Signalling through JAKs and STATs

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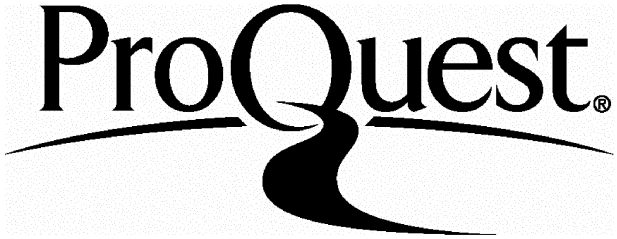

To my family and my friends.

Abstract

Cytokines normally activate the JAK/STAT and additional signalling pathways in a modular fashion through different receptors. There appear to be additional JAK pathways and cross talk between pathways. A mammalian expression and purification system was established to identify phosphorylation sites in JAK1 (Janus Kinase 1) which may modulate activity and/or mediate additional protein-protein interactions and to find new proteins, which are recruited by human Jak1. Highly purified hJAK1 and different analytical mass spectrometry approaches were used to identify potential serine and threonine sites. The functional significance of any sites identified was analysed by mutagenesis and following assaying of different aspects in JAK/STAT signalling. The system was modified to co-purify constitutively and/or inducibly associated proteins.

The binding of interferons (IFNs) to their receptors leads to the phosphorylation and activation of signal transducers and activators of transcription (STATs) and their translocation from the cytoplasm to the nucleus. We could show that IFN-α and -γ signalling and STAT1 translocation are independent of the actin cytoskeleton or microtubules. Using fluorescence loss in photobleaching (FLIP) and fluorescence recovery after photobleaching (FRAP) experiments the mobility of a fusion protein of STAT1 with green fluorescent protein (STAT1-GFP) was compared with that of GFP and protein kinase C (PKC)-GFP. In IFN-γ treated and control cells cytoplasmic STAT1-GFP shows high, energy independent, mobility comparable to that of freely diffusible GFP. A random walk model for movement of STAT1 from the plasma membrane to the nuclear pore is, therefore, indicated. Nuclear STAT1-GFP showed similar high mobility, with exclusion from nucleoli, consistent with high rates of association and dissociation of STAT1-DNA and/or -protein complexes in the nucleoplasm of the cell.

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Table of Contents

Table of Figures

Table of Tables

Abbreviations

6TG	6-thioguanine
ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin (protease free)
CBP	CREB-binding protein
CMV	Cytomegalovirus
cpm	counts per minute
DMSO	dimethyl sulphoxide
dsRNA	double stranded RNA
DTT	dithiothreitol
DMEM	Dulbecco's modified Eagle's medium
ECL	enhanced chemi-luminescence
EDTA	ethylenediamine tetra-acetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol-bis(b-aminoethylehter)N,N,N',N'-tetra-acetic acid
EMCV	encephalomyocarditis virus
EMSA	electrophoretic mobility shift assay
ERK	extracellular regulated kinase
FACS	fluoresence activated cell scanner
FCS	foetal calf serum
FERM	4.1/ezrin radixin USFmoesin
FLIP	fluorescent loss in photobleaching
FRAP	fluorescent recovery after photobleaching
FRET	fluorescent resonance energy transfer
GAS	Interferon γ activated sequence

GFP	green fluorescent protein
gpt	guanine phosphoribosyltransferase
GTP	guanosine triphosphate
HAT	hypoxanthine, aminopterin, thymidine medium
HEPES	N-2-hydroxyethypiperazine-N'-2-ethanesulphonic acid
HLA	human leukocyte antigen
HPLC	high performance liquid chromatography
HPRT	hypoxanthine phosphoribosyltransferase
h	hours(s)
ICRF	Imperial Cancer Research Fund
IFN	Interferon
IL	Interleukin
IRF	interferon regulatory factor
IRS1	insulin receptor substrate 1
ISG	interferon stimulated gene
ISGF3	interferon stimulated gene factor 3
ISRE	interferon stimulated response element
IU	international units
JAK	Janus kinase/just another kinase
JH	Jak homology
kb	kilobase
kD	kilodalton
KLH	keyhole lympet hemocyanin
LB	Luria Broth
LMB	Leptomycin B
LPS	lipopolysaccharide
m.o.i	multiplicity of infection

MAPK	mitogen activated protein kinase
MHC	major histocompatibility complex
NES	nuclear export sequence
NLS	nuclear localisation sequence
NP-40	Nonidet P40
OSM	Oncostatin M
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming units
PI3K	phosphatidylinositol 3-kinase
PIPES	1, 4-piperazidinediethandsulphonic acid
PMSF	phenlmethylsulphonlfluoride
PVDF	polyvinylidene difluoride
rpm	revolutions per minute
s	seconds
SDS	sodium dodecyl sulphate
SH	src homology domain
SIE	Serum Inducible Element
STAT	signal transducer and activator of transcription
TAE	Tris-acetate/EDTA buffer
TBE	Tris-borate/EDTA buffer
TBST	Tris-buffered saline with 0.1% Tween20
TE	10mM Tris/HCL, 1mM EDTA
Tris	2-amino-2(hydroxymethyl)-1,3-propandiol
tRNA	transfer RNA
UTP	uridine 5'-triphosphate

Chapter 1: Introduction

1.1 Interferons

Interferons (IFNs) were first identified as factors secreted by cells in response to viral infections (Isaacs and Lindenmann, 1957 and reviewed in de Maeyer and de Maeyer-Guignard, 1991). IFNs are divided into two groups reflecting to which of the two IFN receptor complexes they bind. Both receptor complexes signal through JAK/STAT pathways (Janus Kinase / Signal Transducer and Activator of Transcription). Different cells secrete different IFN subtypes in response to a variety of stimuli, like virus infection, bacterial endotoxin (LPS), double-stranded RNA (dsRNA) and immunostimulatory cytokines. IFN-α is mainly produced by lymphocytes, monocytes and macrophages. The sources for IFN-β are fibroblasts and some epithelial cells. Producers of IFN-γ are T-cells and NK-cells (Callard and Gearing, 1994). It is believed, that most cells produce IFN-α/β if challenged with virus or doubled stranded RNA.

All Type I IFNs bind the same receptor, but induce signals of different intensity, the biological significance of which is unknown (Foster et al., 1996). They are subdivided into three groups. In humans, there are 14 functional genes (and one pseudogene) for the α_I-IFN group. The α_{II}-IFN group has only IFN-ω as a member (with an additional five pseudogenes). The third group is comprised of only IFN-β. All secreted forms of Type I IFNs are comprised of 165 or 166 amino acid residues (except IFN-ω which has 172 residues) and some are glycosylated monomers. The genes for the human Type I IFNs each encode a single exon and are localised as a cluster on the short arm of chromosome 9 in humans.

IFN-γ, the ligand of the Type II or IFN-γ receptor complex, is the only known member of the Type II IFN. IFN-γ is a 143 amino acid glycosylated protein encoded as a single copy gene on chromosome 12. Its active form is a homodimer.

1.2 Biological responses to the interferons

The responses to IFNs can be divided into three categories: antiviral, antiproliferative/apoptotic and immunomodulatory (reviewed in Kalvakolanu and Borden, 1996). The phenotype of the response is dependent on cell type, co-stimulatory factors and the subset of genes induced.

1.2.1 Antiviral response

IFNs induce an antiviral state in cells, which improves the cells ability to react to virus infection, viral proteins and/or double strand RNA (Boehm et al., 1997; Samuel, 1991; Sen and Ransohoff, 1993). Antiviral proteins induced by IFNs including the dsRNA-dependent protein kinase (PKR), dsRNA dependent 2'-5' oligoadenylate synthetase (2-5 OAS), guanylate binding protein (GBP), Mx proteins and 9-27 protein inhibit virus replication via a variety of mechanisms. IFN-α/β are the main activators of antiviral responses and are themselves induced by virus. The induction and secretion of IFN-α/β is important for the protection of neighbouring tissue. IFN-γ also induces an antiviral response, but its expression is restricted and it is not induced directly by virus. Type I receptor knockouts are therefore more affected by virus infection, than Type II receptor knockouts (Vandenbroek et al., 1995).

1.2.2 Antiproliferative and apoptotic response

The regulation of cell-cycle arrest, proliferation and differentiation by IFNs is (reviewed in Clemens and McNurlan, 1985; Kimchi, 1992; Sangfelt et al., 2000). IFNs initiate an antiproliferative state either at the beginning of G_1 or S-phase (Balkwill and Oliver, 1977; Lin et al., 1986). IFNs induce cyclin-dependent kinase inhibitors (CKIs) p15, p19, p21 and p27 which leads to antiproliferative effects and at least the induction of p21 is mediated by STAT1 (Chin et al., 1996). Furthermore, transcriptional active STAT1 is required for the antiproliferative effects of IFN-α, IFN-γ and epidermal growth factor (EGF) (Bromberg et al., 1998; Bromberg et al., 1996). IFN-γ regulates genes encoding death associated proteins (DAPs) which are necessary to induce apoptosis in HeLa cells (Deiss et al., 1995; Inbal et al., 1997; Levy-Strumpf et al., 1997). However, constitutively active STAT3 protects cells from serum withdraw, UV or STAT1 induced apoptotic effects (Shen et al., 2001). IFNs can induce both pro-apoptotic and anti-apoptotic responses in several cell types alone or in combination with other stimuli like TNFα (Kumar et al., 1997), LPS and dsRNA (Der et al., 1997). Both the antiproliferative and the apoptotic responses may be related to antiviral responses and be mediated by antiviral proteins (Zhou et al., 1997).

1.2.3 Immunomodulation

IFNs are major players in the regulation of the immune system (reviewed in Boehm et al., 1997; Vandenbroek et al., 1995). Much has to be learned and only some major facts are reviewed here. All IFNs upregulate the surface expression of MHC class I proteins which present peptides of intracellular pathogens to CD8 positive T-cells to initiate a cytotoxic immune response. IFN-γ also induces the expression of MHC class II proteins, which present exogenous peptides on mainly professional antigen presenting cells (APCs), and are recognised by CD4 positive T-cells. Dependent on signalling

through additional cytokines, this leads to either an inflammatory (TH1) or humoral immune response (TH2). Type I IFN up-regulate IL12 receptor β2 during differentiation of human T-cells (Th1) (Rogge et al., 1997). IFN-γ itself in conjunction with IL-12 favours an inflammatory response by pushing naive T-cells to develop into TH1 T-cells. IFN-γ also induces proteins which are important for the processing and presentation of peptides on either MHC class I or II, for example LMP2/7, HLA-DMAα/β and TAP1/2.

Additionally, IFNs regulate the activity of other immune cells. In macrophages IFN-γ induces genes such as inducible nitric oxide synthase (iNOS) and indoleamine 2,3-dioxygenase (IDO), major players in the response to intracellular parasites. In B-cells IFN-γ induces immunoglobulin class-switching, important for the humoral immune response.

1.3 Gene regulation by the interferons

1.3.1 Gene regulation: overview

IFNs can induce, inhibit and regulate gene expression in a direct way (primary response). Primary responses do not require protein synthesis, take place rapidly upon IFN treatment and involve pre-existing signalling molecules like receptor chains, JAKs, STATs and other regulatory proteins. The primary response and the consequent translation of regulatory factors leads to secondary responses, which take place much later after IFN treatment (>3h). Up-regulation of MHC-class II for example, requires the transcription factor CIITA, which itself requires gene products of the primary response such as IRF1 (reviewed in Stark et al., 1998).

The IFNs induce pleiotropic responses both in individual cells and cellular systems (e.g. Immune, haematopoetic and/or neural systems). This is reflected in the number of genes regulated by the IFNs. Over 200 IFN-γ inducible genes were already

known in 1997 (Boehm et al., 1997) and new techniques including oligonucleotide microarrays (Der et al., 1998) and new information from the sequence of the genome (Ewing and Green, 2000; Liang et al., 2000; Roest Crollius et al., 2000) suggests around 600 genes regulated by IFN.

1.3.2 Interferon response elements

Two types of element are characteristic for most IFN-stimulated genes (Darnell et al., 1994; Schindler and Darnell, 1995).

1. The IFN-stimulated response element (ISRE) is a typical site in promoters of IFN-α/γ inducible genes. The ISRE has a consensus sequence defined as GAAANN(N)GAAA (Levy et al., 1988). The major inducer of ISREs is the IFN-α/γ activatable ISGF3, a complex containing STAT 1, STAT2 and p48 (IRF9) (Qureshi et al., 1995). p48 itself is up-regulated by IFN, virus or dsRNA. An IFN-γ inducible isoform can also be induced through the interaction of STAT1 and p48 (ISGF3) with this type of element (Reid et al., 1989 and below).

2. GAS elements are loosely palindromic and the consensus is TTNNNNNAA. The symmetrical or palindromic nature of the elements is be consistent with the binding of dimeric STAT complexes binding to the sites (Stark et al., 1998).

1.3.3 Genetic analysis of the JAK/STAT pathway in response to interferon

To identify proteins involved in interferon signal transduction, two IFN selectable cell systems based on human fibrosarcoma (HT1080) cells were established and treated with the frame shift mutagen ICR191 (reviewed in Pellegrini and Schindler, 1993).

The 2fTGH system (Pellegrini et al., 1989) are hypoxanthine phosphorybosyl transferase (hprt) negative HT1080 cells stably transfected with the bacterial gene for guanosine phosphoribosyl transferase (gpt, homologue of hprt) under the control of the IFN-α/β inducible 6-16 promoter. Cells expressing gpt and/or HPRT are susceptible to the drug 6-thioguanine (6TG) and die. Hence, 2fTGH cells which do not respond to IFN-α/β after mutagenesis with ICR191 can survive in medium containing 6TG. Importantly, gpt and hprt negative cells can not recycle guanine nucleotides and therefore die in the presence of aminopterin, an inhibitor of *de novo* purine synthesis. If hypoxanthine and thymidine are present, cells expressing gpt or HPRT can survive aminopterin treatment via the salvage pathway. Thus, 2fTGH, the mutants and complemented mutants could be selected for intact or disrupted IFN-α/β signal transduction.

The 2C4 system (Watling et al., 1993) is based on IFN-γ inducible expression of the cell-surface marker CD2. HT1080 cells were stably transfected with the CD2 gene under control of the IFN-γ inducible 9-27 promoter and a clone chosen which shows good expression levels of CD2 (and endogenous MHC class II) in response to IFN-γ. After mutagenesis cells were selected by FACS for unresponsiveness or responsiveness to IFN-γ.

Mutants in eight complementation groups were obtained (table 1.1.)

Table 1.1 Complementation groups of IFN signalling mutants

Complementation group	Response to IFN			Complemented by
	α	β	γ	
Mutants found in the 2fTGH system				
U1	-	-	+	Tyk2
U2	-	-	+/-	P48 (IRF9)

U5	-	-	+	IFNAR-2
U6	-	-	+	STAT2
Mutants found in the 2C4 system				
γ1	+	+	-	IFNGR-2, JAK2
γ2	+	+	-	JAK2
Mutants found in the 2fTGH and the 2C4 system				
U3	-	-	-	STAT1α/β
U4	-	-	-	JAK1

The mutants have provided invaluable in identifying the JAKs and components of the signal transduction pathway for the IFNs and established the essential role of JAK/STAT pathways in signal transduction in response to the interferons and other cytokines. Examples include the demonstration of the dependence on TYK2 and JAK1 for IFN-α and JAK2 and JAK1 for IFN-γ signalling. As well as confirmation of a central role for STAT1 in response to the IFNs (Muller et al., 1993a; Muller et al., 1993b; Velazquez et al., 1992; Watling et al., 1993).

The mutants also provide a negative background for structural and functional analysis of the complementing proteins. For examples STAT1 negative U3A cells have been extremely used by the Darnell and Stark groups for structural and functional analysis of STAT1 (for example Heim et al., 1995; Horvath and Darnell, 1996; Shuai et al., 1993). The JAK1 negative U4A, JAK2 negative γ2A and TYK2 negative U1A cells have been used extensively for analysis of the JAKs (for example Briscoe et al., 1996; Kohlhuber et al., 1997). Knock-out mice of most of the JAK/STAT signalling components confirmed the mutant cell data and the importance of the JAK/STAT signalling pathway in cytokine signalling.

Here the U4A and U3A cells were used as negative backgrounds for analysis of tagged or mutant JAK1 and STAT1.

1.4 The JAK/STAT pathway

1.4.1 The JAKs

The JAK family of protein tyrosine kinases were first identified by screens for novel kinases (Wilks et al., 1991). There are four known members of the human JAK family: JAK1, 2, 3 and TYK2 (Firmbach-Kraft et al., 1990; Harpur et al., 1992; Johnston et al., 1994; Silvennoinen et al., 1993; Wilks, 1989; Witthuhn et al., 1994). They range in mass from 120kD to 140kD, and contain seven regions of homology: JH1 – JH7 (Fig 1.1 and Harpur et al., 1992). JH1 is the C-terminal tyrosine kinase domain and adjacent to this is a homologous pseudokinase domain (JH2). The pseudokinase domain is thought to be involved in intramolecular regulation, but has no kinase activity. Point Mutations in the JH2 domain of JAK2 and TYK 2 resulted in a constitutively active kinase (Luo et al., 1997; Yeh and Pellegrini, 1999). Classical deletion, point mutation and chimera analyses have established a requirement for regions within the N-terminal JH3-7 domains for interactions with the receptors at the cell membrane (for example Cacalano et al., 1999; Gauzzi et al., 1996; Kohlhuber et al., 1997).

There are conserved regions and amino acids in the kinase domain known to be important for kinase activity. The first tyrosine in the EYY motif (position hJAK1^{1021}, hJAK2^{1006}, hJAK3^{979} and hTYK2^{1053}) has to be phosphorylated for catalytic activity of the JAKs (Feng et al., 1997; Gauzzi et al., 1996). The second tyrosine is necessary for total activation. The ATP-binding loop (glycine rich loop) upstream of the EYY motif is highly conserved. It contains a lysine (position hJAK1^{897}, hJAK2^{882}, hJAK2^{855} and hTYK2^{930}) which forms a salt bridge with a conserved downstream glutamic acid and

co-ordinates the γ-phosphate of ATP (Hanks et al., 1988 and personal communication, Gavin Fox, ICRF). Mutation of this lysine produces a kinase negative JAK molecule (Briscoe et al., 1996). Hydrophobic residues in the ATP binding loop are arranged to form a pocket for the adenosine ring in ATP (personal communication, Gavin Fox, ICRF).

Apart from the JAK homology domains SH2, SH3, PTB or pleckstrin homology (PH) domains have been suggested but not functionally confirmed. The JAK domains JH7-4 show homology to the FERM (4.1/ezrin-radixin-moesin) domain (Girault et al., 1999 and reviewed in Chishti et al., 1998). Other proteins containing a FERM domain are known to interact with transmembrane proteins or signalling molecules involved in the Rho pathway. It is likely that the FERM domain in the JAKs has a similar function.

