTGTCCCAA TCGGGCACTG
151  GATGCCAGTC TGTCCCCTGT CATCCTGGGC GTTCAAGGAG GAAGCCAGTG
201  CCTATCTTGT GGGACAGAGA AAGGGCCAAT TCTGAAACTT GAGCCAGTGA
251  ACATCATGGA GCTCTACCTC GGGGCCAAGG AATCAAAGAG CTTACCTTC
301  TACCGGCGGG ATATGGGTCT TACCTCCAGC TTCGAATCCG CTGCCTACCC
351  AGGCTGGTTC CTCTGCACCT CACCGGAAGC TGACCAGCCT GTCAGGCTCA
401  CTCAGATCCC TGAGGACCCC GCCTGGGATG CTCCCATCAC AGACTTCTAC
451  TTTCAGCAGT GTGACTAG

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Figure 5.2.24 Coding sequence of murine IL-1F5. The DNA sequence of murine IL-1F5 is shown in blocks of 10 residues from the 5' end to the 3' end. The position of the sequence against which the forward and reverse primers were designed are underlined, while the position the restriction site for the restriction enzyme SacI is marked in red with the cleavage site marked with an arrow. The PCR product using the primer sequences designated here is 424bp, while digestion with SacI yields two fragments of size 238bp and 184bp.

around the expected size of 424 bp was amplified using a touchdown PCR from cortex of mouse brain, and in primary neuronal cells stimulated for 48 hours with 10 ng/ml IL-1 (Figure 5.2.25, lanes 1 and 13). Expression was not however detectable in primary neuronal cells in the resting state or stimulated with LPS, TNF, glutamate or β -amyloid for 48 hours (lanes 12 and 14-17). Neither was expression detectable in the cerebellum (lane 2), in primary microglial cells, either untreated or stimulated with LPS for 48 hours (lanes 3 and 4), in CAD murine neuronal cells, either resting or stimulated with IL-1, TNF or LPS for 48 hours (lanes 18-21), or in the neuroblastoma cell line, SK-N-SH (lane 10). A band of the same size was detected in rat lung (lane 24) and weakly in J774 cells stimulated for 48 hours with 200 μ M PMA (lane 8), but not in mouse lung or liver, or rat liver (lanes 22-23 and 25), or either in resting J774 cells or those treated with IL-1 or TNF for 48 hours (lanes 5-7). Sequencing confirmed that the amplified band was indeed murine IL-1F5 (Figure 5.2.26). Furthermore, IL-1F5 mRNA could be detected in the neuroblastoma cell line neuro2a, the astrocytoma cell line 132N1 and the glioblastoma-astrocytoma cell line U78MG, which confirms its expression in brain derived cell lines (Figure 5.2.27). The identity of the PCR product obtained was confirmed using a SacI restriction digest. The IL-1F5 gene has a SacI site at position 263 and the PCR primers amplify between base pairs 25 and 448, thus digestion with SacI yields two bands of 184bp and 238bp (Figure 5.2.28).

5.2.13 Effects Of Stimulation of Brain-Derived Cell Lines with IL-1F5 and IL-1F6.

Given the detected expression of IL-1F5 in the cortex of mouse brain and the brain derived cell lines, it was decided to screen brain derived cell lines for activation of NF κ B and other IL-1-like signals in response to treatment with IL-1F5. The material used for these experiments was recombinant IL-1F5, purified as described in Chapter 4. The astrocytoma cell line 1321N1 was tested for responses using EMSA with an NF κ B specific oligonucleotide. However no activation of NF κ B in response to IL-1F5 at doses up to 1 μ g/ml was detected, although IL-1 itself activates NF κ B at a dose of 10 ng/ml (Figure 5.2.29). Similar results were seen with the glioblastoma-astrocytoma cell line

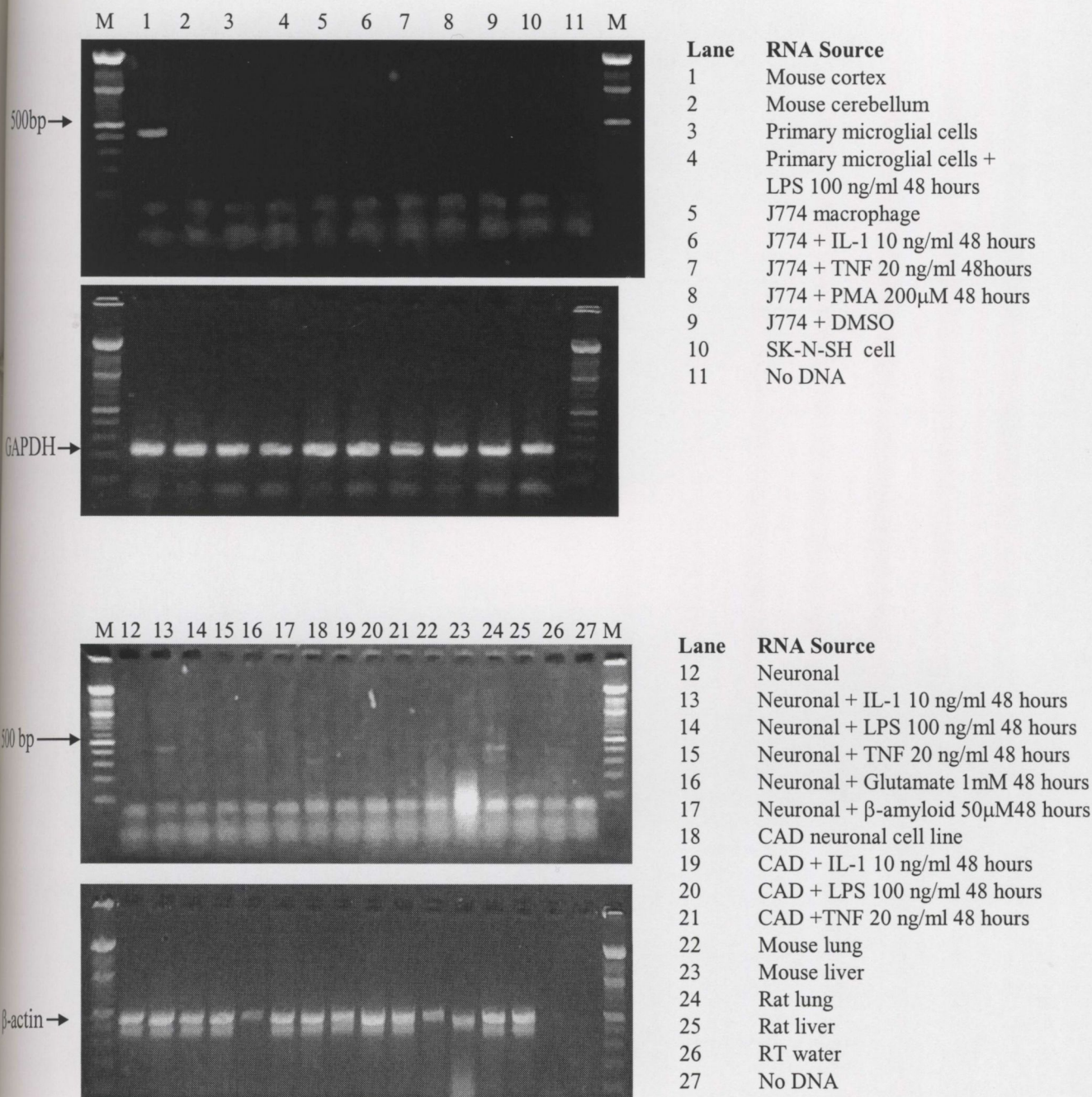
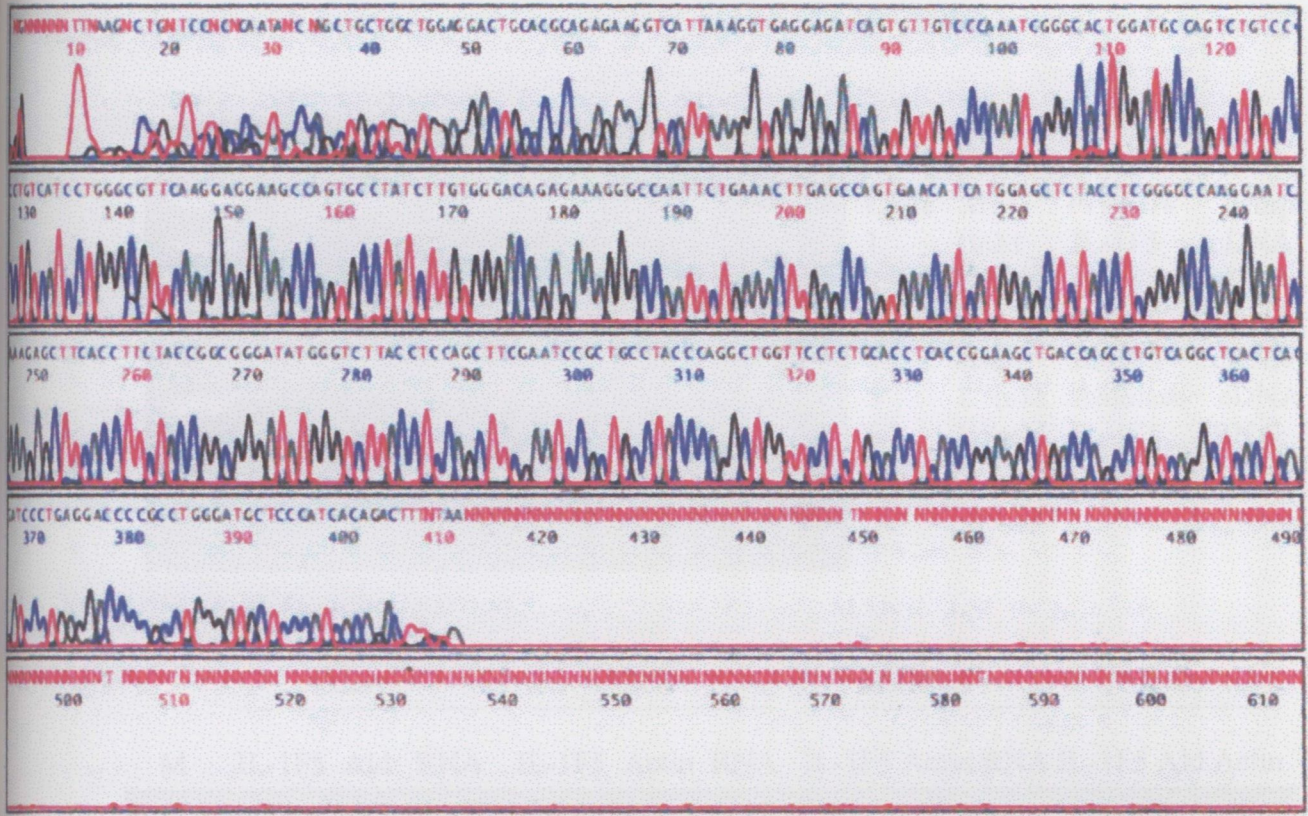


Figure 5.2.25 RT-PCR analysis of IL-1F5 expression. RNA was prepared from indicated tissues, and cells from brain and peripheral sources. 250 ng RNA was reverse transcribed to cDNA and primers designed against the coding region of murine IL-1F5 were used to amplify any IL-1F5 message present. The product detected in the cortex corresponds to the expected size of 424bp and its identity was confirmed by sequencing. A faint product was also detected in PMA stimulated J774 cells, IL-1 stimulated primary neuronal cells and rat lung. Control PCRs to test the integrity of the RNA preparation were included and carried out using primers either to GAPDH (239 bp) or β-actin (493 bp).

Figure 5.2.26 Sequencing of IL-1F5 PCR product. An ABI Prism Sequencer was used to determine the sequence of the PCR product amplified from mouse cortex using primers directed against the coding sequence of IL-1F5. A) Readout from the sequencing experiment. B) The coding sequence of murine IL-1F5; underlined is the section of sequence which corresponds to that determined from the sequencing of the PCR product. The position of the PCR primer sites in the coding region are in italics.

A)



B)

1 ATGGTTCTGA GTGGGGCACT ATGCTTCCGA ATGAAGGATT CAGCCTTGAA

51 GGTACTGTAT CTGCACAATA ACCAGCTGCT GGCTGGAGGA CTGCACGCAG

101 AGAAGGTCAT TAAAGGTGAG GAGATCAGTG TTGTCCCAA TCGGGCACTG

151 GATGCCAGTC TGTCCCCTGT CATCCTGGGC GTTCAAGGAG GAAGCCAGTG

201 CCTATCTTGT GGGACAGAGA AAGGGCCAAT TCTGAACTT GAGCCAGTGA

251 ACATCATGGA GCTCTACCTC GGGGCAAGG AATCAAAGAG CTTCACCTTC

301 TACCGGCGGG ATATGGGTCT TACCTCCAGC TTCGAATCCG CTGCCTACCC

351 AGGCTGGTTC CTCTGCACCT CACCGGAAGC TGACCAGCCT GTCAGGCTCA

401 CTCAGATCCC TGAGGACCCC GCCTGGGATG CTCCCATCAC AGACTTCTAC

451 TTTCAGCAGT GTGACTAG

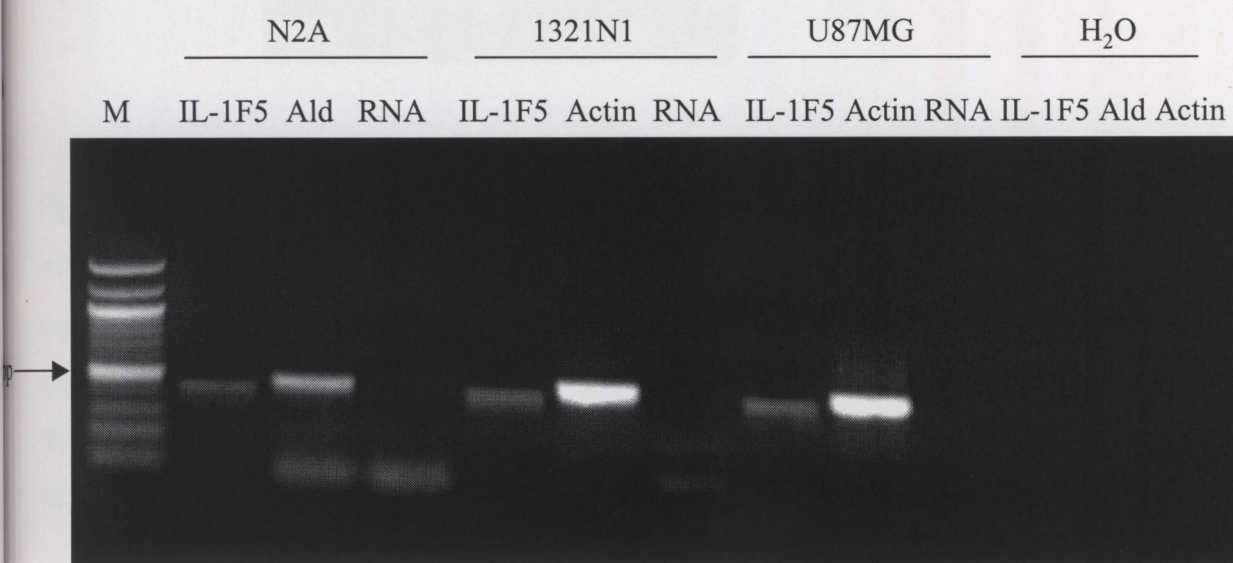
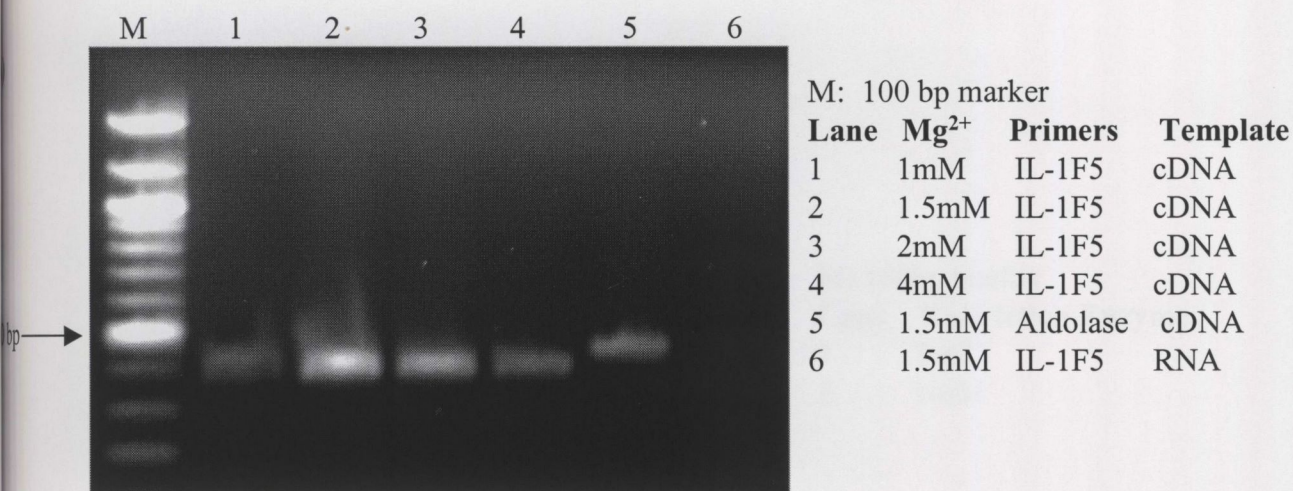


Figure 5.2.27 RT-PCR of IL-1F5 in brain derived cell lines. A) 250 ng mRNA was prepared from neuro2a cells and reverse transcribed to cDNA. DNA was amplified by PCR using primers to IL-1F5 and aldolase. Also included in the PCR was a genomic DNA control with non-reverse transcribed RNA. B) 250 ng RNA prepared from the indicated cell lines was reverse transcribed and amplified by PCR using primers to IL-1F5 and either aldolase for murine cells or actin for human cells at 1.5 mM Mg²⁺. As controls non-reverse transcribed RNA, as well as water were included in the PCR. The expected size for each PCR product is 424 bp for IL-1F5, 442 bp for aldolase and 441 for actin.

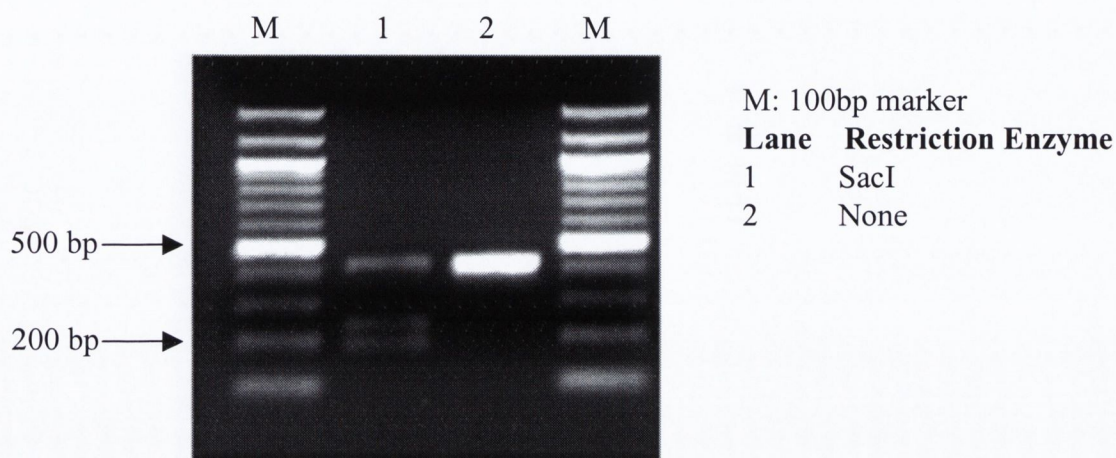


Figure 5.2.28 SacI digestion of PCR product from neuro2a cell line. The product resulting from the PCR of neuronal cell line, neuro2a using IL-1F5 primers was subjected to a SacI restriction enzyme digest at 37°C for 1 hour, or incubated at the same conditions with no restriction enzyme. The resulting products were separated on a 1% agarose, stained with ethidium bromide and visualised under UV light. The reaction in which the SacI was included produced two digested bands around the expected weights of 184 and 238 bp.

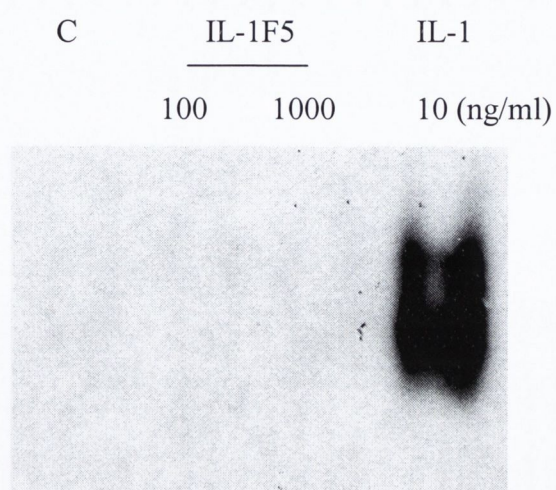


Figure 5.2.29 Effect of IL-1F5 on activation of NFκB in 1321N1 astrocytoma cell line. Cells seeded at 1×10^5 cells/ml were grown in 6 well plates for 48 hours and treated for 30 minutes with indicated doses of IL-1F5 or IL-1. Nuclear extracts were prepared and equal amounts of total protein were examined for NFκB activation. NFκB-DNA complexes are shown. Result is representative of two independent experiments.

U87MG, which also showed no inhibitory effect of IL-1F5 on IL-1 induced NF κ B activation (Figure 5.2.30). In addition, the neuroblastoma cell line, neuro2a, was also assayed for activation of NF κ B using EMSA. While these cells were not responsive to IL-1, they were responsive to TNF, which activates NF κ B at doses of 40 ng/ml. However, IL-1F5 does not activate NF κ B in this cell line at doses up to 1 μ g/ml (Figure 5.2.31) or at various time points from 5 minutes to 2 hours using 500 ng/ml IL-1F5 (Figure 5.2.32). Although some experiments seemed to show a lower shifted band which appeared to be responsive to treatment with IL-1F5, this is likely to be due to unspecific binding of nuclear protein to the oligonucleotide probe. The band also appears in samples incubated with radioactively labelled mutant NF κ B probe, which indicates that the binding protein is not recognising the NF κ B specific sequence GGACTT, which is mutated to CGACTT in the mutant oligonucleotide (Figure 5.2.33). However, the upper band that appears upon TNF stimulation is not sensitive to incubation with unlabelled excess mutant probe (Figure 5.2.34a, lanes 10-13 and figure 5.2.34b, lane 3), but is sensitive to incubation with excess unlabelled wildtype probe (Figure 5.2.34a, lanes 6-9). This upper band is also supershifted by antibodies to the p50 and p65 subunits of NF κ B, confirming its identity as NF κ B (Figure 5.2.34a, lanes 1-5). Furthermore, the lower band is also competed for by excess amounts of other transcription factor consensus sequence oligonucleotides, including Ets and PEA, as well as their respective mutant forms, while the upper band is not (Figure 5.2.34b, lanes 4-7). This indicates that this lower band is not a result of specific binding to these oligonucleotides either, and is thus likely due to non-specific binding of nuclear proteins to the NF κ B oligonucleotide.

The neuro2a cells were also tested for other IL-1 like responses after treatment with IL-1F5. IL-1F5 was tested for its ability to induce degradation of I κ B. However, at doses up to 1 μ g/ml, or at time points from 15 minutes to 2 hours at a dose of 500 ng/ml no degradation could be seen (Figure 5.2.35), whereas 50 ng/ml of TNF did cause degradation of I κ B after a 30 minute stimulation (Figure 5.2.36). In addition IL-1F5 was unable to cause processing of p105, which leads to the release of the p50 subunit of the

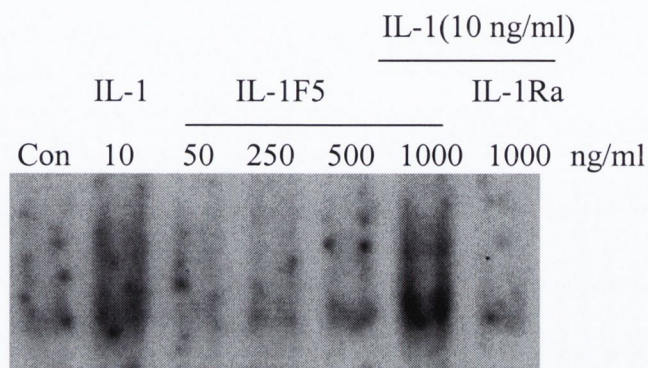


Figure 5.2.30 Effect of IL-1F5 on activation of NFκB in U87MG glioblastoma-astrocytoma cell line. Cells seeded at 1×10^5 cells/ml were grown in 6 well plates for 48 hours and treated either alone for 30 minutes with indicated doses of IL-1F5 or IL-1, or pre-treated for 1 hour with 1 μ g/ml of IL-1F5 or IL-1Ra before stimulation with 10 ng/ml IL-1 for 30 minutes. Nuclear extracts were prepared and equal amounts of total protein were examined for NFκB activation. NFκB-DNA complexes are shown. Result is representative of two independent experiments.

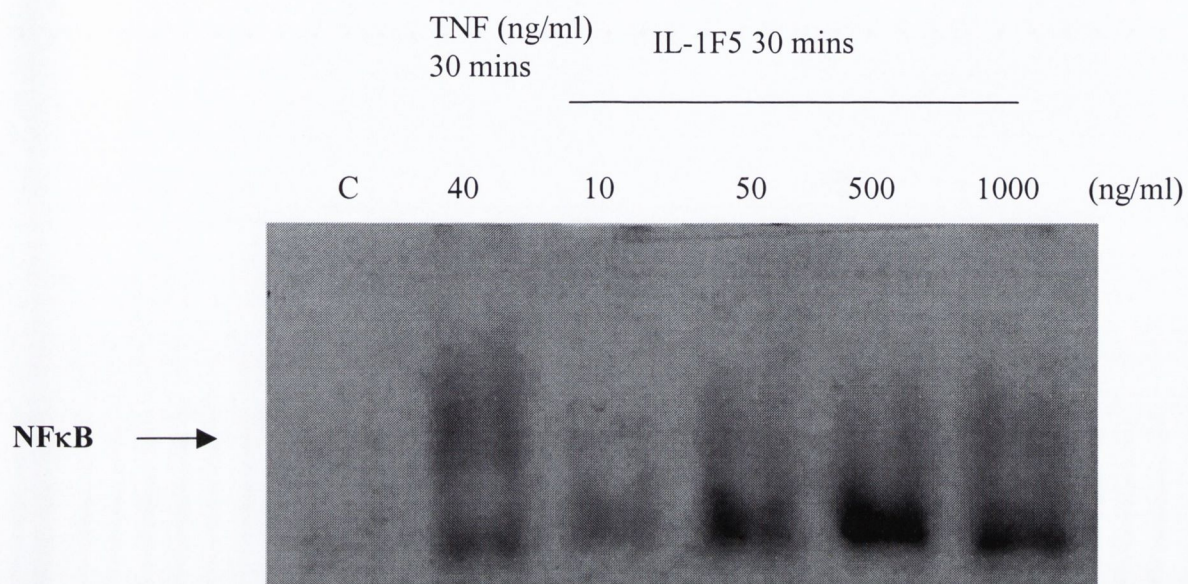


Figure 5.2.31 Activation of NFκB in neuro2a cell line in response to IL-1F5 and TNF. Cells seeded at 1×10^5 cells/ml were grown in 6 well plates for 48 hours and pre-treated for 1 hour with indicated doses of IL-1F5 or TNF before treatment with 1 ng/ml of IL-1 for 30 minutes. Nuclear extracts were prepared and equal amounts of total protein were examined for NFκB activation. NFκB-DNA complexes are shown with an arrow. Result is representative of three independent experiments.

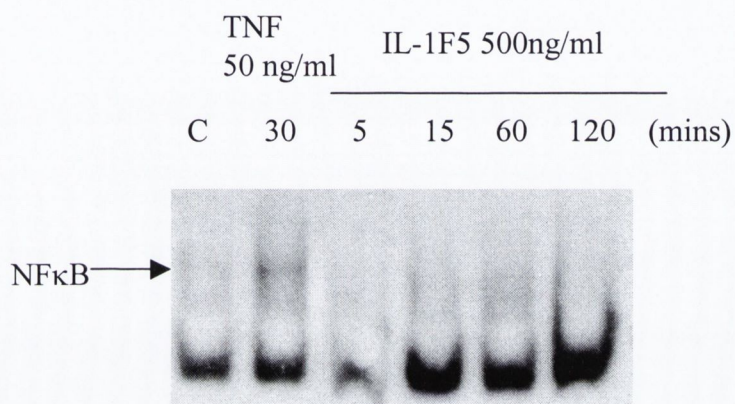


Figure 5.2.32 Time course of the activation of NFκB DNA binding activity in neuro2a cell line in response to IL-1F5. Cells were seeded at 1×10^5 cells/ml in a 6 well plate and grown for 48 hours. Cells were stimulated with 500 ng/ml IL-1F5 for indicated times and with 50 ng/ml TNF for 30 minutes. Nuclear extracts were prepared and equal amounts of total protein were examined for NFκB activation. NFκB-DNA complexes are shown. Result is representative of three independent experiments.

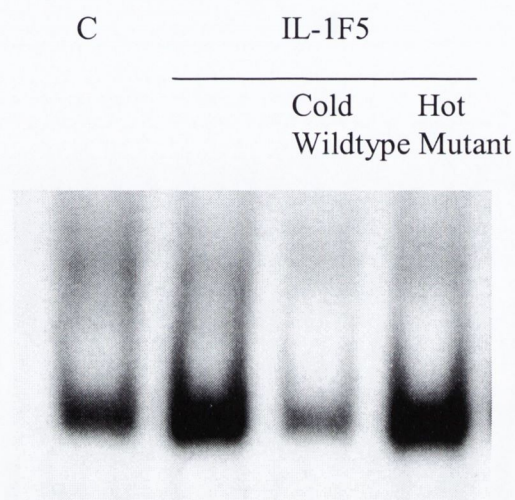


Figure 5.2.33 Wildtype competition and mutant oligonucleotide binding properties of lower complex in neuro2a nuclear extracts assayed by EMSA. 4 μ g of nuclear extract from either untreated or IL-1F5 treated neuro2a cells were incubated as indicated either with excess unlabelled NF κ B oligonucleotide or with radioactively labelled mutant NF κ B oligonucleotide, and examined for protein-DNA binding activity. Protein-DNA complexes are shown. Result is representative of two independent experiments.

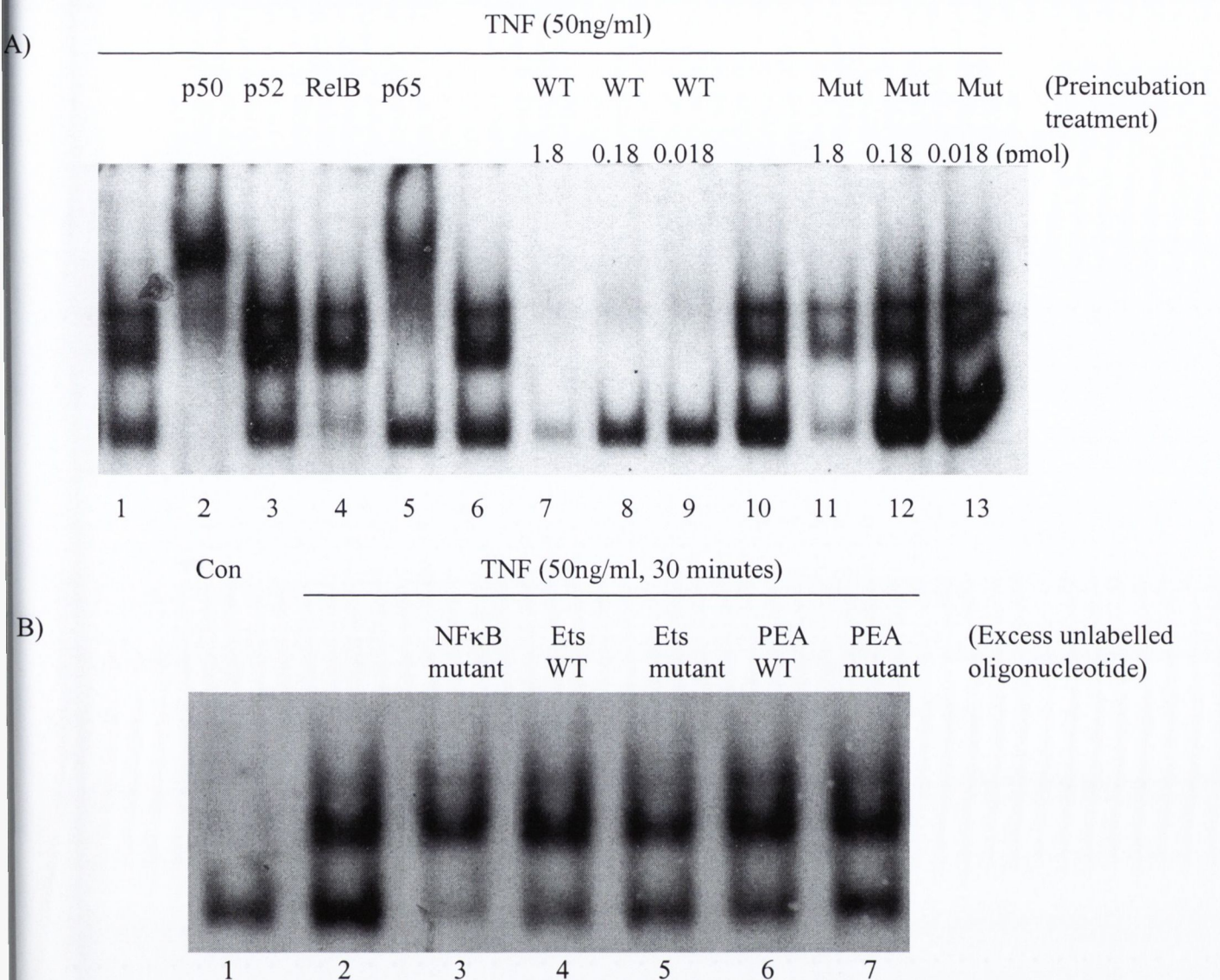


Figure 5.2.34 Competition and supershift studies of neuro2a nuclear extracts assayed by EMSA using NFκB oligonucleotide. A) 4 μg of nuclear extract from TNF treated neuro2a cells were preincubated with either 1 μl of indicated antibodies to NFκB subunit proteins and incubated on ice for 30 minutes (lanes 2-5), or preincubated at room temperature for 30 minutes with either 1.8, 0.18 or 0.018 pmol unlabelled wildtype or mutant probe (lanes 7-9 and 11-13) prior to incubation with radioactively labelled NFκB oligonucleotide and examined for protein-DNA complexes. B) 4 μg of nuclear extract from TNF treated neuro2a cells were preincubated with 1.8 pmol of indicated unlabelled oligonucleotides, followed by incubation with radioactively labelled wildtype NFκB oligonucleotide and examined for protein-DNA complexes. Result is representative of two independent experiments.

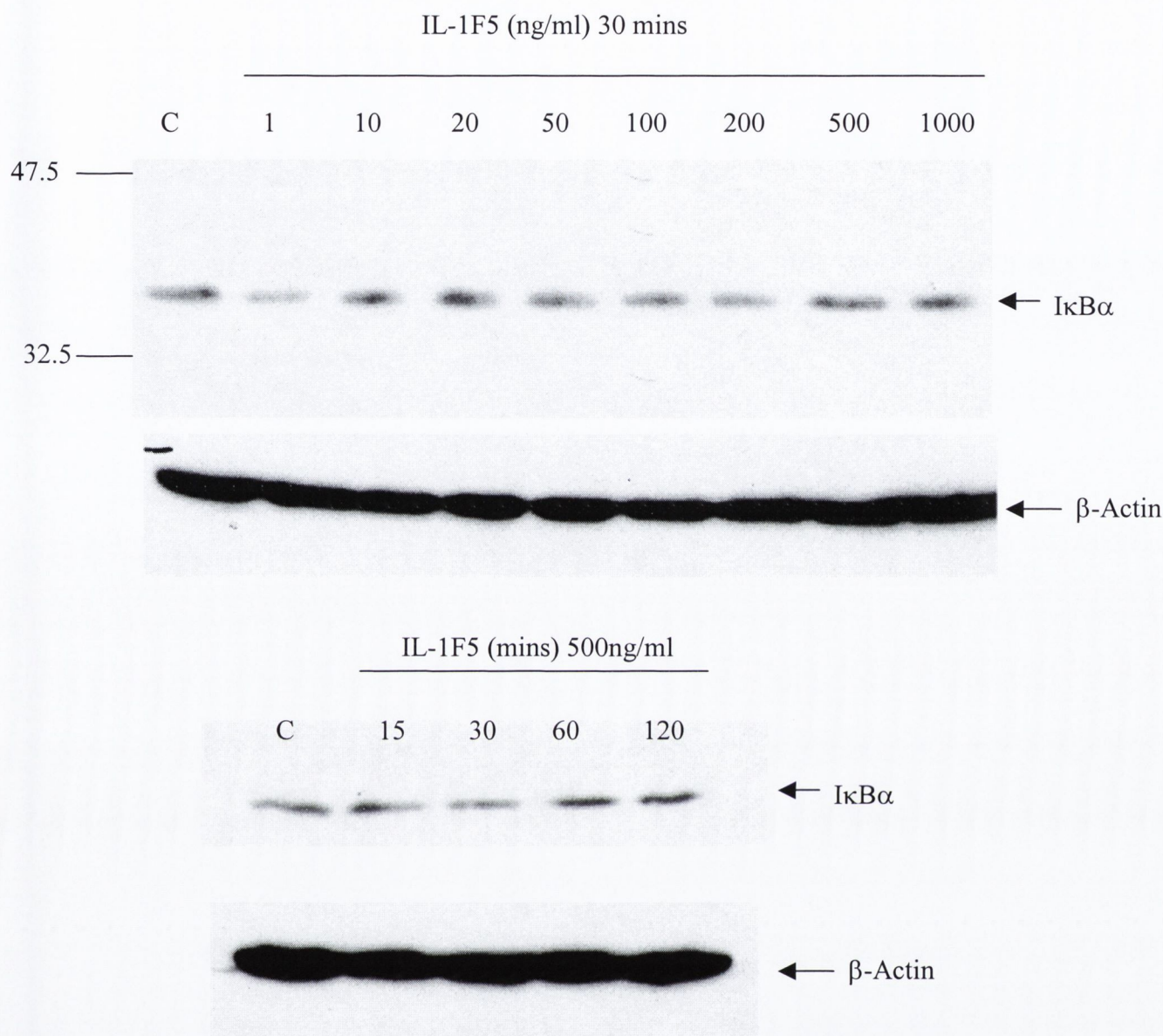


Figure 3.2.35 Lack of IkB degradation in neuro2a cell line in response to IL1-F5 treatment in time course and dose response assays. Neuro2a cells were seeded at 1×10^5 cells/ml and grown in 6 well plates for 48 hours before treatment as indicated with IL-1F5. Cells were washed with ice cold PBS, lysed in 150 μ l lysis buffer and the DNA sheared by repeated passing of the sample through a 21 gauge needle. Samples were boiled and separated on 10% polyacrylamide gel, transferred to nitrocellulose and blotted with anti-IkB antibody and developed using chemiluminescence. Identical samples were also analysed for β -actin content as a loading control. Result is representative of three independent experiments.

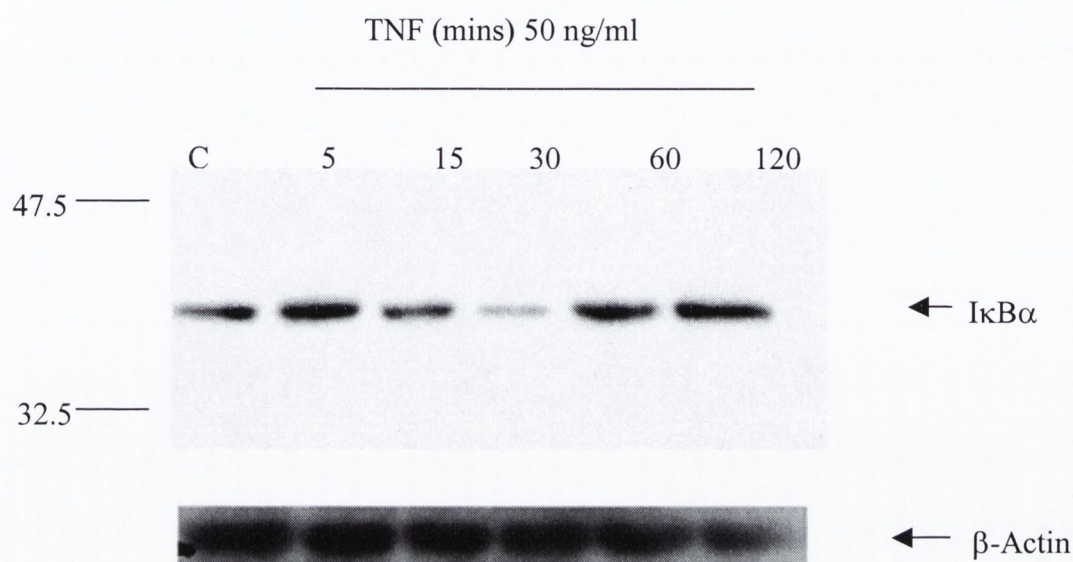


Figure 5.2.36 IκB degradation in response to TNF treatment in time course and dose response assays. Neuro2a cells were seeded at 1×10^5 cells/ml and grown in 6 well plates for 48 hours before treatment as indicated with TNF. Cells were washed with ice cold PBS, lysed in 150 μ l lysis buffer and the DNA sheared by repeated passing of the sample through a 21 gauge needle. Samples were boiled and separated on 10% polyacrylamide gel, transferred to nitrocellulose and blotted with anti-IκB antibody and developed using chemiluminescence. Identical samples were also analysed for β-actin content as a loading control.

NFκB dimer, at doses from 1 ng/ml to 500 ng/ml, or at times from 5 minutes to 2 hours at a dose of 500 ng/ml (Figure 5.2.37).

To test the ability of IL-1F5 to activate the transcriptional activity of NFκB, an NFκB dependent luciferase construct was transfected into neuro2a cells. Tests showed that TNF could efficiently drive this construct, with the greatest fold increase in luciferase activity seen when 0.625 ng of NFκB-luciferase and 0.31 ng of thymidine kinase promoter-renilla luciferase, which is used to standardise results according to transfection efficiency. Using these conditions, a 6 hour treatment with TNF resulted in an eight fold increase in luciferase activity over untreated samples (Figure 5.2.38). Under the same conditions, the effect of treatment with IL-1F5 was examined, however no activation was seen at doses up to 500 ng/ml after 4, 6, or 24 hours of treatment (Figure 5.2.39). Examining the activation of MAP kinases in addition to NFκB activation was carried out using an IL-8 promoter-luciferase reporter construct. Using doses of between 0.3 ng and 1.25 ng of IL-8 promoter luciferase construct did not significantly alter the fold activation that could be detected with 50 ng/ml TNF, therefore 0.625 ng of the construct was used, as for the NFκB-luciferase construct (Figure 5.2.40). When treated for 24 hours with IL-1F5, at doses up to 500 ng/ml, the neuro2a cells showed no activation of the IL-8 promoter (Figure 5.2.41). Finally, treatment of these cells with IL-1F5 failed to produce any significant changes in the level of phosphorylation of p42/44 over a range of times tested from 5 minutes to 1 hour (Figure 5.2.42). However, PMA, which is a strong activator of p42/44, also failed to increase the phosphorylation state of p42/44. Despite the fact that the cells were serum starved in 0.5% serum for 48 hours prior to stimulation the cells still exhibited a high basal level of p42/44 phosphorylation and this may be the reason why activation of the MAP kinase was difficult to detect.

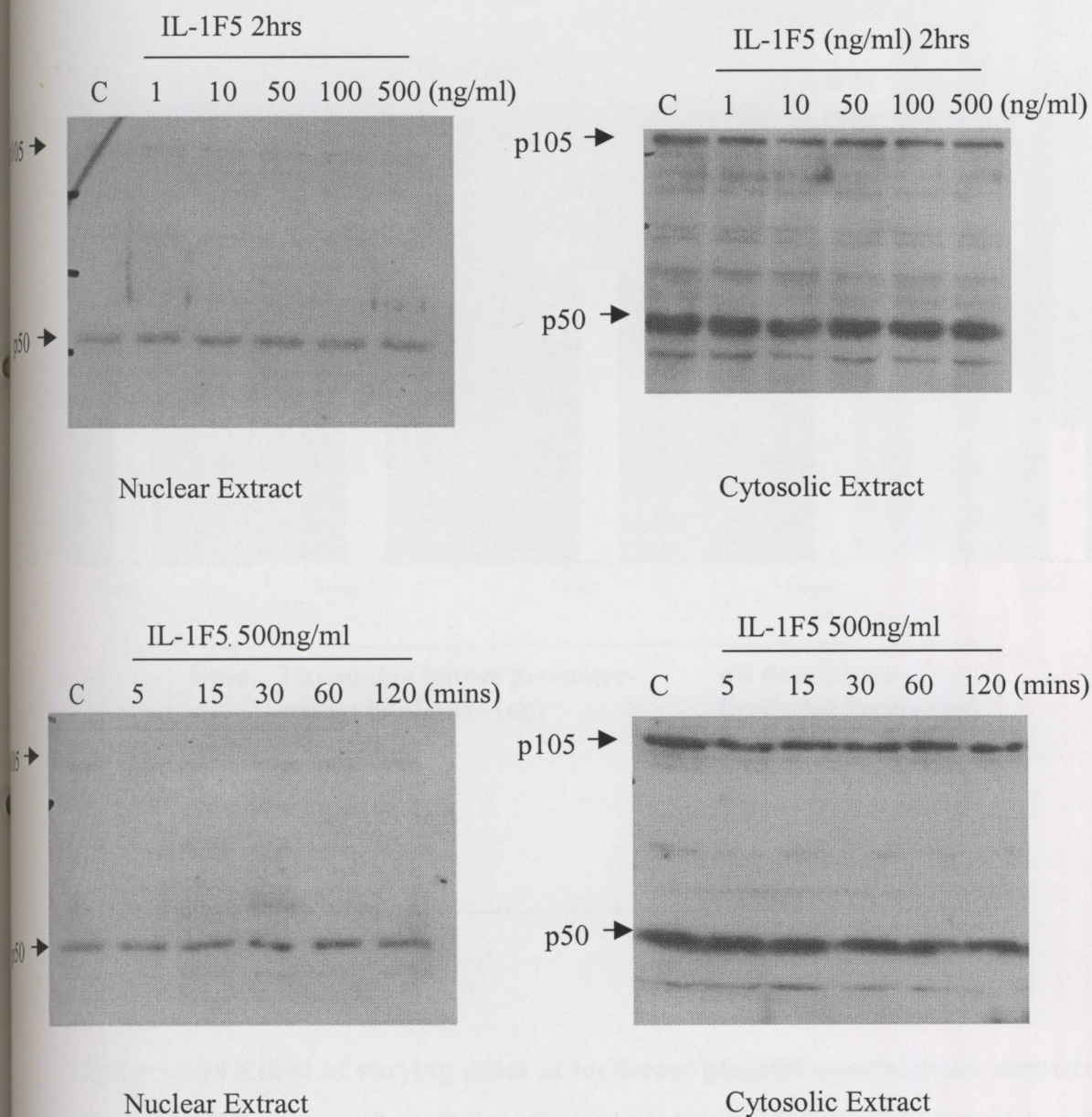
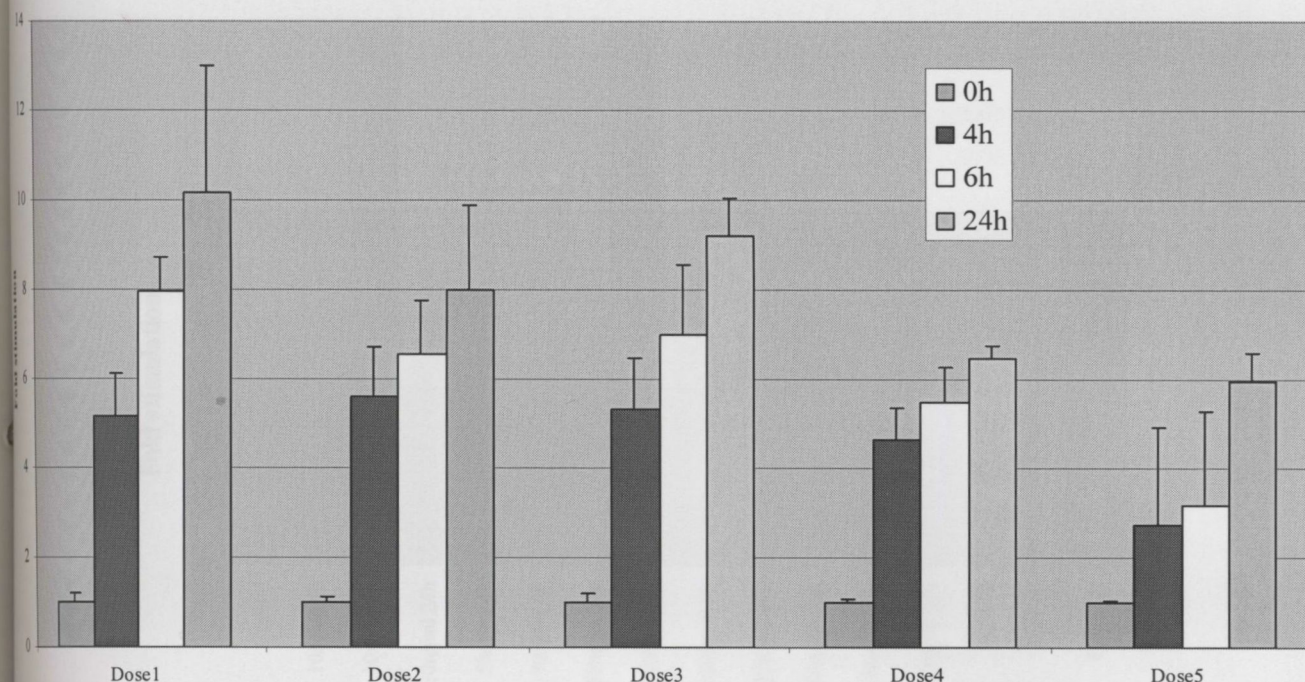


Figure 5.2.37 Effect of IL-1F5 on p105 degradation in time course and dose response assays. Neuro2a cells were seeded at 1×10^5 cells/ml and grown in 6 well plates for 48 hours before treatment as indicated with IL-1F5. Cells were washed with ice cold PBS and nuclear and cytosolic extracts were prepared. Samples were equalised for protein content and separated on 10% polyacrylamide gels, transferred to nitrocellulose and blotted with anti-p105/p50 antibody and developed using chemiluminescence. Result is representative of two independent experiments.



Dose	Thymidine kinase promoter-renilla luciferase (ng)	κ B dependent-firefly luciferase (ng)
1	0.31	0.625
2	0.625	1.25
3	1.25	2.5
4	2.5	5
5	5	10

Figure 5.2.38 Effect of varying doses of luciferase plasmid constructs on measured effects of TNF on neuro2a cell line. Cells seeded at 1×10^5 cells/ml in 200 μ l in a 96 well plate were grown for 24 hours before transfection using Gene Juice with κ B dependent-luciferase plasmid and the constitutively active thymidine kinase promoter-renilla luciferase plasmid. Cells were left for a further 24 hours before stimulation in triplicate with indicated doses of TNF for 4, 6 or 24 hours. Cells were lysed and assayed for firefly luciferase activity as a measure of κ B dependent activity. Results represent mean values of triplicate samples standardised for transfection efficiency with renilla luciferase activity. Result is representative of two independent experiments.

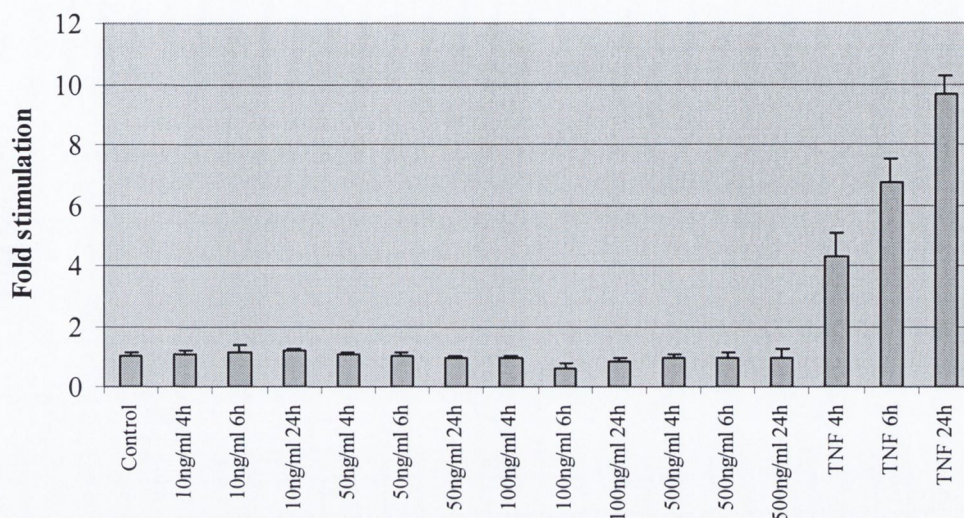
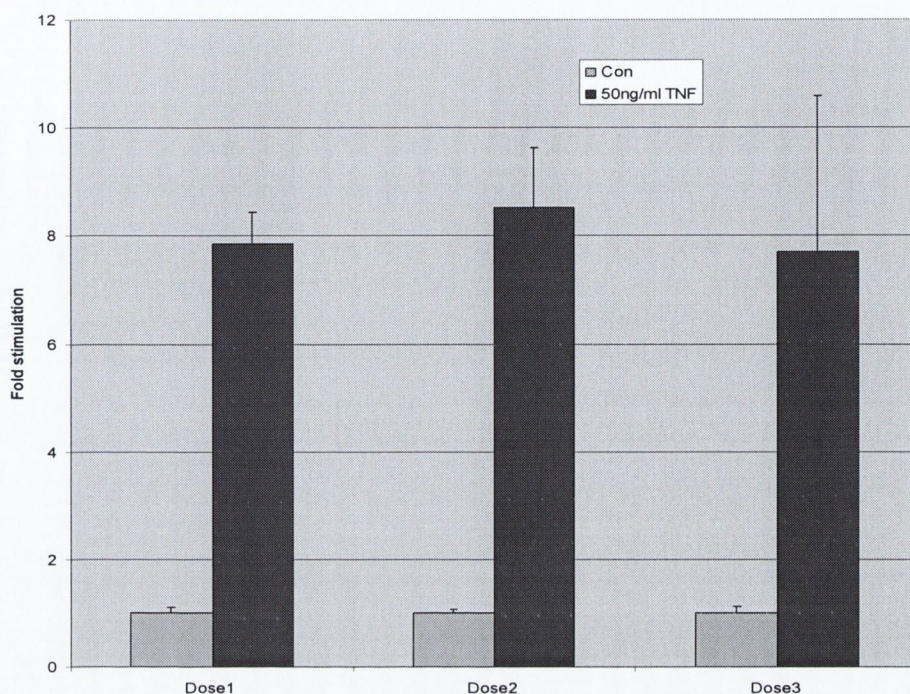


Figure 5.2.39 Effect of IL-1F5 on activation of κ B dependent luciferase in neuro2a cell line. Cells seeded at 1×10^5 cells/ml in 200 μ l in a 96 well plate were grown for 24 hours before transfection using Gene Juice with NF κ B dependent γ -luciferase plasmid and thymidine kinase promoter-renilla luciferase plasmid. Cells were left for a further 24 hours before stimulation in triplicate with indicated doses of IL-1F5 or TNF for 4, 6 or 24 hours. Cells were lysed and assayed for firefly luciferase activity as a measure of κ B dependent luciferase activity. Results represent mean values of triplicate samples standardised for transfection efficiency with renilla luciferase activity. Result is representative of two independent experiments.



Dose	Thymidine kinase promoter renilla luciferase (ng)	IL-8 promoter firefly luciferase (ng)
1	1.25	0.31
2	1.25	0.625
3	1.25	1.25

Figure 5.2.40 Effect of varying doses of IL-8 promoter luciferase constructs on measured effects of TNF on neuro2a cell line. Cells seeded at 1×10^5 cells/ml in 200 μ l in a 96 well plate were grown for 24 hours before transfection using Gene Juice with IL-8 promoter-luciferase plasmid and the constitutively active thymidine kinase promoter-renilla luciferase plasmid. Cells were left for a further 24 hours before stimulation in triplicate with 50 ng/ml TNF for 6 hours. Cells were lysed and assayed for firefly luciferase activity as a measure of IL-8 promoter activity. Results represent mean values of triplicate samples standardised for transfection efficiency with renilla luciferase activity. Result is representative of two independent experiments.

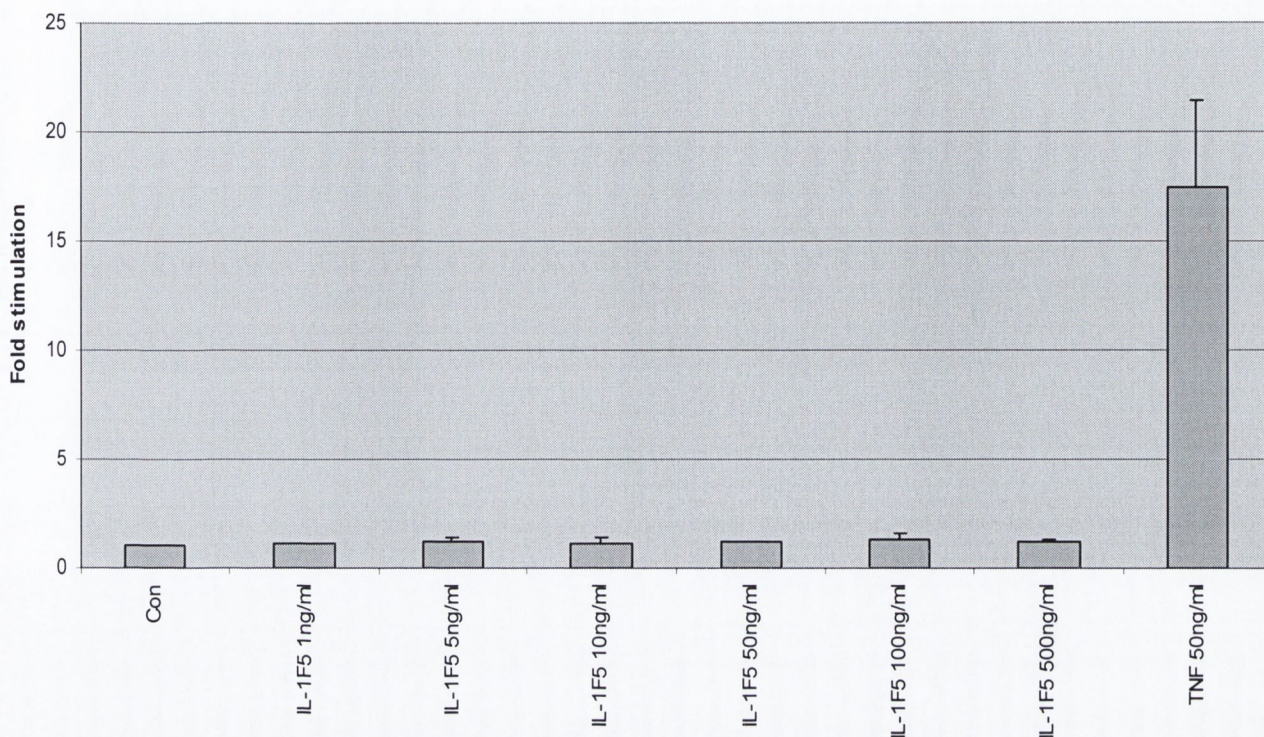


Figure 5.2.41 Effect of IL-1F5 on activation of IL-8 promoter luciferase in neuro2a cell line. Cells seeded at 1×10^5 cells/ml in 200 μ l in a 96 well plate were grown for 24 hours before transfection using Gene Juice with IL-8 promoter-luciferase plasmid and thymidine kinase promoter-renilla luciferase plasmid. Cells were left for a further 24 hours before stimulation in triplicate with indicated doses of IL-1F5 or TNF for 6 hours. Cells were lysed and assayed for firefly luciferase activity as a measure of IL-8 promoter luciferase activity. Results represent mean values of triplicate samples standardised for transfection efficiency with renilla luciferase activity. Result is representative of two independent experiments.