JAKs are constitutively associated with their receptors. These interactions are thought to be mediated through the juxta-membrane cytoplasmic domain of the receptor chain. The Type I cytokine receptor chains contain a conserved motif (Box 1 and 2) in this region, which is thought to be the interacting site (Murakami et al., 1991). JAKs can also interact with receptors having their own kinase activity like PDGF (Vignais et al., 1996) and EGF (David et al., 1996; Leaman et al., 1996) These receptors also activate STATs, the role of the JAKs in these receptor complexes remains to be elucidated.

1.4.2 The STATs

The STATs function both as signal transducers and transcription factors downstream of the JAKs and have transcriptional activity. Seven mammalian STAT genes (STATs 1 to 6 including STATs 5A and B) have been identified (Akira et al., 1994; Fu, 1992; Fu et al., 1992; Hou et al., 1994; Quelle et al., 1995; Schindler et al., 1992a; Wakao et al., 1994; Yamamoto et al., 1994; Zhong et al., 1994). The proteins range in size between 84kD and 113kD. STATs 1, 2, 3, 5 and 6 are ubiquitously expressed. STAT4 is predominantly expressed in the testis and cells of the immune

system. STATs 1, 3 and 4 are differentially spliced. STAT1 has two splice variants, the 91kD STAT1α and the 84kD STAT1β, which lacks the C-terminal transactivation domain. STAT3β lacks the 55 amino acid C-terminal domain of STAT3α and, instead, has a seven amino acid C-terminal 'domain' also without the serine phosphorylation site.

The STATs have some common features, reflected by related structural and functional properties (Fig. 1.2 and reviewed in Shuai, 2000)). The crystal structure of a STAT1 homodimer (lacking the N-teminus and the transactivation domain) bound to DNA has been solved and confirmed functional data (Chen et al., 1998). The N-terminal region of the STAT is thought to be involved in interactions between neighbouring STAT dimers bound to tandem promoter sites (Vinkemeier et al., 1996) and to mediate binding to histone acetyltransferase proteins such as CBP and p300 (Zhang et al., 1996). The neighbouring coiled coil region is implicated in interactions with other STATs and transcriptional partners including p48 and/or c-Jun. Downstream of this is the DNA-binding region followed by a linker region. The linker region contains an SH3-like domain and is essential for transactivation by STAT1 (Yang et al., 1999). Adjacent to the linker region is an SH2 domain containing the highly conserved tyrosine phosphorylation site in STATs. The SH2 domain is required for recruitment of STATs to phosphorylated receptor chains and dimerisation via the phospho-tyrosine in the SH2 domain of the interacting STAT. The C-terminal domain is the transactivation domain. STAT1β is missing the 38 C-terminal amino acids of this domain and therefore does not activate transcription (Muller et al., 1993b). With the exception of STAT2 and 6, this domain contains a serine phosphorylation site which for many promoters mediates maximum transactivation (reviewed in Decker and Kovarik, 2000)). Like the N-terminal region, this domain is implicated in interactions with CBP and p300 (Bhattacharya et al., 1996; Zhang et al., 1996).

Recent studies on STAT1 have revealed three functional leucine-rich nuclear export signals (NES). Two are located in the coiled coil domain within residues 197-205 (Mowen and David, 2000) and 308-315 (Begitt et al., 2000). The third lies in the DNA-

binding domain within residues 392-413 (McBride et al., 2000). Crucial residues for each of these export signals are present in all STATs.

No classical nuclear localisation signal (NLS) has been identified for the STATs. Retention of non-activated STAT1 in the cytoplasm does not reflect anchoring or inhibition of shuttling (McBride et al., 2000). Indirect evidence for a NLS is provided by the observed interaction of activated STAT1 with importin NP-1, which initiates translocation through the nuclear pore (Sekimoto et al., 1997).

1.4.3 A general model for JAK/STAT signalling

A schematic representation for a general model of JAK/STAT signal transduction is shown in figure 1.3. The order of molecular interactions is:

(1) Upon ligand binding, the receptor associated JAKs become activated by auto- and transphosphorylation and phosphorylate the receptor.

(2) STATs are recruited to the receptor phospho-tyrosines via their SH2 domains (Heim et al., 1995). There are exceptions to this: STAT5can be recruited to JAK2 at the gp130 subunit of the IL-6 receptor (Fujitani et al., 1997) and STAT1 is recruited through the N-terminal domain of STAT2 to the Type I IFN receptor (see below and (Improta et al., 1994; Leung et al., 1995). STATs are then tyrosine phosphorylated by the JAKs.

(3) STATs are released, dimerise, are serine phosphorylated (see section 1.4.7) and migrate to the nuclear pore (see also Chapter 4).

(4) The dimers are translocated through the nuclear pore, which at least in the case of STAT1 involves the importin NP-1 (Sekimoto et al., 1997).

(5) The STAT dimers with or without co-factors (which associate anywhere between release from the receptor complex and the nucleus) and co-activators form transcription complexes which bind the appropriate promoter sites and induce transcription.

(6) A nuclear phosphatase dephosphorylates the STATs (David et al., 1993; Haspel and Darnell, 1999), thus promoting disassembly of the transcriptional complex.
(7) Interaction with CRM-1 mediates the nuclear export of STATs through the nuclear pore into the cytosol. STATs are again available for further rounds of activation.

Class I and II cytokine receptors signal through JAK/STAT pathways (reviewed in Leonard and O'Shea, 1998). Class I receptors include the IFN and IL-10 receptors. Class II receptors are utilised by the majority of the cytokines and several growth factor receptors (reviewed in Ihle et al., 1995; Johnston et al., 1996). Examples of cytokines and growth factors which signal through JAK/STAT pathways are given in table 1.2 (Haque and Williams, 1994).

Table 1.2 Signalling through the JAK/STAT pathways

Ligand	JAKs activated	STATs activated
IFN family		
IFNα/β	JAK1, TYK2	STAT1, STAT2, STAT3
IFNγ	JAK1, JAK2	STAT1
IL-10	JAK1, TYK 2	STAT1, STAT3
gp130 family		
IL-6	JAK1, JAK2, TYK2	STAT1, STAT3
IL-11	JAK1, JAK2	STAT1, STAT3
IL-12	JAK2, TYK2	STAT3, STAT4
Leukaemia inhibitory factor	JAK1, JAK2	STAT1, STAT3
Oncostatin M	JAK1, JAK2	STAT1, STAT3
Ciliary neurotrophic factor	JAK1, JAK2, TYK2?	STAT1, STAT3
Granulocyte colony stimulating	JAK1, JAK2,	STAT1, STAT3

factor	TYK2	
Gamma c family		
IL-2	JAK1, JAK3	STAT1(?), STAT3, STAT5
IL-4	JAK1, JAK3	STAT5
IL-7	JAK1, JAK3	STAT5
IL-9	JAK1, JAK3	STAT5
IL-13	JAK1, TYK2	STAT6
IL-15	JAK1, JAK2, JAK3	STAT5
gp140 family		
IL-3	JAK2	STAT5
IL-5	JAK2	STAT5
GM-CSF	JAK2	STAT5
Growth Hormone family		
Growth hormone	JAK2	STAT1, STAT3, STAT5
Erythropoietin	JAK2	STAT5
Prolactin	JAK2	STAT1, STAT5
Receptor Tyrosine Kinase family		
Epidermal growth factor	JAK1, JAK2	STAT1, STAT3
Platelet-derived growth factor	JAK1, JAK2	STAT1, STAT3
Colony-stimulating factor-1	JAK1, JAK2	STAT1, STAT3

1.4.4 JAK/STAT signalling through the Type I interferon receptor

The Type I IFN-α/β receptor (Fig. 1.4) has two subunits designated IFN-α/β receptor 1 and 2 (IFNAR1 and 2). IFNAR2 is expressed as three different splice variants (Novick et al., 1994), a soluble form (IFNAR2a) and two transmembrane forms (IFNAR2b and c). Only IFNAR2c is fully functional as it is the only form which can complement IFNAR2 negative U5A cells (Lutfalla et al., 1995). TYK2 is constituvely

associated with IFNAR1 (Colamonici et al., 1994a; Colamonici et al., 1994b) and phosphorylates it *in vitro*, while JAK1 is constitutively associated with IFNAR2 (Pestka, 1997). Monomeric Type I IFN binds the receptor and oligomerises the two chains to initiate signalling (reviewed in Stark et al., 1998). There is evidence that STAT2 initially interacts with IFNAR2 via non-SH2-phosphotyrosyl interactions then translocates to IFNAR1 following ligand binding and subsequent oligomerisation of the receptor chains (Yan et al., 1996). STAT1 is believed to interact with the receptor via STAT2 (Improta et al., 1994; Leung et al., 1995). STAT1 and STAT2 heterodimerise after tyrosine phosphorylation at positions 690 and 701 respectively. In the nucleus the heterodimer in conjunction with p48, to form ISGF3 (IFN-stimulated gene factor 3) binds IFN-α/β response elements (ISREs) in the promoters of inducible genes. Type I IFNs activate STAT1 homodimers, which induce transcription through GAS elements, more typical for IFN-γ regulated genes.

1.4.5 JAK/STAT signalling through the interferon γ receptor

The Type II (IFN-γ) receptor (reviewed in Bach et al., 1997; Farrar and Schreiber, 1993 and Fig. 1.5) is a heterodimer, consisting of IFN-γ receptor chains 1 and 2 (IFNGR1 and 2). IFN-γ homodimers bind to IFNGR1, assembling a complex of four receptor chains. JAK1 is constitutively associated with IFNGR1, and JAK2 with IFNGR2 (Kotenko et al., 1995). Following receptor ligation, the JAKs phosphorylate IFNGR1 at tyrosine 440 (Greenlund et al., 1994; Igarashi et al., 1994) and recruit STAT1 through its SH2 domain (Greenlund et al., 1995). IFNGR2 does not undergo tyrosine phosphorylation. Its known function is to recruit JAK2, activation of JAK2 initialises signalling. STAT is tyrosine phosphorylated at position 701 (Schindler et al., 1992b; Shuai et al., 1992; Shuai et al., 1993), homodimerises (Darnell et al., 1994) and initiates transcription via promoters containing GAS sites. STAT1 homodimers can also

associate with p48 forming a complex analogous to ISGF3. This complex interacts with IFN-γ inducible ISREs, as is the case for the 9-27 gene (Reid et al., 1989).

A kinase negative JAK1 (JAK1 K>E) expressed in U4A cells can support ligand mediated phosphorylation of JAK1 and 2, whilst a kinase negative JAK2 K>E cannot. This suggests that JAK2 activation is upstream of JAK1 activation and that JAK2 phosphorylates JAK1. JAK1 K>E can induce gene transcription, but not an antiviral state (Briscoe et al., 1996).

1.4.6 JAK/STAT signalling through Oncostatin M (OSM) receptors

(OSM was used in this thesis for the analysis of JAK1 phosphorylation.) OSM a member of the IL-6 family of cytokines can bind two different receptor complexes (Fig. 1.6). The Type I receptor complex comprising gp130 and the LIFR β chain and the Type II receptor complex comprising gp130 and the OSM receptor chain (Gearing and Bruce, 1992; Mosley et al., 1996). The OSM Type II receptor complex activates JAK1, JAK2 and TYK2 (Auguste et al., 1997) which induces the transcriptional activity of STAT1, 3 and 5 (Auguste et al., 1997; Kuropatwinski et al., 1997). JAK1 is required for the phosphorylation of gp130 and the activation of STAT1, 3 and 5. In the absence of any single JAK protein the remaining two JAKs are phosphorylated, demonstrating that OSM signalling does not involve a sequential cascade of JAK activation (J. Smith, PhD, thesis, 1997).

1.4.7 Serine phosphorylation of STATs

Serine phosphorylation of the STATs reflects a link to other pathways which have yet to be fully defined. Several kinases have been identified as possible mediators of such a phosphorylation. It is likely that different kinases are involved for different

STATs in different cell types and in response to different ligands (Decker and Kovarik, 2000). The p38 MAP kinase has been the prime candidate, but its role in IFN signalling remains debatable. Published data supports the activation of p38 in response to Type I IFNs (Uddin et al., 1999), but not to IFN-γ (Kovarik et al., 1999). MAP kinases recognise serine or threonines with a proline in +1 and also preferably in the –2 position (Schaeffer and Weber, 1999). STATs have such motifs and the observed *in vitro* interaction of STAT1 and the MAP kinase ERK after IFN-α/β (David et al., 1995b) supports the hypothesis that a MAP kinase phosphorylates the STATs. The Ras/MAPK pathway is required for full transcriptional activation through STAT1 and STAT3 (Wen et al., 1995), but this may also support the report of p38 MAPK-dependent phosphorylation of histones as reported for the PML gene promoter in response to Type I IFN (Uddin et al., 2000).

1.4.8 Down-regulation of the JAK/STAT pathway

Phosphorylation of signalling components of the JAK/STAT pathway peaks between 30min and 3 hours and IFN induced gene transcription also peaks after a few hours, indicating tight control of the pathway. Down-regulation of the JAK/STAT pathway has been connected with three protein families: phophatases, suppressors of cytokine signalling (SOCS), which can also be induced via the JAK/STAT pathway, and protein inhibitors of activated STAT (PIAS) (reviewed in Starr and Hilton, 1999; Yasukawa et al., 2000).

Treatment of cells with the tyrosine phosphatase inhibitor pervanadate increases phosphorylation of signalling components of the JAK/STAT pathway following stimulation, and can even activate the pathway in resting cells. This suggests an involvement of phosphatases in the regulation of the pathway (David et al., 1993; Haque et al., 1995). The SH2-containing phosphatases 1 and 2 (SHP-1 and 2) associate with

cytokine receptors and can dephosphorylate specific JAKs and STATs. The presence of mutant SHP-1 with no phosphatase activity leads to increased JAK1 and STAT1 activation upon IFN-α treatment (David et al., 1995a; Haque et al., 1997). The role of SHP-1 is best demonstrated, however, by the 'moth-eaten' mouse, which lacks SHP-1, has multiple haematopoietic and immunological abnormalities (Shultz et al., 1997), and has been used to study the role of SHP-1 in JAK/STAT signalling (David et al., 1995a). A negative effect on EGF and IFN-γ signalling by a catalytically inactive SHP-1 mutant has also been reported (You and Zhao, 1997). Results from SHP-2 -/- mice show that SHP-2 is a negative regulator of the JAK/STAT pathway after IFN stimulation (You et al., 1999). In haematopoietic cells the protein tyrosine phosphatase CD45 negatively regulates cytokine signalling and dephosphorylates the JAKs *in vivo* and *in vitro* (Irie-Sasaki et al., 2001). Regulation of STAT function is also associated with a nuclear tyrosine phosphatase, yet to be identified (David et al., 1993; Haspel and Darnell, 1999).

The cytokine-inducible SH2-containing protein (CIS) was the first member of the SOCS family to be identified (Yoshimura et al., 1995). SOCS1 was isolated and differently named by several groups as SOCS1 (Starr et al., 1997), JAB (JAK-binding protein) (Endo et al., 1997) or STAT-inducible STAT inhibitor (SSI) (Naka et al., 1997). To date there are eight members of the SOCS family, SOCS1-7 and CIS. Characteristically, the SOCS proteins contain an SH2-domain in the middle of the protein and an adjacent 40 amino acid C-terminal SOCS box motif. SOCS proteins are rapidly induced upon STAT activation by cytokines. Constitutively expressed SOCS1 inhibits signalling in response to several ligands. Interestingly, additionally to the members of the SOCS family other protein families also contain a C-terminal SOCS box. These include proteins containing ankyrin like repeats, WD40 domains and Ras-like GTPases (Hilton et al., 1998; Kile et al., 2000).

Several mechanisms how the SOCS-proteins inhibit the JAK/STAT pathway have been described. CIS was first known to bind to phospho-tyrosine motifs on the

EPO and IL3 receptor and inhibit JAK recruitment (Yoshimura et al., 1995). SOCS1 may inhibit JAK substrate phosphorylation by direct interaction with JAKs (Endo et al., 1997; Naka et al., 1997; Starr et al., 1997; Yasukawa et al., 1999). It has been proposed that a region in SOCS1 and 3 which mimics the JAK activation loop, a substrate for JAK/JAK phosphorylation, may interact with the JAKs to inhibit substrate binding. CIS is thought to compete with JAK substrates (as STAT5) for binding to the activated receptor (Matsumoto et al., 1997; Yoshimura et al., 1995). SOCS proteins have also been implied in the recruitment of the SHP-2 phosphatase to the activated receptor complexes. SOCS inhibition is not restricted to the primary signal, but can inhibit subsequent signals. IL-10 desensitises cells to IFN-γ signalling through induction of SOCS3 (Ito et al., 1999). This is an example of a synergistic effect, as IL-10 also down-regulates MHC-class II surface expression in macrophages, and the inhibition of IFN-γ signalling prevents the new synthesis of MHC-class II. This demonstrates complexity and cross talk in signal processing.

Degradation of JAK/STAT signalling molecules is probably also involved in down-regulation of the JAK/STAT pathway. STAT1 is ubiquitinylated and degraded by the proteasome (Kim and Maniatis, 1996), and the activation of the JAK/STAT pathway by IL-3 in presence of proteasome inhibitors is prolonged (Callus and Mathey-Prevot, 1998). Furthermore, it has been reported that the SOCS box, although not essential for direct inhibition of the cytokine induced JAK/STAT activation (Nicholson et al., 1999; Yasukawa et al., 1999), is interacting with the elongin B and C complex which may couple SOCS proteins and their substrates to the proteasomal protein degradation pathway (Kamura et al., 1998; Zhang et al., 1999). Consistent with this, a constitutive active JAK2-TEL fusion protein is degraded by the proteasome on co-expression of SOCS1 (Kamizono et al., 2001). These results indicate that the JAKs are connected to the proteasomal degradation pathway via the SOCS.

The PIAS proteins are constitutively expressed and interact with STAT molecules to inhibit their function. PIAS/STAT interactions are thought to require STAT phosphorylation and each PIAS interacts with a certain STAT such as PIAS1 with STAT1 and PIAS3 with STAT3 (Chung et al., 1997).

Additional mechanisms which likely regulate JAK/STAT pathways include receptor endocytosis, inhibition by soluble receptors, modulatory phosphorylations (Chapter 3) and methylations (Mowen et al., 2001).

1.5 Additional signalling pathways

Additional JAK-dependent signalling pathways have been characterised which are activated in response to different ligands. The MAP kinase pathway (also discussed in section 1.4.7) potentially links the JAK/STAT pathway to a variety of signalling cascades. IFN-α activates the cytosolic phopholipase A_2 ($cPLA_2$) in a JAK1-dependent manner which leads to the release of arachadonic acid (Flati et al., 1996). Inhibitors of cPLA2 block the activation of ISGF3 in response to IFN-α but not to IFN-γ. Further analysis implicates the MAP kinase p38 in the activation of cPLA2 (Goh et al., 1999).

Raf-1 is activated through JAK1 in response to IFN-γ, IFN-β and OSM (Stancato et al., 1997) independently of $p21^{ras}$ in the case of IFN-γ.