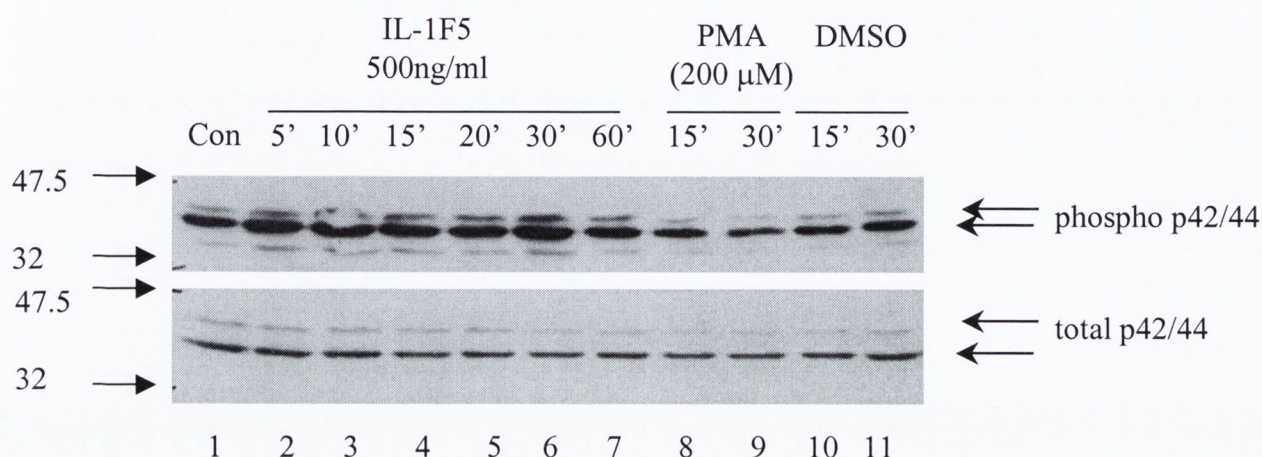


Figure 5.2.42 The effect of IL-1F5 on the activation of MAP kinase p42/44 in neuro2a cell line. Cells seeded at 1×10^5 cells/ml were grown in 6 well plates overnight before the growth medium was changed to 0.5% (v/v) serum. The cells were grown for a further 48 hours before being treated for indicated times with 500 ng/ml IL-1F5, as well as 200 μ M PMA and equivalent volumes of DMSO. Total cell lysates were prepared and passed through a 21 gauge needle to shear DNA. Proteins were separated on a 12% polyacrylamide gel, transferred to nitrocellulose and probed by western blot analysis using anti-p42/44 and anti-phospho p42/44 antibodies. Total p42/44 acts as a loading control. Result is representative of three independent experiments.

5.3 Discussion.

5.3.1 The Effect of IL-1F5 and IL-1F6 on IL-1 Induced Cellular Responses.

IL-1Ra is capable of competitively inhibiting IL-1 activity by binding to IL-1RI (Arend et al., 1998). Due to the homology between IL-1F5, IL-1F6 and IL-1Ra, the possibility that these proteins might act in a similar fashion was investigated. Because the proteins used in these investigations were novel proteins with unknown biological activity, it was important to confirm the integrity of the preparations used. Both Coomassie and western blot examination of the proteins showed single bands around the expected molecular weight, with a higher molecular weight of IL-1F5 possibly due to glycosylation of the protein. The absence of lower molecular weight bands indicates that the preparations are not degraded and assuming correct folding should be biologically active.

Many lines of evidence from this study suggest however, that IL-1F5 and IL-1F6 do not act in a fashion similar to IL-1Ra. Firstly, IL-1F5 and IL-1F6 were unable to inhibit IL-1 induced nuclear translocation and DNA binding of NF κ B in four different cell types all known to express IL-1RI and be responsive to IL-1. No inhibitory activity was seen at concentrations up to 1000 fold that of IL-1. In comparison, IL-1Ra was capable of inhibiting IL-1 activity at concentrations 100 fold that of IL-1. In addition, IL-1F5 and IL-1F6 are unable to significantly inhibit transcriptional activation of NF κ B at levels 100 fold that of IL-1, as assayed in the gene reporter system in T24 cells. In comparison, at levels 10 fold that of IL-1, IL-1Ra was clearly able to strongly inhibit IL-1 induced transcriptional activation of NF κ B. There was a slight blunting effect seen on the activity of IL-1 when cells were pre-treated with either IL-1F5 or IL-1F6. However, several lines of evidence suggest that this may not be a specific effect. Firstly, similar concentrations of IL-1F5 and IL-1F6 were not capable of causing significant inhibition of IL-1 induced transactivation of the p65 subunit of NF κ B in T24 cells. This pathway would be a major target for any signal likely to cause regulation of NF κ B activity. Regulation of the transactivation pathway by IL-1, via Rac and the MAP kinases leads to alteration of the phosphorylation state of p65, which in turn regulates the transcriptional activity of NF κ B

independent of its DNA binding activity (Jefferies et al., 2001). The E-selectin promoter used in these gene reporter assays has four transcription binding sites, three bind NF κ B and the other is an NF-ELAM-1 binding site which contains the binding motif for ATF2/c-Jun transcription factors (Nawa et al., 2000). These transcription factors have been shown to be essential components in the activation of the E-selectin promoter and are themselves activated by the MAP kinases p38 and JNK (Tamaru and Narumi, 1999). However, in Saos-2 cells, neither IL-1F5 nor IL-1F6 were able to cause inhibition of IL-1 induced activation of either p38 or JNK as measured by their phosphorylation state. Neither were they able to cause inhibition of p38 phosphorylation in T24 cells. Therefore, it is difficult to explain the blunting effect of the IL-1 induced activation the E-selectin promoter, certainly it is an effect that does not mimic that of IL-1Ra treatment and therefore, if it is a real effect, it may be as a result of some subtle mechanism of inhibition.

Taken together these data indicate that IL-1F5 and IL-1F6 were unable to inhibit multiple IL-1 induced activities when applied extracellularly to cells in high concentrations. This suggests that IL-1F5 and IL-1F6 are unable to bind IL-1 receptors in a manner similar to that of IL-1Ra. These data are in agreement with that published by Barton et al. (2000) who likewise could find no IL-1Ra like activity for IL-1F5.

5.3.2 The Effect of IL-1F5 and IL-1F6 on IL-18 Induced Activation of NF κ B.

Given the similarity between IL-1 and IL-18 (Dinarello et al., 1998) it was possible that IL-1F5 and IL-1F6 might act as IL-18 antagonists. Studies showed that in EL4.NOB-1 cells 100 ng/ml IL-18 could activate NF κ B as assayed using EMSA. However, pre-treatment with either IL-1F5 or IL-1F6 showed no inhibition of this signal. Since only a ten fold excess of the novel IL-1s was used in these experiments, the possibility remains that higher doses to IL-1F5 and IL-1F6 may inhibit IL-18. Thus studies completed provide no evidence that IL-1F5 or IL-1F6 can competitively bind to the IL-18 receptor. Again, this is in agreement with the data published by Barton et al. (2000), who could find no antagonism of IL-18 signalling by IL-1F5. Together these data indicate that

neither IL-1F5 nor IL-1F6 can act as antagonist for the IL-1 or IL-18 systems. However the possibility remains that they could have such activities through other members of the IL-1R family. Indeed, Debets et al. (2001) report that IL-1F5 can inhibit IL-1F9 induced NF κ B activation in IL-1Rrp2 transfected Jurkat cells. However, direct binding of IL-1F5 to IL-1Rrp2 was not shown, and the result remains subject to confirmation by these and other techniques.

5.3.3 Activating Effects of IL-1F5 and IL-1F6.

Although there was no evidence for any inhibitory effects of IL-1F5 and IL-1F6 it was very possible that they act as agonists at the cell surface. Indeed, examination of the amino acid sequence shows certain traits which indicate that this indeed could be the case (see Chapter 3). If this were true it could be expected that they would activate similar pathways to those activated by IL-1, akin to the manner in which IL-1 and IL-18 have similar signalling pathways (see section 1.5.8). However, no stimulatory effect was detected in terms of NF κ B nuclear translocation and DNA binding in HeLa, EL4.NOB.1, T24, Saos-2, HL-60 or K562 cell lines, transcriptional activation in T24 cell line, STAT3 activation in T24 and Saos-2 cell lines or MAP kinase activation in T24 and Saos-2 cell lines. In all cases concentrations were used which were well above the concentration at which IL-1 would normally elicit its effects. Furthermore, the possibility that IL-1F5 and IL-1F6 might be ligands for the orphan receptor T1/ST2 was also examined by using two cell lines that are known to express the receptor, however this also failed to yield any positive data in two different cell lines used. Although chimeric proteins of the extracellular domain of IL-1RI fused to the intracellular domain of T1/ST2 have been shown to activate NF κ B (Mitcham et al., 1996), studies in our laboratory suggest T1/ST2 does not activate NF κ B (Brint et al., 2002). The chimera may activate NF κ B by recruiting the IL-1RAcP. Therefore, other signals such as p38 or JNK activation could also be investigated in these cell lines. These data suggest that the receptors for IL-1F5 and IL-1F6 are not present in the cell lines used, at least at the plasma membrane. It remains a possibility that in a physiological setting these proteins may act intracellularly. Like IL-1 itself, the novel proteins lack any leader sequence recognised to target it for

secretion. However, IL-1F5 has been shown to be secreted from the trophoblastic cell line, JEG-3, suggesting it does indeed act as an extracellular protein (Barton et al., 2000).

5.3.4 Expression Patterns of IL-1F5

In order to try to localise attempts to find functional activity for the novel IL-1s it was decided to screen for expression of IL-1F5 and use sites of expression as target cells rather than use cell lines which were known to respond to IL-1. Towards this end IL-1F5 was found to be expressed well in murine cortex but not other brain regions such as the cerebellum. Furthermore, expression could also be detected in several brain derived cell lines including the neuroblastoma cell line, neuro2a, the astrocytoma cell line 132N1 and the glioblastoma-astrocytoma cell line U87MG. When IL-1F5 was tested for functional activity in neuro2a cells, as in previous assays, no agonist activity for the protein could be found. Antagonist activity against IL-1 itself could not be tested in these cells since they were found to be unresponsive to treatment with IL-1. Agonist activity was tested using several different approaches and assays including EMSA to detect nuclear translocation of NF κ B, gene reporter assays to detect transcriptional activity of NF κ B as well as AP-1 transcriptional activity using both a κ B-luciferase construct and an IL-8 promoter-luciferase construct, and also western blotting of p105 to detect processing and activation of the p50 NF κ B subunit, I κ B to detect degradation of I κ B, and p42/44 to detect phosphorylation and activation of the MAP kinase. Other brain derived cell lines such as the astrocytoma cell line 1321M1 and U87 MG were also assayed for responsiveness to IL-1F5 but no activity could be detected in these cell lines either. In addition to this, work carried out in collaboration with Nancy Rothwell (University of Manchester) showed no activation in response to treatment with up to 100 ng/ml IL-1F5 in terms of release of IL-6, activation of MAP kinases, or activation of NF κ B in mixed glial cultures used at 13 days *in vitro*. IL-1 activated all the above responses, but IL-1F5 failed to inhibit this activity at a ten fold excess concentration.

5.3.5 Concluding Remarks

It is possible that the lack of activity of IL-1F5 and IL-1F6 described above was due to the proteins being defective. Initially the proteins were derived from over-expression systems in human HEK293 cells. Although this method is used for the production of many functional proteins, these cells may be unable to produce fully functional IL-1F5 and IL-1F6. In later experiments recombinantly produced protein was used. This protein was examined by circular dichroism to evaluate whether secondary structure elements were present in the purified product as an indication that the protein was properly folded, this indeed was found to be the case (see Chapter 4). Also, the same material was used to produce crystals and solve the structure of IL-1F5; this indicates that the protein was indeed properly folded (see Chapter 6). The possibility of misfolded material in the earlier experiments however, is difficult to rule out until some biological activity for the proteins has been established.

The assays used in these experiments described here, all concentrated on looking for signals that mimic those activated by IL-1 itself. It could be the case however, that IL-1F5 and IL-1F6 do not activate any of these signals, but has distinct signals of their own. To this end it would be worth, for future investigations to look at other, more global signals that would give an indication of IL-1F5 or IL-1F6 activity. Such experiments might include examining the ability of IL-1F5 and IL-1F6 to induce protein tyrosine phosphorylation, which can be achieved in a western blot assay, or looking at the ability of IL-1F5 to bind to cell surfaces. This assay could be carried out using fluorescently labelled IL-1F5 or IL-1F6. Upon addition of labelled protein to cells, and after washing, cells can be measured for increases in their fluorescence as an indicator of binding. Since this assay examines the obligatory first step in the activation of signal transduction, receptor binding, it relegates the possibility that the wrong downstream signal is been examined.

In the absence of any biological activity that could give clues to the function of IL-1F5 it was decided to pursue examination of the structure of the protein by crystallographic methods.

Chapter 6. Determination of the Crystal Structure of Murine IL-1F5.

6.1 Introduction

The crystal structure of human IL-1 β has been solved by crystallographic methods to 2 Å (Finzel et al., 1989; Priestle et al., 1988a; Veerapandian et al., 1992) and by NMR (Clore et al., 1991). In addition, the structure of murine IL-1 β has also been solved to 2.8 Å (van Oostrum et al., 1991), and this structure shows that the paralogue IL-1 β proteins have almost identical folds, which superimpose with an RMS of 0.86 Å. The structure of IL-1 α has also been solved, with the C α trace publicly available in the Protein Database (Graves et al., 1990), while the structure of IL-1Ra has been solved to 2 Å by crystallographic methods (Schreuder et al., 1995; Vigers et al., 1994) and by NMR (Stockman et al., 1994). The structures of both IL-1 β and IL-1Ra in complex with IL-1RI are also known (Schreuder et al., 1997; Vigers et al., 1997). All these structures have given insights into the mechanism of binding and activity of IL-1 and IL-1Ra. Extensive site directed mutagenesis backs-up the data derived from the structural analysis, as well as giving other insights into residues which may be important contributors to both the structural integrity of the IL-1 proteins and their ability to interact with the IL-1RAcP. The structure of IL-1 β and IL-1Ra in complex with IL-1RI indicates that these two molecules interact with the receptor using similar regions. Therefore, using this knowledge, along with the structure of IL-1F5, insights can be gained as to which region of the structure are likely to be important for conferring receptor recognition and specificity and functionality to the protein.

Murine IL-1F5 is 29% identical to murine IL-1 β at the amino acid level and as discussed in chapter 3 is very likely to have a similar 3 dimensional structure. Therefore, IL-1 β could act as a good template for solving the phase problem for IL-1F5 by molecular replacement. In this method, the phases from the IL-1 β structure are used as a first

estimate to solve the structure of IL-1F5. Before this can be achieved however, both the orientation and the position of IL-1F5 within its unit cell must first be solved. Once a first estimation of the phase is obtained, a model of the structure can be built and refined and new phases obtained. Through an iterative process of refinement and phase determination, the model structure can be improved until no further improvement is achieved and a low R-factor is obtained as an indication that the model very closely approximates the true structure of the proteins. These methods were used to solve the structure of murine IL-1F5.

6.2 Results and Discussion

6.2.1 Crystallisation of IL-1F5

Murine recombinant IL-1F5 purified as described in Chapter 4 was used to screen potential crystallisation conditions for the protein using Hampton Research Crystal Screen 1 and 2. This involved screening of 98 different conditions with varying salts and precipitants and at varying concentrations and pHs (Table 6.2.1). Crystal screen 1 contained 50 conditions described by Jancarik and Kim (1991) as those that have been most commonly reported to crystallise a wide variety of proteins. The remaining 48 are more 'exotic' variations on the first 50. The conditions used for crystallisation was that of vapour diffusion by the hanging drop method. In this method of crystallisation, a small volume of concentrated protein is mixed with an equal volume of precipitant (typically 1-2 μ l) on a coverslip and suspended over a reservoir of the precipitating solution of much greater volume (typically 1ml). The coverslip is sealed over the reservoir well with wax to prevent equilibration with the outside environment. Nucleation occurs firstly through supersaturation, which is achieved by diffusion of water away from the drop, through air to the more hygroscopic reservoir solution. As nucleation proceeds macromolecular concentration falls, allowing for conditions that favour crystal growth rather than nucleation (Figure 6.2.1).

Protein, at a concentration of 5 mg/ml, which was dialysed into water, was used in the crystallisation trials, which were set up in duplicate for incubation at both 4°C and room

Crystal Screen 2

Sample:	5mg/ml IL-1F5		
Buffer:	Water		
Reservoir Volume:	1ml		
Drop:	2 μ l		
Temperature:	22°C and 4°C		
		22°C	4°C
1. 10% PEG 6000, 2.0 M Sodium Chloride		Precipitate	Precipitate
2. 0.5 M Sodium Chloride, 0.01M CTAB, 0.01M Magnesium Chloride		Clear	Clear
3. 25% Ethylene Glycol		Clear	Clear
4. 35% Dioxane		Precipitate	Precipitate
5. 5% iso-Propanol, 2.0 M Ammonium Sulfate		Quasi	Quasi
6. 1.0 M Imidazole pH 7.0		Clear	Clear
7. 10% PEG 1000, 10% PEG 8000		Clear	Clear
8. 10% Ethanol, 1.5 M Sodium Chloride		Clear	Clear
9. 2.0 M Sodium Chloride, 0.1M Na Acetate pH 4.6		Clear	Clear
10. 30% MPD, 0.1M Na Acetate pH 4.6, 0.2 M Sodium Chloride		Precipitate	Clear
11. 1.0 M 1,6 Hexanediol, 0.1M Na Acetate pH 4.6, 0.01M Cobalt Chloride		Precipitate	Clear
12. 30% PEG 400, 0.1M Na Acetate pH 4.6, 0.1M Cadmium Chloride		Precipitate	Precipitate
13. 30% PEG MME 2000, 0.1M Na Acetate pH 4.6, 0.2 M Ammonium Sulfate		Quasi	Quasi
14. 2.0 M Ammonium Sulfate, 0.1M Na Citrate pH 5.6, 0.2 M K/Na Tartrate		Quasi	Quasi
15. 1.0 M Lithium Sulfate, 0.1M Na Citrate pH 5.6, 0.5 M Ammonium Sulfate		Quasi	Clear
16. 2% Polyethyleneimine, 0.1M Na Citrate pH 5.6, 0.5 M Sodium Chloride		Clear	Clear
17. 35% tert-Butanol, 0.1M Na Citrate pH 5.6		Precipitate	Clear
18. 10% Jeffamine M-600, 0.1M Na Citrate pH 5.6, 0.01M Ferric Chloride		Clear	Clear
19. 2.5 M 1,6 Hexanediol, 0.1M Na Citrate pH 5.6		Clear	Clear
20. 1.6 M Magnesium Sulfate, 0.1M MES pH 6.5		Clear	Quasi
21. 2.0 M Sodium Chloride, 0.1M MES pH 6.5, 0.2 M Na/K Phosphate		Clear	Clear
22. 12% PEG 20,000, 0.1M MES pH 6.5		Clear	Clear
23. 10% Dioxane, 0.1M MES pH 6.5, 1.6 M Ammonium Sulfate		Clear	Quasi
24. 30% Jeffamine M-600, 0.1M MES pH 6.5, 0.05 M Cesium Chloride		Precipitate	Clear
25. 1.8 M Ammonium Sulfate, 0.1M MES pH 6.5, 0.01M Cobalt Chloride		Quasi	Quasi
26. 30% PEG MME 5000, 0.1M MES pH 6.5, 0.2 M Ammonium Sulfate		Clear	Clear
27. 25% PEG MME 550, 0.1M MES pH 6.5, 0.01M Zinc Sulfate		Precipitate	Precipitate
28. 1.6 M Sodium Citrate pH 6.5		Clear	Quasi
29. 30% MPD, 0.1M Hepes pH 7.5, 0.5 M Ammonium Sulfate		Precipitate	Clear
30. 10% PEG 6000, 0.1M Hepes pH 7.5, 5% MPD		Small crystals	Clear
31. 20% Jeffamine M-600, 0.1M Hepes pH 7.5		Precipitate	Clear
32. 1.6 M Ammonium Sulfate, 0.1M Hepes pH 7.5, 0.1M Sodium Chloride		Quasi+crystal	Clear
33. 2.0 M Ammonium Formate, 0.1M Hepes pH 7.5		Clear	Quasi
34. 1.0 M Sodium Acetate, 0.1M Hepes pH 7.5, 0.05 M Cadmium Sulfate		Precipitate	Clear
35. 70% MPD, 0.1M Hepes pH 7.5		Clear	Precipitate
36. 4.3 M Sodium Chloride, 0.1M Hepes pH 7.5		Clear	Light precipitate
37. 10% PEG 8000, 0.1M Hepes pH 7.5, 8% Ethylene Glycol		Clear	Clear
38. 20% PEG 10,000, 0.1M Hepes pH 7.5		Clear	Clear
39. 3.4 M 1,6 Hexanediol, 0.1M Tris pH 8.5, 0.2 M Magnesium Chloride		Precipitate	Clear
40. 25% tert-Butanol, 0.1M Tris pH 8.5		Precipitate	Clear
41. 1.0 M Lithium Sulfate, 0.1M Tris pH 8.5, 0.01M Nickel (II) Chloride		Clear	Clear
42. 12% Glycerol, 0.1M Tris pH 8.5, 1.5 M Ammonium Sulfate		Quasi+precipitate	Clear
43. 50% MPD, 0.1M Tris pH 8.5, 0.2 M Ammonium Phosphate		Precipitate	Quasi
44. 20% Ethanol, 0.1M Tris pH 8.5		Precipitate	Precipitate
45. 20% PEG MME 2000, 0.1M Tris pH 8.5, 0.01M Nickel (II) Chloride		Precipitate	Clear
46. 20% PEG MME 550, 0.1M Bicine pH 9.0, 0.1M Sodium Chloride		Clear	Clear
47. 2.0 M Magnesium Chloride, 0.1M Bicine pH 9.0		Clear	Clear
48. 10% PEG 20,000, 0.1M Bicine pH 9.0, 2% Dioxane		Clear	Clear

Table 6.2.1 Continued

Crystal Screen 1

Sample:	5mg/ml IL-1F5		
Buffer:	Water		
Reservoir Volume:	1ml		
Drop:	2µl		
Temperature:	22°C and 4°C		
		22°C	4°C
1. 30%MPD, 0.1M Na Acetate pH 4.6, 0.02 M Calcium Chloride		Precipitate	Clear
2. 0.4 M K ₂ Na Tartrate		Crystal	Clear
3. 0.4 M Ammonium Phosphate		Clear	Clear
4. 2.0 M Ammonium Sulfate, 0.1M Tris HClpH 8.5		Quasi	Quasi
5. 30%MPD, 0.1M Na Hepes pH 7.5, 0.2 M Sodium Citrate		Precipitate	Plate
6. 30%PEG 4000, 0.1M Tris HClpH 8.5, 0.2 M Magnesium Chloride		Clear	Small crystals
7. 1.4 M Sodium Acetate, 0.1M Na Cacodylate pH 6.5		Clear	Small crystals
8. 30%iso-Propanol, 0.1M Na Cacodylate pH 6.5, 0.2 M Sodium Citrate		Precipitate	Clear
9. 30%PEG 4000, 0.1M Na Citrate pH 5.6, 0.2 M Ammonium Acetate		Clear	Clear
10. 30%PEG 4000, 0.1M Na Acetate pH 4.6, 0.2 M Ammonium Acetate		Clear	Clear
11. 1.0 M Ammonium Phosphate, 0.1M Na Citrate pH 5.6		Clear	Clear
12. 30%iso-Propanol, 0.1M Na Hepes pH 7.5, 0.2 M Magnesium Chloride		Precipitate	Precipitate
13. 30%PEG 400, 0.1M Tris HClpH 8.5, 0.2 M Sodium Citrate		Clear	Clear
14. 28%PEG 400, 0.1M Na Hepes pH 7.5, 0.2 M Calcium Chloride		Ppt+plate crystal	Clear
15. 30%PEG 8000, 0.1M Na Cacodylate pH 6.5, 0.2 M Ammonium Sulfate		Clear	Clear
16. 1.5 M Lithium Sulfate, 0.1M Na Hepes pH 7.5		Quasi	Quasi
17. 30%PEG 4000, 0.1M Tris HClpH 8.5, 0.2 M Lithium Sulfate		Clear	Clear
18. 20%PEG 8000, 0.1M Na Cacodylate pH 6.5, 0.2 M Magnesium Acetate		Clear	Clear
19. 30%iso-Propanol, 0.1M Tris HClpH 8.5, 0.2 M Ammonium Acetate		Precipitate	Light Precipitate
20. 25%PEG 4000, 0.1M Na Acetate pH 4.6, 0.2 M Ammonium Sulfate		Clear	Clear
21. 30%MPD, 0.1M Na Cacodylate pH 6.5, 0.2 M Magnesium Acetate		Precipitate	Clear
22. 30%PEG 4000, 0.1M Tris HClpH 8.5, 0.2 M Sodium Acetate		Clear	Clear
23. 30%PEG 400, 0.1M Na Hepes pH 7.5, 0.2 M Magnesium Chloride		Clear	Clear
24. 20%iso-Propanol, 0.1M Na Acetate pH 4.6, 0.2 M Calcium Chloride		Clear	Clear
25. 1.0 M Sodium Acetate, 0.1M Imidazole pH 6.5		Clear	Clear
26. 30%MPD, 0.1M Na Citrate pH 5.6, 0.2 M Ammonium Acetate		Precipitate	Clear
27. 20%iso-Propanol, 0.1M Na Hepes pH 7.5, 0.2 M Sodium Citrate		Clear	Clear
28. 30%PEG 8000, 0.1M Na Cacodylate pH 6.5, 0.2 M Sodium Acetate		Clear	Clear
29. 0.8 M K ₂ Na Tartrate, 0.1M Na Hepes pH 7.5		Quasi	Clear
30. 30%PEG 8000, 0.2 M Ammonium Sulfate		Precipitate	Precipitate
31. 30%PEG 4000, 0.2 M Ammonium Sulfate		Precipitate	Quasi
32. 2.0 M Ammonium Sulfate		Quasi	Quasi
33. 4.0 M Sodium Formate		Clear	Clear
34. 2.0 M Sodium Formate, 0.1M Na Acetate pH 4.6		Clear	Clear
35. 1.6 M Na ₂ KPhosphate, 0.1M Na Hepes pH 7.5		Quasi	Clear
36. 8%PEG 8000, 0.1M Tris HClpH 8.5		Ppt+small crystal	Clear
37. 8%PEG 4000, 0.1M Na Acetate pH 4.6		Clear	Clear
38. 1.4 M Sodium Citrate, 0.1M Na Hepes pH 7.5		Quasi	Quasi
39. 2%PEG 400, 0.1M Na Hepes pH 7.5, 2.0 M Ammonium Sulfate		Quasi	Clear
40. 20%iso-Propanol, 0.1M Na Citrate pH 5.6, 20%PEG 4000		Clear	Clear
41. 10%iso-Propanol, 0.1M Na Hepes pH 7.5, 20%PEG 4000		Clear	Clear
42. 20%PEG 8000, 0.05 M Potassium Phosphate		Precipitate	Clear
43. 30%PEG 1500		Clear	Clear
44. 0.2 M Magnesium Formate		Clear	Clear
45. 18%PEG 8000, 0.1M Na Cacodylate pH 6.5, 0.2 M Zinc Acetate		Precipitate	Precipitate
46. 18%PEG 8000, 0.1M Na Cacodylate pH 6.5, 0.2 M Calcium Acetate		Clear	Clear
47. 2.0 M Ammonium Sulfate, 0.1M Sodium Acetate pH 4.6		Quasi	Quasi
48. 2.0 M Ammonium Phosphate, 0.1M Tris HClpH 8.5		Quasi	Clear
49. 2%PEG 8000, 1.0 M Lithium Sulfate		Clear	Precipitate
50. 15%PEG 8000, 0.5 M Lithium Sulfate		Precipitate	Precipitate

Table 6.2.1 Crystal screen formulations. The 98 different conditions screened for their ability to crystallise IL-1F5 are shown. The conditions of the drops three weeks after the trial was set up is recorded. Those conditions marked in red were considered promising for further investigation.