Type I IFNs, OSM and IL-4 activate insulin receptor substrate 1 (IRS1) and phosphatidyl inositol 3'-kinase (PI3 kinase), which may feed into a wide spectrum of signalling pathways, in a JAK dependent manner (Burfoot et al., 1997).

The data above are intrigues examples for the involvement of the JAKs in additional signalling events and led to the analyses of JAK1 associated proteins and modulatory phosphorylation in chapter 3.

1.6 Markers for and mediators of intracellular trafficking

1.6.1 The Green Fluorescent Protein (GFP)

The Northern Atlantic organism *Aequorea victoria* can emit green fluorescent light from photocytes at the margin of its umbrella. The fluorescent light is generated by activation of two proteins, aequorin and the green fluorescent protein (GFP) (reviewed Tsien, 1998). Upon calcium binding, aequorin emits blue light, which in turn excites GFP to fluoresce green. GFP contains 238 amino acids (Prasher et al., 1992) and has a size corresponding to 27-30kD on SDS-PAGE (Chalfie et al., 1994). Its crystal structure has been solved (Ormo et al., 1996; Fig. 1.7). The protein fold consists of an 11-stranded β barrel with a coaxial helix. The chromophore forms from the central helix by cyclisation and oxidation of the three amino acids Ser65, Tyr66 and Gly67 (Heim et al., 1994; Prasher et al., 1992; Fig. 1.8). This posttranslational modification (maturation) occurs within 2-4 hours after synthesis and is probably autocatalytic. The wildtype GFP has two absorption maxima, a major peak at 395nm and a minor at 475nm (Cubitt et al., 1995). The emission of the green light is at 508nm. Genetic engineering has changed the fluorescence properties of the GFP. The Ser65 to Thr (S65T) mutant (Heim et al., 1995) has a single excitation peak at 475nm and six-fold stronger fluorescence. This mutant matures four times faster than the wildtype. Nowadays there are many modified green fluorescent proteins (blue fluorescent protein, cyan fluorescent protein, yellow fluorescent protein) as well as fluorescent proteins from other organisms, like the red fluorescent protein from *dicosoma*.

Proteins tagged with a fluorescent protein are used in many techniques to study their localisation, functions and interactions. Examples include:

1. In reporter constructs for transfection and expression
2. Localisation of proteins in cells
3. As calcium sensors

4. Fluorescence loss in photobleaching (FLIP)

5. Fluorescence recovery after photobleaching (FRAP)

6. Fluorescence resonance energy transfer (FRET)

7. Fluorescence lifetime imaging microscopy (FLIM)

Here FLIP and FRAP (reviewed White and Stelzer, 1999 and Material and Methods) were used to compare the mobility of a STAT1-GFP fusion protein with GFP and PKC-GFP (Chapter 4).

1.6.2 Microtubules, kinesins and dyneins

Microtubules extend throughout the cytoplasm and localise membrane-bound organelles and other components in the cell. Microtubules are cylindrical structures formed from α- and β-tubulins. Which are encoded by at least six independent genes. The microtubules are built from 13 linear protofilaments of alternating α- and β-tubulin and are bundled in parallel. Microtubuli are polar structures, with a fast growing end (plus) localised towards the plasmamembrane and a slow growing end (minus) embedded in the centrosome. They are labile structures with a half life of a microtubulus of 10min, compared to a half life of 20 hours for tubulin (Alberts et al., 1994).

Two protein families are responsible for movement along the microtubuli. Kinesins and dyneins are protein complexes of two heavy chains and several light chains. The heavy chains contain the ATP binding 'head', which contains the ATPase activity and the site to interact with microtubuli. Kinesins move towards the plus end of microtubule and dyneins towards the minus end of tubulin. The mechanism kinesins and dyneins use to transform the energy of ATP into movement along the microtubuli is not known. Both proteins are involved in mitosis, meiosis, movement of organelles and vesicles (Alberts et al., 1994).

Recently published data shows, that the transcription factor p53 is physically associated to microtubuli. After DNA damage p53 moves into the nucleus in a dynein dependent fashion (Giannakakou et al., 2000). Here, work with microtubuli disrupting drugs, has shown that this is not the only mechanism for the translocation of transcription factors into the nucleus: STAT1 movement is independent of dynein (see Chapter 4).

1.7 Objectives

The overall objectives of this thesis were to identify phosphorylation sites in hJAK1 which modulate JAK1 dependent pathways in response to cytokines. Therefore, bacterial expression systems, for analysing *in vitro* phosphorylation, and mammalian expression systems, for investigating *in vivo* phosphorylation, were established. These systems were also used to study interactions of hJAK1 with proteins involved in JAK1 signalling.

An additional project was to analyse the dependence of JAK/STAT signalling on the cytoskeleton, as well as, the mobility of STAT1 in the cytosol and the nucleus. The latter was done using FLIP (Flourescent Loss In Photobleaching) and FRAP (Flourescent Loss After Photobleaching) analyses with a functional STAT1-GFP fusion protein.

Fig. 1.1 The general structure of the JAK family proteins

Regions of homology are indicated by coloured boxes and linker in the regions are represented by gaps.

Fig. 1.2 The general structure of the STAT family proteins

The upper panel shows the structure of a STAT1 dimer (without N-terminal and transactivation domains; Chen et al., 1998); the structure is taken from the home page of the group: http://www.rockefeller.edu/kuriyan/pdb/stat/figure2a.jpg).

The bottom panel shows the general domain structure of all STAT molecules and domains are coloured as in the structure above. The two known phosphorylation sites are indicated with 'tyrosine' or 'serine'. Regions and proteins that interact with these regions are shown below the full length STAT.

Figure 1.1

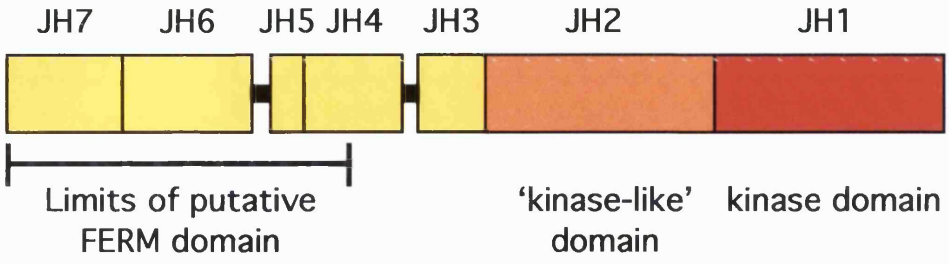


Figure 1.2

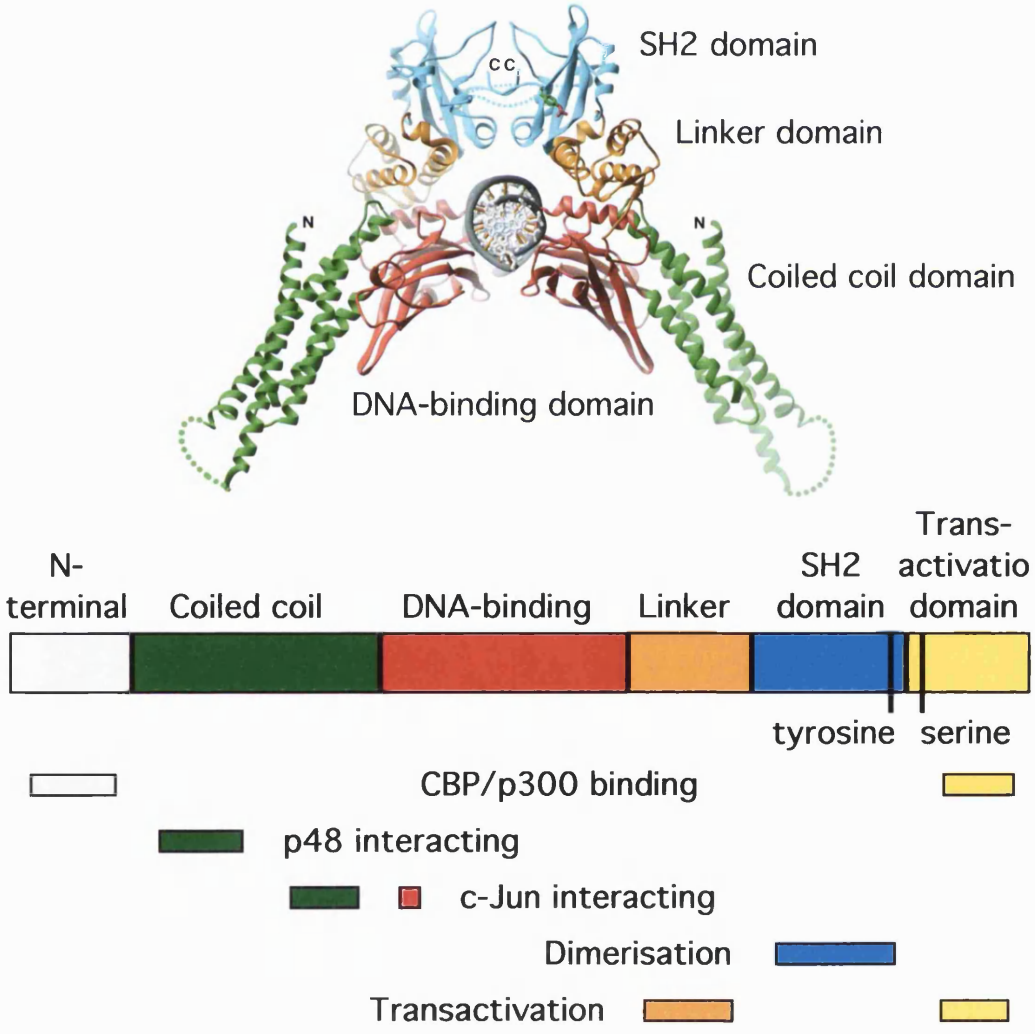


Fig. 1.3 A minimal model for cytokine signalling through the JAK/STAT pathway

The model is explained in detail in Chapter 1.4.3. The symbols are explained in the box on the bottom right. Phosphorylated proteins are marked with a red P.

Figure 1.3

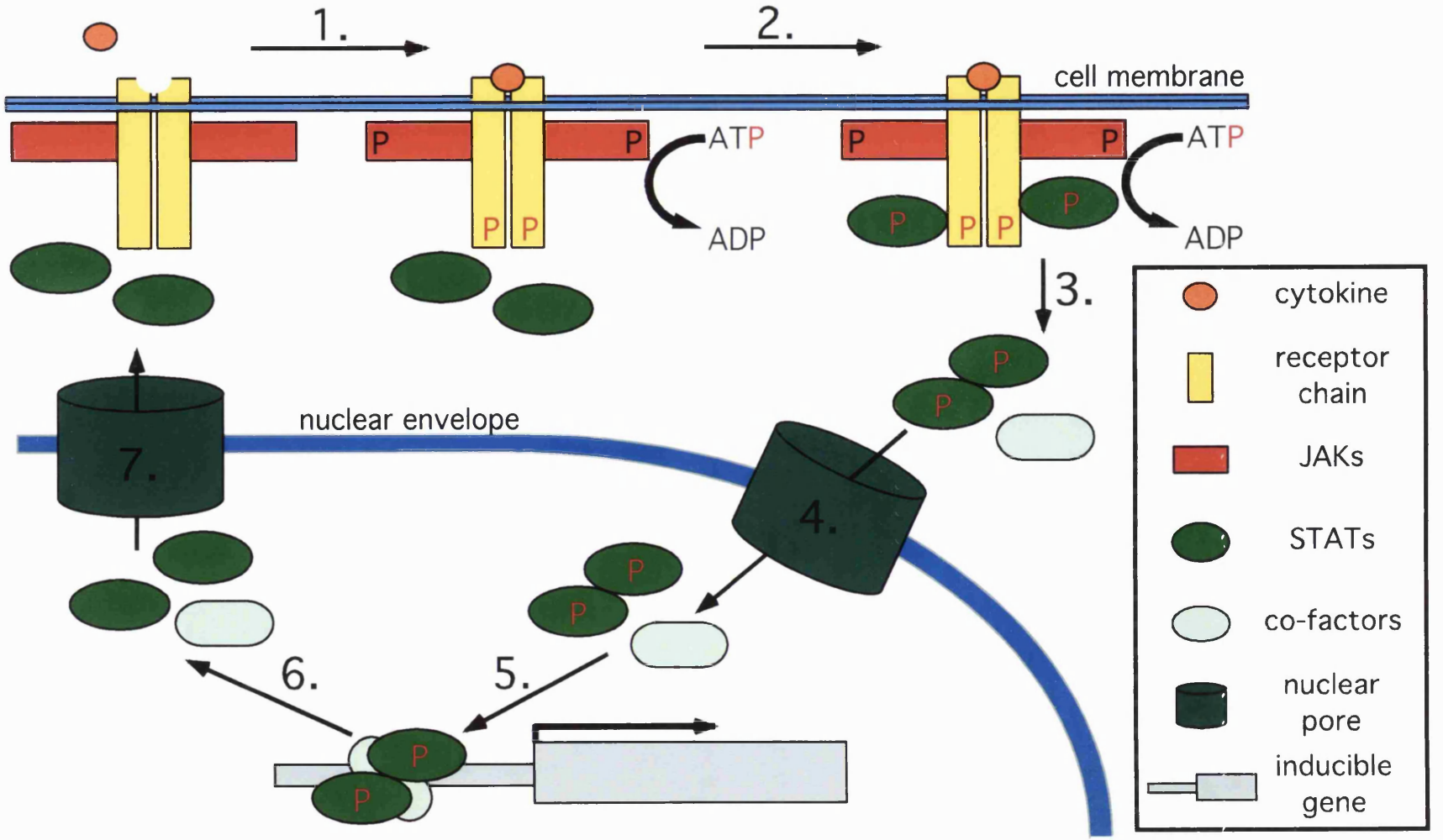

1.
2.
cell membrane
ATP
ADP
P
3.
nuclear envelope
4.
5.
6.
7.
cytokine
receptor chain
JAKs
STATs
co-factors
nuclear pore
inducible gene

Fig. 1.4 Model for the Type I interferon receptor

Fig. 1.5 Model for the Type 2 interferon receptor

Fig. 1.6 Model for the Oncostatin M receptor

Figure 1.4

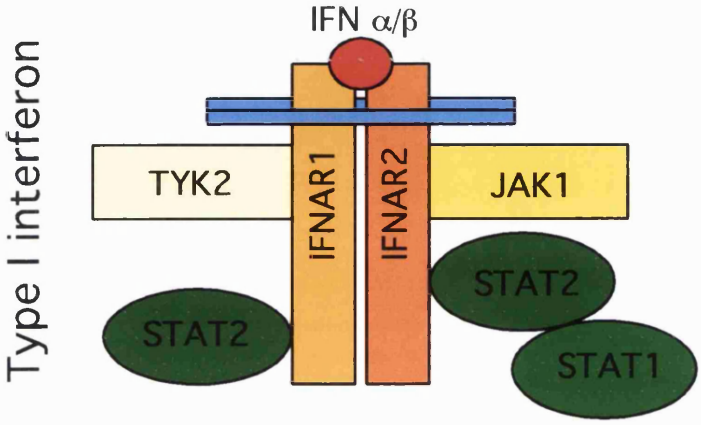


Figure 1.5

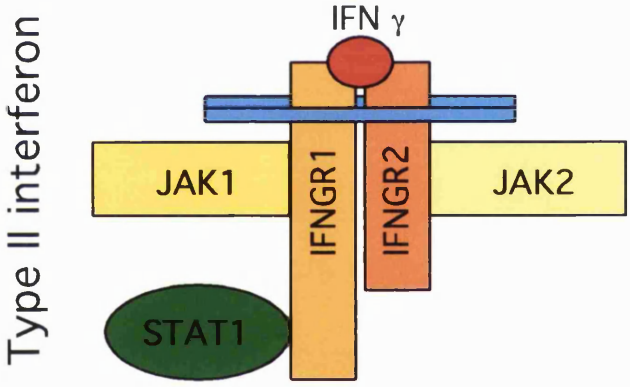


Figure 1.6

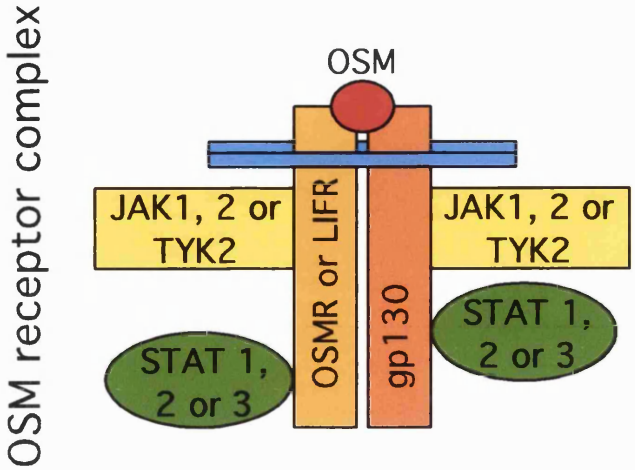


Fig. 1.7 Structure of the Green Fluorescent Protein (GFP)

The structure of the green fluorescent protein was taken from the internet page: http://www.rcsb.org/pdb/cgi/explore.cgi?job=graphics&pdbId=1EMA&page= (Ormo et al., 1996)

Fig 1.8 Dehydration-dehydrogenation mechanism for the formation of the chromophore in GFP

The top panel shows the position of the chromophore forming amino acids in the DNA-sequence of GFP. The bottom panel shows the mechanism proposed by Cubitt et.al. (Cubitt et al., 1995) for the intramolecular biosynthesis of the GFP chromophore.