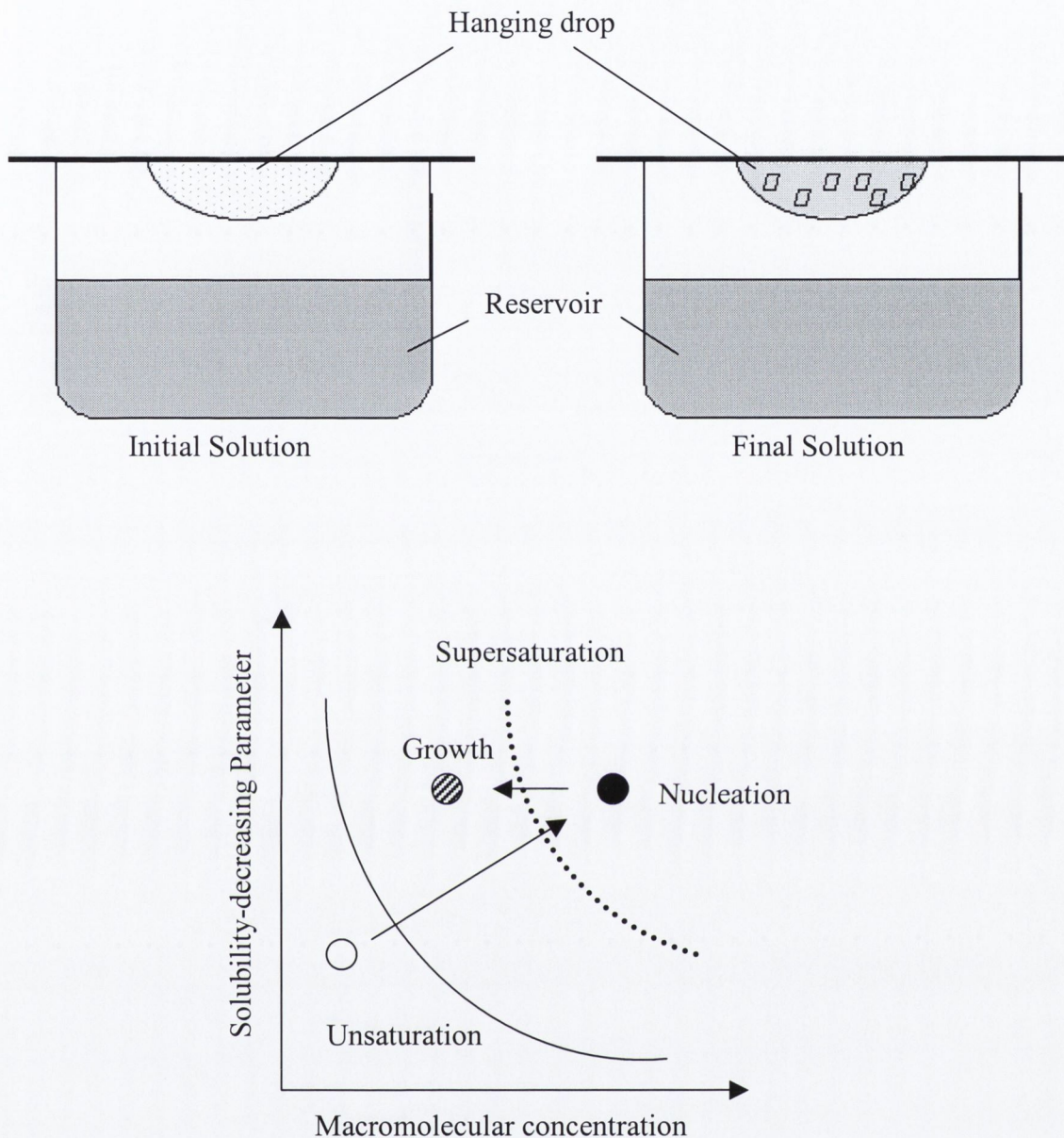


Figure 6.2.1 Schematic diagram of hanging drop vapour diffusion. Protein-precipitant drops are hung over a precipitant reservoir and sealed with wax. Vapour diffusion occurs inducing supersaturation that sustains nucleation (filled circle). As protein concentration decreases conditions move to favour crystal growth (hatched circle). (Adapted from Weber in *Methods in Enzymology* 276, 13-22.)

temperature. In order to try and maintain as constant a temperature as possible, the trials at room temperature were stored under a polystyrene box. Initial examination of the trials after one and three weeks showed six conditions that showed potential for crystal growth. These were a) 0.4 M K, Na tartrate, b) 2.0 M ammonium sulphate, 0.1 M Tris-HCl pH 8.5, c) 28% PEG 400, 0.1 M Na HEPES pH 7.5, 0.2 M Calcium Chloride d) 1.8 M ammonium sulphate, 0.1 M MES pH 6.5, 0.01 M cobalt chloride, e) 10% PEG 6000, 0.1 M HEPES pH 7.5, 5% MPD and f) 1.6 M ammonium sulphate, 0.1 M HEPES pH 7.5, 0.1 M sodium chloride (Red in table 6.2.1). All of these occurred in trials set up at room temperature. Conditions A and F showed crystal growth, which was also accompanied by heavy precipitate, while condition C, had plate crystals with no precipitate. There is always the possibility that crystals obtained are salt crystals from the precipitating solution or remnants from purification steps. Therefore to examine if these crystals were protein crystals or not they were stained with a blue Izit dye. This dye is absorbed by protein crystals due to its ability to be able to penetrate the solvent channels that exist within protein crystals, which due to the large size of the protein molecules do not pack together tightly. Salt crystals, on the other hand do pack together very tightly and consequently do not absorb the dye. Using this method it was seen that crystals grown in 0.4M K/Na tartrate tetrahydrate, as well as 1.6 M ammonium sulphate, 0.1 M HEPES pH 7.5, 0.1 M sodium chloride both stained blue with Izit dye (Figure 6.2.2) but the plates that grew in 28% PEG 400, 0.1 M Na HEPES pH 7.5, 0.2 M calcium chloride did not. In addition, many small crystals could be seen in conditions with 10% PEG 6000, 0.1 M HEPES pH 7.5, 5% MPD, while quasi crystals were observed in conditions with 1.8 M ammonium sulphate, 0.1 M MES pH 6.5, 0.01 M cobalt chloride, and also 2.0 M ammonium sulphate, 0.1 M Tris-HCl pH 8.5 (See Table 6.2.2 for summary).

Therefore, the conditions described above were chosen as the starting point for further investigation into conditions that would yield bigger, single crystals of IL-1F5. Towards this end conditions such as pH or salt concentration were varied slightly from the starting conditions. The manner in which conditions were varied is given in Table 6.2.3. Crystals were obtained from precipitant with 10 mM CoCl, 0.1 M HEPES pH 7 and 1.5 M ammonium sulphate as well as 0.1 M KCl, 0.1 M HEPES pH 7.5 with 1.5 M, 1.3 M and

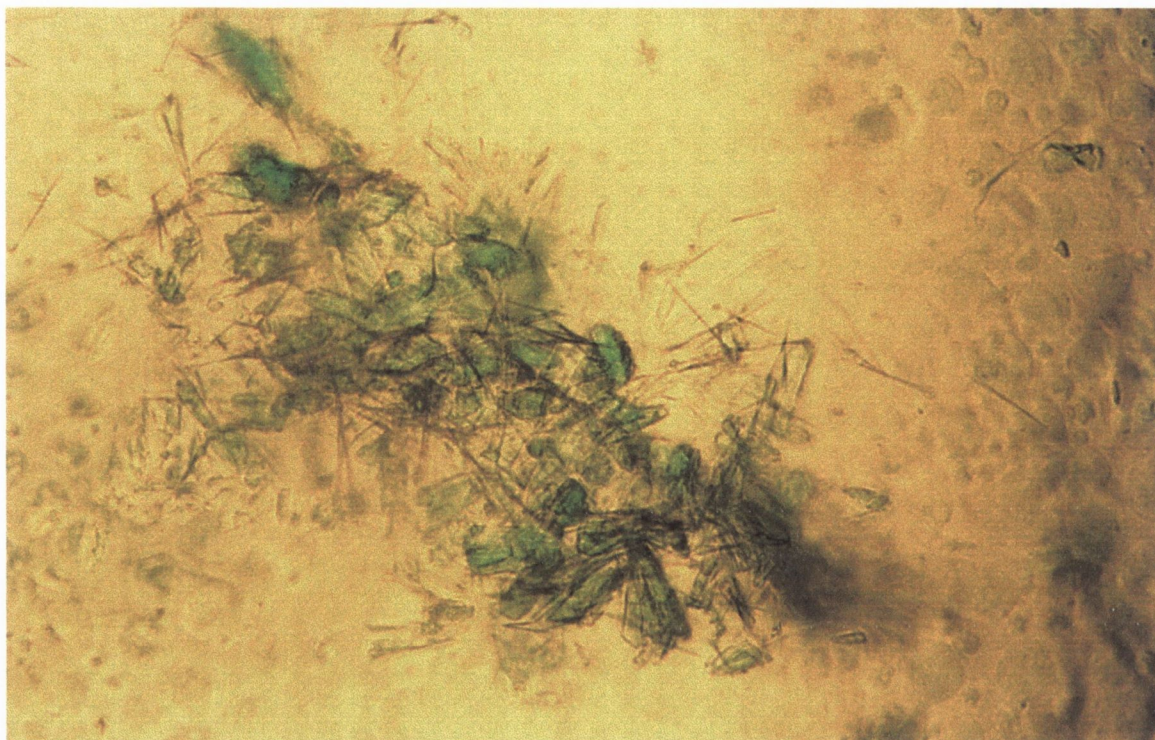


Figure 6.2.2 Initial crystals stained with Izit dye. Crystals grown from 2 μ l of 5 mg/ml IL-1F5 in water mixed with an equal volume of precipitant solution containing 1.6 M ammonium sulphate, 0.1 M HEPES pH 7.5, 0.1 M NaCl and set up as a hanging drop above a reservoir of the same precipitant solution. Crystals were stained by applying 2 μ l of a 1 in 10 dilution of Izit dye (Hampton Research) to the drop.

Condition	Salt	Buffer	Precipitant	Observation
A			0.4 M K/Na tartrate tetrahydrate	Crystal plus heavy precipitate
B		0.1 M Tris HCl pH 8.5	2.0 M ammonium sulphate	Quasi crystals
C	0.2 M CaCl ₂	0.1 M HEPES pH 7.5	28% v/v PEG 400	Plates
D	0.01M CoCl ₂ hexahydrate	0.1 M MES pH 6.5	1.8 M ammonium sulphate	Quasi crystals
E		0.1 M HEPES pH 7.5	10% w/v PEG 6000, 5% v/v MPD	Small crystals
F	0.1 M NaCl	0.1 M HEPES pH 7.5	1.6 M ammonium sulphate	Crystal plus heavy precipitate

Table 6.2.2 Conditions from initial Crystal Screen trials which showed promising potential for growth of IL-1F5 crystals.

Condition	Precipitant	Alteration	Observation
A	0.4 M K/Na tartrate tetrahydrate	0.38 M, 0.36 M, 0.34 M, 0.32 M, 0.3 M K/Na tartrate tetrahydrate	No crystals
B	0.1 M Tris HCl pH 8.5, 2.0 M ammonium sulphate	pH 7.5, pH 7.2, pH 7.0	No crystals
C	0.2 M CaCl ₂ , 0.1 M HEPES pH 7.5, 28% v/v PEG 400	26%, 24%, 22%, 20%, 18% v/v PEG 400	No crystals
D	0.01M CoCl ₂ hexahydrate, 0.1 M MES pH 6.5, 1.8 M ammonium sulphate	pH 6.8, pH 7, pH 7.2, HEPES with 1.8 M and 1.5 M ammonium sulphate	Single crystals in 0.01M CoCl ₂ hexahydrate, 0.1 M HEPES pH 7, 1.5 M ammonium sulphate
E	0.1 M HEPES pH 7.5, 10% w/v PEG 6000, 5% v/v MPD	0.1M KCl, LiCl, Mg acetate	No crystals
F	0.1 M NaCl, 0.1 M HEPES pH 7.5, 1.6 M ammonium sulphate	1.5 M, 1.3 M, 1.2 M, 1.1 M, 1.0 M ammonium sulphate with 0.1M NaCl, LiCl, KCl	Twining and disordered crystals with LiCl. 3, 6 and 9 crystals in 1.5 M, 1.3 M and 1.2 M ammonium sulphate and 0.1 M KCl.

Table 6.2.3 Conditions used for second round of IL-1F5 crystallisation trials.

1.2 M ammonium sulphate. To ensure that the crystals obtained were indeed IL-1F5 crystals, one was taken, washed and crushed and added to sample buffer, boiled and run on an acrylamide electrophoresis gel against purified recombinant IL-1F5 (Figure 6.2.3). This showed that the crystals grown were indeed IL-1F5. These crystals were used in a further round of crystallisation trials as a seed stock. Since nucleation and growth of crystals occur under different conditions it is often useful to separate the two processes. This can be achieved by seeding methods, which introduce preformed crystals into equilibrated drops that sustain crystal growth. In microseeding, crystals are taken and washed in unsaturated solution in order to remove misorientated protein molecules or other matter whose inclusion may inhibit crystal growth. Crystals are then crushed into small seeds, which are then introduced into less saturated drops to allow the growth of a few large crystals (Figure 6.2.4). Making serial dilutions of the seed stock can control the number of seeds introduced into the new drop, or streaking methods can be used to introduce an unspecified number of seeds. Macroseeding can also be carried out using a single washed crystal, which is then transferred to a less saturated solution for further growth. Single crystals were obtained in 0.1 M KCl, 0.1 M HEPES, pH 7.5, 1.3 M ammonium sulphate using microseeding methods, with seeds streaked into drops that had been allowed to equilibrate for 2 hours prior to the addition of seeds. A typical crystal from these conditions grew to the full size of 0.2 mm x 0.08 mm x 0.1 mm at 18°C in 10 to 14 days (Figure 6.2.5). Crystals were flash frozen in a liquid nitrogen stream at 77 K in cryo-protectant containing the precipitant and 25% glycerol.

One such crystal diffracted to 1.5 Å at a wavelength of 0.934 Å at the ESRF in Grenoble, France. The crystal belongs to the trigonal space group $P3_221$ and has unit cell dimensions of $a=78.93$ Å, $c=69.74$ Å. There is one molecule in the asymmetric unit with corresponding solvent content of 62.2% and Matthews' coefficient of 3.28 Å³/Da. Since this value is at the higher end of the frequency distribution for most proteins this may indicate that there are more than one molecules in the asymmetric unit, this is however not the case (Matthews, 1968). Concomitant with this derivative protein with selenomethionine incorporated into it was also produced and purified from *E.coli* cells in case it were needed to solve the phase problem by the Multiwavelength Anomalous

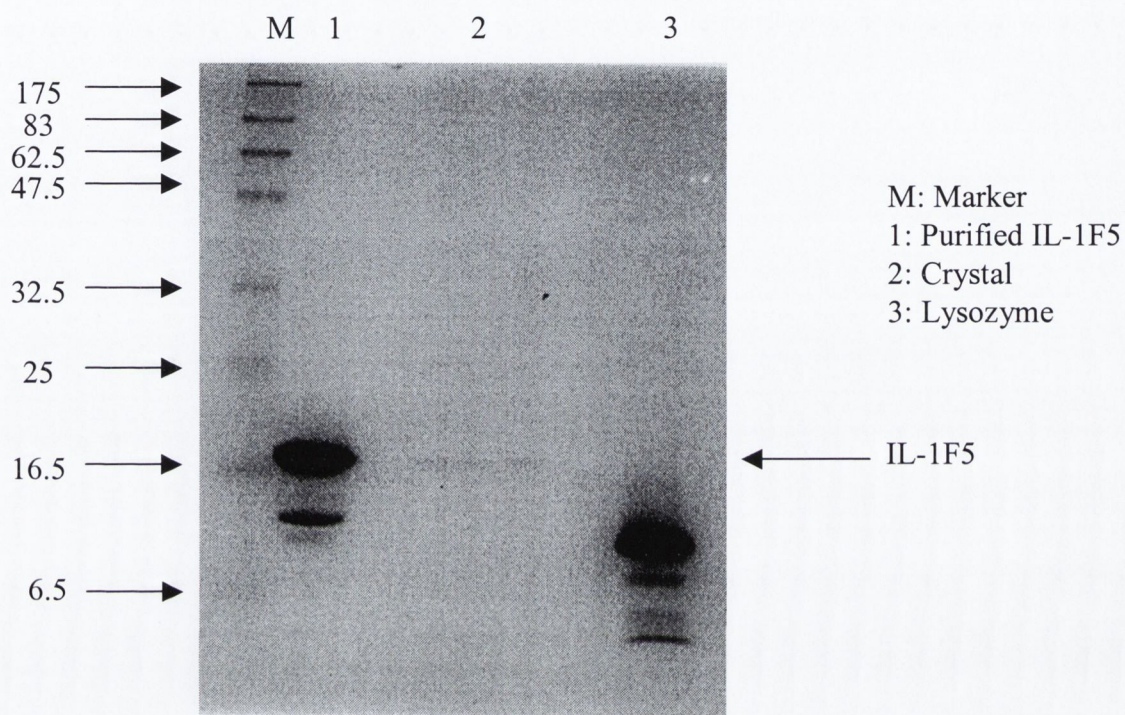


Figure 6.2.3 IL-1F5 crystal analysed by SDS-PAGE. A crystal grown by the hanging drop method was crushed and resuspended in 20 μ l sample buffer and boiled for 5 minutes at 100°C. The sample was then run on a 15% polyacrylamide gel and stained for protein with Commassie blue.

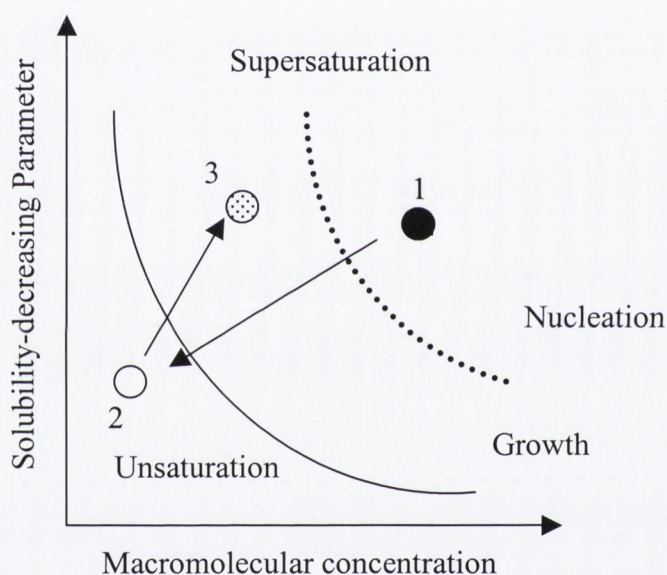
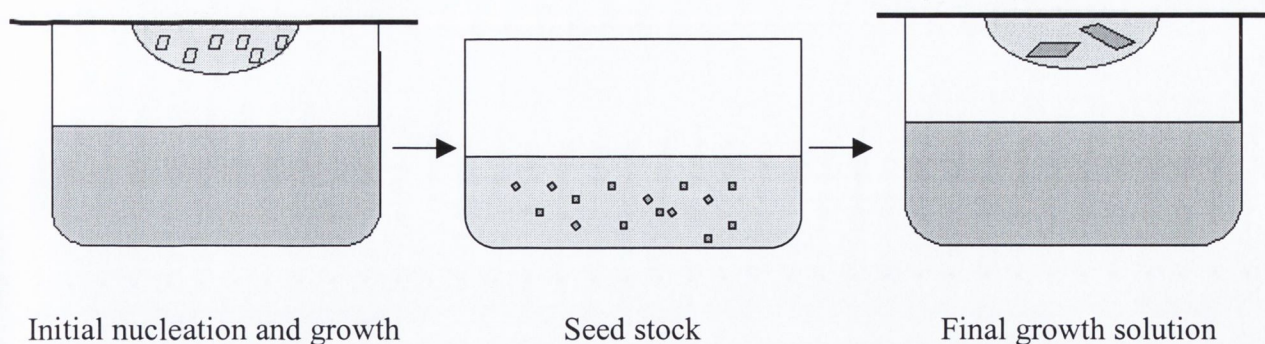


Figure 6.2.4 Schematic diagram of crystallisation by microseeding. A single crystal from the initial crystallisation experiment is taken and placed in an unsaturated solution. The crystal is then crushed and a seed stock is made, which is then transferred to the final growth solution to produce a few large crystals. The phase diagram shows the transfer of the crystal from a solution the favours nucleation (filled circle) to an unsaturated solution (open circle) and finally to a solution that favours crystal growth (dotted circle). (Adapted from Weber in *Methods in Enzymology* 276, 13-22.)

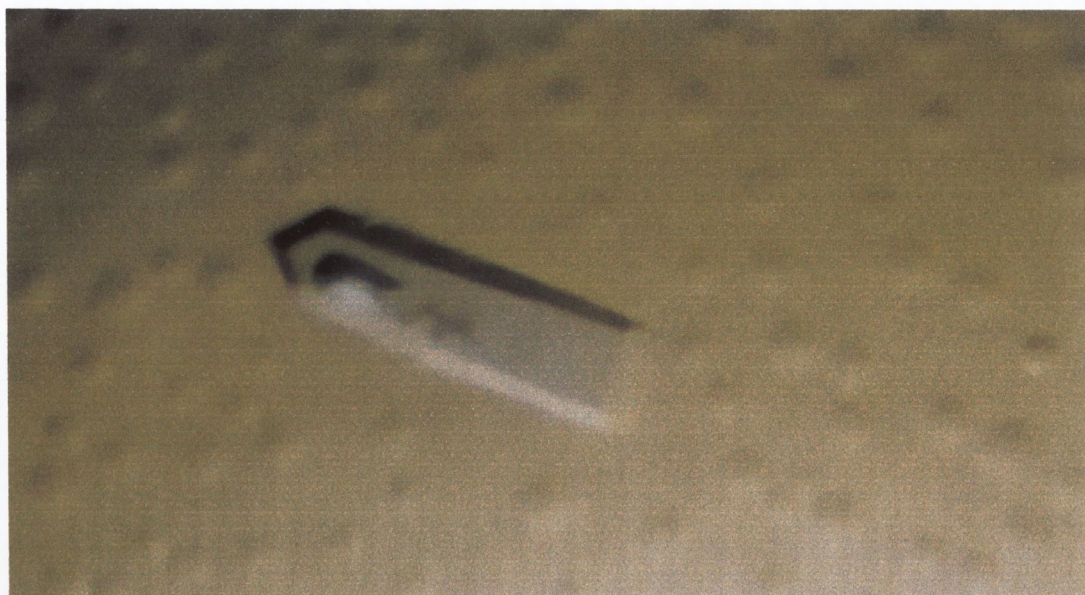


Figure 6.2.5 Native crystal of IL-1F5. Single crystal of IL-1F5 grown from 2 μ l of 5 mg/ml IL-1F5 in water mixed with an equal volume of precipitant solution containing 1.5 M ammonium sulphate, 0.1 M KCl and 0.1 M HEPES pH 7.5 and set up as a hanging drop above a reservoir of the same precipitant solution.

Diffraction (MAD) method. The same purification protocol was used to purify the derivative protein and the incorporation of selenomethionine was confirmed using mass spectroscopy (Figure 6.2.6). However, this protein failed to yield any crystals under the same conditions of crystallisation as used for the native protein. Optimisation of conditions for growth of selenomethionine derivative protein crystals was not pursued however, as molecular replacement was sufficient to solve the structure.

6.2.2 Phase Determination and Refinement

Data analysis, phase determination by molecular replacement with murine IL-1 β , and model building and refinement was carried out in collaboration with Xue Yuan Pei and Nick Gay in the University of Cambridge Biochemistry Department.

An initial model of IL-1F5 with a correlation of 60.0% and an R-factor of 53.6% using murine IL-1 β (2mib) as a search model was obtained by following a rigid body and B overall refinement in AMoRe (Navaza, 2001). The loops connecting β -strands 3 and 4, β -strands 7 and 8 and also β -strands 11b to 12 were manually built using X-ray data and refined against the standard geometry library in the program O (Jones et al., 1991). The final model of IL-1F5 has an R-factor of 21.2% and a free R-factor of 20.2%. 182 water molecules in total were found in the structure. The structure has a well ordered electron density for the entirety of the main chain. There are a few residues including Glu72, Lys73 and Glu137, which have disordered sidechains. The model geometry was checked using PROCHECK (Laskowski, 1993) and SFCHECK (CCP4, 1994) (Table 6.2.4). There are no residues with phi and psi-angles either in the disallowed region or the generously allowed region. 90% of the residues are in the favourable region, according to the limits defined by Ramachandra (Ramakrishnan and Ramachandran, 1965) (Figure 6.2.7). The phi, psi and chi angles of each amino acid type also show that the majority fall into the allowed regions (Figure 6.2.8). Only Gly105 has an unfavourable phi, psi conformation, while only Ile36 has an unfavourable chi1-chi2 conformation. The mainchain bond lengths also fall within mean values as defined by Engh and Huber (1991) (Figure 6.2.9). Furthermore, amino acids with large side chains do not show large

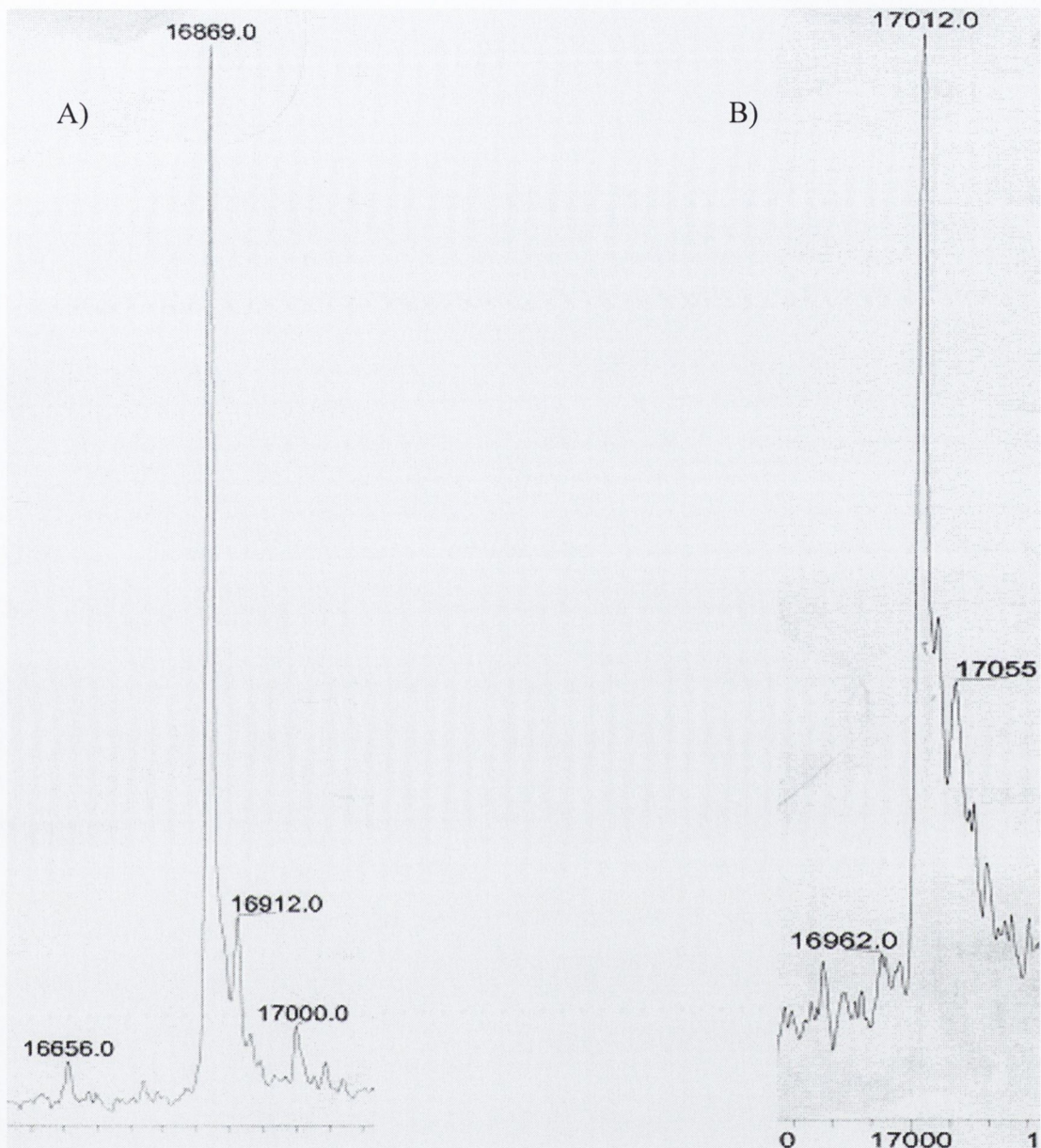


Figure 6.2.6 Mass spectroscopic analysis of native and selenomethionine forms of IL-1F5. A) The molecular weight of 16869 Da is consistent with the predicted molecular weight of the native form of the protein with the first methionine removed. B) The molecular weight of the derivative form is determined to be 17012 Da, a difference of 143 Da from the native form. Selenium (mass number 79) has a molecular weight difference of 47 from sulphur (mass number 32) which indicates that the three remaining methionines have been substituted.

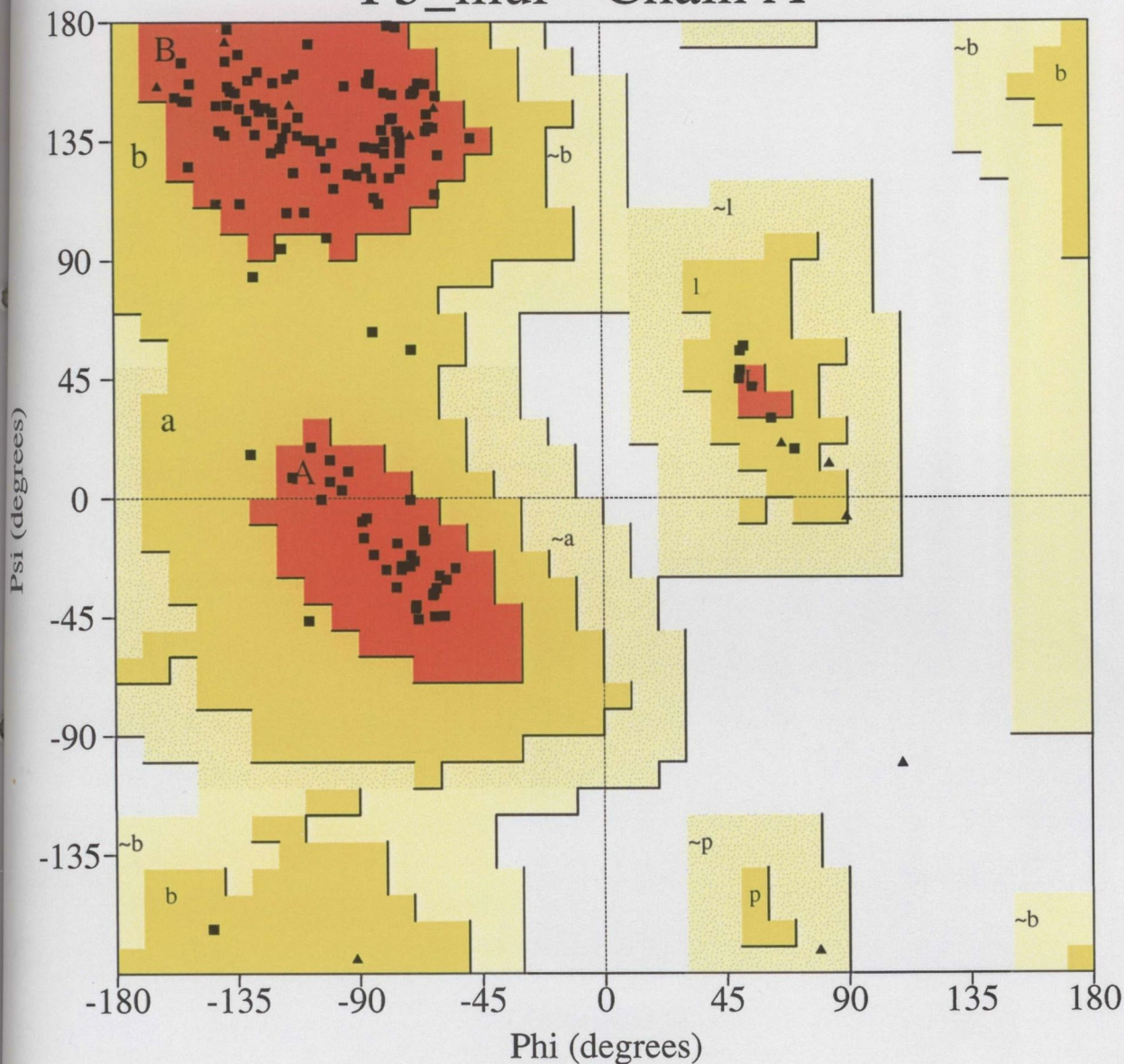
Table 6.2.4 X-ray structure determination

Data collection and processing		Refinement statistics	
Space group	P3221	Resolution (Å)	39.5 – 1.6
Cell dimension (Å)	78.9, 78.9 69.7, 90, 90, 120	R factor (%)	21.2
Resolution (Å)	39.5 – 1.58	R free factor (%)	20.2
Wavelength (Å)	0.934	weighted r.m.s.d from ideality	
No of unique reflection	40560	Bond length (Å)	0.005
Mean redundancy (outer shell)	6.5 (7.0)	Bond angle (degree)	1.4
Overall Rsym (outer shell) (%)	7.8 (47.8)	Dihedral angle (degree)	24.7
I/σ(I) (outer shell)	4.7 (1.4)	Total no. of protein atoms	1185
B overall (by Patterson) (Å ²)	24.4	No. of waters	182
		Mean B value, protein (Å ²) (main chain / side chain)	19.8 / 23.1
		Mean B value, water (Å ²)	37

Figure 6.2.7 Ramachandran plot of IL-1F5. The phi, psi angles of all amino acids are plotted and designated as being in the most favoured (red), allowed (yellow), generously allowed (pale yellow) or disallowed regions (white).

Ramachandran Plot

F5_mur - Chain A



Plot statistics

Residues in most favoured regions [A,B,L]	118	92.2%
Residues in additional allowed regions [a,b,l,p]	10	7.8%
Residues in generously allowed regions [~a,~b,~l,~p]	0	0.0%
Residues in disallowed regions	0	0.0%
-----		-----
Number of non-glycine and non-proline residues	128	100.0%
Number of end-residues (excl. Gly and Pro)	4	
Number of glycine residues (shown as triangles)	12	
Number of proline residues	10	
-----		-----
Total number of residues	154	

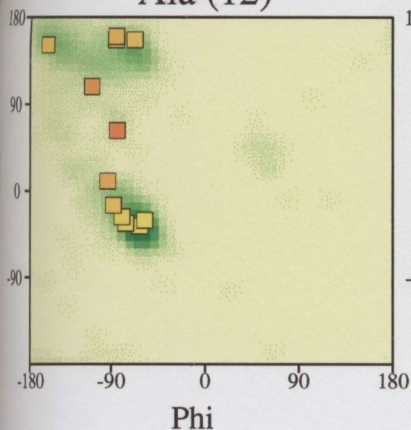
Based on an analysis of 118 structures of resolution of at least 2.0 Angstroms and R-factor no greater than 20%, a good quality model would be expected to have over 90% in the most favoured regions.

Figure 6.2.8 Phi, psi and chi angles of individual amino acids. The bond angles of each amino acid type is given. Those falling within the favourable regions, shown by green shading, are depicted with yellow squares, those in less favourable and unfavourable regions are depicted by orange and red squares respectively.

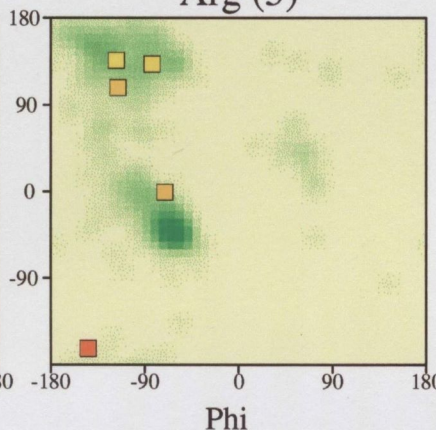
Ramachandran plots for all residue types

F5_mur - Chain A

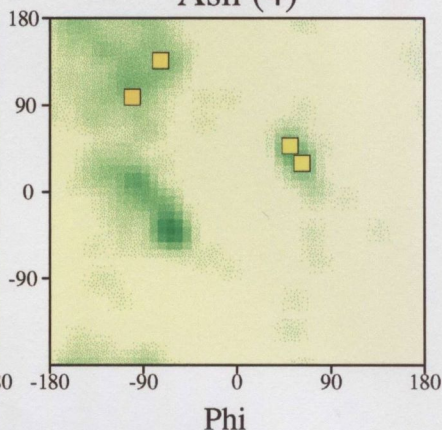
Ala (12)



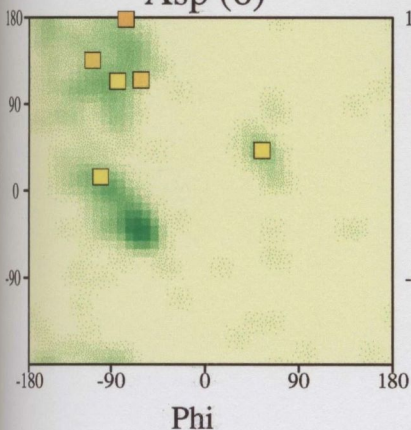
Arg (5)



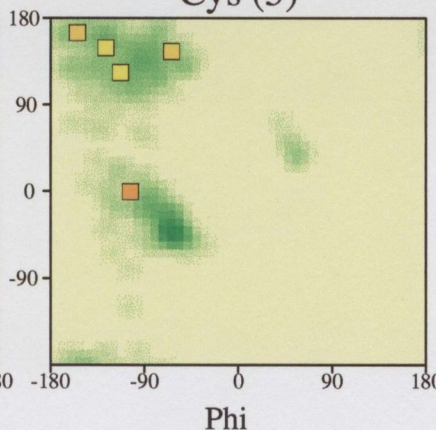
Asn (4)



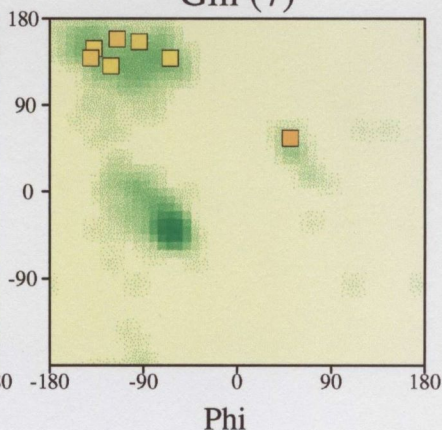
Asp (6)



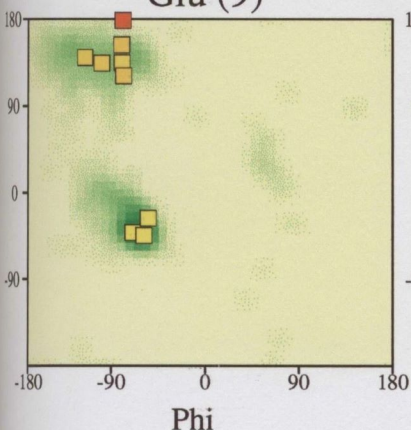
Cys (5)



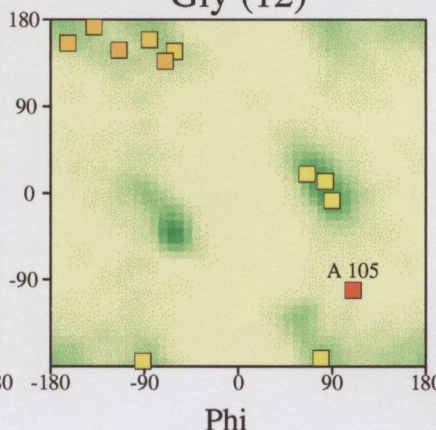
Gln (7)



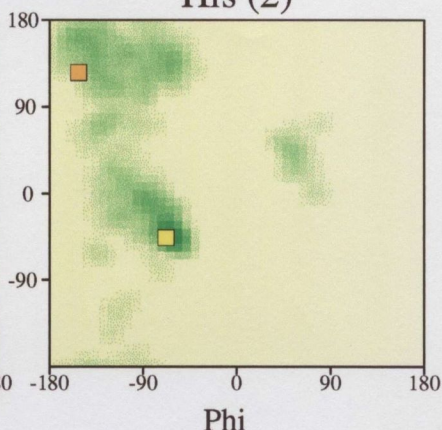
Glu (9)



Gly (12)



His (2)



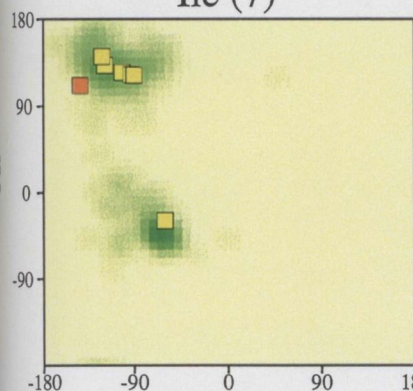
Numbers of residues are shown in brackets. Those in unfavourable conformations (score < -3.00) are labelled. Shading shows favourable conformations as obtained from an analysis of 163 structures at resolution 2.0Å or better.

Figure 6.2.8 Continued.

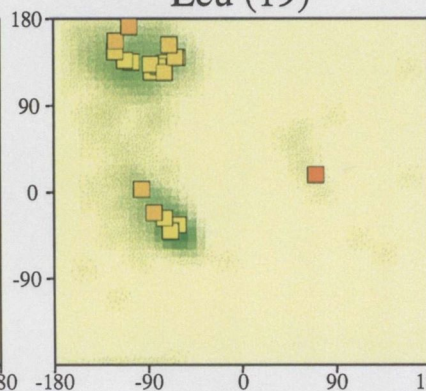
Ramachandran plots for all residue types

F5_mur - Chain A

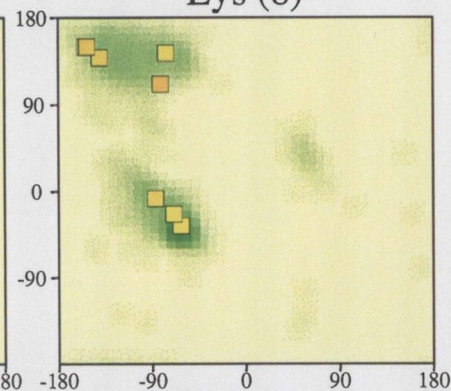
Ile (7)



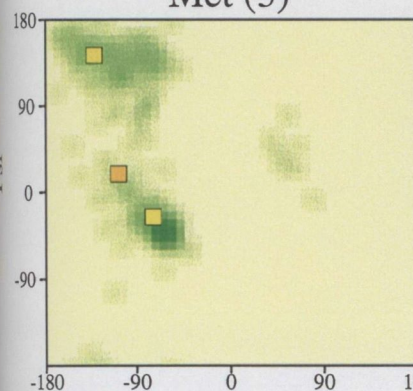
Leu (19)



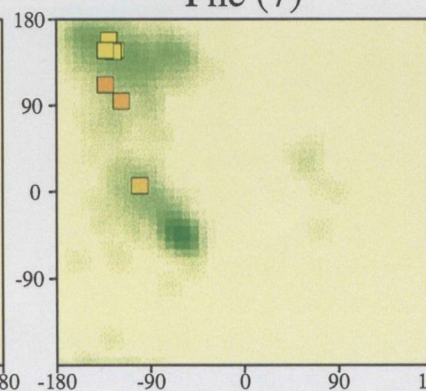
Lys (8)



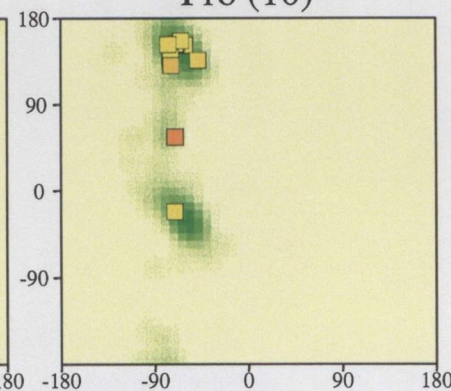
Met (3)



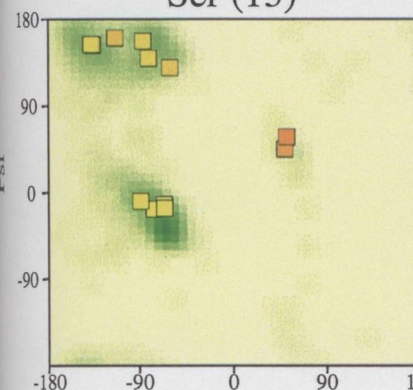
Phe (7)



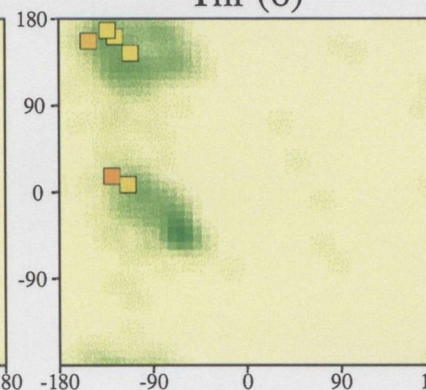
Pro (10)



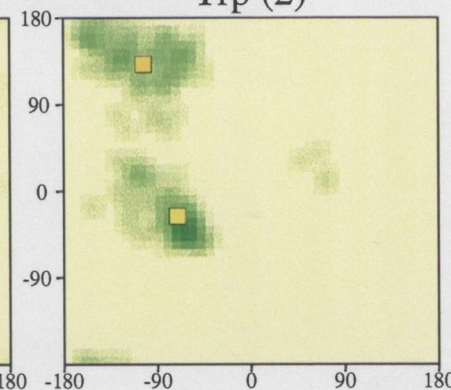
Ser (13)



Thr (6)



Trp (2)



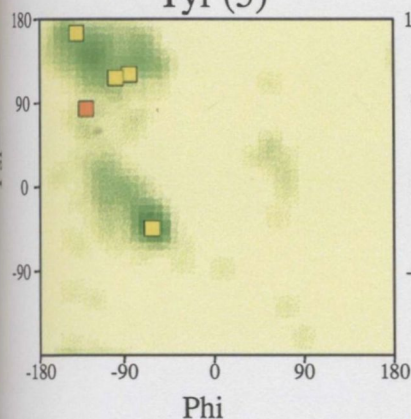
Numbers of residues are shown in brackets. Those in unfavourable conformations (score < -3.00) are labelled. Shading shows favourable conformations as obtained from an analysis of 163 structures at resolution 2.0Å or better.

Figure 6.2.8 Continued.

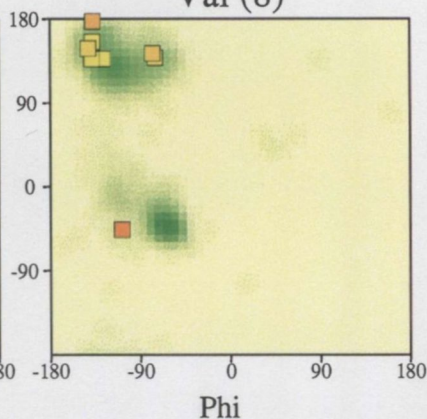
Ramachandran plots for all residue types

F5_mur - Chain A

Tyr (5)



Val (8)

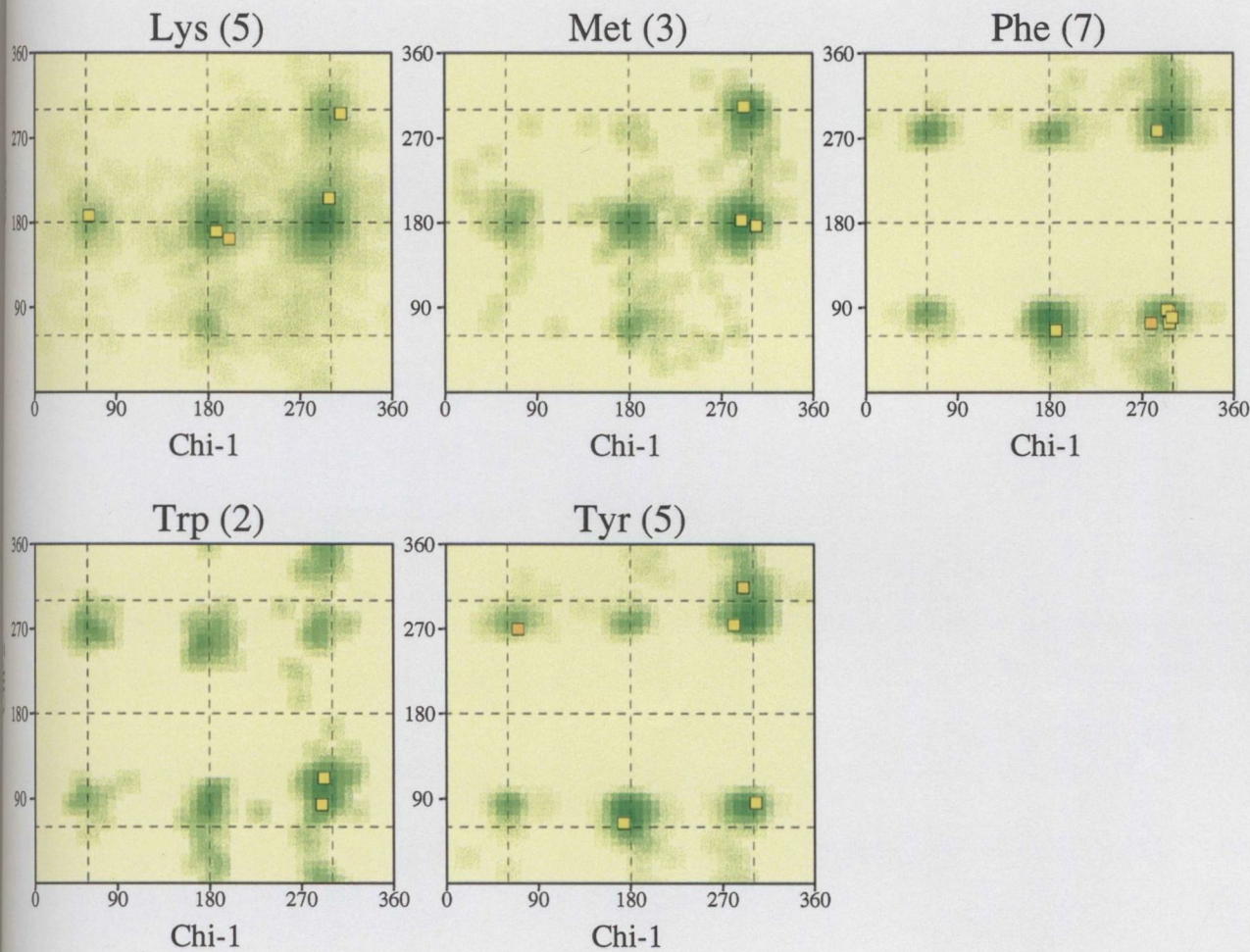


Numbers of residues are shown in brackets. Those in unfavourable conformations (score < -3.00) are labelled. Shading shows favourable conformations as obtained from an analysis of 163 structures at resolution 2.0Å or better.

Figure 6.2.8 Continued.

Chi1-Chi2 plots

F5_mur - Chain A

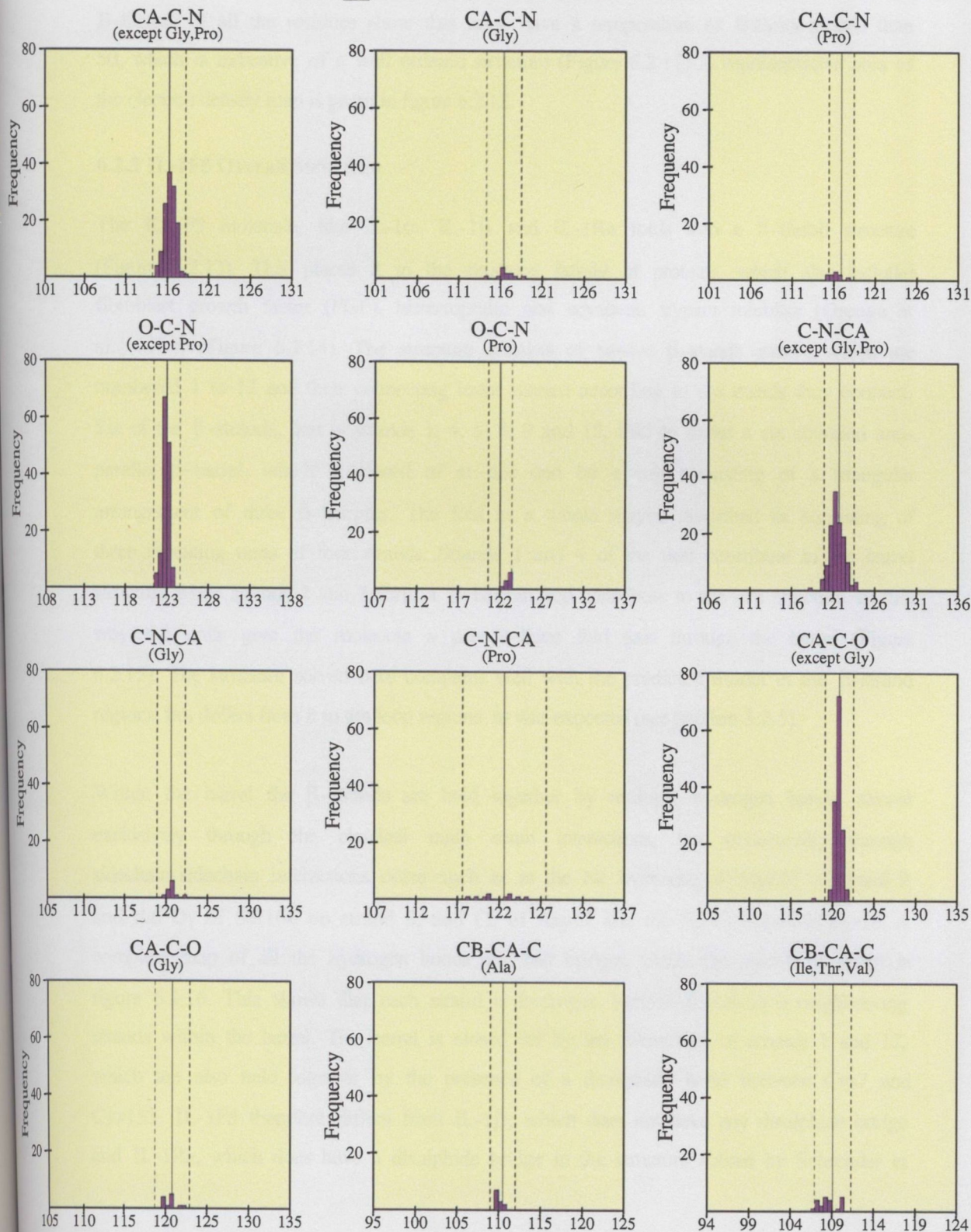


Numbers of residues are shown in brackets. Those in unfavourable conformations (score < -3.00) are labelled. Shading shows favourable conformations as obtained from an analysis of 163 structures at resolution 2.0Å or better.

Figure 6.2.9 Mainchain bond lengths in IL-1F5. The frequency of the the bond lengths for each amino acid sub-type is shown, with mean and standard deviation lengths depicted by solid and dashed lines respectively.

Main-chain bond angles

F5_mur - Chain A



Black bars > 2.0 st. devs. from mean.
Solid and dashed lines represent the mean and standard deviation values as per Engh & Huber small-molecule data.

deviations from a planar conformation (Figure 6.2.10). In addition, a plot of the average B-factors of all the residues show that none have a temperature or B-factor greater than 50, which is indicative of a well ordered structure (Figure 6.2.11) A representative area of the electron density map is given in figure 6.2.12.

6.2.3 IL-1F5 Overall Structure

The IL-1F5 molecule, like IL-1 α , IL-1 β and IL-1Ra folds into a β -trefoil structure (Figure 6.2.13). This places it in the structural family of proteins, which also includes fibroblast growth factor (FGF), histactophilin and soyabean trypsin inhibitor (Orengo et al., 1994) (Figure 6.2.14). The structure consists of twelve β -stands, which are numbered 1 to 12 and their connecting loops named according to the stands they connect. Six of the β -stands, that is strands 1, 4, 5, 8, 9 and 12, fold to make a six stranded anti-parallel β -barrel, which is closed off at one end by a cap consisting of a triangular arrangement of three β -hairpins. The fold as a whole maybe described as consisting of three repeating units of four strands. Strands 1 and 4 of the unit contribute to the barrel structure while strands 2 and 3 form a β -hairpin and contribute to the cap structure. In this way the units give the molecule a pseudo-three fold axis through the barrel (Figure 6.2.15). The structure solved here compares well with the predicted model in the β -strand regions, but differs from it in the loop regions, as was expected (see section 3.2.5).

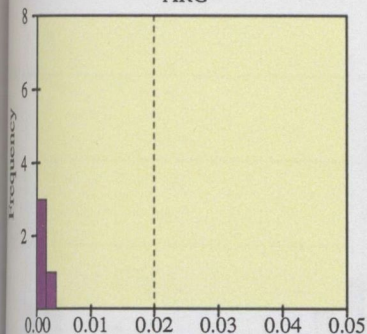
Within the barrel the β -strands are held together by multiple hydrogen bonds, almost exclusively through the classical main chain interactions, but occasionally through sidechain-sidechain interactions occur such as at the N ϵ hydrogen of Arg101 of strand 9 and the O γ of Ser108 on strand 8, and O δ of Asp12 and the N ζ hydrogen of Lys16. A complete map of all the hydrogen bonds and salt bridges within the protein is given in figure 6.2.16. This shows that each strand is hydrogen bonded to both of its neighbouring strands within the barrel. The barrel is closed off by the interaction of strands 1 and 12, which are also held together by the presence of a disulphide bond between Cys7 and Cys153. IL-1F5 therefore differs from IL-1 β , which does not have any disulphide bridge and IL-1Ra, which does have a disulphide bridge in the structure solved by Schreuder et

Figure 6.2.10 Distance from planarity of large and aromatic amino acid side chains.

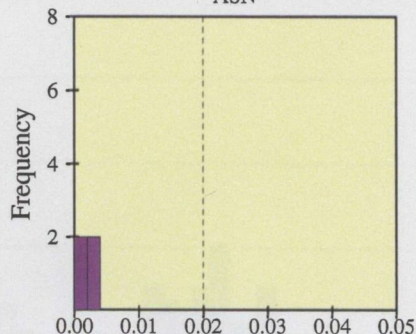
RMS distances from planarity

F5_mur - Chain A

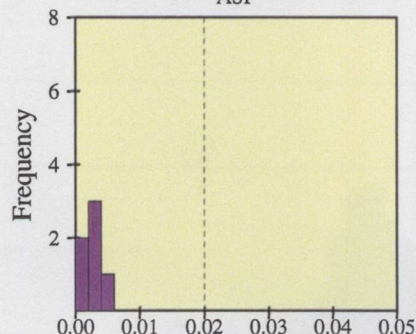
ARG



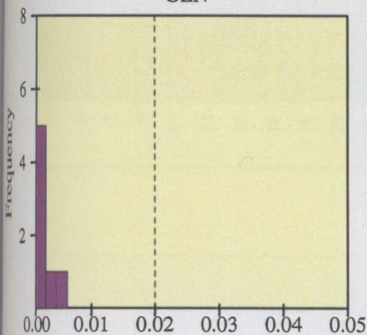
ASN



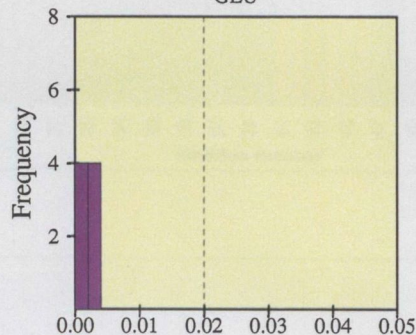
ASP



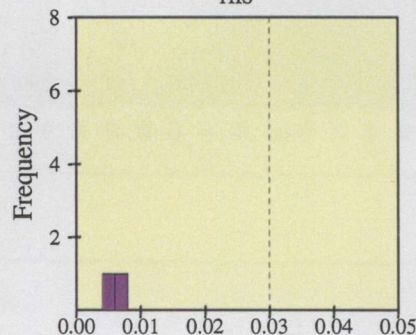
GLN



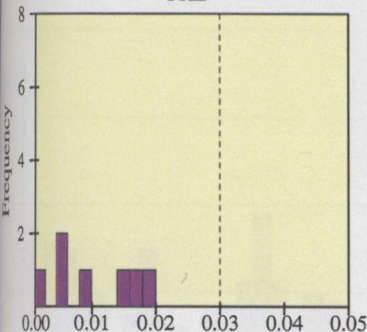
GLU



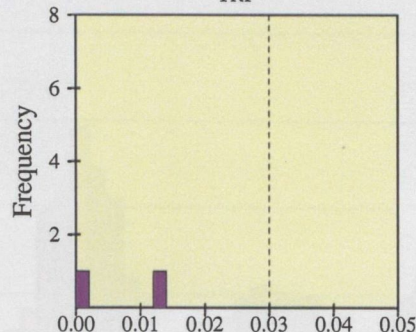
HIS



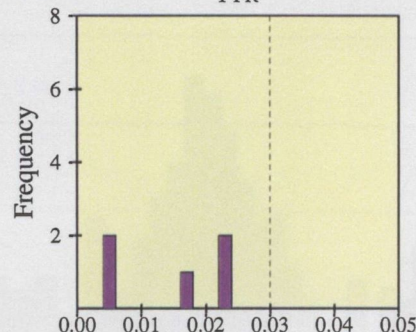
PHE



TRP



TYR



Histograms showing RMS distances of planar atoms from best-fit plane.
Black bars indicate large deviations from planarity: RMS dist > 0.03 for rings, and > 0.02 otherwise.