Figure 1.7

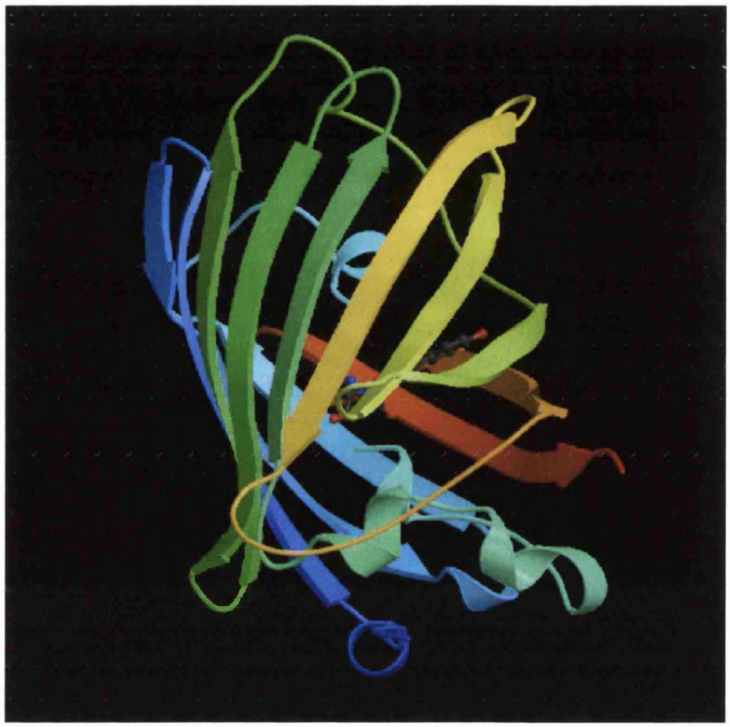
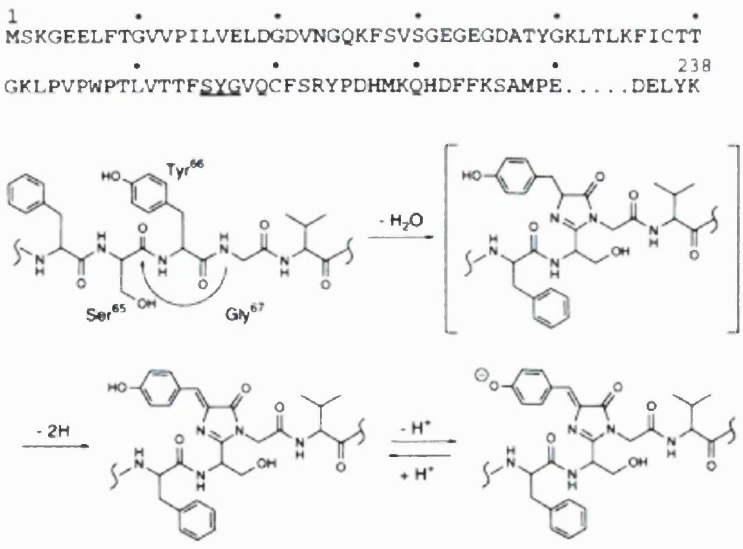


Figure 1.8



Chapter 2: Materials and Methods

2.1 Chemicals

Unless stated otherwise, chemicals were supplied by Sigma Chemical Company Ltd.

2.2 Tissue Culture

2.2.1 Adherent cells

Adherent cells were cultured as a monolayer in Dulbecco's Modified Eagles Medium (DMEM) containing penicillin and streptomycin (ICRF Media Production) and supplemented with 10% (v/v) foetal calf serum (heat-inactivated at 56°C for 30min) and 2mM glutamine. Cells carrying selectable resistance markers were grown in the presence of 250μg/ml Hygromycin B (Calbiochem) or 700μg/ml G418/Neomycin (GIBCO) as appropriate for maintenance. Typically cells were passaged 1:10 as follows: monolayers were rinsed in versene (0.02% (w/v) EDTA in PBSA), detached in versene containing 1% trypsin, and resuspended in DMEM/10% serum for replating. Cells for experiments were grown and treated in the absence of selection drugs. All disposable plastic tissue culture plates were obtained from Falcon.

2.2.2 Suspension cells

Suspension cells were cultured in RPMI (3.7%) containing penicillin and streptomycin (ICRF Media Production) and supplemented with 5% (v/v) foetal calf serum. Cells carrying selectable resistance markers were grown in the presence of the appropriate antibiotic (see above). Cells were grown in spinner flasks which were

gassed with 10% CO_2. Cells were grown to a density of 6×10^5 cells/ml and subsequently diluted to 2×10^5 cells/ml.

2.2.3 Transient and stable transfection

Transient and stable transfection of expression constructs into adherent cells was performed using Superfect transfection reagent from Qiagen. Briefly, the plasmid was resuspended in 100μl DMEM/μg plasmid and mixed with 6μl Superfect per μg plasmid, vortexed and left for 10min at room temperature. DMEM 10% FCS was added to give an appropriate volume (1.5ml per 6cm dish or 2.5ml per 10cm dish) and the transfection mix warmed to 37°C. Monolayers of cells were incubated with the mix for up to 4h, then "washed" for 10min in DMEM 10% FCS three times and left overnight to recover. The next morning cells were split as required for the experiment or put under drug selection and single cell cloned.

Suspension cells were transfected by electroporation using a BioRad electroporator. 1×10^7 cell were washed in phosphate buffered saline pH7.4 (PBS) and resuspended in 0.5ml DMEM with 10μg plasmid. Cells were electroporated at 220–280V and 96μF and left for 5–10min at room temperature and then transferred in 100ml RPMI 3.7%. The following day cells were put under drug selection and 14 days later single cell cloned.

2.2.4 Freezing cells

Cells were stored in liquid nitrogen in 90% heat-inactivated serum (v/v) and 10% dimethyl sulphoxide (v/v; DMSO). To revive frozen cells a vial was warmed to 37°C and the contents placed into a sterile universal tube containing 10ml DMEM/10% FCS.

Cells were pelleted by low speed centrifugation, resuspended in fresh DMEM/10%FCS and plated out as required.

2.2.5 [^{32}P]-labelling of HeLa-S3/hJAK1-M6H

Hela-S3/hJAK1-M6H cells were washed and resuspended in RPMI (3.7%) minus phosphate (ICRF) and supplemented with 2-5% (v/v) foetal calf serum (dialysed against 25mM HEPES pH7.4 and 150mM NaCl). The cells were transferred in a spinner flask in a concentration of 1×10^7 cells/ml in the presence of 0.5mCi/ml [^{32}P]-phosphate (Amersham). Cells were incubated at 37°C for 4h and then stimulated with 150ng/ml OSM for 20min. Cells were harvested and washed in 25mM HEPES pH7.4 and 150mM NaCl before lysis and purification (section 3.4.2).

2.2.6 Stable cell lines

Table 2.1 Stable cell lines

Name	Comment	Source
U4A	see introduction	S. Pellengini
U4A/hJAK1-M6H	HAT-selected & Neo	B.F. Lillemeier
U4A/hJAK1-M12H	HAT-select., Neo & single clones	B.F. Lillemeier
U4A/hJAK1-MT12H	HAT-selected & Neo	B.F. Lillemeier
U4A/hJAK1(T1001>A)-M12H	single clone & Neo	B.F. Lillemeier
U4A/hJAK1(T1013>A)-M12H	single clone & Neo	B.F. Lillemeier
U4A/hJAK1(T889>A)-M12H	single clones & Neo	B.F. Lillemeier
U4A/hJAK1(T889>D)-M12H	single clones & Neo	B.F. Lillemeier
U4A/hJAK1-GFP	FACS-sorted & Neo , free GFP	B.F. Lillemeier
2fTGH	see introduction	S. Pellengini

2fTGH/hJAK1-M6H	single clones & Neo	B.F. Lillemeier
2fTGH/hJAK1-M12H	single clones & Neo	B.F. Lillemeier
HeLa-S3		ICRF
HeLa-S3/hJAK1-M6H	single clones & Neo	B.F. Lillemeier
HeLa-S3/hJAK1-M12H	single clones & Neo	B.F. Lillemeier
K562		ICRF
K562/hJAK1-M6H	single clones & Neo	B.F. Lillemeier
U3A	see introduction	S. Pellengini
U3A/STAT1		N.A. Broughton
U3A/STAT1-GFP	HAT-selected	B.F. Lillemeier
U3A/STAT1 (N460>A)	single clone & Neo	B.F. Lillemeier
U3A/STAT1 (K336>A)	single clone & Neo	B.F. Lillemeier
2C4	see introduction	D. Watling
2C4/STAT1-GFP	FACS-sorted	B.F. Lillemeier
2C4/EGFP	FACS-sorted & Neo	B.F. Lillemeier
2C4/PKC-GFP	FACS-sorted & Neo	B.F. Lillemeier
Vero		ICRF
Vero/GFP	FACS-sorted & Neo	B.F. Lillemeier
Vero/PKC-GFP	FACS-sorted & Neo	B.F. Lillemeier

2.2.7 Cytokines , inducers and drugs treatment

Table 2.2 Cytokines

	spec. activity	**work.-conc.**	**comment**	**source**
hIFN-α (Wellferon)	3×10^8IU/mg	1×10^3IU/ml	purified mixture	Welcome Research

hIFN-γ	3×10^7IU/mg	1x103IU/ml	recombinant	Dr. G.R. Adolf Boehringer Ingelh.
hu. Oncostatin M		100ng/ml	recombinant	R&D Systems

Staurosporine, Nocodazole and Cytochalasin D were dissolved in DMSO and used at final concentrations of 500nM, 5μg/ml and 4μg/ml, respectively. Cells were cultured in sodium azide (0.05%) and 2-deoxyglucose (50μM) for 20min prior to experiments to deplete ATP.

2.3 Cellular assays

2.3.1 Fluorescence activated cell scanner (FACS) analysis

Cells to be analysed were detached in versene, washed with PBS and resuspended in ice-cold DMEM. All subsequent steps were performed at 4°C to prevent aggregation or internalisation of the antigen. The monoclonal antibody used was phycoerythrin-conjugated anti HLA-DR (Becton-Dickinson) diluted 1 in 20 in DMEM. The cells were stained for 45min, washed twice with PBSA and then fixed in PBSA containing 1% paraformaldehyde in preparation for analysis. FACS analysis was carried out using Becton-Dickinson FACScan scanner and Cell Quest analysis software.

2.3.2 Cytopathic antiviral assay

This assay was used to characterise the response of cells pre-treated with ligand and challenged with virus - in this case, the picornavirus Encephalomyocarditis virus (EMCV) - assaying the ability of the ligand to reduce the cytopathic effect of the virus (Familletti et al., 1981). Cells were plated at a density of 10^5/well in 24-well plates and

allowed to settle. The medium was then changed with the addition of IFN where appropriate. Following overnight incubation the medium was removed and 0.5 plaque-forming units (pfu) of EMCV per cell was added. After 24h - at which stage the control, virus-infected, untreated cells had lysed and detached from the bottom of the wells - the plates were washed once with PBSA and fixed in formal saline for 30min, then washed again in PBSA, and 0.00075% Giemsa added to the wells to stain those cells which survived the virus infection.

2.3.3 Confocal analysis and bleaching

Cells were plated on glass coverslips and fixed with 4% paraformaldehyde in PBS for 20min at room temperature. Cells were permabilised with 0.2% Triton X-100 in PBS for 5min, washed in PBS and blocked with 0.2% fish skin gelatine in PBS for 1h. The slides were incubated for 20min with the primary antibody, washed 3 times with PBS, incubated with Cy3 labelled secondary antibody (P. Bastiaens, ICRF, London, UK), washed 3 times with PBS and mounted on glass slides with Mowiol (Calniochem). Living cells were analysed in glass bottom tissue culture dishes (MatTek Corporation) at 37°C in DMEM with low bicarbonate (2.2g $NaHCO_3$/l), no fluorescent agents and 25mM HEPES pH7.4. An Axiovert 100M confocal microscope equipped with argon and helium-neon lasers (Zeiss, Germany) was used for analysis. Green fluorescence was detected at λ>505nm after excitation at λ=488nm. Cy3 labelled antibodies were detected at λ>560nm after excitation at λ=543nm. Endosomes were labelled by internalisation of Cy3 labelled mouse IgG (50μg/ml) in living cells. To bleach GFP-tagged proteins in living cells, a small region of the cytoplasm or the nucleus was scanned with maximum laser power for the times indicated.

2.3.4 Fluorescence recovery after photobleaching

A boxed region in the cytoplasm or the nucleus was bleached and the effect on the fluorescence in this box versus four adjoined boxed regions was quantitated. The later regions were averaged to compensate for local variations. This data was plotted in a graph to compare the recovery of fluorescence in the different regions (through diffusion of GFP or GFP-fusion proteins into the boxed area). Any immobile fraction of the GFP-tagged proteins can be determined using the formula %-immobile = $[(F_{Bafter}/F_{Bbefore}) - (F_{Aafter}/F_{Abefore})]\times 100$. F_A is the fluorescence in the bleach box and F_B is the average fluorescence in the boxes outside this region. The fluorescence is determined before and after bleaching, and whereas both F_A and F_B will decrease due to equal bleaching of the mobile fraction, any immobile fraction will be bleached only for F_A (Cole et al., 1996; Edidin et al., 1976). For all data presented the average of 10 measurements is presented.

2.3.5 Quantification of STAT1-GFP in 2C4 cells

To quantify the total fluorescence in single cells, cells expressing GFP-tagged STAT1 were sectioned in Z-stacks of 0.5μm height. The fluorescence intensity of each stack was corrected for the background fluorescence and multiplied with the area for the stack to give the relative fluorescence of the stack. The relative fluorescence of a cell was the sum of the fluorescence of all stacks in the cell. The average fluorescence of all measured cells was set equal to the ratio of STAT1-GFP to STAT1 in the FACS sorted population, determined by Western blotting. From these data the ratios of STAT1-GFP to STAT1 in each cell were calculated.

2.4 Bacterial Expression of hJAK1-M6H

The bacterial strain FB810 (Bl-21 reA$^-$) or FB810 pLys (containing a construct expressing T7-lysozyme) were transformed by electroporation with the different Pet30a constructs. T7-lysozyme is a natural inhibitor of T7 polymerase and reduces the background expression from the UV lacZ promoter due to endogenous lactose. FB810 bacteria were inoculated in a 100ml culture containing 300μg/ml kanamycine and grown overnight at 37°C. The next morning, the culture was spilt 1 in 20 in the presence of 30μg/ml kanamycine and shaken at 25°C until an OD_{600} of 0.4-0.6. The culture was then induced with 1mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3–4h.

2.5 Molecular Biology

2.5.1 DNA manipulation, preparation and sequencing

Molecular biology procedures such as restriction digestions, ligations and polymerase chain reactions (PCR) were performed using enzymes, buffers and reagents from Boehringer Mannheim, Promega or New England Biolabs.

Plasmid DNA was propagated in the XL-1 Blue strain of *E.Coli*. Bacteria were transformed by electroporation using a BioRad electroporator, and positive clones selected and cultured in the presence of 50μg/ml ampicillin or kanamycin.

Miniprep DNA from overnight cultures derived from a single colony was prepared using a Qiagen Biorobot 9600 workstation operated by the ICRF Equipment Park. DNA was sequenced using the ABI Prism Dye Terminator Cycle Sequencing kit (Perkin Elmer). Sequence information was obtained using an ABI 377 sequencer, again operated by the ICRF Equipment Park

Large-scale preparation of plasmid DNA was carried out using Qiagen Maxi-prep kits (Qiagen) following the manufacturers instructions. For each prep a single

colony from an agar plate, or a stab from a glycerol stock, was inoculated into 250ml of LB medium containing 50μg/ml ampicillin and cultured at 37°C overnight.

2.5.2 Mutagenesis

A schematic representation of the mutagenesis strategy is given in Fig. 2.1. The mutagenesis-primers contained the mutation for the amino acid exchange and a newly induced restriction site which is in neither of the PCR products of the first step. The 5' and 3' PCR products contain single restriction sites 5' and 3' of the mutated sequence, respectively. The product of the two PCR reactions was digested with the newly introduced restriction site. The two fragments were ligated to the full-length insert and digested with the 5' and 3' restriction sites. The insert was gel-purified, using the Qiagen extraction kit, and cloned into the original vector, which was also digested with the two 5' and 3' restriction enzymes and gel purified.

2.5.3 Agarose gel electrophoresis

DNA products were analysed on agarose gels. Gels were prepared in 1×TAE (40mM Tris-acetate pH7.5, 2mM EDTA). Ethidium bromide was added to a final concentration of 1μg/ml allowing visualisation of DNA with a transilluminator.

2.5.4 Plasmids

Table 2.3 Plasmids

Name	Details	Source
pcDNA3,1	Amp^r,Neo^r	Invitrogen
pcDNA3.1-Myc-His C	Amp^r,Neo^r	Invitrogen
PBSK	Amp^r	Stratagene

Pet30a	Kan^r	Novagen
Pet30a/ PCS (+ KpnI, PacI, PmeI)	Kan^r	B.F. Lillemeier
pEGFP-N2	Kan^r,Neo^r	Clontech
pEGFPnls	Kan^r,Neo^r	Clontech
hJAK1/pcDNA1	Amp^r,Neo^r	A.Harpur & A. Wilks
hJAK1-M6H/pcDNA3.1	Amp^r,Neo^r	B.F. Lillemeier
hJAK1-M12H/pcDNA3.1	Amp^r,Neo^r	B.F. Lillemeier
hJAK1(T1001A)M12H/pcDNA3.1	Amp^r,Neo^r,extra SpeI	B.F. Lillemeier
hJAK1(T1013A)-M12H/pcDNA3.1	Amp^r,Neo^r,extra NheI	B.F. Lillemeier
hJAK1(T989A)-M12H/pcDNA3.1	Amp^r,Neo^r,extra BspEI	B.F. Lillemeier
hJAK1(T989D)-M12H/pcDNA3.1	Amp^r,Neo^r,extra BspEI	B.F. Lillemeier
hJAK1-MT12H/pcDNA3.1	Amp^r,Neo^r	B.F. Lillemeier
hJAK1-M6H/Pet30a	Kan^r	B.F. Lillemeier
hJAK1-M12H/Pet30a	Kan^r	B.F. Lillemeier
hJAK1-EGFP	Kan^r,Neo^r	B.F. Lillemeier
hJAK2/pcDNA3	Amp^r,Neo^r	J. Ihle
hJAK2-M12H/pcDNA3.1	Amp^r,Neo^r	B.F. Lillemeier
HJAK3/pBSK	Amp^r	J. O'Shea
HJAK3/Pet30A	Kan^r	B.F. Lillemeier
HTYK2/pRC	Amp^r,Neo^r	S. Pellegrini
hSTAT1/pRcCMV	Amp^r,Neo^r	J. E. Darnell
hSTAT1-GFP/pMBC	Amp^r	M.Köster & H. Hauser
hSTAT1-N460A/pRcCMV	Amp^r,Neo^r	J. E. Darnell
hSTAT1-K336A/pRcCMV	Amp^r,Neo^r	J. E. Darnell

hPKCα-GFP/pcDNA3	Amp^r,Neo^r	T. Ng & P. Parker

2.5.5 Primers and linkers

Table 2.4 Primers and linkers

Name	2.5.6 Sequence	Comment
CMV5'S	GATGTACGGGCCAGATATACGCG	Sequencing, PCR
T7pcDNA3.1	TAATACGACTCACTATAGGG	Sequencing, PCR
ASpcDNA3.1	TAGAAGGCACAGTCGAGG	Sequencing, PCR
hJAK1SEQ1	GAGCTCCAAGAAGACTGAGGTGAAC	Sequencing, PCR
hJAK1SEQ2	CCTCTCCTTGATGCCAGCTCACTG	Sequencing, PCR
hJAK1SEQ2-AS	CAGTGAGCTGGCATCAAGGAGAGG	Sequencing, PCR
hJAK1SEQ3	GTGAAATACTTGGCTACCTTGGAAAC	Sequencing, PCR
hJAK1SEQ4	GACAACAAGAAAATGGAACTGAAGCT	Sequencing, PCR
hJAK1SEQ5	GTCTGCACGGTTCGGACCGCAGC	Sequencing, PCR
hJAK1SEQ6	CACAGGGATATTTCCCTGGCCTTC	Sequencing, PCR
hJAK1SEQ7	CTAGGCAAGAATGCATTGAACGAATC	Sequencing, PCR
hJAK1SEQ8	CCCACACATTTTGAGAAGCGCTTCCT	Sequencing, PCR One mutation
SFGPR1 (hJ1)	GCTAAAATATGCCGTTCAGATTTGTAAGG	Sequencing, PCR
SFGPR2 (hJ1)	AGATTGCATTAAACATTCTGGAGCATACC	Sequencing, PCR
Pet28a-r	CGGGCTTTGTTAGCAGCCG	Sequencing, PCR
Pet28a-f	GCAGCGGCCTGGTGCCGC	Sequencing, PCR
JAK1HISP1	GGATCGAGGTACCGCATGCCATGGCTTTCTGTGC TAAAATG	Cloning, PCR
JAK1HISP2	CTGTGAAGCGGCCGCTTTTAAAAGTGCTTCAAAA	Cloning, PCR