Average B-Factor of Mainchain and Sidechain Atoms

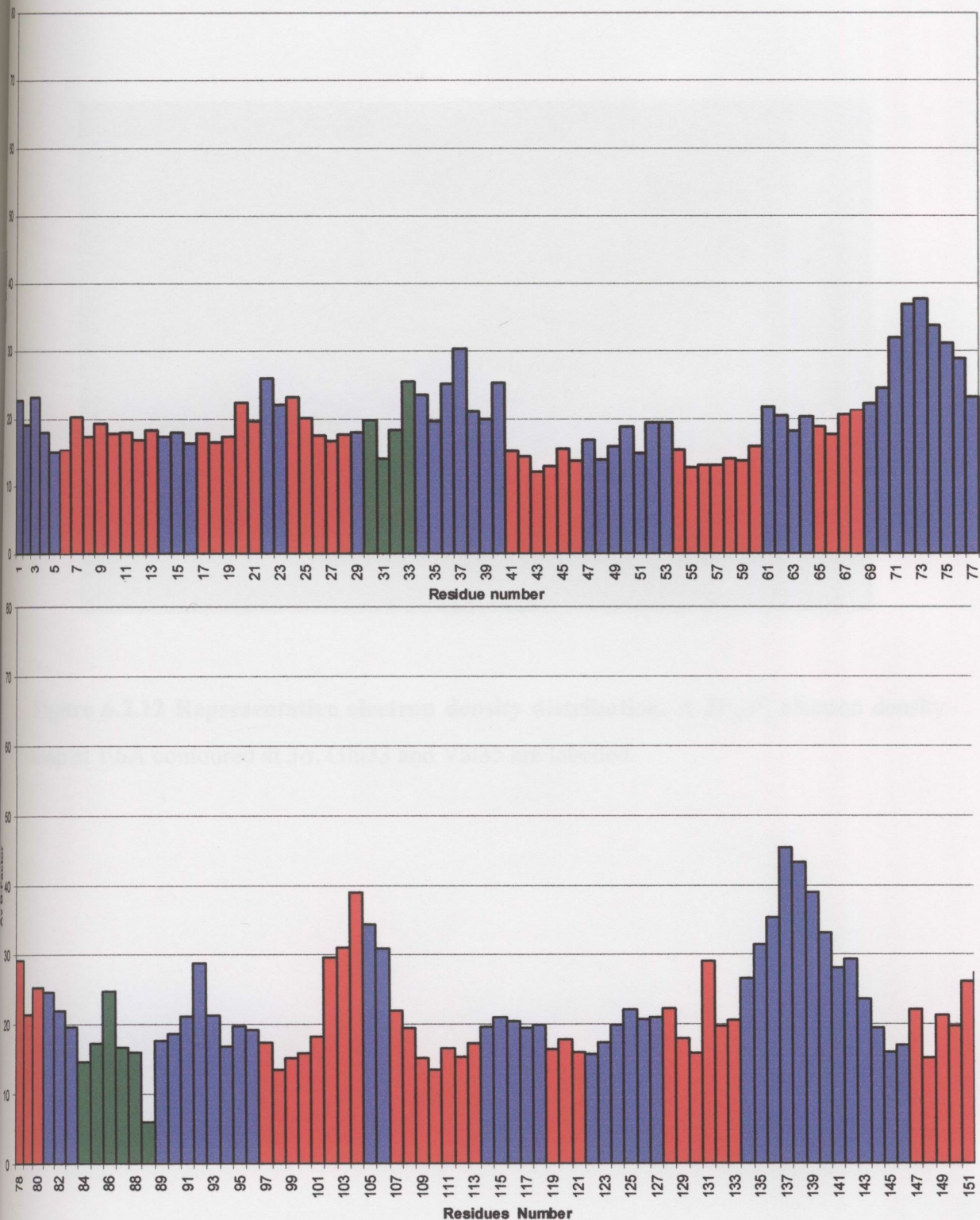


Figure 6.2.11 B-plot of IL-1F5 structure. Residues that form part of β -strands are shown in red, while those that are involved in helical structures are shown in green, other residues are shown in blue.

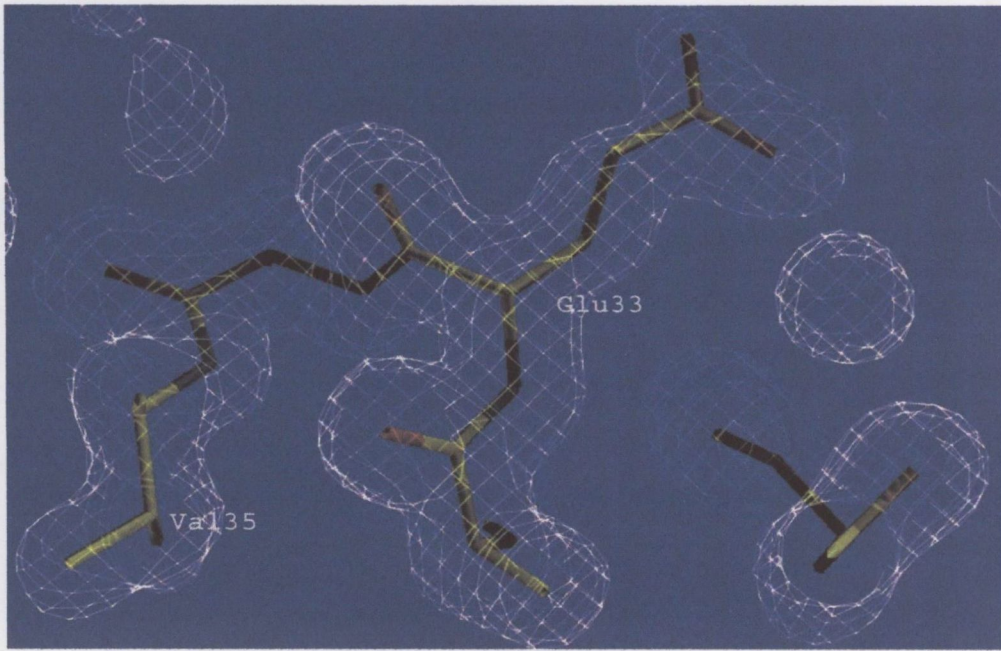
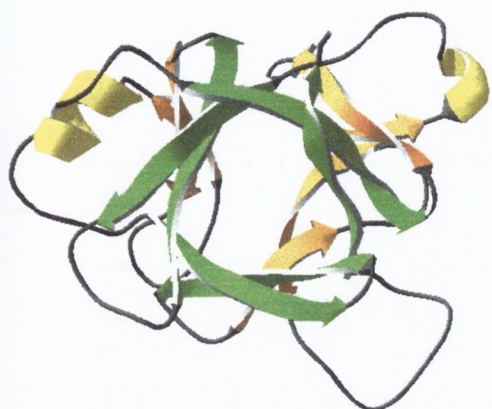


Figure 6.2.12 Representative electron density distribution. A $2F_o - F_c$ electron density map at 1.6Å contoured at 3σ . Glu33 and Val35 are labelled.



Open end of barrel

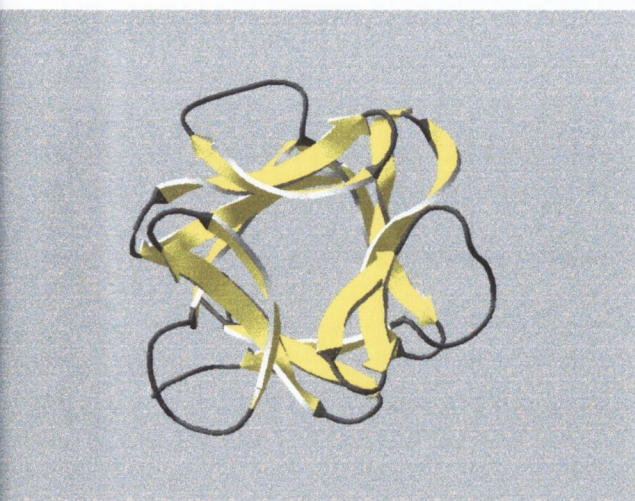


Closed end of barrel

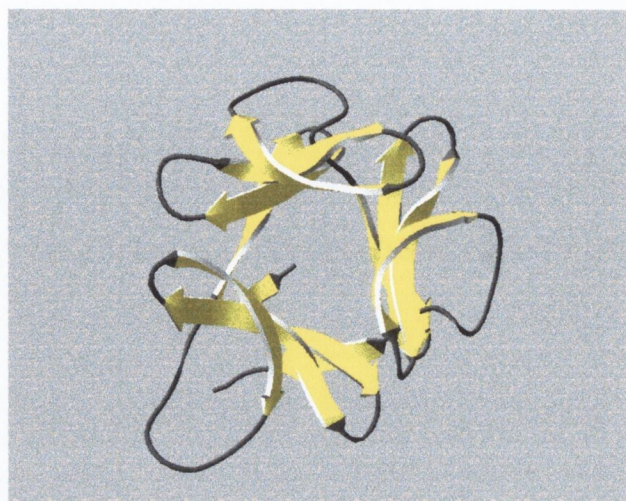


Perpendicular to barrel axis

Figure 6.2.13 Structure of murine IL-1F5 in ribbon diagram representation. The protein is viewed from three different angles; looking down the barrel axis from both the open and closed end and also perpendicular to the axis. The β -strands which contribute to the barrel are coloured green, while those that contribute to the cap are orange. Helices are coloured yellow.



Fibroblast Growth Factor



Hisactophilin



IL-1 β

Figure 6.2.14 Three members of the β -trefoil family of proteins. All the members of the protein structural family, represented here by fibroblast growth factor, hisactophilin and IL-1 β , share the same structural topology of twelve β -strands joined by connecting loops. Six of the strands form a β -barrel, while the remaining six form a cap structure at one end of the barrel. All proteins are viewed down the barrel axis with the closed end nearest the viewer.

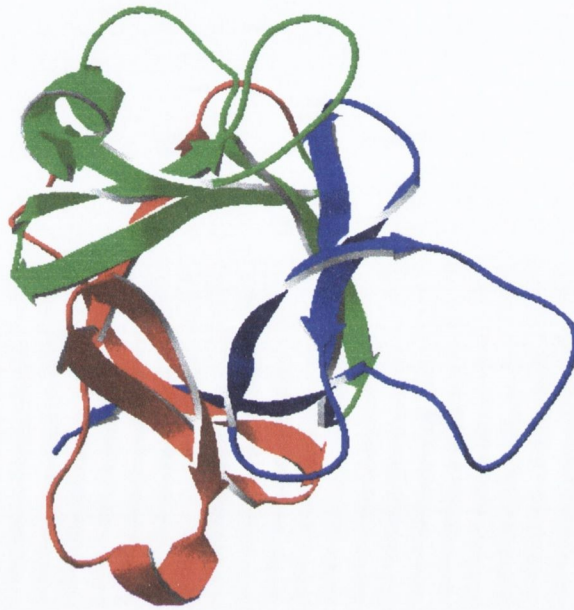
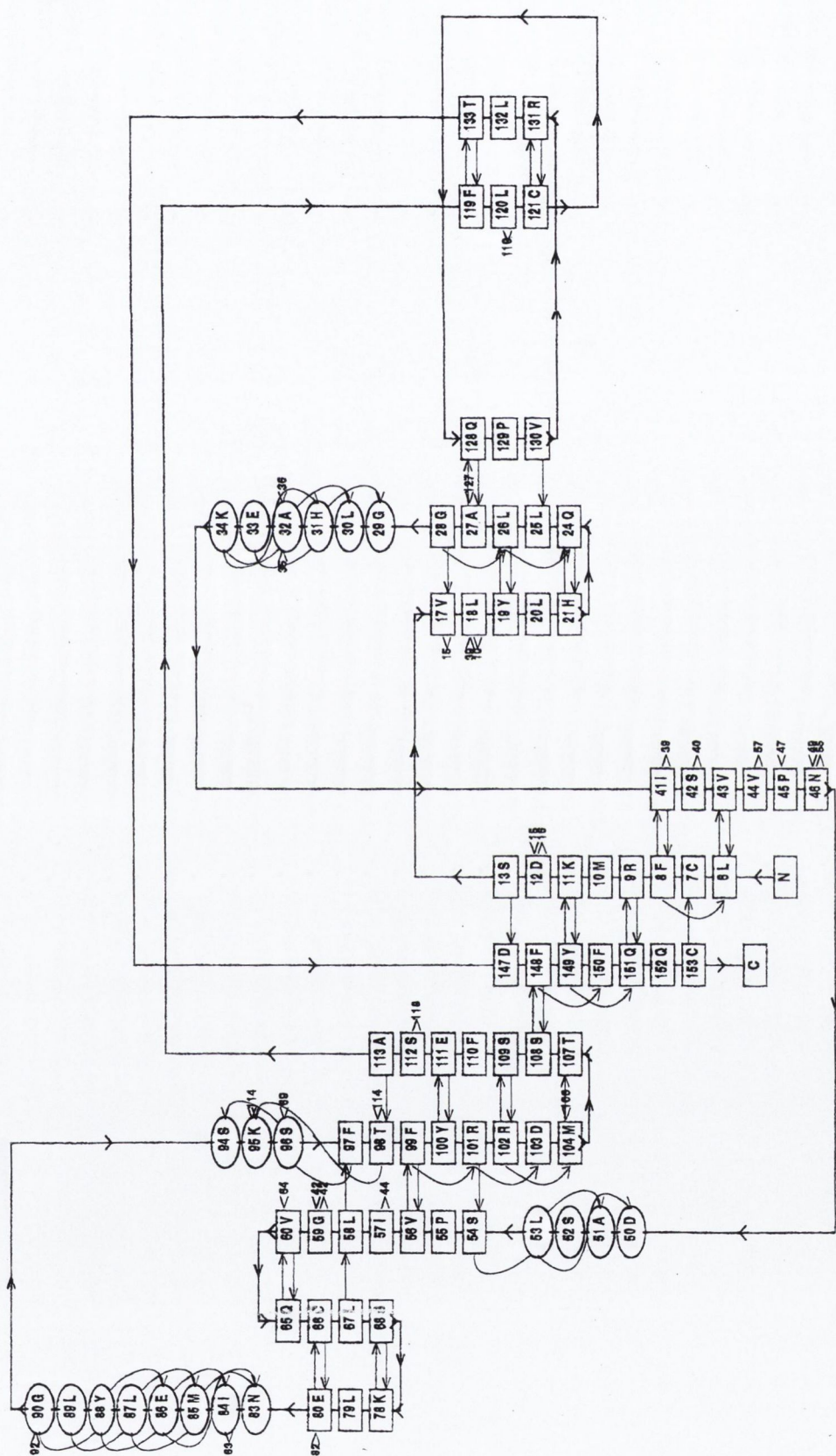


Figure 6.2.15 Pseudo-threefold symmetry of IL-1F5. Each unit of the three fold symmetry is coloured red, green and blue. Two strands from each unit contribute to the β -barrel and two contribute to the cap structure. The protein is viewed down the barrel axis with the closed end nearest the viewer.

Figure 6.2.16 Hydrogen bonding map of IL-1F5. Residues involved in β -strands are shown in boxes, while those involved in helical structures are shown in circles. Hydrogen bonds are represented by arrows between residues, or by arrow heads followed by the residue number of the bonding partner.



al. (1995) but is in a different location between Cys69 and Cys116. The disulphide of IL-1F5 may therefore play a role in helping to stabilise the β -barrel and indeed the structure seems to be quite resistant to guanidine-HCl unfolding (see chapter 4, figure 4.2.24). In addition to hydrogen bonds between strands there are some hydrogen bonds that anchor loop regions also. In this way, loop 3-4 is connected to strand 2 via a mainchain hydrogen bond between Leu18 and Glu39, strand 3 also links to loop 10-11 via another mainchain hydrogen bond between Ala27 and Asp127.

The barrel is packed by three layers of sidechains, pointing into its interior (Figure 6.2.17). These are contributed by alternate residues on the strands each of which contributes 3 residues to the interior layers, with the exception of strand 9, which does not contribute any residue to the bottom layer. This bottom layer is quite variable in its residue composition due to its exposure at the end of the barrel. The other two layers however fill the interior with mainly large hydrophobic residues. The only polar residues to occur in these layers are Ser108 and Arg101. Arg101 contributes to the middle layer but its sidechain reaches out in to the solvent. The top layer of residues interact also with the cap structure, in particular the residues of Ile41, Phe99 and Phe148 can be seen to point away from the barrel up into the cap structure. These two interior layers have a volume of $1358 \text{ \AA}^3$, which compares well with those of IL-1 β , which have a volume of $1345 \text{ \AA}^3$. The residues are therefore large enough to fill the barrel formed by the six β -strands, which has a mean diameter of $15.5 \text{ \AA}$ (Figure 6.2.18). This comparable to the mean diameter of $16 \text{ \AA}$ for other β -trefoil proteins reported by Murzin et al. (1992).

The cap structure is also held together by hydrogen bonds between the upper (relative to the barrel) strands 3 and 11. Strand 7, which contributes the third upper strand would be expected to form bonds with strands 3 and 11 but is too short to do so. There is however a side chain-side chain interaction between Lys78 of strand 7 and Asn23 of the short loop preceding strand 2. Two layers of three residues each, pack the cap structure; strands 2, 6 and 10 each contribute a leucine to the bottom layer, while strands 3, 7 and 11, contribute one residue each to the top layer, a leucine from strands 3 and 7 and a valine from strand 11 (Figure 6.2.19). It is these residues, which interact with the residues pointing up from

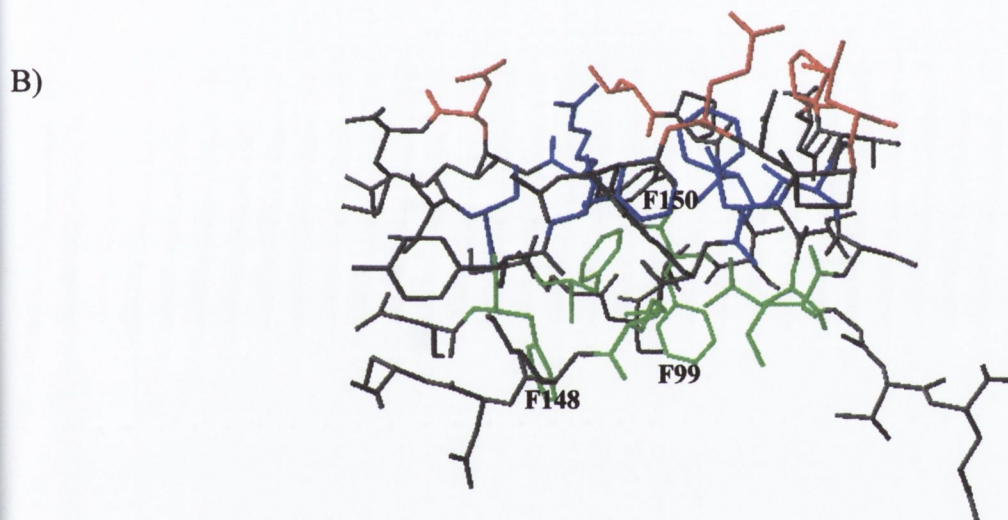
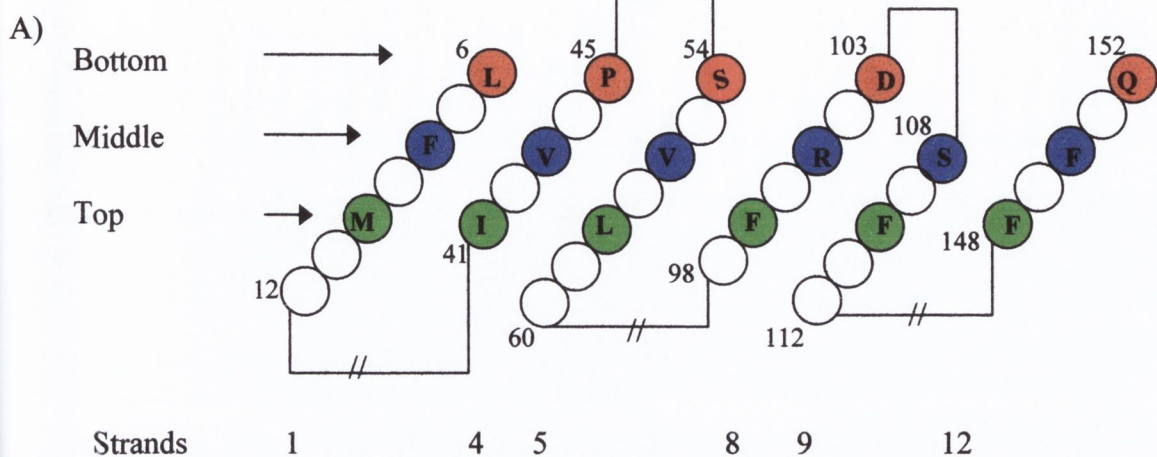


Figure 6.2.17 Schematic diagram and stick representation of the β -strands which form the barrel in IL-1F5. A) Residues which contribute to the bottom (solvent exposed) layer are coloured red, those that contribute to the middle layer are coloured blue and those that contribute to the top (packed against cap) are coloured green. All are labelled with their respective amino acid one letter codes. β -Strand numbers are also indicated. (Adapted from Murzin *et al.*, 1992.) B) Stick diagram of backbone and sidechain of residues involved in β -barrel formation. Colour scheme is the same as for 'A' with residues Phe99, Phe148 and Phe150 are labelled.



Figure 6.2.18 Diameter of the six-stranded β -barrel of IL-1F5. The $C\alpha$ traces of the β -strands which contribute to the β -barrel are depicted, with a representative selection of diameter measurements shown. Figures represent diameter in Å. The mean diameter of the barrel is calculated as 15.5 Å.

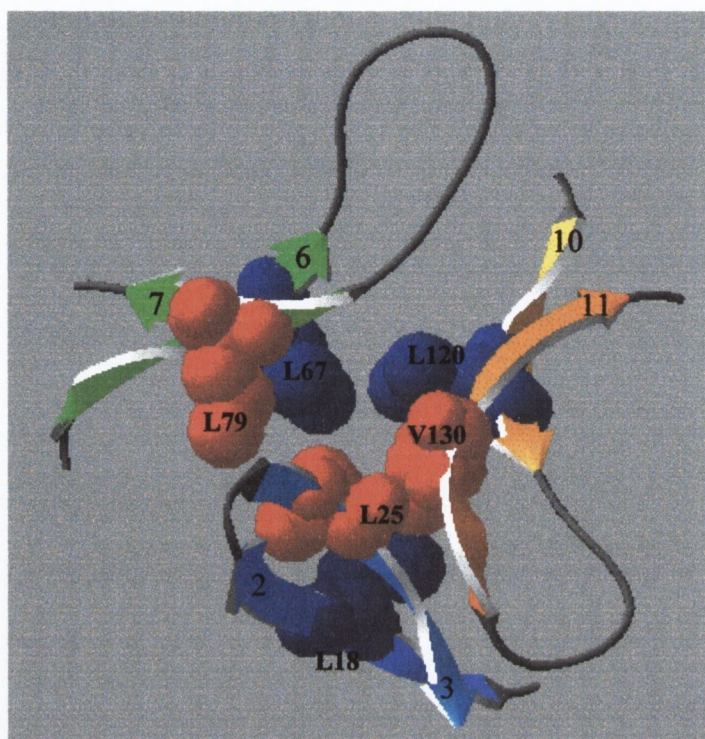


Figure 6.2.19 The cap structure of IL-1F5. β -Strands are shown by ribbons and coloured blue for strands 2 and 3, green for strands 6 and 7 and orange for strands 10 and 11. Residues that contribute to the two layers of the cap are shown in spacefilling. They are coloured blue for the bottom layer and red for the top layer and are labelled according to residue number and one letter amino acid code.

the barrel causing the two regions of the structure to interlock. The residues of this cap structure interior are the most highly conserved across the family of β -trefoil proteins. Murzin et al. (1992) report that for the proteins they examined (IL-1 β , FGF and ETI) all had either leucines or valines in this position, with just one exception. IL-1F5 does not deviate from this requirement for small and medium sized hydrophobic residues in this region, with all residues contributing to the cap interior being either leucines or valines.

6.2.4 Loop Conformations

The loops connecting the β -strands vary considerably in length and structure throughout the molecule. Within the repeating unit however, the arrangement of the loops can be described as consisting of hairpins connecting strands 1 to 2 and 2 to 3, with a longer and more variable loop connecting strands 3 to 4. Repeating units 1 and 2, and 2 and 3 are also connected by β -hairpins, giving a total of 11 loops. Of the 8 loop that form hairpin turns all belong to classifications previously described and based on mainchain ϕ , ψ angles as well as the number of hydrogen bonds linking the anchor residues of the turn (Table 6.2.5) (Lewis et al., 1973; Sibanda et al., 1989).

Of particular interest is the presence of two helical structures; helix 1, an α -helix which consists of residues Leu30 to Lys34 of loop 3-4 and helix 2 at the end of loop 7-8 from residues Ile84 to Leu89. The helix in loop 3-4 is located in a region that is close to the symmetry related molecule. However, the packing distance between the symmetry related molecules is always greater than 4 Å for those residues that are part of the helix, with the exception of Lys34, which is involved in a non-hydrogen bond interaction with Ser54 (Table 6.2.6). The residues in IL-1F5 adjacent to helix 1 that do contact the symmetry related molecule at 3.5 Å are Glu125, Glu128 and Trp141 (Table 6.2.6). In the helical region there are hydrophobic interactions to the symmetry related molecule, however there are no water molecules at this interface and the water molecules that stabilise the mainchain and sidechain conformation of helix 1 are on the solvent accessible area. These water molecules form an extensive and well ordered hydrogen bonding network; the mainchain conformation of Glu33 is stabilised by water28 and 142, with the

Loop	type
1-2	2:2 I
2-3	2:2 I'
3-4	
4-5	8:10
5-6	4:6
6-7	5:7
7-8	
8-9	2:4
9-10	5:7
10-11	9:9
11-12	

Table 6.2.5 Classification of β -turns in IL-1F5. β -turns are defined according to the number of residues in the loop and the hydrogen bonding pattern of residue i (Sibanda *et al.*, 1989). Type I and type I' turns are defined according to the ϕ , ψ angles of residues in position $i+1$ and $i+2$ (Lewis *et al.*, 1973).

Contact region on IL-1F5m	Residue	Symmetry related molecule	Residue	Distance (Å)
Helix 1	Tyr19 OH	X	Leu2 Cδ2	3.52
	His21 Nδ1	X	Leu2 Cδ2	3.82
	His21 Cγ	X	Leu6 Cδ2	3.63
	Leu26 Cδ1	X	Leu2 Cδ2	3.77
	Leu30 Cγ	X	Ala51 Cβ	3.90
	Leu30 Cδ2	Z	Ala48 Cβ	3.69
	His31 Cβ	X	Pro45 Cβ	3.74
	His31 Ne2	X	Ala51Cβ	3.64
	His31 Cδ2	X	Pro55 Cγ	3.93
	Lys34 Cβ	X	Ser54 Oγ	3.25
	Val35 Cγ1	X	Leu6 Cδ1	3.69
	Val35 Cγ1	X	Phe8 Cζ	3.83
	Val35 Cγ1	X	Val43 Cγ1	3.83
	Val35 Cγ2	X	Pro45 Cγ	3.97
	Val35 Cγ2	X	Val46 Cγ1	3.65
	Ile36 Cγ2	X	Phe8 Cζ	3.93
	Ile36 Cγ2	X	Gln152 Ne2	3.86
	Pro124 Cβ	Z	Val1 Cγ2	3.78
	Glu125Oε1	Y	Glu125 Oε1	3.45
	Glu125 Oε2	Y	Gln128 Ne2	2.5
	Gln128 Oε2	Y	Glu125 Oε2	2.93
	Glu125 Oε1	Z	Val1 Cγ1	3.2
	<i>Trp141 Cδ2</i>	Z	<i>Met85 Cβ</i>	3.4
	Trp141 Cζ2	Z	Asn83 Nδ2	3.62
	Asp142 Cβ	Z	Val1 Cγ2	3.9
	Asp142 Oδ1	Z	Ser3 Cβ	3.52
Helix 2	Leu2 Cδ2	B	Tyr19 OH	3.6
	Leu2 Cδ2	B	His21 Nδ1	3.73
	Leu2 Cδ2	B	Leu26 Cδ1	3.82
	<i>Leu6 Cδ1</i>	<i>B</i>	<i>Val35 Cγ1</i>	3.33
	Leu6 Cδ2	B	His21 Cδ2	3.62
	Phe8 Cζ	B	Val35 Cγ1	3.86
	Val43 Cγ1	B	Val35 Cγ1	3.84
	Pro45 Cβ	B	His31 Cβ	3.76
	Pro45 Cγ	B	Val35 Cγ2	3.74
	Ala51 Cβ	B	Leu30 Cγ	3.91
	Ala51 Cβ	B	His31 Ne2	3.63
	Ser54 Oγ	B	Lys34 Cβ	3.4
	Pro55 Cγ	B	His31 Cδ2	3.93
	Val1 Cγ2	F	Pro124 Cβ	3.8
	Val1 Cγ2	F	Asp142 Cβ	3.89
	Val1 Cγ1	F	Glu125 Oε1	3.2
	Ser3 Cβ	F	Asp142 Oδ1	3.52
	Ala48 Cβ	F	Leu30 Cδ2	3.76
	Asn83 Nδ2	F	Trp141 Cζ2	3.61
	<i>Met85 Cβ</i>	<i>F</i>	<i>Trp141 Cδ2</i>	3.4
	Leu49 Cd2	E	Asp50 Oδ2	3.71
	Asp50 Od2	E	Leu49 Cδ2	3.71
	Asp50 Od1	E	Glu93 Oε2	3.79
	Ser52 Cβ	E	Glu93 Oε2	3.52
	Glu93 Oε2	E	Asp50 Oδ1	3.79
	Glu93 Oε2	E	Ser52 Cβ	3.53

Table 6.2.6 Contact residues between symmetry related molecules. Three molecules, X, Y, Z are contacted around helix 1 and three, B, E, F are contacted around helix 2 Only residues with bonding distances ≤ 4 Å are listed. Hydrogen bonds are highlighted in bold while hydrophobic bonds are highlighted in italic.

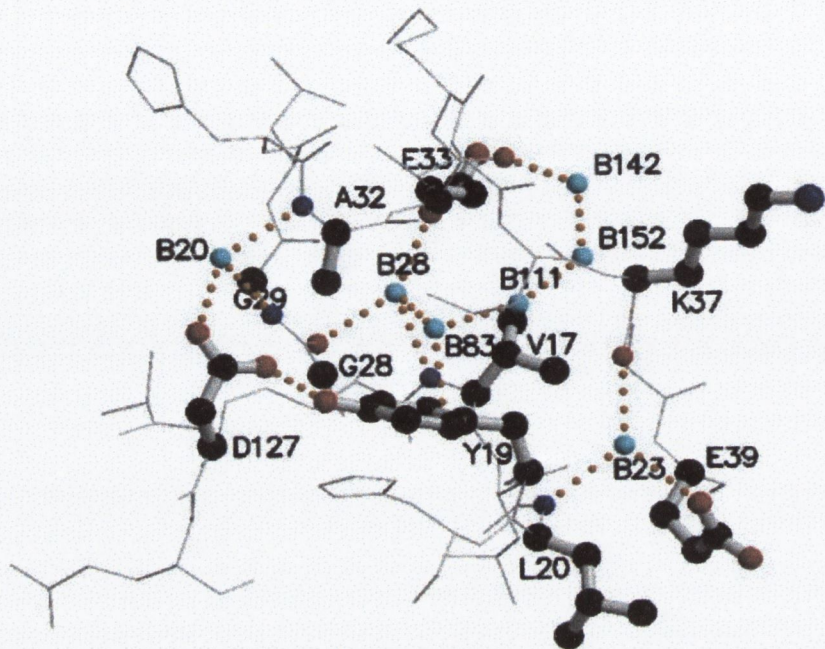
hydrogen-bonding network between Gln33 O and water142 further translated through water152, water111 and water83 to Val17 O. Water28 continues the network to Gly28 O and also Val17 N, and back to Glu33 O ϵ 1. Lys37 N and Glu39 O ϵ 1 are bonded via water 23, which also anchors the loop to the β -barrel through the interaction of Leu20 N on β strand 2. The mainchain conformation of the beginning of the loop is mediated by water 20, which forms hydrogen bonds to Ala32 N and Gly29 N and Asp127 O δ 2 and the network continues through Asp127 O δ 1 to Tyr19 OH of β -strand 2 (Figure 6.2.20). Therefore it is unlikely that these water molecules will be removed upon ligand-receptor complex formation and the strength of the network indicates there is a well ordered structure to the loop, implicating this helix as a contributor to receptor specificity. Both IL-1 β and IL-1Ra also have a helix in the same loop region, though in a different position; in IL-1 β this helix comprises residues Gln34 to Gln38 and residues Pro39 to Leu42 in IL-1Ra. In the case of IL-1 β the helix is also present in the structure solved by NMR techniques (Clare et al., 1991).

Loop 7-8, which also has a unique α -helix between residues Ile84 and Leu89 is likewise stabilised by multiple hydrogen bonds to water (Figure 6.2.20b and 6.2.20c). This hydrogen-bonding network mediates the connection of the loop to the framework of the protein. The α -helix of this loop region is also close to the interface between symmetry related molecules, however there is only one interaction between a residue of this helix and the symmetry related molecule, where Met85 is involved in a hydrophobic interaction with Trp141 of the symmetry related molecule (Table 6.2.6). Therefore as in the case with helix 1, this structure is unlikely to be induced by crystal packing. Because of the unique conformations of these loops they are likely to contribute to the receptor binding specificity of IL-1F5 (see below).

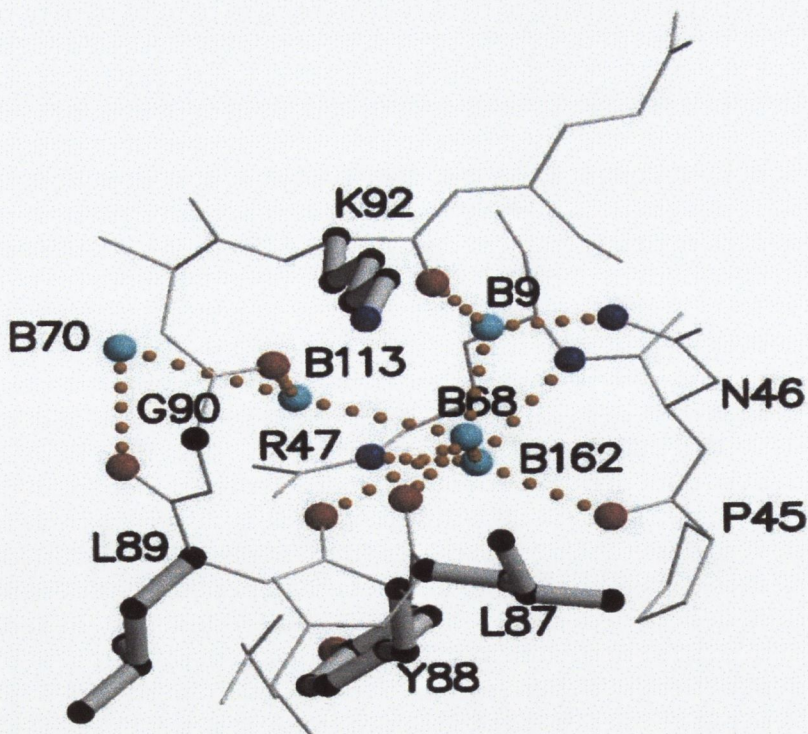
6.2.5 Comparison to IL-1 β and IL-1Ra

A structural alignment of murine IL-1F5 with human IL-1 β , IL-1Ra and acidic and basic FGF is given in figure 6.2.21. The core residues of the related molecules are remarkably

A) loop 3-4



B) loop 7-8



C) loop 11-12

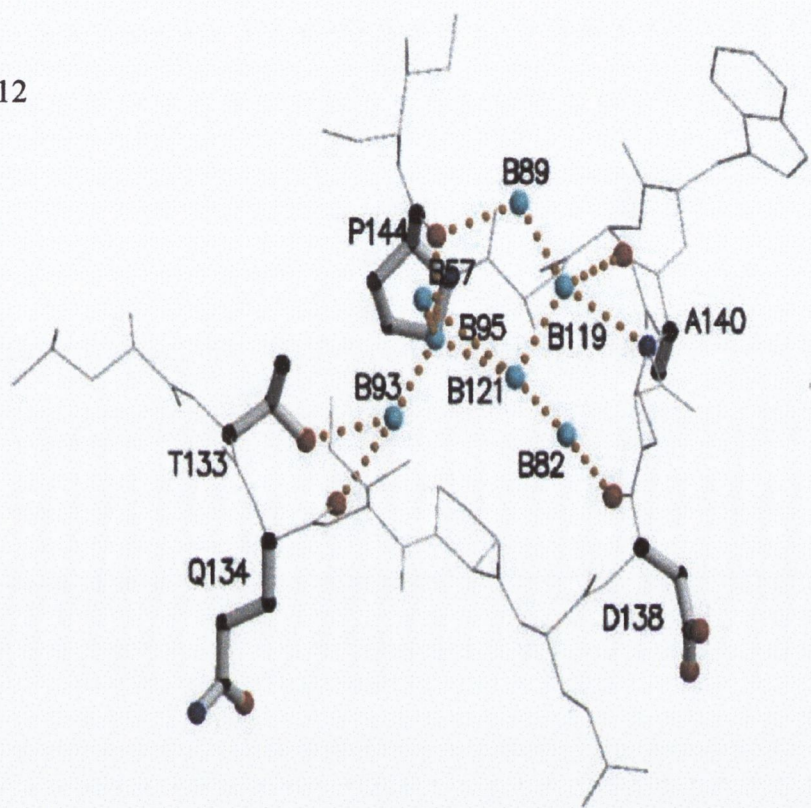


Figure 6.2.20 Hydrogen bonding network around selected loop regions a) helix1 b) C terminal of loop7-8 and c) loop11-12. The sidechains of residues involved in hydrogen bonding to water are represented by ball and stick., hydrogen bonds are represented by a dotted orange line and water molecules are labelled with the letter B. Water, nitrogen, oxygen and carbon are coloured green, blue, red and black respectively. Diagrams were generated using MOLSCRIPT and RASTER3D (Esnouf, 1997).

similar and superimpose very well (Figure 6.2.21, marked by star symbol). Of the twelve residues that pack the α -barrel of IL-1F5 nine are identical to those found in IL-1Ra and the remaining three are similar. They all also have similar χ_1 angles. Five of the residues are identical to those found in IL-1 β ; the remaining seven are similar and again have similar χ_1 angles. All the residues that pack the cap structure core are identical to those found in IL-1 β , and all except Leu18 are identical to those in IL-1Ra where Phe23 occurs. This re-iterates the concept that the hydrophobic core provides the framework onto which variable loop regions, which confer specificity to the molecules, are built.

As expected from the discussion above the overall structure of IL-1F5 is very similar to IL-1 β and IL-1Ra (Figure 6.2.22). Taking only β -strands into account IL-1F5 can be superimposed on IL-1 β using mainchain C α atoms to a RMSD of 0.78 Å, while it can be superimposed on IL-1Ra to an RMSD of 0.79 Å. If loop regions are also included in the calculation then the RMSD for IL-1 β and IL-1Ra is increased to 1.02 Å and 1.04 Å respectively. Five major regions of divergence occur from the structure of IL-1 β : 1) the N-terminal regions are displaced by as much as 7.5 Å from each other before the β -strands superimpose (Figure 6.2.23). 2) In loop 3-4 IL-1F5 has a helix like IL-1 β but the helix precedes that of IL-1 β , they do not superimpose and they are of a different nature; IL-1 β has a 3_{10} helix while IL-1F5 has a short α -helix (Figure 6.2.24). This difference may occur due to the presence of the small glycine as opposed to the large histidine of IL-1 β , which would not allow the formation of the helical structure. Evidence from the structure of IL-1 β and IL-1Ra receptor complexes indicates that this helix is located in a region that is likely to be involved in receptor interaction (Schreuder et al., 1997; Vigers et al., 1997). Indeed at the apex of the helix the sidechain of His31 extends into the solvent, exposed and available for interactions with receptor residues. Neighbouring His31 are Glu33 and Lys34, which are also available for other polar interactions with the receptor. 3) Loop 6-7 is shorter by one residue in the hairpin of IL-1F5 causing the tips of the loops to diverge such that they are 6 Å apart, with that of IL-1F5 pointing down towards the β -barrel (Figure 6.2.25). 4) Loop 7-8, the longest loop in IL-1F5 varies considerably from that of IL-1 β ; helix 2 is located in this region and is a feature that is absent from IL-1 β .

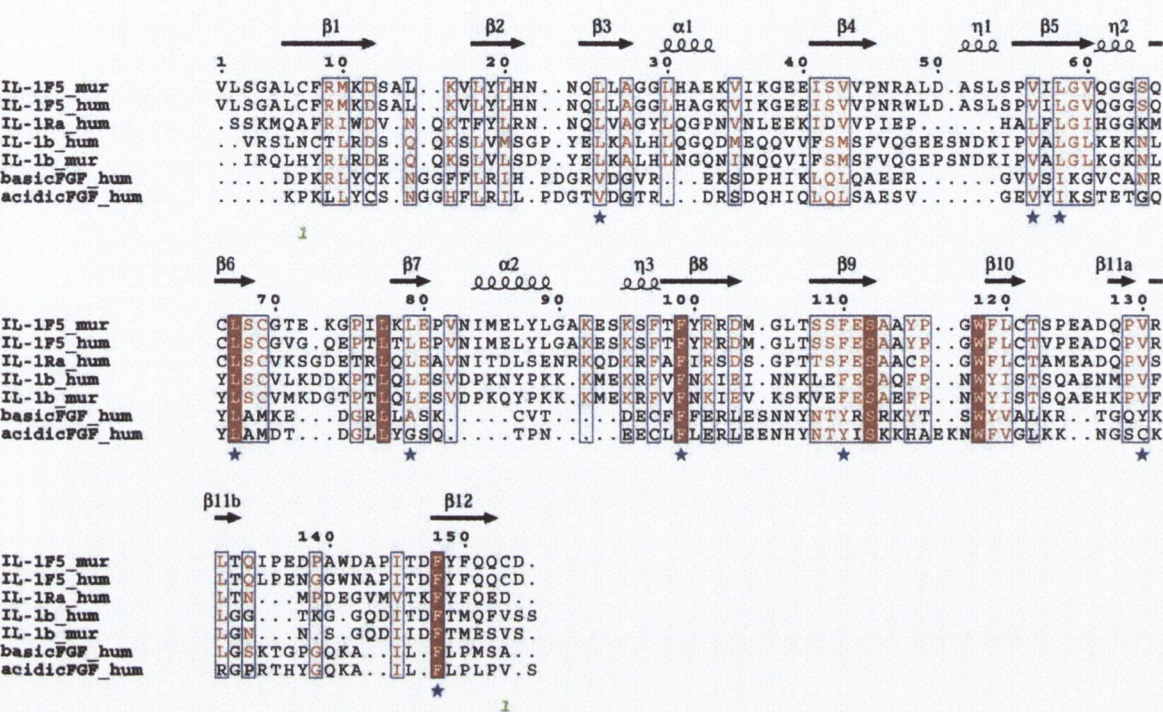
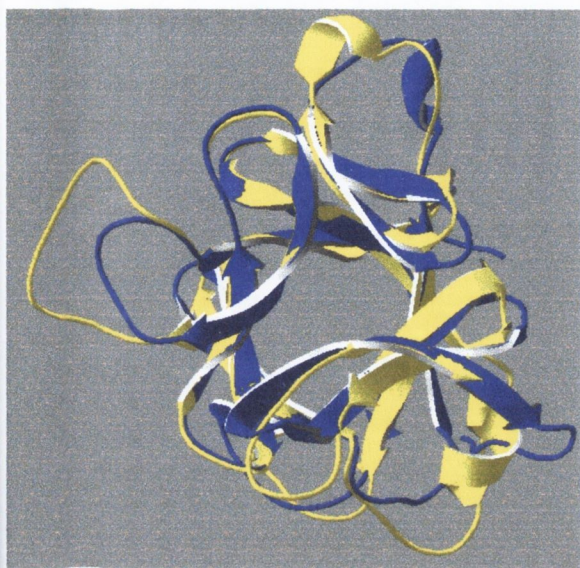
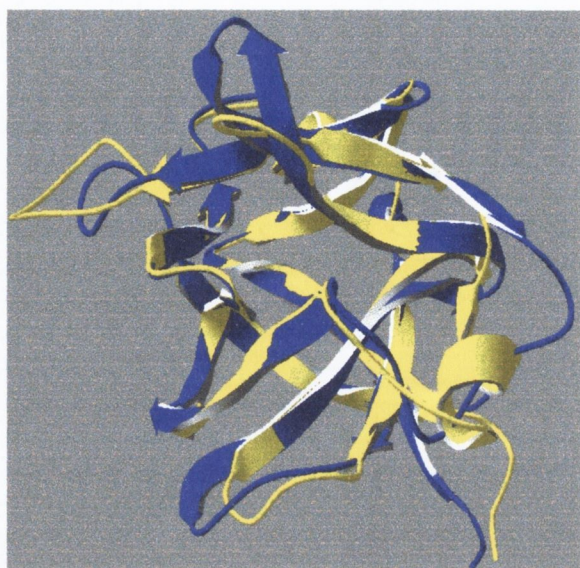


Figure 6.2.21 Structure based sequence alignment of murine IL-1F5 with six other members of the β -trefoil family. IL-1Ra_hum, human IL-1Ra (1IRP), IL-1b_hum, human IL-1 β (1I1B), IL-1b_mur, murine IL-1 β (2MIB), basic FGF_hum, human basic fibroblast growth factor (2FGF), acidic FGF_hum, human acidic fibroblast growth factor (2AFG), the sequence of human IL-1F5 (IL-1F5_hum) is also included. β -strands and helical structures of IL-1F5 are indicated with arrows and coils respectively and numbered according to their succession. The location of the disulphide in IL-1F5 is also indicated by the green digit 1, and conserved residues that contribute to the hydrophobic core are highlighted with a blue star. Residues are coloured according to percentage equivalence.

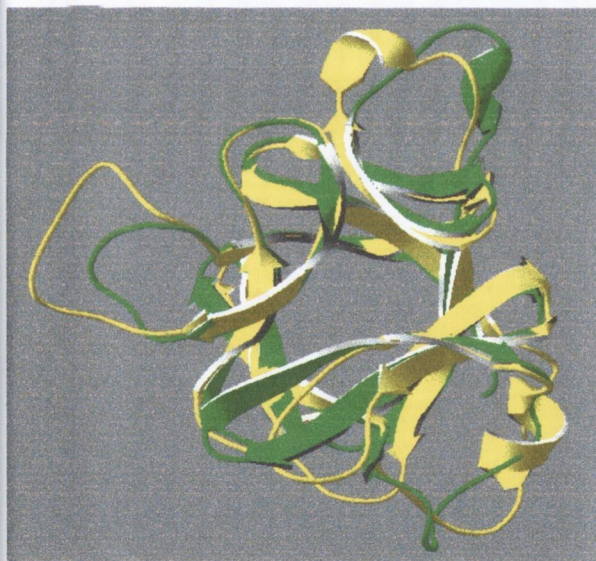
A)



B)



C)



D)

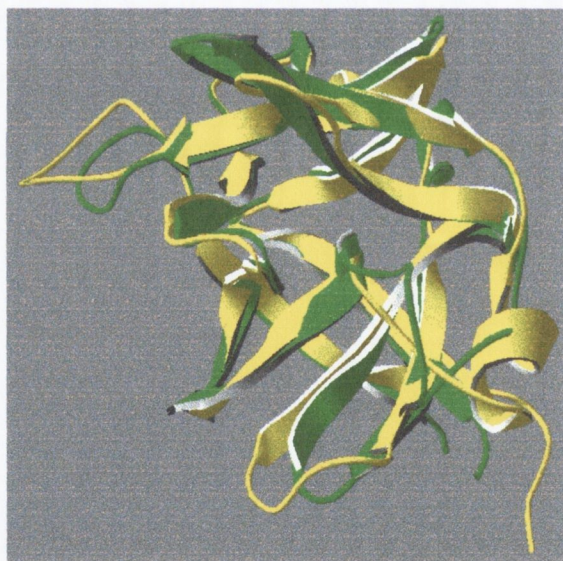


Figure 6.2.22 Superimposition of IL-1F5 on IL-1 β and IL-1Ra. Ribbon diagram representation of the structure of IL-1F5 (yellow) superimposed on that of IL-1 β (blue and IL-1Ra (green). Molecules are viewed, down the barrel axis with the cap structure nearest the viewer (A and C) and perpendicular to the β -barrel with loop 4-5 facing the viewer (B and D).

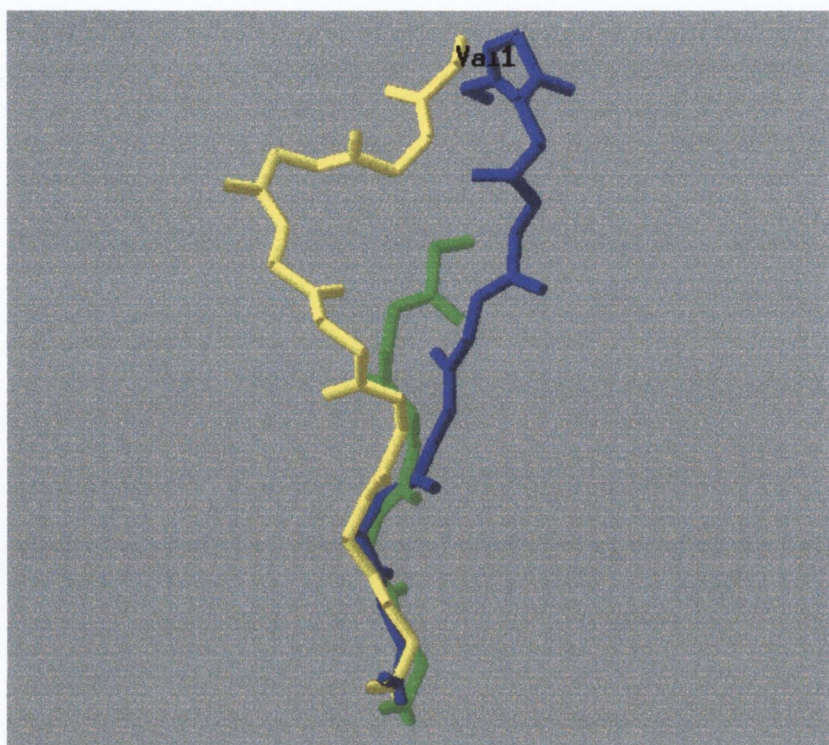


Figure 6.2.23 Comparison of N-terminal region of IL-1F5 (yellow), IL-1 β (blue) and IL-1Ra (green). Only the backbone of each amino acid is presented with Val 1 of IL-1F5 labelled.

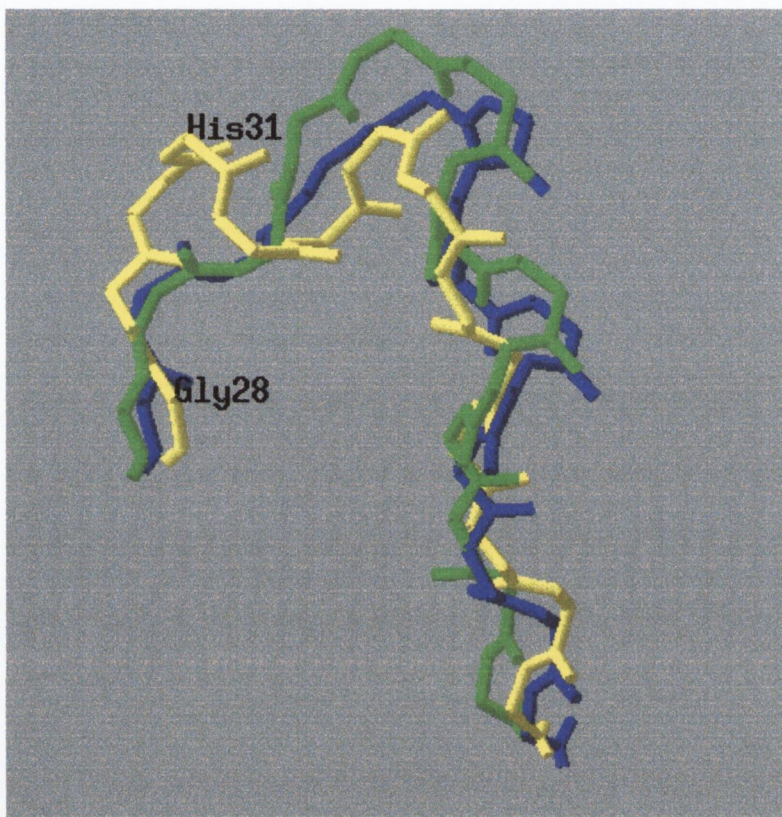


Figure 6.2.24 Comparison of loop 3-4 of IL-1F5 (yellow), IL-1 β (blue) and IL-1Ra (green). Only the backbone of each amino acid is presented with Gly28 and His31 of IL-1F5 labelled.

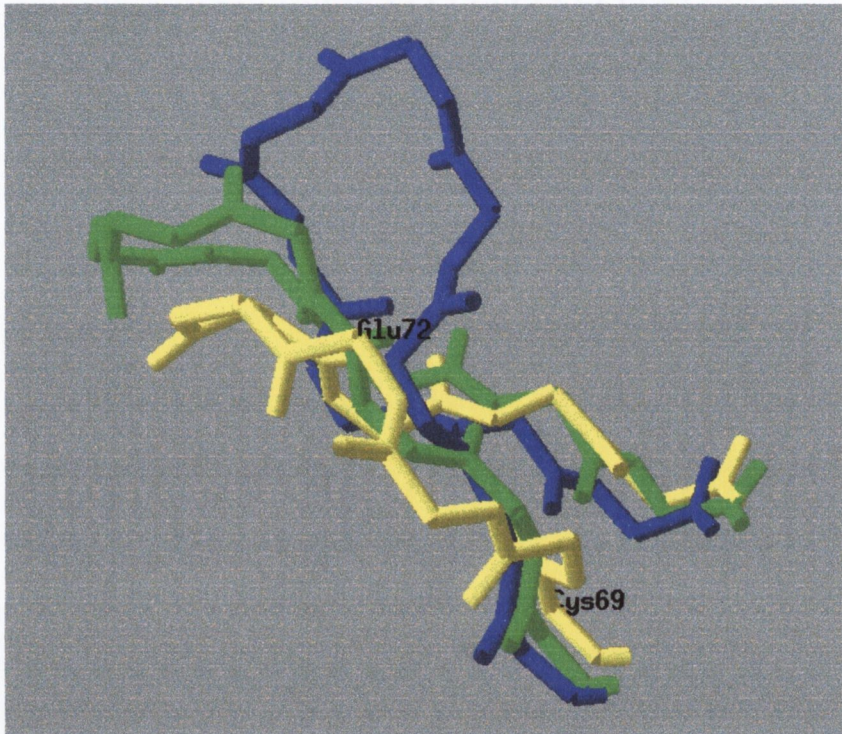


Figure 6.2.25 Comparison of loop 6-7 of IL-1F5 (yellow), IL-1 β (blue) and IL-1Ra (green). Only the backbone of each amino acid is presented with Cys69 and Glu72 of IL-1F5 labelled.

(Figure 6.2.26). There is a hydrophobic patch, similar to one found in IL-1Ra and consisting of residues Val82, Leu87 and Phe97, and also contributed by Val44 and Ile51, that is not present in IL-1 β . The hydrophobic forces in this region stabilise the helix structure. However, in IL-1 β the introduction of a tyrosine at the position equivalent to Leu87 and also Ser45 at the position equivalent to Val44, disrupts the hydrophobic patch. The presence in IL-1 β of a proline at the position equivalent to Ile84 allows for the structural differences seen between the proteins in this region. This loop in IL-1 β contains the polar residues Lys93 and Lys94, which contact domain 3 of IL-1RI. In IL-1F5 there several polar residues, such as Tyr88, Lys 92 and Glu93 whose sidechains are solvent exposed and would be accessible to interact with domain 3 of the receptor, however the structural diversity here is likely to lead to specificity in receptor recognition between the proteins. 5) Loop 11-12 of IL-1F5 has a four residue insertion in it that extends this loop further out into the solvent perpendicular to the barrel axis and level with the barrel/cap interface (Figure 6.2.27).

Extending this comparison to IL-1Ra, then divergences occur at the similar locations; in particular the presence of a tyrosine at the position equivalent to Gly29 does not allow the formation of a helix like that in IL-1F5, and here a 3_{10} helix occurs as in IL-1 β . This tyrosine, like the histidine of IL-1 β , introduces a different molecular and electrostatic surface to IL-1Ra, and this is likely to contribute to receptor specificity between the proteins. Furthermore, in loop 7-8 IL-1Ra does not have the same α -helical structure as IL-1F5. The introduction of a serine at the position equivalent to Tyr88 prevents this. In IL-1F5 this Tyr88, conjugated with Arg47, points towards the solvent and is a potential site of receptor interaction. In addition to these differences IL-1Ra also differs from IL-1F5 in that loop 45 is shorter in IL-1Ra, causing IL-1F5 to mimic the equivalent loop in IL-1 β and point more out into the solvent perpendicular to the barrel axis (Figure 6.2.28). The length of this loop has been shown to influence the antagonist properties of IL-1Ra, and when the β -bulge of the loop from IL-1 β is inserted into IL-1Ra it gains agonist characteristics (Greenfeder et al., 1995b), while replacement of the loop in IL-1 β with that from IL-1Ra causes IL-1 to lose its agonistic properties (Boraschi et al., 1995). This loop interacts with domain 3 of the receptor in IL-1 β , the movement of which is

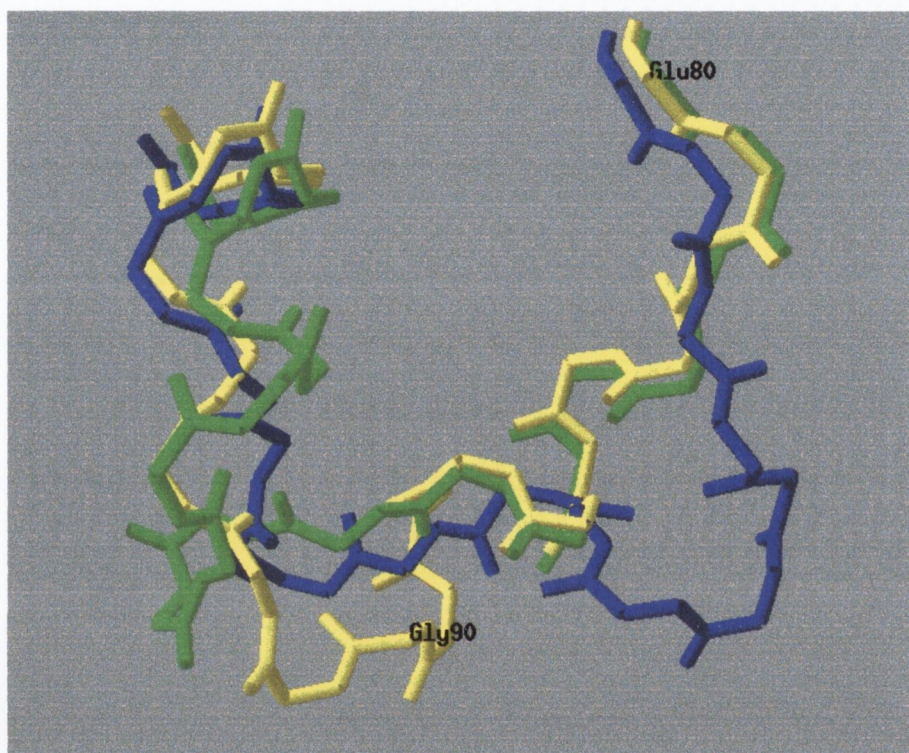


Figure 6.2.26 Comparison of loop 7-8 of IL-1F5 (yellow), IL-1 β (blue) and IL-1Ra (green). Only the backbone of each amino acid is presented with Glu80 and Gly90 of IL-1F5 labelled.

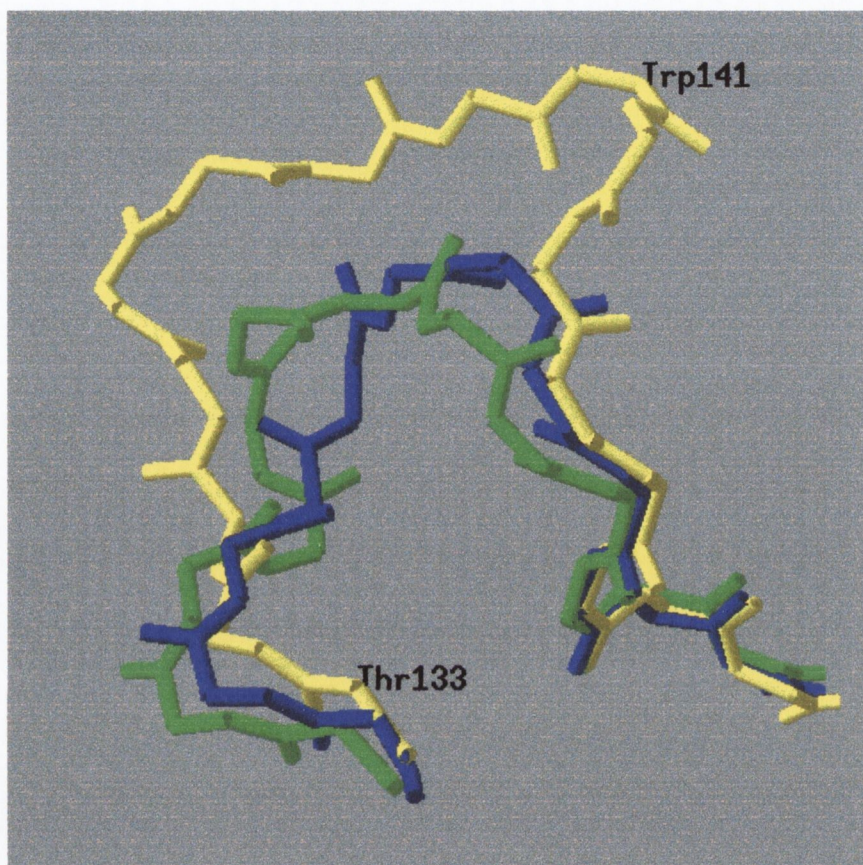


Figure 6.2.27 Comparison of loop 11-12 of IL-1F5 (yellow), IL-1 β (blue) and IL-1Ra (green). Only the backbone of each amino acid is presented with Thr133 and Trp141 of IL-1F5 labelled.