	TCCTTC	
Nde-hJAK1-S	GATATACATATGGCTTTCTGTGCTAAAATGAG	Cloning, PCR
12-His-PmeI-AS (hJ1)	GACTATGTTTAAACTCAATGGTGATGATGGTGG TGATGGTGATGGTGATGATGACCG	Cloning, PCR
hJ2 clone M12H-S	GACTGGTACCGTTAACCAAAGTCTTGCCACAAG	Cloning, PCR
hJ2 clone M12H-AS	CGAATGGGTGACCTCCAGCCATGTTATCCCTTAT TTG	Cloning, PCR
hJ1S1001>A-S & AS	CAAGAAATGTACTAGTTGAGGCTGAACACCAAG	Cloning, PCR
hJ1T1013>A-S & AS	GAGACTTCGGGCTAGCCAAAGCAATTG	Cloning, PCR
hJ1T889>A-S & AS	CAGGTATGATTCCGGAAGGGGACAATGCAGGGG AGCAG	Cloning, PCR
hJ1T889>D-S & AS	GTATGATCCGGAAGGGGACAATGATGGGGAGCA GG	Cloning, PCR
KpnI-XhoI-S & AS	GCATACGGTACCTCGACTCGAGTGTCTC	Adapter
XbaI-XhoI-S & AS	GCGTGTTCTAGAAACTTTAAGAAGGAGATATAC ACTCGAGATCTGG	Adapter
NdeI-XhoI-S & AS	GCGTCCATATGACTTAGCCCTCGAGATCTGG	Adapter
Pet-PCS-S & AS	TCTAGAAGAAGGAGATATAGGTACC TTAATTAA GTTTAAAC Ggatcc	Adapter, PCR, 5'phosphate
TEV-AgeI-S & AS	aCCGGTGAAAACCTGTACTTCCAGTCAGACTA CGATATCCCAACGACTAccggt	Adapter, PCR, 5'phosphate

BOLD = amino acid motif or mutation

Underlined = restriction sites (small letters are not part of primer)

2.6 Biochemistry

2.6.1 Preparation of cell extracts

Whole cell extracts for protein analysis were prepared in "Schindler" lysis buffer. Cells were washed in ice-cold PBSA, resuspended in lysis buffer, scrapped and transferred to an Eppendorf tube, vortexed and incubated on ice for 20min. Samples were centrifuged at 20,000×g for 5min and the supernatant removed for analysis.

The concentration of protein in the extracts was determined using BioRad's DC protein assay, based on the Lowry method of protein determination.

Table 2.5 'Schindler' lysis Buffer

'Schindler' lysis Buffer	
Tris-HCL pH8.0	50mM
NaCl	150mM
Glycerol	10%
Na_3VO_4	1mM
NaF	25mM optional for P-Ser/Thr analysis
EDTA	0.1mM
NP-40	0.5%
aprotinin	3μg/ml
leupeptin	1μg/ml

2.6.2 Immunoprecipitation

Matched Schindler extracts were incubated with 1μg of antibody (table 2.9) for from 4h to overnight on a rotating wheel. 50μl of a 50% suspension of protein G- and A-sepharose beads in 1×Schindler lysis buffer added for the last hour. Subsequently, the

beads were washed twice with lysis buffer and resuspended in 1×SDS-PAGE loading buffer. Samples were boiled for 5min before loading onto a gel.

Table 2.6 SDS-PAGE sample buffer

1 × SDS-PAGE sample buffer	
Tris-HCl pH6.8	100mM
glycerol	10%
SDS	3%
DTT or β-mercaptoethanol	100mM or 450mM
Bromphenol Blue	0.05%

2.6.3 Gel electrophoresis of proteins

Denaturing polyacrylamide gel electrophoresis (PAGE) of immunoprecipitated proteins or extracts was based on the method of Laemmli (Laemmli, 1970). The same gels were used to resolve immunoprecipitated proteins or whole cell extracts. A stacking gel containing 5% (v/v) acrylamide (37.5:1 acrylamide:bis-acrylamide), 125mM Tris-HCl pH6.8 and 0.1% (w/v)SDS was cast over a separating gel of between 6% and 10% acrylamide (depending on the size of the proteins of interest) containing 0.375M Tris-HCl pH8.8 and 0.1% SDS. The running buffer for gels contained 25mM Tris-base, 186mM Glycine and 0.1% SDS. The pre-stained markers that were run in parallel with the proteins to monitor their size were High-range Molecular Weight Markers from Amersham Pharmacia.

2.6.4 Staining of SDS-PAGEs with silver, zinc or coomassie

SDS-PAGE gels were stained with either the silver or zinc stain (for mass spectrometry) kit from BioRad according to the manufactures instructions. Gels were also stained with 45% methanol, 10% acetic acid and 0.2% Coomassie Brilliant Blue R250 and washed in the same solution without the die.

2.6.5 Western transfer of proteins

A transfer buffer containing 25mM Tris-base, 186mM glycine and 10% (v/v) methanol was used. Gels were equilibrated in transfer buffer and transferred to PVDF membrane (Immobilon P, Millipore) using a wet blotter (IDEA Scientific Company) for 1 hour.

2.6.6 Western blot analysis

The Table 2.9 below lists the antibodies used for Western-blot analysis of cell proteins. Immediately following transfer, membranes were equilibrated in TBST (10mM Tris-HCl pH7.4, 75mM NaCl, 1mM EDTA pH8.0, 0.1% Tween-20). Membranes were then blocked overnight in 5% (w/v) BSA dissolved in TBST containing 20mM Na_3VO_4 and 0.01% sodium azide as a preservative. To analyse tyrosine phosphorylation of proteins the primary antibody was diluted in 1% BSA, TBST, 20mM, Na_3VO_4 and 0.01% sodium azide, whilst for other analysis vanadate was omitted. Primary antibodies were diluted 1:2000 unless stated below and incubated for 2h at room temperature. Between incubation with primary and secondary antibodies the membranes were washed three times in TBST for 5min. The secondary antibody for phosphotyrosine analysis was diluted in 1% BSA in TBST, including vanadate but in the absence of azide, whilst the secondary antibody for other primary antibodies was diluted in 5% (w/v) skimmed milk

powder in TBST. Secondary antibodies were horseradish peroxidase-conjugated (Amersham) anti-rabbit, mouse or goat, and were used at 1:2000 dilution, and incubated for 30-45min at room temperature. After incubation with secondary antibody the membranes were washed extensively for at least 3h in TBST. Electrochemiluminescence (ECL) was used to visualise the proteins using solutions from Amersham. Following ECL and autoradiography, the membranes were stripped overnight in 2.5M glycine, pH2.5 and then reprobed as required.

2.6.7 Purification of membrane associated proteins

Cells were harvested by low speed centrifugation and resuspended in Schindler lysis buffer without glycerol or NP-40. The suspension was added to a ball-bearing homogeniser which had been pre-chilled on ice. The cells were dounced twelve times and then centrifuged at 4°C at 6500rpm. The supernatant was ultracentrifuged (100000×g, 4°C), and the new supernatant removed. The pellet containing membrane-associated cell protein was solubilised in Schindler buffer without NP-40 and glycerol, but with 1.5M NaCl. The suspension was dounced again and ultracentrifuged. The supernatant, containing membrane-associated proteins, was removed for analysis by SDS-PAGE and Far-Western.

2.6.8 Cleavage of hJAK1-MT12H with the TEV-protease

The resin bound hJAK1-MT12H was transferred into 100μl TEV-cleavage Buffer and 20U TEV protease (GibcoBRL) were added. The resin was incubated with the protease for 30 – 60min at 30°C on a rotor. Following this, the resin was spun out at 2000rpm for 1min and the supernatant contained the free cleaved 'hJAK1-M-'.

Table 2.7 TEV-cleavage Buffer

TEV-cleavage Buffer	
Tris-HCl pH7.8	50mM
β-mercaptoethanol	5mM
NaCl	100mM
Glycerol	5%
NP-40	0.25%
imidazole	40mM

2.6.9 Coupling of monoclonal antibody to protein A-sepharose

The monoclonal antibody (2mg) was mixed with 1 volume PBS, centrifuged at 14,000rpm for 15min, added to 1ml protein G beads (Fastflow sepharose protein G, Pharmacia) and incubated at 4°C for 3h. Beads were washed three times in 15ml PBS and two times in 15ml Na_3Bo_4 buffer (200mM H_3Bo_4/NaOH, pH9.0) at room temperature. Cross linking was carried out by incubating the beads with dimethylsuberimidate in Na_3Bo_4 buffer at room temperature for 30min. Beads were washed twice with 10ml 200mM ethanolamine pH8.0 and then incubated with 10ml 200mM ethanolamine pH8.0 at room temperature for 2h to quench excess dimethylsuberimidate. Beads were washed three times with 15ml PBS/0.05% Tween-20, 2 times with neutral elution buffer (ICRF antibody production), three times with PBS and stored as 50% slurry in PBS and 0.01% azide.

2.6.10 Preparing of bacterial lysates

After induction with IPTG, the bacteria were transferred to 4°C and washed with ice-cold PBS. The bacteria were pelleted and transferred into different lysis buffers (see

Fig. 3.8) and lysozyme was added (tip of spatula). The bacterial suspension was left for 20min on ice. After incubation the lysate was sonicated 3 times on ice for 1min at maximum amplitude. Alternatively, the bacterial lysate was freeze thawed for 3 times in liquid nitrogen before sonication. The debris and the DNA were pelleted at 35000×g and the supernatant contained the solubilised protein.

2.6.11 *In vitro* translation of proteins

HJAK1 was *in vitro* translated using TNT coupled reticulocyte lysate (Promega), different T7-promotor constructs and redivue [^{35}S]-methionine (Amersham). For Far-Western analysis the hJAK1-M6H/Pet30a construct was used. The *in vitro* translation was performed according to the manufactures instructions.

2.6.12 Coupling of peptide to KLH and Affigel-10

The peptides were coupled to Imject maleimide activated keyhole lympet hemocyanin (Pierce) through their N-terminal cystine. The coupling reaction was done following the manufacturers instruction. The suspension was then pelleted by centrifugation (6500rpm) and the supernatant was dialysed against 83mM sodium phosphate pH7.2 and 900mM NaCl. The dialysed solution and the pellet were combined and used for injection into rabbits.

Affigel-10 was purchased from BioRad. Peptide was coupled to the resin according to the manufacturers instruction.

Table 2.8 Peptides

Peptide-name	Sequence	Comment
hJ1 1007-1017	CIGDFGLTKAIE	+/- N-term. biotinylation

hJ1 1007-1017 (P-Thr)	CIGDFGL(**P-Thr**)KAIE	+/- N-term. biotinylation
hJ1 844-894	CPEGDNTGEQVA	+/- N-term. biotinylation
hJ1 844-894 (P-Thr)	CPEGDN(**P-Thr**)GEQVA	+/- N-term. biotinylation
hJ1 1033	E**YY**TVK	EYY-motif see introduction
hJ1 1033 P1	E(**P-Tyr**)YTVK	EYY-motif see introduction
hJ1 1033 P2	E**Y**(**P-Tyr**)TVK	EYY-motif see introduction
hJ1 1033 P1/P2	E(**P-Tyr**)$_2$VK	EYY-motif see introduction

BOLD = possible or known phosphorylation site

Underlined = cysteine is not part of hJAK1, but necessary for coupling reaction with maleimide activated KLH

2.6.13 Antibodies

Table 2.9 Antibodies

Name	Antigen	Type	Source
sheepαmouse (HRP)	mouse IgG	Fab-fragment	Amersham
donkeyαrabbit (HRP)	rabbit IgG	Fab-fragment	Amersham
sheepαmouse (Cy3)	mouse IgG	sheep polyclonal	P. Bastiaens
PY20	phospho-tyrosine	mouse monoclonal	Affiniti
4G10	phospho-tyrosine	mouse monoclonal	UBI
αP-Ser	phospho-serine	rabbit polyclonal	Zymed
αP-Thr	phospho-threonine	mouse monoclonal	Zymed
9E10	αMyc-Tag	mouse monoclonal	ICRF
αHis-Tag	6×histidine	mouse monoclonal	R&D System
M8	JAK1aa566–865	rabbit polyclonal	A. Ziemeicki
M7	JAK1aa566–865	rabbit polyclonal	A. Ziemeicki

SCαJAK1	JAK1	rabbit polyclonal	Santa Cruz
αJAK2N	N-terminal of JAK2	rabbit polyclonal	A. Ziemeicki
αJAK2C	C-terminal of JAK2	rabbit polyclonal	A. Ziemeicki
SCαJAK2	JAK2	rabbit polyclonal	Santa Cruz
SCαTYK2	TYK2	rabbit polyclonal	Santa Cruz
p91	STAT1	rabbit polyclonal	B. Williams
αSTAT1	STAT1	mouse monoclonal	Novacastra
αPY701	P-Tyr701 in STAT1	mouse monoclonal	NEB
SCαSTAT2	STAT2	mouse monoclonal	Santa Cruz
αGFP	GFP	mouse monoclonal	ICRF
HLA-DR (+phycoerythrin)	MHC-class II	mouse monoclonal	Becton-Dickinson

2.7 Electrophoretic mobility shift assay (EMSA) analysis of DNA-binding protein complexes.

2.7.1 Probe labelling

200ng of the appropriate oligonucleotide containing a protein binding sequence was incubated with 10 units of T4 Polynucleotide Kinase (New England Biolabs) in kinase buffer (70mM Tris-HCl pH7.6, 10mM $MgCl_2$, 5mM DTT) with 5μCi of [γ^{32}P]-ATP for 30min at 37°C. 200ng of complementary oligonucleotide and 100mM NaCl were added to the reaction mixture, which was then heated to 95°C for 5min and allowed to cool slowly in a water bath whilst annealing took place. NAP-5 Sephadex G-25 columns (Pharmacia Biotech) were used to purify the labelled double-stranded oligonucleotides from unincorporated label and unannealed oligo's as follows. The column was washed with TE (10mM Tris-HCl pH7.5, 1mM EDTA) and the probe was

added to the column. The column was eluted with TE and eluate fractions collected. A scintillation counter was used to identify the first fraction with a high activity, containing the labelled probe. (specific activity of the probe $\approx 2.5 \times 10^4$ cpm/μg input probe)

Table 2.10 EMSA-probes

Probe	Sequence
β–Casein	5`GGATTGAATTCCTAGAAATCT3`
hSIE	5`GTCGACATTTCCCGTAAATC3`

2.7.2 Mobility-shift assay

Extracts made in Schindler buffer were assayed in the presence of 2.5mg/ml BSA, 500μg/ml tRNA, 4% (w/v) Ficoll (Pharmacia), 100mM NaCl, 10mM HEPES pH7.9, 0.1mM EGTA, 5% (v/v) glycerol and 0.5mM DTT.

Matched extracts were preincubated with polydI:dC for 10min at room temperature to eliminate non-specific DNA-binding. Extracts were incubated with probe for 20min and then loaded onto 6% non-denaturing polyacrylamide gels (6% 37.5:1 acrylamide:bis-acrylamide, 1×TGE - 25mM Tris-base, 250mM Glycine, 1mM EDTA) and run at 200 volts. A marker of xylene cyanol blue was added to one lane, which was used to monitor the distance the samples had run. Once run far enough, the gels were dried for autoradiography.

2.8 RNase protection analysis

2.8.1 RNA preparation

Cells were washed in ice-cold PBSA, resuspended in RNA extraction buffer (150mM NaCl, 10mM Tris-HCl pH7.9, 1.5mM $MgCl_2$, 0.65% NP40), vortexed, incubated on ice for 5min and then centrifuged at 20,000×g for 5min at 4°C. The supernatant was removed and adjusted to 0.5% (w/v) SDS, 1mM EDTA. The proteins and DNA were extracted using an equal volume of acid-buffered phenol (Tris-HCl pH4.3)/chloroform/isoamylalcohol (25:24:1), and then once more with chloroform/isoamylalcohol (24:1). RNA was precipitated with 100% ethanol and 3M NaOAc pH5.0, centrifuged for 30min at 20,000×g, washed with 70% ethanol and resuspended in deionised water. The OD at 260nm was measured using a spectrophotometer, and converted to concentration on the basis that 1 OD unit = 40μg ssRNA.

2.8.2 Probe labelling

Plasmid DNA containing template DNA for the probe was linearised with the appropriate restriction enzyme to cut at the distal end of the probe template. The digest was checked on an agarose gel, cleaned by phenol/chloroform extraction, ethanol precipitated and resuspended in deionised water.

1μg of template DNA was used in each labelling together with 100μCi [α^{32}]-UTP (Amersham, Bucks), 10mM DTT, 15mM GTP, ATP and CTP (Amersham Pharmacia Biotech), 1μl per reaction RNasin (Promega), 1× transcription buffer (Boehringer), and 1μl of the appropriate RNA polymerase (Promega). Reactions were incubated at 37°C for 2h and then ribonuclease-free DNase (Boehringer) was added for 15min. 80μl of STE (10mM Tris-HCl pH7.2, 1mM EDTA, 0.1% SDS (w/v) was then

added and the mixture was extracted with phenol/chloroform/isoamyl alcohol. The probe was precipitated with 2.5M NH_4OAc, tRNA and 100% ethanol, resuspended in STE, precipitated again and resuspended in 40μl STE. RNase protection probes used are listed in table 2.11 (specific activity of the probe $\approx 2 - 5 \times 10^6$ cpm/μg input DNA)

Table 2.11 RNase protection probes

Probe	Protected fragment	Reference
Actin	130b	(Ackrill et al., 1991)
9-27	160b	(Ackrill et al., 1991)
6-16	190b	(Ackrill et al., 1991)
IRF1	175b	(Maruyama et al., 1989)
p48	270b	(Veals et al., 1992)

2.8.3 Hybridisation and mapping

13μg of RNA was dried in a speedvac and resuspended in 24μl deionised formamide. A cocktail of probes and hybridisation buffer was made up and added to the RNA; final hybridisation conditions were 80% formamide, 40mM PIPES pH6.4, 400mM NaCl and 1mM EDTA. Samples were heated to 95°C for 5min and placed at 47°C overnight to hybridise. Unprotected RNA was digested in 10mM Tris-HCl pH7.5, 5mM EDTA, 300mM NaCl, 0.5% SDS (w/v), 40μg/ml RNase A and 2μg/ml $RNaseT_1$ (both Boehringer) at 37°C for 30min. 45μg/ml Proteinase K was added for 15min and protein was extracted in phenol/chloroform/isoamyl alcohol. RNA was precipitated with tRNA and 100% isopropanol, and resuspended in 85% formamide, 0.1% (w/v) each of Bromophenol Blue and Xylene Cyanol. Samples were heated at 95°C for 5min, run on a 6% denaturing gel (6.5% 19:1 acrylamide:bis-acrylamide, 0.5×TBE - 45mM Tris-borate, 1mM EDTA - 7M urea) and dried for autoradiography.

2.9 Mass-Spectrometry

2.9.1 Tryptic digest of protein

Destained gel particals are incubated with 10mM DTT and 25mM $(NH_4)_2CO_3$ pH8.0 at 50°C for 45min. The solution is replaced with 50mM iodacetic acid dissolved in 25mM $(NH_4)_2CO_3$ pH8.0 and then washed with 25mM $(NH_4)_2CO_3$ pH8.0/50% acetonitrile. The gel pieces are then dried in a Speed-Vac. Gep pieces are rehydrated in 25mM $(NH_4)_2CO_3$ pH8.0 containing 50ng trypsin and incubated at 37°C overnight. The supernatant is removed and the gel piece extracted twice with 50% acetonitrile/5% TFA. The extract is dried in a Speed-Vac and resuspended in water.

2.9.2 Purification of peptides using ZipTip$_{MC}$ (IDA resin) or ZipTip$_{C18/\mu C18}$

Desalting and concentration of peptides for increased MALDI-TOF MS sensitivity and resolution was done with ZipTip $_{C18/\mu C18}$ (Millipore). Enrichment of phosphopeptides was obtained using the ZipTip$_{MC}$ (Millipore). The tips were used according to the instructions of the manufacturers.

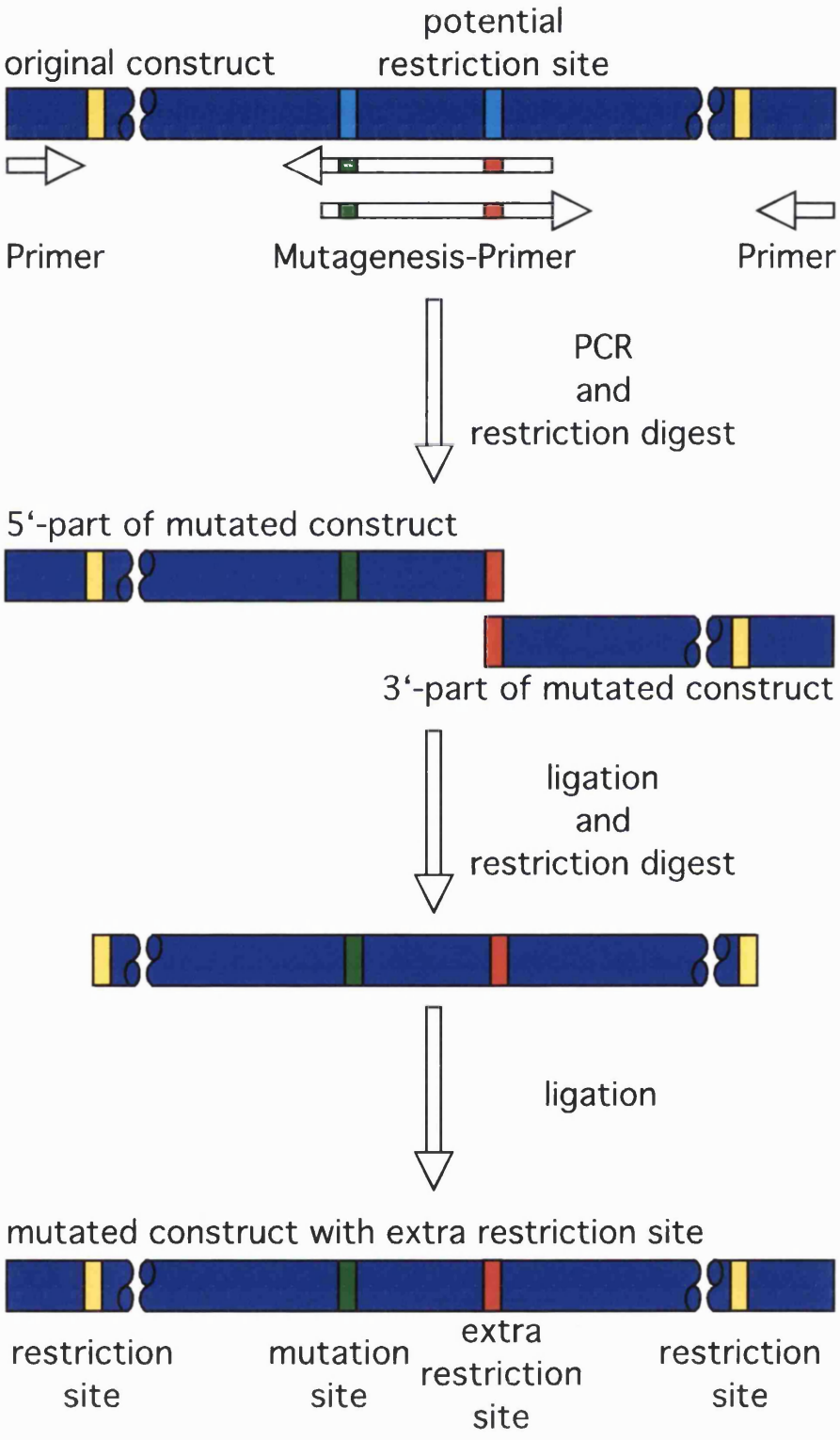
2.9.3 Mass-Spectrometry, reverse phase HPLC and Edman degradation

Mass-Spectrometry, reverse phase HPLC and Edman degradation were done in collaboration with the Mass Spectrometry unit of the ICRF. The reverse phase HPLC and the Edman degradation were established and performed by Nick Totti (ICRF).

Fig. 2.1 Mutagenesis strategy used to mutate residues in hJAK1-M12H

Mutations were introduced into cDNAs by PCR. A primer containing the mutation for the amino acid exchange (green) and an additional restriction site (red), which has to be unique to the fragment of the cDNA used for mutagenesis, is designed. The primer is synthesised in sense and antisense orientation. Two additional primers are used, the sense primer is upstream and the antisense primer is downstream of two restriction sites (yellow), later used to re-introduce the mutated insert into the original construct. (All primers are shown in the first panel). Each mutagenesis primer is used with the corresponding second primer in a PCR reaction to obtain two PCR fragments. Both fragments contain the mutation (green), the new restriction site (red) and either a 5'- or 3'-part of the original construct. The fragments are digested at the new restriction site (red and second panel). They are ligated to obtain the full-length insert, which is subsequently digested at the 5'- and 3'-restriction site (yellow and third panel). The insert is cloned back into the original cDNA to encode the mutated form of the protein (fourth panel).

Figure 2.1


potential
restriction site
original construct
Primer
Mutagenesis-Primer
Primer
PCR
and
restriction digest
5'-part of mutated construct
3'-part of mutated construct
ligation
and
restriction digest
ligation
mutated construct with extra restriction site
restriction
site
mutation
site
extra
restriction
site
restriction
site

Chapter 3: JAK1 Interactions and Phosphorylation

3.1 Introduction

Cytokine treatment leads to activation of the JAK/STAT as well as additional signalling pathways in a modular fashion. There also appear to be alternative activators of the STATs, additional JAK-mediated pathways and cross talk between JAK/STAT and other pathways. In addition, the same ligand can trigger a different spectrum of JAK/STAT activation in different cell types. Possible mechanisms whereby specificity might be generated include cell type specific adaptors, scaffolding or anchoring proteins. The modulation of the activatability, activity or availability (recruitment to receptor or subcellular localisation) of pathway components could also play a role in signal specificity. This may be due to changes in tyrosine and/or serine/threonine phosphorylation in response to different cytokines and/or cross talk between signalling pathways. JAKs have been reported to interact directly with a number of signalling molecules, like Grb2 (Giorgetti-Peraldi et al., 1995) and STAT5 (Improta et al., 1994; Leung et al., 1995). Whether such interactions are mediated through tyrosine phosphorylation and SH2 domains versus other protein/protein interactions remains to be established.

The overall objectives of this chapter was to identify unknown phosphorylation sites in hJAK1 which may modulate activity and/or mediate additional protein-protein interactions and to find new proteins which are recruited to complexes containing hJAK1. For this, recombinant hJAK1 was purified and new *in vitro* assays to analyse JAK1 interactions and phosphorylation were established.

3.2 Results

3.2.1 hJAK1 expression systems

3.2.1.1 hJAK1 expression constructs

Three different tags were fused to the C-terminus of hJAK1 (Fig. 3.1 and table 3.1). The Myc-6×His tag (M6H) from the mammalian expression vector pcDNA3.1/Myc-His (Material and Methods) and two others based on it. The second tag, Myc-12×His (M12H), had the original six histidines replaced with a stretch of twelve histidines by PCR. The third tag was the Myc-12×His tag (MT12H) with a TEV-cleavage site introduced between the Myc tag and the twelve histidines. This was done using an existing restriction site and a synthetic linker encoding the proteolytic cleavage site.

Table 3.1 Tags of hJAK1 constructs

name of tag	Amino acid sequence
Myc-6×His (M6H)	5'-hJAK1-AAARGHPFEQKLISEEDLNMHTGHHHHHH•-3'
Myc-12×His (M12H)	5'-hJAK1-AAARGHPFEQKLISEEDLNM-HTGHHHHHHHHHHH•-3'
Myc-Tev-12×His (MT12H)	5'-hJAK1-AAARGHPFEQKLISEEDLNMHTG-ENLYFQSNYNIPTTTGHHHHHHHHHHHH•-3'

• = Stop codon

All JAK1 constructs were initially cloned in the mammalian expression vector pcDNA3.1. For bacterial expression the constructs were subcloned into the bacterial expression vector Pet30a. For expression in the bacteria strain FB810 the 5'-untranslated

region of the wildtype cDNA had to be removed by PCR. It inhibits the translation of the protein, probably due to the long stretch of non-coding sequence between the ribosome binding site of the vector and the start codon of the cDNA. The Pet30a construct without the untranslated 5'-region also had the highest yield for *in vitro* translation of hJAK1 using reticulocyte lysates (see section 1.2.3.2).

During the cloning and sequencing of the different expression constructs, several differences in the sequence of the wildtype hJAK1 cDNA compared to its published sequence were observed and further investigated. The nucleotide sequence differences and their effect on the amino acid sequence of hJAK1 are summarised in table 3.2.

Table 3.2 Sequencing analysis of hJAK1

bp-position in new sequence	**difference**	**amino acid exchange and position in new sequence**
274	C > T	Arg 92 > Trp
1012	G > C	Asp 338 > His
1065	A > T	silent
1067	T > A	Phe 356 > Tyr
1681	C > T	silent
2526	C > T	silent
2535	C deletion	Ser 845 – Arg - Lys – Lys - Asn – Gln – Pro > Ser 845 – Glu – Lys – Lys- Pro - Ala
2538	A deletion	see 2535
2552	C deletion	see 2535
2580	G > A	silent
2659	GGG insertion	extra Gly 886
3345	G > A	silent

3354	A > G	silent

3.2.1.2 The tagged hJAK1 are functional signalling molecules

To verify that all tagged constructs were functional, the mammalian expression constructs were stably expressed in the JAK1 negative cell line U4A (Introduction). As the cell line is derived from 2fTGH cells (Introduction), it was possible to use HAT selection to select and maintain populations expressing the constructs. Survival in HAT medium was indicative of functionality. The cell populations were also checked for an antiviral response after treatment with IFN-α or –γ (Fig. 3.2); the induction of HLA-class II after IFN γ (Fig. 3.3); the induction of representative sets of IFN-α and IFN–γ-inducible genes (Fig. 3.4) and the phosphorylation of signalling components involved in IFN-α and –γ signalling (Fig. 3.5). All were comparable to the responses in wildtype cells. Therefore, all of the tagged constructs function as wildtype cDNA. Furthermore, sequential immunoprecipitation with anti-Myc tag and anti JAK1 antibodies indicated no detectable cleavage of the Myc-His tags (data not shown).

3.2.1.3 Mammalian cell expression systems for hJAK1

Wildtype cell lines were used for expression of recombinant hJAK1 to avoid artefacts in later analyses, due to the mutagenised genetic background in the U4A cells. 2fTGH cells were transfected with all mammalian expression constructs, as they are a well characterised system for analysis of the JAK/STAT pathway. In addition, suspension cells were used as an expression system to facilitate growth of large numbers of cells, to obtain high yields of protein. For this purpose HeLa-S3 and K562 cells were transfected with the hJAK1-M6H and the hJAK1-M12H constructs. All cell lines were analysed by Western-Blot for normal phosphorylation of signalling components after

IFN treatment. The HeLa-S3/hJAK1 cell lines turned out to be the most useful expression systems, as it allows to work with high numbers of cells and the cells are easy to handle in experimental procedures. The JAK/STAT phosphorylation profile of HeLa-S3/hJAK1-M6H is shown as an example in figure 3.6.

3.2.1.4 Bacterial Expression systems of hJAK1

The bacterial strains FB810 or FB810 pLys were used to express the IPTG inducible Pet30a constructs. The bacterial expression systems with constructs containing the 5'-untranslated region of the hJAK1 cDNA did not to express any hJAK1 protein after induction. After removal of the 5'-untranslated region high amounts of recombinant hJAK1 were obtained. Maximum level of expression were reached 5h post induction (starting at OD_{600} = 0.6) with 1mM IPTG at 37°C (data not shown). The recombinant protein proved insoluble for all of the growth or lysis conditions tested if expressed at 37°C.

The induction at the lower temperature of 25°C reduced the amount of recombinant protein, but still resulted in an acceptable expression level of recombinant hJAK1 (Fig. 3.7). The bacterial strain containing the pLys construct expressed higher amounts of the recombinant protein (Fig. 3.7). If expressed at 25°C small amounts of the protein were soluble, depending on the growth and lysis conditions (Fig. 3.8; Material and Methods). The highest relative level of soluble recombinant protein was obtained in buffers with high salt concentrations (>150mM NaCl), high detergent concentrations (1% NP-40) under strongly reducing conditions (1mM DTT or at least 10mM β-Mercaptoethanol) from bacteria induced at OD_{600} = 0.4.

The ICRF mass spectrometry unit in the ICRF confirmed by peptide map analysis of a single SDS-PAGE protein band that the induced protein was hJAK1 (data not shown).

3.2.2 Purification of hJAK1

3.2.2.1 Purification from wildtype systems

To avoid possible overexpression artefacts, including high background phosphorylation, purification of wildtype endogenous hJAK1 from HeLa-S3 cells with an antibody affinity column was initially attempted.

Polyclonal rabbit serum (M8 from A. Ziemiecky) against JAK1 (amino acids 566–865 fused to GST) was purified with Protein A affinity columns and bound to Affigel 10. hJAK1 was loaded by circulating Schindler lysate from HeLa-S3 cells over the JAK1 antibody column system over night at 4°C and washed extensively with lysis buffer.

To elute the bound JAK1 several methods were tried. Eluting the column with high pH (25mM Na_2CO_3 pH 11.0; Fig. 3.9) resulted in a distinct elution in a small amount of buffer. However, high yield of the protein could not be obtained. There was also a very high background of contaminating proteins, which co-eluted with hJAK1. These were detected by running the fractions on SDS-PAGE, followed by silver stain (data not shown). Experiments using pH gradients failed, as no eluted protein was detectable (data not shown).

The chaotropic salt KSCN, at a constant concentration 500mM showed high potential in eluting hJAK1 from the antibody column. The amount of eluted contaminant was, however, also extremely high (data not shown). A gradient of KSCN eluted JAK1 as a smear in every fraction. Again the amount of eluted JAK1 was relatively high, but was also very dilute and contaminated with non-specific protein (Fig. 3.10 and data not shown).

Low pH or alternative salt elution resulted in high contamination with non-specific protein and/or low recovery of JAK1. All attempts to purify wildtype JAK1 failed due to poor elution profiles from the polyclonal affinity column.

3.2.2.2 Metal affinity purification of recombinant hJAK using histidine tags

Initial purification of hJAK1-M6H from the transfected 2fTGH cells showed that the affinity of a native hJAK1 with a six histidine tag for either Ni-NTA or Talon resins was not sufficiently high enough to get efficient binding. In addition, once bound the affinity was not high enough for stringent washes with imidazole (data not shown). Binding to the column only occurred with imidazole concentrations below 5mM and the protein already eluted at imidazole concentrations as low as 15mM. These characteristics were not sufficient to differentiate binding of the tagged hJAK1, metal binding or highly phosphorylated proteins.

Replacing the stretch of six histidines with twelve increased the affinity of the tag. Again the 2fTGH system was used to optimise the binding and elution of hJAK1-M12H. Using the longer tag, more stringent binding conditions could be used (300mM NaCL, 1% NP-40 and 10% glycerol; see Material and Methods). It was possible to get binding in buffers containing 10mM imidazole (Fig. 3.11), which was sufficient to bind most of the JAK1-M12H, with less endogenous protein. Intensive washing with up to 40mM imidazole did not elute bound hJAK1-M12H and was stringent enough to elute the majority of contaminants (Fig. 3.11). Continuous washing with buffers containing 50mM or more imidazole resulted in the loss of binding (Fig. 3.11). Results were similar for the Ni-NTA and the Talon resins. The Talon resin had slightly lower affinity for the tag, but also less non-specific binding. Even the best conditions, however, yielded insufficient and insufficiently pure hJAK1-M12H for the intended experiments. For example, SDS-PAGE analysis of the eluted hJAK1-M12H did not yield the sufficiently resolved band necessary for mass spectrometry. In addition, the amount of non-specific proteins binding to recombinant hJAK1-M12H or the resin was to high, to permit the identification of specific JAK1-associated proteins.

A common method to increase the efficiency of purification is to use a proteolytic cleavage site between the tag and the protein. A TEV-cleavage site was introduced between the Myc and the 12×His tag. Despite optimisation of the protease buffer conditions the cleavage of the proteolytic site was never complete (Fig. 3.12). In addition a large fraction of the cleaved hJAK1-M remained bound to the metal affinity resin. The amount of cleaved but bound protein varied dependent on the cleavage conditions. Many experiments gave the impression that conditions which improved cleavage also increased the non-specific binding of the cleaved hJAK1-M to the column.

Having solubilised a portion of the bacterially expressed recombinant hJAK1-M12H, I attempted to purify it using the conditions optimised as above. As the expression levels of hJAK1-M12H in the bacterial system are much higher, it was expected that competition from bacterial protein would be less important. Hence, making it possible to purify relatively large amounts of highly purified hJAK1-M12H. Calculation of the amount of soluble hJAK1-M12H in the solubilisation experiments indicated that milligram amounts of purified protein might reasonably be expected. The purification from the bacterial lysates was very clean (Fig. 3.13) and on silver staining SDS-PAGE analysis contaminating proteins were not detected in the size range of hJAK1-M12H and only minor amounts of contaminating protein in lower size ranges were detected (data not shown). This allowed a final purification by size exclusion chromatography. However the increased scale of the solubilisation and/or purification resulted in a very low yield of hJAK1-M12H: the yield was of the order of micrograms from 50 to 500 ml of bacterial culture.