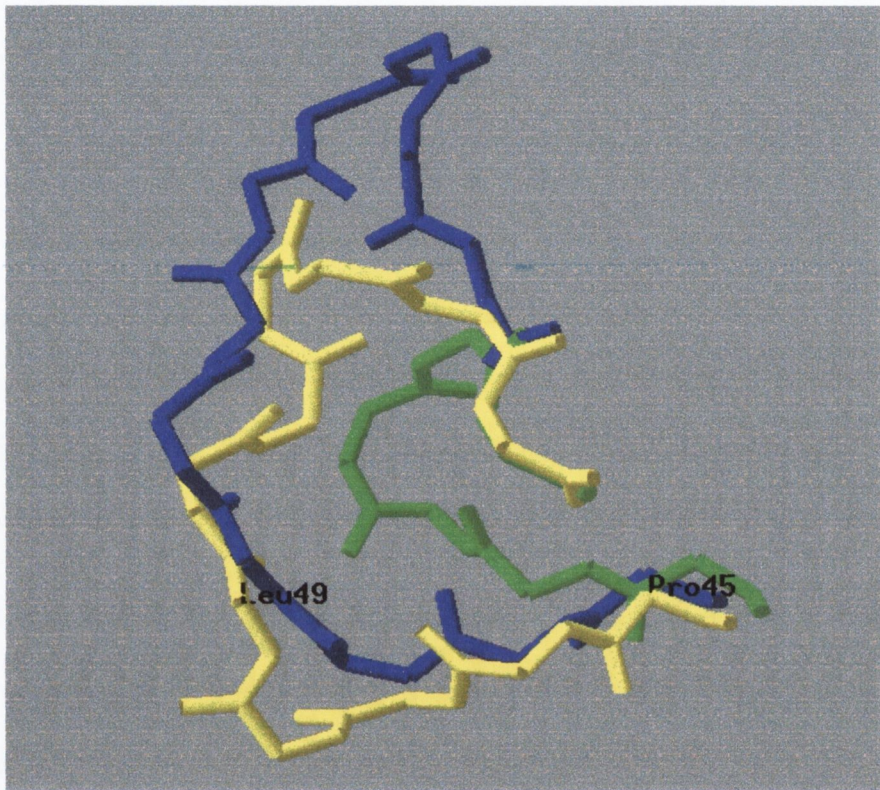


Figure 6.2.28 Comparison of loop 4-5 of IL-1F5 (yellow), IL-1 β (blue) and IL-1Ra (green). Only the backbone of each amino acid is presented with Pro45 and Leu49 of IL-1F5 labelled.

associated with IL-1RAcP recruitment (see section 1.6.8); IL-1F5 may therefore be able to mimic the interaction of IL-1 β in such a manner as to allow recruitment of an accessory protein.

The divergence in structure occurs predominantly in regions known to be important for receptor binding (Evans et al., 1995; Labriola-Tompkins et al., 1991; Palla et al., 1993; Simon et al., 1993). Regions of divergence not implicated in IL-1RI-like binding may therefore be involved in accessory protein binding; one such candidate region would be loop 11-12. Residues from this loop, including Gln134 to Asp147, form a solvent accessible groove filled with water, with residues Glu137, Asp138 and Trp141 solvent exposed, all of which through electrostatic or hydrophobic interactions could act as accessory protein binding residues. The loop is adjacent to Asp147 in IL-1F5, and the equivalent residues in IL-1 β and IL-1Ra have been shown to contribute to agonist and antagonist properties of the molecules and in a way that does not affect binding to IL-1RI (Ju et al., 1991). This residue is therefore implicated in accessory protein interaction. Modelling of the binding of the IL-1RAcP to the IL-1RI/IL-1 β complex indicates that binding may occur via the back of the complex rather than the front (Casadio et al., 2001). Loop 11-12 in conjunction with Asp147 may therefore produce a binding surface for an accessory protein that is distinct to that found in IL-1 β . Like loops 3-4 and 7-8, loop 11-12 is also stabilised by a water hydrogen-bonding network (Figure 6.2.20). The mainchain oxygens of Thr133, Gln134, Asp138, Ala140 and Pro 144 are all stabilised by waters, while the mainchain N of Ala140 and Thr133 O γ 1 are also stabilised by water molecules. This assumes that IL-1F5 is indeed able to recruit an accessory protein and act as an agonist. Comparing the electrostatic potential of the surfaces in this region around residue 147 it is seen that IL-1Ra has a relatively featureless electrostatic surface in this region, whereas in IL-1 β there is a large negative potential due to the presence of Asp145, surrounded by smaller positive patches contributed by Lys138 and Lys109. This is mimicked on the surface of IL-1F5 by the presence of Asp147 and Arg104 (Figure 6.2.29). Electrostatic properties of proteins are well known as major contributors, along with steric and hydrophobic properties, to molecular recognition of other proteins or ligands (Warwicker, 1989; Wells et al., 1987). Therefore the similarity of the electrostatic

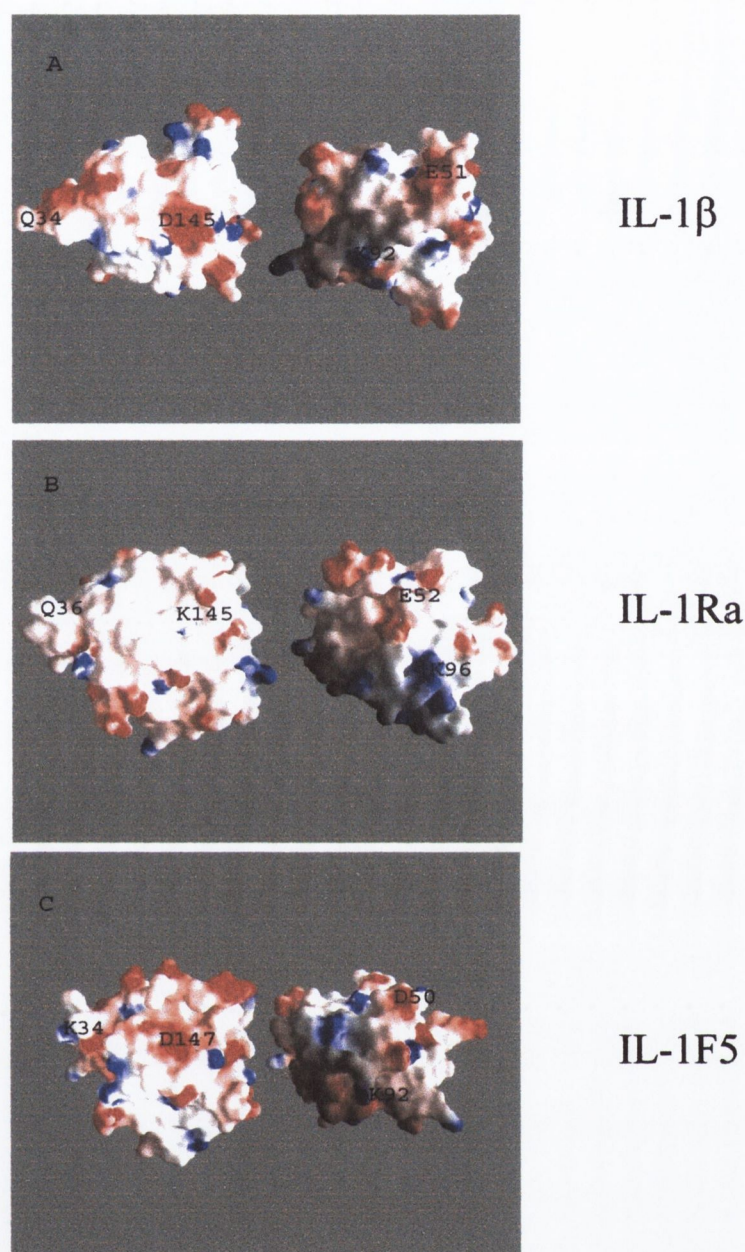


Fig. 6.2.29 Electrostatic potential surface maps of A)IL-1 β , B)IL-1Ra, C)IL-1F5. Negative potential is coloured red and positive potential blue. Greatest saturation is at -10 and $+10$ kT. Molecules are viewed both perpendicular to the barrel axis, with the face exposed in the ligand/receptor complex in the case of IL-1 β and IL-1Ra shown and centred on the residue at the start of β -strand 12 (left) and from the face that interacts with domain 3 of receptor in the case of IL-1 β and IL-1Ra (right). Diagrams were generated using GRASP (Nicholls et al., 1991).

patterns on the surfaces of IL-1 β and IL-1F5 around the functionally important aspartate residue may be an indication that both are indeed designed to bind an accessory protein. The potential sites of receptor interaction on IL-1F5 mentioned here are depicted in figure 6.2.30. Although here is evidence to suggest that IL-1F5 acts as an antagonist to IL-1F9 (Debets et al., 2001), the lack of direct binding shown for IL-1F5 to IL-1R α leaves open that possibility that it could produce this effect by the generation of a negative signal, rather than acting as an inhibitor of IL-1F9 receptor binding.

All the key residues mentioned above are also conserved in human IL-1F5, which is 92.2% identical at the amino acid level to murine IL-1F5. Therefore the conclusions drawn about murine IL-1F5 can also be extended to the human form of the protein.

6.2.6 Modelling of Receptor Binding

By superimposing the structure of IL-1F5 onto that of IL-1 β complex to IL-1RI, sites that preclude binding due to sidechain clashes could be identified. Table 6.2.7 shows the residues of IL-1F5 that might interact with IL-1RI and those that make clashes are highlighted in bold and can be compared with Table 6.2.7b where residues involved in IL-1 β binding to IL-1RI are listed. All the residues that make clashes are with sidechains of receptor residues and they all occur in loop regions where unique conformations as compared to IL-1 β occur. These include loops 3-4, 4-5 and 7-8, all of which are involved in binding in IL-1 β and also loop 10-11 and 11-12 where Glu125 and Trp141 make clashes with domain 2 of the receptor. Furthermore, when the program GRAMM (Katchalski-Katzir et al., 1992) was used to evaluate possible binding conformations of IL-1F5 to IL-1RI no interaction could be found that did not introduce clashes between receptor and ligand (Table 6.2.7c). The best conformation out of 100 possibilities searched produced clashes that occurred in similar areas of IL-1F5 as compared to the superimposed model. In addition it also had clashes in loop 6-7 and the beginning of strand 6. This is as a result of IL-1F5 being docked with a 180° shift perpendicular to the linker of domain 2 and 3 of the receptor. These results indicate that IL-1F5 would not be able to bind to IL-1RI, which is in agreement with studies which show that IL-1F5 cannot

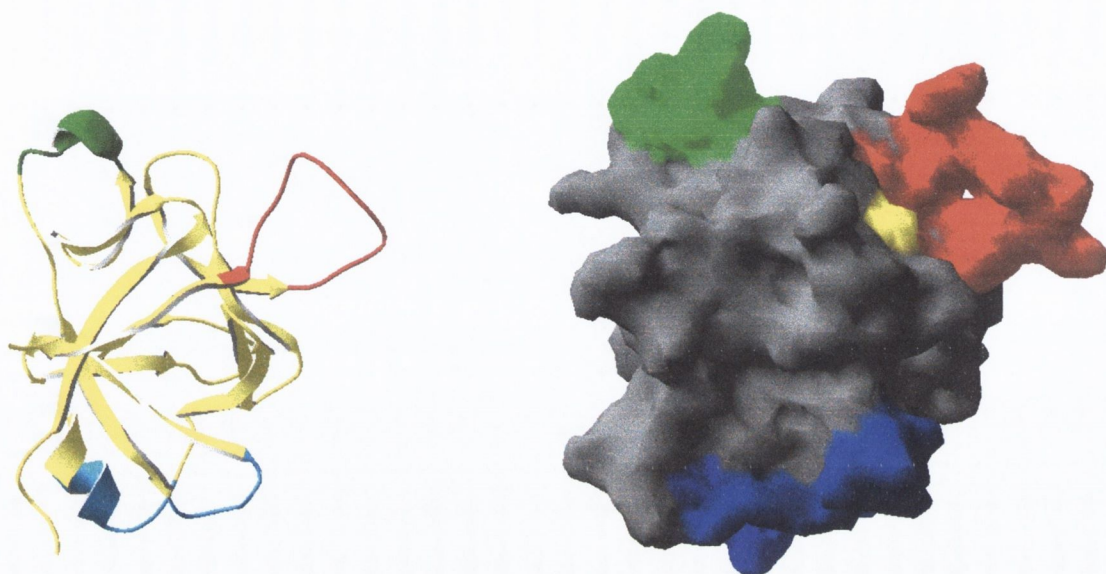


Figure 6.2.30 Potential sites of receptor interaction on IL-1F5. IL-1F5 is shown in ribbon (left) and molecular surface (right) representations, with predicted sites of interaction to domain 1 and 2 shown in green, to domain 3 shown in blue and to the accessory protein shown in red. The position of Asp147 is shown in yellow in the molecular surface representation.

a)

IL-1F5 Residue	IL-1RI (1itb) residue	Distance (Å)
Val1 Cγ2	Asp260 Oδ1	3.1
Val1 Cγ1	Tyr261 Cδ1	3.5
Arg9 NH1	Lys114 Nζ	3.0
Leu15 Cδ1	Gln113 Oε1	2.7
Leu30 Cγ	Phe111 Cγ	0.1
Leu30 Cβ	Pro126 Cγ	3.4
His31 Cγ	Leu15 Cδ1	0.5
His31 Nε2	Ser17 Oγ	3.1
His31 Nδ1	Phe111 Cζ	3.1
His31 Cβ	Tyr127 OH	2.68
Ala32 Cβ	Ile13 Cδ1	3.3
Val35 Cδ	Ile13 Cγ1	2.3
Val35 Cγ2	Ile14 Cδ1	3.4
Ile36 Cδ1	Ile13 Cβ	2.95
Arg47 Cδ	Tyr261 OH	2.6
Ala51 Cε	Tyr242 OH	2.8
Ala51 Cβ	Lys298 Nζ	1.8
Tyr88 OH	Tyr261 Cδ1	2.86
Leu89 Cβ	Glu252 Oε2	2.7
Ala91 Cβ	Asp251 Oδ2	3.5
Ala91 Cβ	Asp253 Oδ2	1.1
Asp103 Oδ2	Thr300 Cδ2	2.56
Leu106 Cδ2	Pro116 Cγ	3.4
Glu125 Oε2	Glu129 Cδ	1.6
Trp141 Cζ2	Val162 Cγ1	2.58
Trp141 Cδ1	Lys161 Nζ	0.76
Gln151 Nε2	Leu201 Cδ1	3.2

b)

IL-1F5 Docked Residue	IL-1RI (1itb) residue	Distance (Å)
Leu30 Cβ	Ile13 Cγ1	1.68
His31 Cε1	Ile14 Cδ	3.12
Glu33 Oε1	Ile13 Cδ	3.45
Lys34 Cε	Phe111 Cε1	2.3
Val35 Cγ2	Lys112 Nζ	2.12
Ile36 Cγ2	Lys114Nζ	2.88
Lys37 Cε	Lys114 Cβ	2.29
Ser64 Cβ	Ser238 Cβ	2.07
Thr71 Cγ2	Glu252 Oε2	2.17
Pro81 Cγ	Ser263 Oγ	3.26
Val82 Cβ	Ile240 Cγ2	2.45
Asn83 Cβ	Ile240 Cδ	3.03
Asn83 Oδ1	Thr300 Cγ2	3.13
Glu86 Oε1	Ile240 Cγ1	2.53
Glu86 Cδ	Tyr242 Cε1	1.75
Glu86 Cβ	Lys298 Cε	1.51
Lys92 Cδ	Val249 Cγ1	1.87
Lys92 Nζ	Glu259 Oε2	2.84
Lys92 Nζ	Tyr261 OH	3.4
Leu106 Cγ	Pro31 Cβ	2.85
Trp141 Cβ	Glu10 Oε2	3.29
Tyr149 OH	Asn30 Cβ	3.34

c)

IL-1β Residue	IL-1RI (1itb) residue	Distance (Å)
Arg4 NH1	Ser262 Oγ	2.95
Arg4 NH2	Leu275 Cδ2	3.2
Arg11 NH2	Lys114 Nζ	2.75
Gln14 Oε1	Arg163 Nε	3.3
Glu25 Oε2	Asn30 Cδ	3.5
Lys27 Nζ	Glu11 Oε2	2.85
Gln32 Oε2	Phe111 Cζ	3.3
Glu51 Cγ	Lys 298 Nζ	3.1
Asn53 Nδ2	Ile303 Cγ1	3.2
Lys93 Nζ	Glu259 Oε3	3.1
Lys94 CB	Glu252 Oε1	3.2
Glu105 Oε2	Thr300 Oγ1	3.3
Asn108 Nδ2	Asn204 Nδ2	2.8
Glu128 Cγ	Tyr127 Cε2	3.35
Glu128 Cβ	Glu129 Oε1	3.0

Table 6.2.7. Contact residues of IL-1RI. The residues of IL-1RI which make contact with a) IL-1F5 superimposed on IL-1β in the receptor ligand complex (1ITB), b) IL-1F5 docked to the receptor using GRAMM (Katchalski-Katzir *et al.*, 1992) c) IL-1β. Only residues with bonding distances $\leq 3.5\text{\AA}$ are listed. Those with bonds $\leq 2.5\text{\AA}$ are highlighted in bold.

bind IL-1R1 (Smith et al., 2000), or that IL-1RI would have to undergo structural changes in order to accommodate the IL-1F5 ligand.

Therefore, the structure of murine IL-1F5 indicates that its loop regions are likely to confer receptor specificity to the protein and that receptor binding may occur in such a way as to induce recruitment of an IL-1RAcP-like protein.

Chapter 7 Final Discussion and Future Perspectives

This study aimed to gain insights into the possible function of the novel IL-1-like cytokines IL-1F5 and IL-1F6. On the basis of sequence homology it could be seen that both of these proteins are related to IL-1 and contain the IL-1 signature motif. However, IL-1F5 shows greatest similarity to IL-1Ra and the structure solved as a result of this work shows that on the basis of a structural alignment human IL-1Ra and murine IL-1F5 share 44% sequence identity. Given this level of sequence identity is it reasonable to assume that the proteins are related in function also. Indeed, both IL-1 β and IL-1Ra are known to fold into the same 12-stranded β -trefoil structure. The bioinformatic analysis carried out suggested that IL-1F5 has a similar secondary structure to IL-1, consisting of the 12 β -strands required for the β -trefoil. Meanwhile, hydropathy plots indicated that IL-1F5 had similar hydrophobicity characteristics to IL-1Ra, this would suggest that the amino acid sequence would fold in such a way that the relationship between the secondary structure elements would be similar. This again suggested that IL-1F5 could adopt the same or similar structure to IL-1 and IL-1Ra.

However, the question still arises as to whether the greater sequence identity with IL-1Ra can be taken as an indication that the proteins also share functional similarities. Knowledge of the structures of IL-1 β and IL-1Ra complexed to IL-1RI show that the majority of residues of functional importance are within the loop structures of the proteins. This is consistent with the theory that in the β -trefoil structure, it is the loop regions that are important for conferring functional specificity to the proteins, while the β -strands provide the structural framework onto which the variable loops are built (Murzin et al., 1992). This is also evidenced by the fact that the members of the β -trefoil family, although sharing the same structure have little or no functional similarities. Therefore, a comparison of loop structure sequences may give a more meaningful insight into the relationship of the IL-1 proteins in terms of functional relatedness. When IL-1Ra and IL-1F5 were compared in this way their sequence homology dropped to 28%, which

suggested that, although the two proteins are definitely closely related, their functions may have diverged. Other sequence information also suggested that IL-1F5 could act as an agonist, specifically the presence of an aspartate residue in the position equivalent to one found in IL-1 β and IL-1 α , which when mutated to a lysine, as found in IL-1Ra, converts them to antagonists. This residue is implicated in the ability of IL-1 to recruit the IL-1RAcP, which is essential for the activation of signal transduction (Cullinan et al., 1998). The presence of the same residue in IL-1F5 may be an indication that this protein is also able to recruit an accessory protein and activate a signal transduction pathway. Furthermore, the absence of a deletion in the alignment of the protein family at the position of loop 4-5 in IL-1F5 is also the loss of a feature that is important for the antagonistic properties of IL-1Ra. This loop is known to interact with domain 3 of IL-1RI and a shortening of it in IL-1 β reduces biological activity (Boraschi et al., 1995; Simoncsits et al., 1994) or its insertion into the K145D mutant of IL-1Ra increases the mutant's agonist properties (Greenfeder et al., 1995b). Therefore the presence of an equivalent loop in IL-1F5 was another indication that it may function as an agonist.

I set out to test whether IL-1F5 and a second novel IL-1, IL-1F6 could functionally mimic IL-1Ra in cell based assays. These assays tested the ability of the protein to inhibit intracellular signals known to be initiated by IL-1, including the ability of IL-1 to cause translocation of NF κ B to the nucleus and to induce NF κ B regulated gene expression, as well as the ability of IL-1 to induce activation of various MAP kinases. None of these experiments suggested that IL-1F5 could inhibit IL-1 activity. Furthermore, the proteins were also tested for their ability to inhibit IL-18 signalling, but again no activity was detected. These data suggested that IL-1F5 could not act in a way similar to IL-1Ra. Furthermore, experiments to assay the ability of IL-1F5 to activate signals in a manner similar to IL-1 also lead to negative results. Together these results suggested that IL-1F5 could not bind to the IL-1RI or IL-18R in a functional manner. Similar results were obtained when the same assays were carried out using IL-1F6.

These data also highlight the need for caution when predicting function based solely on amino acid sequence identity. The data clearly show that despite the sequence homology

between IL-1F5 and IL-1Ra, IL-1F5 is unable to act as a novel IL-1 antagonist. Also, the fact that no IL-1-like signal could be detected in these cells suggested that the cell types used do not express the receptor for either of the novel proteins. However, the homology to IL-1 itself, although clearly established does not guarantee that the proteins will activate similar signalling pathways and the possibility that the cells do express the receptor but generate signals unlike those produced by IL-1RI cannot be ruled out. One way of overcoming this problem might be to look for the generation of global signals such as the generation of newly phosphorylated proteins upon addition of the ligand to cells or to assay cells for the ability to bind the ligands in a specific way.

Given the lack of effect in the IL-1 responsive cells tested, it was decided to try and localise the expression of IL-1F5 and use this as point from which to examine the possible biological effects of IL-1F5. Reports suggest that IL-1F5 is well expressed in the placenta and skin (Barton et al., 2000; Debets et al., 2001; Smith et al., 2000) and I, in addition, found good expression of IL-1F5 in murine cortex and could also find expression in several brain derived cell lines such as the neuroblastoma cell line, neuro2a, the astrocytoma cell line, 1231N1 and the astrocytoma-glioblastoma cell line U87MG. However, as before, IL-1F5 showed no activity in terms IL-1 like responses in these cell types. Again the possibility remains that signals unlike those activated by IL-1 are initiated by IL-1F5.

IL-1F5, along with the other novel IL-1 family members, has been identified solely on the basis of its DNA sequences, and with the sequencing of the human, and other genomes, it is likely to be joined by many other proteins identified with no known function. This work highlights the need to use sequence information with caution, and would argue against the hasty assumption of function based on the function of the closest related protein. In depth knowledge of the structure-function properties of the related proteins is required to use the sequence information to the full. One should ask therefore, which aspects of the sequence are absolutely required for conservation, for example the β -strands of the IL-1s, or the ATP binding site of a kinase, and which regions of the sequence are likely to give functional diversity to the protein, for example the loop

structures of the IL-1s, or the protein interaction domains of a kinase. In this way, experimental assays can be designed which best approach the question as to the function of the novel proteins.

The lack of evidence as to the functional properties of IL-1F5 still left open the question of whether the protein might act as an agonist or antagonist. Therefore, given that structural and sequence information gave insights in the functional differences between IL-1 β and IL-1Ra, I decided to investigate whether the structure of IL-1F5 might give insights into the functionality of the protein. Crystals diffracting to 1.5 Å allowed the structure to be solved to a resolution of 1.6 Å by molecular replacement methods using IL-1 β as a template and a source of initial phase estimates. The refined structure is of very good quality with little doubt as to the placement of sidechain atoms, which fit the electron density very well. However, as with all crystal structures, it is a static picture of the molecule that does not represent the small fluxes in structures that occur in solution. Nevertheless, the structure of IL-1F5 indicates that it would be unable to bind IL-1RI due to differences in the structure of the loop regions and this supports the data gained from the biological assays. In particular, it can be seen that the introduction of a well defined helical region in loop 3-4 prevents the binding of IL-1F5 through domain 1 and 2 of the receptor, due to clashing of the sidechains of Leu30 and His31 with IL-1RI residues.

There are also several features that support the conclusions made from the sequence analysis that IL-1F5 may act as an agonist. These are that the position of the functionally important aspartate exactly superimposes on that of the IL-1 β , suggesting that is in the correct position and orientation to allow interaction with the accessory protein. Also, the loop between strands 4 and 5 is much longer than that in IL-1Ra, as suggested by the sequence alignment, implying that IL-1F5 could interact with domain 3 of an IL-1 family receptor in a manner that would allow accessory protein recruitment. Finally, examination of the electrostatic potential on the face of the molecule that has the Asp147 at its centre shows that IL-1F5 has a surface potential that mimics that found in IL-1 β but not IL-1Ra. IL-1Ra has a relatively featureless potential on this face, a fact which is likely to be a reflection of its inability to interact with other proteins through this region.

Therefore the strong electrostatic potential of IL-1F5 found in this region is another indication that it could recruit an accessory protein in a manner similar to IL-1 β .

However, this hypothesis goes against a report by Debets et al. (2000) that IL-1F5 can act as an antagonist through IL-1Rrp2 to inhibit the activation of NF κ B by IL-1F9. There are several lines of evidence that call into question this data. One is the lack of receptor binding data, which would prove satisfactorily that the ligand bound the receptor and within an affinity range that would be expected for this family of ligands. Another is the fact that the inhibition seen by IL-1F5 is at a concentration equal to, or even less than that of IL-1F9, where as with IL-1Ra, which has a similar affinity for IL-1RI as IL-1 (Dripps et al., 1991a), concentrations are required to be at least 100 to 1000 fold that of IL-1. Although this can be explained if the affinity of IL-1F5 for IL-1Rrp2 is much greater than that of IL-1F9, this is an unusual situation for an antagonist, which is usually required to be in excess in order to elicit its inhibitory activity. In addition, reports from other groups indicate that IL-1F5 was unable to bind an Fc-fusion of IL-1Rrp2 (Smith et al., 2000). Given the suggested high affinity of IL-1F5 for IL-1Rrp2 a positive result in this assay would be expected, especially since the technique is capable of detecting the binding of IL-18 to IL-18R. Therefore, these considerations leave open the possibility that IL-1F5 could act by generating a negative signal through another endogenous receptor. Furthermore, communication from other labs suggest that although IL-1F9 does seem to be able to activate NF κ B through IL-1Rrp2, the antagonistic effects of IL-1F5 could not be repeated (Sims, J., personal communication), while attempts to model IL-1F5 to IL-1Rrp2 either as an antagonist or an agonist have so far failed (Gay, N.). The exact activity of IL-1F5 therefore remains open to debate.

Although the work described here gives evidence to suggest that IL-1F5 may be able to activate signal transduction through an IL-1R like protein, future work needs to be carried out in order to fully establish the functional properties of this protein. One step towards this would be the identification of a cell line that was responsive to or capable of binding IL-1F5 specifically. To this end it would be possible to fluorescently label the protein and look for the binding capacity of cells as measured by FACS, in a manner that could be

competed for by 'cold' unlabelled ligand. Another approach would be to test IL-1F5 against the remaining IL-1R family members in terms of its ability to bind these receptors. Specifically, IL-1Rrp2, IL-1APL, TIGIRR and SIGIRR could be used in such assays as receptors that are potentially capable of binding IL-1F5. There is the possibility also, that there are other as yet unidentified IL-1R family members that might bind IL-1F5. The structure of IL-1F5 presented here, and the information learned from it could be used to design mutants of IL-1F5 that would be likely to cause it to lose its receptor binding capacity and/or its biological activity. In this way, insights into the functionally determining residues of IL-1F5 would be gained. The structure immediately suggests that residues such as His31, loop 4-5 residues, Tyr88 and Asp147 would be excellent candidates for mutagenesis in such studies.

This work firmly establishes IL-1F5 as a member of the IL-1 family. Based on the crystal structure and sequence information it is suggested that it could act as an agonist to control immune and/or inflammatory responses in a manner similar to IL-1. Given the involvement of immune and inflammatory responses in many human diseases the potential role of a novel cytokine such as IL-1F5 is of great interest towards gaining a fuller understanding of such diseases and also as a novel drug target for therapeutic intervention in these diseases.

Chapter 8 References

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Appendix I IL-1F5 Atomic Coordinates

The coordinates of the crystal structure of murine IL-1F5 are supplied with this text in pdb format. The file can be used to view the structure using a program such as Swiss-PdbViewer, which can be downloaded free of charge from the Expasy website at <http://ca.expasy.org/spdbv/>.

Appendix II Addresses of Suppliers

<i>Amersham International</i>	Export Sales Division Lincoln Place, Green End Aylesbury Bucks HP20 2TP United Kingdom.
<i>BDH Limited</i>	P.O. Box 8 Dagenham Essex RM8 1RY United Kingdom
<i>Boehringer Mannheim GmbH</i>	Biochemica P.O. Box 31 01 20 D-6800 Mannheim 31 Germany
<i>Calbiochem</i>	Freepost Nottingham NG7 2BR United Kingdom
<i>Clonetics Corporation</i>	9620 Chesapeake Drive Suite 201 San Diego CA 92123 USA
<i>European Collection of Animal Cell Cultures</i>	PHLS Center for Applied Microbiology and Research Porton Down Salisbury SP4 OJG United Kingdom
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USA

Sigma Chemical Company Ltd

Fancy Road
Poole
Dorset BH17 7BR
United Kingdom

Appendix III Publications

Publications and abstracts as a result of this work.

Eleanor F. Dunn, Nick J. Gay, Luke A. J. O'Neill and Xue Yuan Pei. High resolution structure of murine IL-1F5 reveals unique loop conformation for receptor binding specificity. *Submitted*.

Dunn, E., Sims, J.E., Nicklin, M.J. and O'Neill, L.A. (2001) Annotating genes with potential roles in the immune system: six new members of the IL-1 family. *Trends Immunol*, **22**, 533-6.

Dunn, E., Bowie, A., and O'Neill, L.A.J. (2000) Bioinformatic analysis of five gene sequences predicted to encode novel IL-1-like cytokines. *European Cytokine Network* 11 (Special Issue) Abstract No. 8003, pg 55.

Dunn, E., Bowie, A., and O'Neill, L.A.J. (14-15th September 2000) Bioinformatic and functional analysis of two novel IL-1-like cytokines. Irish Society for Immunology and Ulster Immunology Group, Joint Scientific Meeting, NUI Maynooth