It was possible to purify the insoluble hJAK1-M12H protein under denaturing conditions (6M guanidinhydrichloride) in relative high amounts. However, it was not possible to refold the protein by dialysis, due to aggregation in the different dialysis buffers (data not shown).

Thus, purification of recombinant hJAK1 using metal-affinity purification and/or bacterial expression systems was not practical.

3.2.2.3 Antibody affinity purification of hJAK1 containing a Myc tag

An alternative method to purify the tagged hJAK1 was to use the monoclonal antibody 9E10 (ICRF) specific to the Myc tag. The 9E10 was cross-linked to protein A/sepharose. To reduce the amount of antibody in the eluate, the resin was pre-washed with a neutral elution buffer. (9E10 is insoluble at low or high pH, personal communication, antibody department, ICRF). In later experiments (mainly for mass spectrometry) the 9E10 antibody was not covalently bound to the protein A/sepharose as there was no need to avoid elution of the antibody. It was necessary to apply lysates of high protein concentration (7 -15mg/ml) to the resin to obtain effective binding of hJAK1-M6H or –M12H. Due to the high antibody affinity, the protein could be bound and washed under very stringent conditions (300mM NaCL, 1% NP-40 and 10% glycerol, 1mM DTT) to obtain highly purified hJAK1. On the other hand less stringent conditions (150mM KCl, 0.5% NP-40, 5% glycerol, 5mM β-mercaptoethanol) could be used in order to maintain interactions with other proteins. Bound proteins were analysed by SDS-PAGE after elution either at pH2.5, neutralised with 1M Tris-base ~pH12.0 and acetone precipitation or for analyses of hJAK1 per se by boiling in SDS-sample buffer. An example of a purified hJAK1-M6H from HeLa-S3 cells is given in figure 3.14 (see section 3.4.1).

The purification using the 9E10 antibody led to the highest and purest yield of hJAK1 protein of all tested expression and purification systems. Therefore, all future analyses of hJAK1 will be performed using this method.

3.2.3 Protein/protein interactions of hJAK1

3.2.3.1 Indications of associated proteins

Immunoprecipitations of signalling components, followed by Western-Blot analysis are often used to assay the induction of phosphorylation in signalling pathways. Hence, inducibly phosphorylated proteins have been detected. Examples include: A protein of approximate 100kD which is tyrosine phosphorylated after IFN-γ treatment, but not after incubation with IFN-α or OSM (Fig. 3.15). It is only seen when the precipitation is performed with lysates from HeLa-S3 cells, but not with HT1080 derived cells. Suggesting cell type specificity. A second co-immunoprecipitated protein of about 100kD is specifically threonine phosphorylated after induction of 2fTGH cells with IFN-α, IFN-γ or OSM (Fig. 3.16). These examples suggest additional signalling molecules in the JAK/STAT pathway, as they do not correspond to proteins previously described.

3.2.3.2 Far-Westerns as an *in vitro* interaction assay

Far-Western analysis is an *in vitro* assay used to defect and/or analyse protein/protein interactions. The general principle is to separate target proteins by SDS-PAGE and blot them onto a PVDF membrane (Fig. 3.17). Target proteins can be single proteins or complex protein mixtures, but to enhance resolution it is advantageous to use less complex mixtures obtained by pre-purifying proteins and/or the use of subcellular fractions (cytosol, plasmamembrane, vesicles, nucleus etc.). The proteins attached to the membrane are renatured. Either by incubation in urea followed by dialysis or incubation with several changes of buffer containing detergent able to replace the protein bound SDS. Complete renaturation never takes place, as the random attachment to the PVDF-membrane inhibits complete refolding. The aim is to renature domains of the protein not

attached to the membrane. The membrane is blocked to inhibit non-specific interactions with BSA or more effectively, with milk powder..

In classical Western-Blots the probe is an antibody, in Far-Westerns the probe is the interacting protein. Probes have to be detected indirectly or by labelling the probe itself. Indirect detection can be via an antibody as in a conventional Western-Blot or for example by streptavidin if the probe is biotinylated. For direct detection radioactive or fluorescent-labelled probe can also be used. An easy way to [^{35}S]-methionine label a probe is to *in vitro* translate the protein. In this work, that was the method of choice. The probe has to be soluble in the interaction and wash buffer. Solubility was controlled through pelleting aggregates using a 100.000×g ultra-centrifugation.

Far-Westerns are very sensitive to the composition of the interaction buffer. Salt, detergents, pH, blocking agent and temperature can be very critical to the *in vitro* reconstitution of interactions. The interactions have to be controlled very carefully as the system can introduce artificial interactions. For example, amino acid motifs which are normally 'hidden inside' a protein, may be exposed in a Far-Western, thus allowing artificial interactions (see also section 3.4.2).

3.2.3.3 JAK-JAK interactions: hJAK1-M6H interactions with JAKs in Far-Westerns

Many kinases including the JAKs are thought to dimerise. To test whether JAK1 interacts in a homotypic fashion, recombinant [^{35}S]-methionine labelled hJAK1-M6H was used as a probe in Far-Westerns utilising JAKs, immunoprecipitated from cells with or without IFN pre-treatment (Figure 3.18). JAK1, JAK2 and TYK2 were precipitated from 2fTGH cells (+/- IFNs), blotted on a PVDF-membrane, renatured by incubation in NP-40 containing interacting buffer (pH7.8), the membrane blocked with boiled and filtered milk and probed with [^{35}S]-methionine labelled hJAK1-M6H. The hJAK1-M6H

interacts strongly with JAK1 and TYK2 and to a lesser extend with JAK2. These interactions are induced by IFN-γ and more strongly by IFN-α. Furthermore, in 2fTGH cells incubation with IFN-α leads to a much stronger phosphorylation of the JAKs than IFN-γ. Therefore, the data suggests a correlation between JAK1/JAK interactions and the state of phosphorylation. To investigate this further, Far-Westerns with dephosphorylated target proteins were performed. For example, JAK1 and TYK2 were precipitated from 2fTGH cells with and without IFN-γ stimulation. For each JAK a sample was incubated with alkaline phosphatase to dephosphorylate the proteins. Independent experiments showed that the treatment with alkaline phosphatase had no effect on the amount of the JAKs in the immunoprecipitations. The Far-Western data in figure 3.19 suggests that the interaction of hJAK1-M6H as a probe with JAK1 or TYK2 is dependent on the phosphorylation of the target proteins, as the treatment with alkaline phosphatase completely abrogated this association. One obvious problem as shown in this Far-Western, was poor reproducibility. In this experiment no inducible interaction by IFN-γ was detectable. This may reflect variety within JAK phosphorylation in the 2fTGH cells. Interestingly, a protein band of approximately 220kD also interacted with hJAK1-M6H in a phosphorylation dependent manner.

As discussed above (section 3.2.3.2), results from Far-Westerns require confirmation by other techniques. As hJAK1-M6H runs at a higher molecular weight in SDS-PAGE compared with its native counterpart, co-immunoprecipitation was tested as a complementary procedure. However, it was not possible to co-immunprecipitate wildtype JAK with the hJAK1-M6H from 2fTGH/hJ1-M6H cells with the Myc tag antibody.

In an alternative approach in collaboration with the Cell Biophysics Laboratory, (ICRF) was made, to study JAK/JAK interactions by fluorescence resonance energy transfer (FRET). Initial results suggested an inducible transient interaction between JAK1 and JAK1. FRET peaked at the same time after treatment with IFN-γ as JAK

phosphorylation (data not shown). This suggested an enzyme/substrate interaction between JAK1 and JAK1, but the initial results could not be repeated or controlled.

3.2.3.4 Proteins interacting with hJAK1-M6H in Far-Western

The Far-Western system was also used in attempts to identify proteins that interact with hJAK1. As a target a subset of cellular proteins was prepared. In cell fractionation experiments it was shown that JAK1 is associated with membranes, probably through its interaction with different transmembrane receptors. Whole membrane was prepared from wildtype or from hJAK1-M6H transfected HeLa-S3 cells. The purpose of using an overexpressing cell line was to increase the amount of proteins attached to hJAK1 in the membrane fraction of these cells. Proteins not tightly bound to the membrane were removed by high salt buffer washes (representing about 20% of the total cellular protein). The target proteins were separated by SDS-PAGE, blotted onto PVDF and renatured in NP-40 buffer (pH 7.8) using milk as a blocking reagent. To identify specific binding, some Far-Westerns were performed in the presence of proteins eluted from HeLa-S3 or U4A (JAK-null) cell membranes. Only the proteins from HeLa-S3 cells, but not from U4A cells should inhibit JAK1-specific interactions.

Surprisingly, the result was different to what was expected. Several bands (indicated by arrows in fig. 3.20) were stronger in the lanes with the target protein from HeLa-S3 cells. Some of the band decreased in intensity if the probe had to compete with proteins from the membranes. There was no significant difference between the inhibition with HeLa-S3 or U4A proteins. Attempts to follow up these results by preparing less complex mixtures of target protein were not successful. Further purification was achieved by sequential elution of membrane protein with increasing salt concentration. The target protein was never pure enough to obtain single bands in silver staining of SDS-PAGE, which correlated with bands in the Far-Westerns. Overall,

although revealing possible JAK/JAK interactions, the Far-Western approach was not sufficiently promising for the detection of novel JAK/protein interactions to pursue further.

3.2.3.5 Co-purification of proteins associated with hJAK1-M6H

Having established simple convenient 'pull-down' purification hJAK1, an obvious experiment was to manipulate the conditions to purify associated proteins. To eliminate most non-specific candidates, the co-purification was always done in parallel from HeLa-S3 and HeLa-S3/hJAK1-M6H cells. All bands, which were unique to the co-purification from HeLa-S3/hJAK1-M6H cells, were due to interactions (specific or non-specific) of hJAK1-M6H with other proteins. Several attempts were undertaken with traditional co-immunoprecipitation buffers. The weaker detergents, digitonin, Brij, CHAPS and Tween, used in these buffer were not stringent enough to reduce the amount of co-purified protein to a workable number. Even stronger detergents like Triton X-100 were unsuccessful. A common feature of these detergents is that they do not dissolve the membrane micro domains, in which JAK1 and cytokine receptors may be located together with different unrelated proteins. NP-40 is a detergent strong enough to solubilise membrane micro domains and proved to be the detergent of choice.

Once more, the pH was crucial for reproducible results. The best buffers found for co-purification were Tris-HCL buffers at pH 7.5 – pH 7.6. One problem with Tris-HCl buffers is the susceptibility to drastic changes of pH with temperature fluctuations. For this reason, the pH had to be titered on ice as the whole experiment was done on ice without changing the temperature at any time. The elution of proteins from a 9E10 (anti-Myc antibody) protein A-sepharose column with low pH and the following precipitation of proteins with acetone seemed to contribute to the purity of the co-purification.

Several trial experiments were performed for optimisation and reproducibility. Figure 3.21 shows an experiment, using the optimised conditions. There are clearly proteins, which appear in the HeLa-S3/hJAK1-M6H, but not the control samples. In this particular experiment there were no interferon inducible interactions. Other experiments have shown IFN inducible interactions. Cell numbers of 2.5 - 4×10^5 cells/ml in medium with 1% FCS (for 15h) seem best to observe inducible interactions and phosphorylation. Table 3.3 shows a list of proteins and their approximate weight, which are targeted for further analysis by mass spectrometry. All of these proteins have a lower molecular weight than hJAK1-M6H. There are proteins of higher molecular weight which associate with hJAK1, which are future candidates for future analyses. Priority is, however, to the smaller proteins, potentially more easily identifiable by mass spectrometry.

Table 3.3 Unidentified proteins associated with hJAK1-M6H in co-purifications

No.	~size [kD]	Comments
1	100	doublet
2	100	increased after IFN-α/γ
3	96	doublet
4	80	IFN-α/γ inducible
5	60	
6	58	
7	50	
8	45	
9	40	
10	35	
11	33	

12	30	Tyr-phosph. after IFN-α/γ
13	28	
14	25	
15	21	

In a single trial experiment using protein chips from Ciphergen some interacting proteins could be detected (table 3.4 and data not shown). The 9E10 antibody was coupled to the chips and used to 'fish' for complexes containing hJAK1-M6H from HeLa-S3 cell lysates (+/- OSM). The associated proteins were then detected by mass spectrometry and their size analysed. The detected proteins may correspond to some of those listed in table 3.3. The more precise size data from this technique may help in their ultimate identification.

Table 3.4 Unidentified proteins associated with hJAK1-M6H on Ciphergen chip

No.	Size [kD]	Inducible with OSM
1	46.06	Yes
2	47,71	---
3	51.49	Yes
4	53.91	---
5	70.56	Yes
6	75.79	Yes

The co-purification of proteins from the HeLa-S3/hJAK1-M6H system looks promising and further analysis is required.

3.2.4 Identification of phosphorylation sites in hJAK1

3.2.4.1 Phosphorylation of hJAK1

JAK1 is a substrate for itself and other JAK tyrosine kinases. It has been demonstrated that the EYY motif becomes phosphorylated after activation. In the published data mutation of the EYY motif inhibits all tyrosine phosphorylation detectable with anti-phospho-tyrosine antibodies (Feng et al., 1997; Gauzzi et al., 1996). It is possible that there is additional EYY dependent tyrosine phophorylation and/or secondary phosphorylation which may be ligand specific or modulated by cross talk. Figure 3.22 shows Western-Blots of immunoprecipitated JAK1 probed with a range of antibodies against phosphorylated amino acids. Tyrosine phosphorylation is inducible by interferons and OSM. A different blot with the same samples probed for threonine phosphorylation shows a good signal for unstimulated and stimulated JAK1. This suggests constitutive threonine phosphorylation of hJAK1, but good controls are difficult when using general antibodies. Apparent constitutive serine phosphorylation of JAK1 was also detected, but the signal was weak (the blot was developed for 16 h). This data are not conclusive, but were certainly consistent with the possible existence of additional phosphorylation sites for JAK1.

3.2.4.2 Identification of phosphorylation sites by peptide mapping

Biochemical identification of additional phosphorylation sites requires purified protein. The HeLa-S3/hJAK1-M6H expression system and the purification of hJAK1 with the 9E10 antibody made it possible to obtain pure protein for analysis it by mass spectrometry. HeLa-S3/hJAK1-M6H cells were serum starved for 18 h and incubated with or without OSM. In these cells this cytokine induces higher JAK1 tyrosine phosphorylation than other ligands (Fig. 3.22) presumably reflecting either a higher

proportion of phosphorylated JAK1 or additional sites. This would make the detection of phospho-peptides more likely and/or of additional interest. hJAK1-M6H was purified and as a control one sample was dephosphorylated with alkaline phosphatase. The samples were separated by SDS-PAGE and single hJAK1-M6H bands digested with trypsin. Eluted peptides were concentrated and transferred into the appropriate buffer using ZIP tips. The samples were analysed for peptides with masses correlating to the size of tryptic fragments from the hJAK1 sequence with an additional mass of 80 Dalton for single phosphorylation or 160 Dalton for double phosphorylation. Figure 3.23 (top) shows the mass spectrometry profile for the three samples for the mass/charge region of 2180 to 2220. There is no detectable difference between the unstimulated and stimulated spectra. There is a certain amount of constitutive phosphorylation in hJAK1, and as mass spectrometry is not a quantitative method, any induced difference may be lost. Importantly the removal of all phosphate groups from hJAK1 resulted in the loss of the signal for this peptide peak (blue trace Fig. 3.23). Comparison of the peptide mass with a list of all theoretical tryptic fragment masses for hJAK1 showed that the peptide corresponds to amino acids 996 – 1014 and had the right mass if a single phosphate group is included. There are two potential phosphorylation sites in the peptide, serine 1001 and threonine 1013 (bottom; Fig. 3.23). This peptide is not completely digested, and in a later experiment, the overlapping C-terminal peptide (amino acids 1007 – 1020) was found to contain a single phosphorylation site as determined by mass spectrometry (data not shown). The two sets of data suggested that threonine 1013 in hJAK1 is a possible phosphorylation site for an unknown serine/threonine kinase. Threonine 1013 is a conserved residue in hu/muJAK1 and hu/mu/ratJAK2, but is replaced with an alanine in JAK3 and TYK2. It is localised at the C-terminal side of a conserved stretch of amino acids. The EYY-motif is located nine amino acids downstream of threonine 1013. An aspartic acid four amino acids upstream of the threonine 1013 is in the highly conserved kinase motif VII (DFG; Hanks et al., 1988), implicated in ATP binding and known to be

essential for the activity of protein kinases in general and JAK1 in particular (Briscoe et al., 1996).

3.2.4.3 Thr1013 - Analysis of a possible phosphorylation site in hJAK1

To test the hypothesis that threonine 1013 in hJAK1 is a phosphorylation site, it was mutated to an alanine in hJAK1-M12H/pcDNA3.1 by PCR. The construct was stably expressed in U4A cells (U4A/hJAK1-M12H (T1013>A)). The cell line was then analysed and compared to U4A cells transfected with wildtype hJAK1-M12H (U4A/hJAK1-M12H (wt)) in several assay systems to confirm threonine 1013 as a phosphorylation site and investigate its function in JAK/STAT signalling.

There was no detectable difference in tyrosine phosphorylation of endogenous JAK1, JAK2 or TYK2 in response to the interferons or OSM between the mutant and the wildtype cell lines (data not shown). Inhibition tyrosine phosphorylation might not have been an expected result, as threonine 1013 is not conserved in all JAKs and would not have been expected to control initial events, in the same way as phosphorylation of the EYY-motif, in the signalling cascade. That said, constitutive phosphorylation of such a residue could a priori differentially modulate JAK activation.

Analysis of STAT1 phosphorylation after IFN-γ treatment suggested a regulatory effect on the kinase activity by threonine 1013. The kinetics of phosphorylation of STAT1 showed the same general profile for both cell lines (Fig. 3.24), peaking at 20min and decreasing over the next 3h. This probably reflects in part the translocation of STAT1 into the nucleus, as Schindler buffer does not lyse or extract the nucleus efficiently. After 8h STAT1 protein is upregulated, relocalised in the cytosol and phosphorylation rises. Both cell lines express the same amount of STAT1 (Fig, 3.24) and wildtype or mutant hJAK1-M12H (data not shown). The threonine 1013 mutant, however, showed a higher level of STAT1 phosphorylation (Fig. 3.24). This may be due

to a regulatory effect of threonine 1013 on kinase activity, but only one clone of U4A/hJAK1-M12H was obtained for analysis and clonal differences between the cell lines cannot be excluded.

DNA oligonucleotide binding assays (EMSAs), after interferon or OSM treatment, showed binding of the different transcriptional complexes to their promoter sites. Binding of STAT1 homodimers to an SIE-probe after IFN-α or IFN-γ stimulation was seen for mutant and wildtype hJAK1-M12H (Fig. 3.25). The same was true for activation with OSM and binding of STAT1 and STAT5 homodimers to a β-casein probe. STAT5/DNA complexes run above STAT1/DNA complexes and are more difficult to detect (Fig. 3.25). Once again a small quantitative enhancement of binding was observed for the T1013>A mutant.

A biological assay of JAK1 function is the induction of an antiviral state in cells treated with interferon. Comparison of the wildtype and the mutant U4A/hJAK1-M12H cells in antiviral assays showed no difference in protection from EMC virus after pre-treatment with IFN-α, IFN-β or IFN-γ (Fig. 3.26).

DNA macro arrays permit to assay the expression of over 100 genes of choice in single experiments. In collaboration with Jörg Schlaak (BRM, ICRF) the expression of 100 known interferon inducible genes after 6 hours IFN-α and IFN-γ (data not shown) treatment was assayed for differences between the two cell lines. No significant difference was observed. Differentials for single genes were not reproducible. The average strength of the response was very similar between the two cell lines.

In an alternative polyclonal rabbit sera against a specific hJAK1 peptide, containing the phosphorylated threonine 1013 were generated. Such sera would potentially allow detection and analysis of the putative phosphorylation site in wildtype cell systems. Two rabbits were immunised with a phospho-peptide (hJAK1 1007-1017, phosphorylated at Thr1013) coupled to maleimide activated keyhole limpet hemocyanin (KLH). The sera recognise the phosphorylated but not the non-phosphorylated form of

the peptide in Dot-Blots (data not shown). Further experiments are required to confirm that there is no recognition of non-phosphorylated JAK1 by the sera.

To date there is no conclusive data proving that threonine 1013 is a phosphorylation site in JAK1. Further analysis will be required to include or exclude a possible role of threonine 1013 in JAK1 signalling.

3.2.4.4 Purification of phospho-peptides by metal affinity chromatography

It was surprising to detect an apparent phospho-peptide from hJAK1-M6H in finger print mass spectrometry as the percentage of phosphorylated protein is probably quite low. It is known that the JAKs pre-associate with cytokine receptors but only JAKs associated with an activated receptor will be phosphorylated. Taking the number of cytokines which utilise the JAK/STAT pathway, it is probably a valid assumption, that only a very small percentage of all JAK molecules are involved in a given response. Using complex peptide mixtures to detect peptides of low representation can be a problem. Other peptides, which are easier to ionise with a positive charge than phospho-peptides, can suppress ionisation of the phospho-peptide and prohibit their detection. Purifying the phospho-peptides with metal affinity resin, due to their high density of negative charge at the phospho amino acid, could remove peptides which ionise more efficiently, and/or increase the concentration of phospho-peptides. Commercial micro-columns were used to bind phospho-peptides from the tryptic digests at low pH. Elution was performed with ammonium hydroxide at very high pH. The method worked with many synthetic phospho-peptides but those which had positive charges next to the phosphate group did not bind to the resin. Using the method to isolate phospho-peptides from tryptic digest of hJAK1-M6H, the purification did not yield in any detectable phosphopeptides. This may be due to the very low amount of phospo-peptides, or to the amino acid sequence surrounding the phosphorylation sites in hJAK1.

3.2.4.5 Identification of phosphorylation sites using [^{32}P] labelling and mass spectrometry

In collaboration with Nick Totty (Mass Spectrometry, ICRF) a further analysis was carried out, using a combination of [^{32}P]-labelling, high resolution reverse phase HPLC and mass spectrometry. A schematic representation of the procedure is shown in figure 3.27. HeLa-S3/hJAK1-M6H cells are labelled with [^{32}P]-phosphate and stimulated with cytokine prior to purification of hJAK1-M6H (as above). The single hJAK1-M6H band from a SDS-PAGE is digested with trypsin and the peptide mixture separated into more than 70 fractions by reverse phase HPLC. Phospho-peptide containing fractions are identified by radioactivity. The radioactive fraction and the first following fraction are subsequently analysed by mass spectrometry. The phosphorylated form of the peptide is rarely detectable but the same or the following reverse phase HPLC fraction should contain the unphosphorylated version of the same peptide. A phosphate group only slightly decreases the retention time of a peptide on a reverse phase HPLC column. The appropriate fractions are also analysed by Edman degradation. After every cycle the eluates are assayed in a scintillation counter. This allows identification of the position of the phosphorylated amino acid in relation to the N-terminus of the phospho-peptide. If the peptide identified by mass spectrometry contains a tyrosine, serine or threonine in the corresponding position it is very likely that this peptide contains a phosphorylation site.

The actual experiment was performed using the HeLa-S3/hJAK1-M6H cells after 20min OSM stimulation. The single band of hJAK from a SDS-PAGE was digested with trypsin and the peptides were separated by reverse phase HPLC. Five fractions contained [^{32}P]-phospho-peptides (Fig. 3.28). Fraction 4 and 6 could not be analysed due the amount of ammonium carbonate ($(NH_4)_2CO_3$) and TFA in the sample. In future experiments this will be removed from the early fractions by several cycles of

drying and resuspension in water methanol mixtures. Fraction 26 and 38 have not yet been analysed. No peptide was detected in fraction 24. This may be due to bad ionisation or a low amount of the [^{32}P]-phospho-peptide. However, in fraction 25 a double charged peptide could be detected (Fig. 3.29). The mass of 1622.6 D correlates with the tryptic fragment mass for amino acids 882-896 of hJAK1-M6H. The peptide contains a tyrosine in position 882 and a threonine in position 889 (Fig. 3.30). If the [^{32}P]-phospho-peptide in fraction 24 is the same peptide as in fraction 25 it is very unlikely that the tyrosine is phosphorylated as a phospho-tyrosine next to the proteolytic cleavage site inhibits cleavage by trypsin. Edman degradation analysis was performed on the peptides in fraction 24. The eluate after the 8 cycle of degradation contained a small amount of [^{32}P] consistent with phosphorylation of a threonine at the eighth position in hJAK1 peptide 882-896 (Fig. 3.30). The signal was weak as the coupling efficiency of the peptide pool to the matrix for Edman sequencing is very poor. The co-incidence of the mass spectrometry and Edman sequencing data, however, strongly suggest that threonine 889 is phosphorylated *in vivo* in HeLa-S3/hJAK1-M6H.

Threonine 889 has been mutated in hJAK1-M12H/pcDNA3.1 and is be expressed in U4A cells. Polyclonal sera in rabbits against the phospho-peptide hJAK1 884-894 are being generated. These reagents will be used to confirm whether or not Thr889 is a phosphorylation site and permit the further analysis of its functional relevance.

3.3 Discussion

3.3.1 Sequence analysis of hJAK1

The differences found between the observed sequence and the published sequence of hJAK1 are all minor. Asp338>His and Phe356>Tyr are amino acid changes which replace published hJAK1 amino acids with those reported for mouse JAK1. The extra Gly 886 is also found in the mouse sequence and is localised in the ATP-binding loop. It brings this conserved motif to the same length as in other JAK sequences. The removal of one amino acid between position Ser845 and Ala850 brings the number of residues to the same number found in the mouse sequence. There are still differences in the amino acid sequence between mouse and human in this region, but if compared with the other JAKs it seems this region is not conserved. The differences either reflect sequencing errors from previous analyses or polymorphism of cDNAs from different genetic origin.

3.3.2 Function, expression and purification of hJAK1

U4A cells with the different forms of Myc-His tags respond normally in the available assays for JAK/STAT signalling. There is a higher constitutive phosphorylation of JAK1 which is also seen with transfected wild-type JAK1. This may be due to the increased amounts of protein (2 to 3 times endogenous JAK1) and therefore easier detection of constitutive tyrosine phosphorylation and/or to an non-specific autophosphorylation of hJAK1 as seen in baculovirus overexpression systems. Even if this makes the detection of inducible phosphorylation more difficult and cells have to be starved before experiments, the advantage of a tagged hJAK1 outweigh the disadvantages.

Soluble hJAK1 from bacteria would have been an excellent reagent. Possible uses include: as a probe for example in *in vitro* Far-Western assays; as a substrate in *in gel* kinase assays to identify and characterise kinases which phosphorylate JAK1; for co-purification of interacting proteins; and for structural analysis of JAK1, including partial proteolytic digesting to identify protein domains for crystallisation and characterisation of the proposed FERM, SH2 and/or SH3 domains. JAK1 expressed in mammalian cells would probably not be obtained in high enough amounts to undertake these kinds of experiments.

The attempt to affinity purify hJAK1 with a polyclonal antibody column probably failed due to the heterogeneous mixture of antibodies and their different affinities to hJAK1 and there are no known monoclonal antibodies to JAK1 available. A good immune response against hJAK1 is unlikely in mice, as the sequence is highly conserved between the two species. It seems that the only way to purify proteins with polyclonal sera is to remove all non-specific antibodies from the sera (using the immunogen) combined with batch elution from the antibody column to avoid tailing of the protein to be purified. This was not practical for JAK1 in light of the limited supply of polyclonal sera available.

Purification with metal affinity resins is usually a very successful method, especially for bacterial expressed protein. Therefore, it was surprising that the binding efficiency and purity in the 2fTGH/hJAK1-M6H system was so poor. There are several possibilities as to why the six-histidine tag did not bind well to the resins. Firstly, the Myc tag N-terminal of the histidines contains a sequence of three acidic residues (EED) which probably represent a high density of negative charge under the binding conditions (pH7.4 – 8.0) and thus lowers the binding efficiency of the histidines. Secondly, the tag may be partially hidden in the protein and/or in a position were steric inhibition to the resin inhibits binding. Thirdly, the tag may be localised next to residues with positive charges within the protein. This would lower the affinity, as it is energetically

unfavourable to have positive charges next to the metal atoms from the resin. The tag with twelve histidines has a much higher affinity to the resins used and is probably suitable in high expressing systems (not mammalian) where large amounts of recombinant protein out compete the binding of non-specific proteins. Consistent with this hJAK1-M12H can be purified under denaturing conditions from the bacterial system. This may prove useful in the future for assays where hJAK1 does not have to be in a native conformation.

The introduction of a TEV-cleavage site N-terminal of the histidine tag provided the potential to cleave hJAK1-MT12H and elute it from the column without contaminating protein. However, cleavage of the TEV site was never complete. This could reflect sub-optimal conditions, steric inhibition and/or a His tag on the TEV-protease itself, used by the manufacturers to purify the protease. The cleavage buffer contained 40mM imidazole but this may not be enough to inhibit immobilisation of the protease, hence inhibition of cleavage. The conditions for cleavage were not optimised further, as the cleaved hJAK-MT12H remained associated with the column despite the presence of detergents, salt and glycerol in the cleavage buffer. The increased temperature for the cleavage reaction may have caused protein aggregation on the column.

The best purification of hJAK1 was achieved using the 9E10 antibody against the Myc tag. The antibody seems to be very specific with low binding of untagged protein. The affinity is high enough to use high detergent, salt and glycerol concentrations. Purified protein from this system is very pure when analysed by different staining methods. The elution with low pH may turn out to be a problem in future experiments. Low pH can change confirmation, hydrolyse phosphorylated aminoacids and/or denature protein. If to, the pH elution could probably be replaced by a neutral elution with a chaotropic salt like KSCN.

The HeLa-S3 expression system combined with 9E10 affinity purification is a good system to obtain recombinant hJAK1 for analytical experiments. It allows the purification of small amounts of hJak1 from untreated and treated mammalian cells, which is important for the identification of phosphorylation sites and interacting proteins. It was not possible to set up a system which permits the expression and purification of the high quantities of hJAK1 which would have been necessary to set up *in vitro* systems using hJAK1 as a probe, substrate or for structural analysis.

3.3.3 Interactions of hJAK1

The established Far-Western system was able to detect possible interactions of JAK1 and from the data to date would be best used to analyse interactions with known target protein like the JAKs. It was not specific enough to observe known or new interactions from complex mixtures of target protein. It was very difficult to identify new interactions by Far-Western. A future possibility might be the use of Far-Westerns only as an assay system to control different biochemical purification steps until the interacting protein is pure enough for identification. Comparison of Far-Western and conventional Western-Blots only promise success if the mixtures of target protein are not too complex for comparison. Another disadvantage in this system is its sensitivity to buffer variations and low reproducibility. Availability of good controls is essential for the interpretation of Far-Western results.

The observed hJAK1-M6H/JAK interactions may have identified an unexpected strength of the Far-Western system. Co-immunoprecipitations between JAK1, 2 and Tyk2 are not yet successful. This may be due to affinity of the interaction, technical problems and/or the interactions have a very short half-life *in vivo*. The preliminary results of the fluorescence resonance energy transfer (FRET) experiments suggest that any such interactions are transient and may reflect kinase/substrate interactions. This

would mean, that it is possible to 'freeze' interactions with a short lifetime in Far-Westerns and observe enzyme/substrate interactions, to which the coupling of protein to the membrane and the statistical representation of all possible conformation of protein domains may contribute. In the case hJAK1-M6H, the interactions with target protein seem to be dependent on phosphorylation of the target protein. The data does not give any indications if the probe or the target protein mimics the enzyme or the substrate. However, from published data it is known that JAKs have to be phosphorylated to be active and are then able to phosphorylate their substrates. This suggests that the target protein in the Far-Westerns mimics the enzyme and may recruit the *in vitro* translated hJAK1-M6H as its substrate, eventually via a phosphorylation site.

The co-purification of associated proteins by changing the buffer conditions of the purification was potentially very successful. The proteins obtained are likely either components of JAK1 containing complexes or proteins which interact non-specifically with hJAK1-M6H itself as they were not co-purified from the non-transfected HeLa-S3 cells. The identification and future analysis of these proteins should distinguish between these possibilities. It is very likely that at least some of the co-purified proteins are part of JAK1 signalling pathways and will provide an interesting field of research.

3.3.4 Identification of phosphorylation sites

The use of [^{32}P] labelled protein, followed by reverse phase HPLC, Edman degradation and mass-spectrometry (Section 3.2.4.5) looks like the most successful way to identify phosphorylation sites. Purification of phospho-peptides via metal-affinity columns may yield to further optimisation but it is still likely that certain peptides will be missed due to their neighbouring amino acids. Following initial identification further characterisation will only be possible with either *in vitro* [^{32}P] phosphorylated protein or a non-radioactive *in vivo* method, as the high amount of [^{32}P]-phosphate used in *in vivo*

phosphorylation makes it impractical to analyse a high number of samples. Alternatively, specific antibodies against the phosphorylation sites will be used for further characterisation.

The extra three fractions containing [^{32}P]-phospho-peptides have the potential to identify phosphorylation sites additional to the EYY-motif and Thr889. Further analysis will be required however to exclude the possibility that they contain longer peptides not completely cleaved by trypsin which contain one of the two identified sites.

The biochemical identification of phosphorylation sites is a very useful tool if a protein contains multiple tyrosines, threonines and/or serines. It is also useful if sequence analysis is not revealing due to a lack of significant sequence homology with well characterised proteins. Where possible, sequence analysis of amino acids to identify conserved residues, known motifs and mechanisms is probably still the faster, simpler and cheaper way to identify potential phosphorylation sites.

Thr1013 as possible phosphorylation site will clearly be investigated further. That said, the additional data do not obviously support the hypothesis that Thr1013 is a real and functional phosphorylation site and the relevance of the quantitative differences in activation between the wild-type and the mutant JAK1 have to be confirmed. The polyclonal rabbit sera against P-Thr1013 may yet establish the *in vivo* phosphorylation of this residue and thus a potential role for this site in JAK function. Due to the detection limits the detection of a phosphorylation site by fingerprinting on the mass spectrometer is a priori unlikely. Furthermore, approximately the same amount of JAK protein was used for the fingerprint and the [^{32}P]-phosphate analysis but it was not possible to detect the phospho-peptide other then by radioactive labelling in the fraction from the reverse phase HPLC. It is surprising therefore, that the threonine1013 peptide was detected. Accordingly it remains possible that signal seen in the fingerprint analysis may be an artefact. Thr1013 is not conserved in JAK3 or TYK2. Thus if a real site, it likely has a function specific to JAK1 and/or JAK2. This could involve regulation of the JAK/STAT

pathway, recruitment of additional factors or any unknown function of JAK1 and JAK2. Presently, there are no full-proof comprehensive assay systems to analyse for all functions for any of the JAKs, making a rigorous conclusion impossible in the absence of a clear cut positive result.

In contrast Threonine 889 is a good candidate for a phosphorylation site. It is present in all JAKs and it is located in a very conserved region (the ATP-binding loop) of the JAKs. Threonine 889 is seven amino acids upstream of lysine 896, which is essential for ATP binding (Hanks et al., 1988) and kinase activity (Briscoe et al., 1996). Most intriguingly, cyclin dependent kinase 2 (CDK2) has a phosphorylation site in its ATP-binding loop which is similar to Thr889. Tyr15 (and probably Thr14) in CDK2 can be phosphorylated via Wee1/Mik1- resulting in inhibition of ATP-binding and loss of kinase activity (Gu et al., 1992). Dephosphorylation by CDC25 allows ATP-binding and activation. If Thr889 has the same function in JAK1, its mutation to an alanine should lead to a lower activation threshold for JAK1 or even a constitutively active JAK1. In contrast its mutation to an aspartic acid may result in a kinase dead JAK1. The analysis of JAK1 with these mutations is in progress and may yield a better understanding of JAK regulation and function.

3.4 Newly Established Protocols

3.4.1 Purification of hJAK1-M6H or –M12H

To purify hJAK1-M6H from HeLa-S3/hJAK-M6H cells are cultivated at concentrations of ≈ 2.5 - 4×10^5 cells/ml. Cells are then serum starved over night in 1% FCS. Starving reduces the background phosphorylation and activation of the JAK/STAT pathway. Depending on availability of the cytokine used for stimulation, cells can be stimulated at the same concentration or be pelleted by low gravity centrifugation (JA-20, Beckmann) and resuspended in concentrations up to 1×10^7 prior to stimulation. Pelleting cell can probably induce the stress pathways and increases background phosphorylation occasionally. After stimulation, the suspension of cells is diluted 1:1 with ice cold PBS (from now on all steps are on ice or at 4°C) and cells are pelleted at 2000rpm (JA-20, Beckman). Normally 2.5 ×10^8 cells are used for a single sample. This amount of cells results in an amount of hJAK1-M6H which is easy to label with [^{32}P] or to detect by any SDS-PAGE staining method. A pellet of 2.5 ×10^8 cells is lysed in ≈ 10ml of either Myc-PB or Myc-CoPB buffer (DTT, β-ME and protease inhibitors are added at his stage to the buffers; table 3.5) for 20min. Nuclei are removed by centrifugation with 12000rpm (JA-17, Beckman). For JAK1 purifications, protein A and 9E10 antibody are added to the lysate (table 3.5). For co-purifications, 9E10/protein A resin is added to the lysate (table 3.5). The lysate is incubated with the antibody on a roller for 18 h. After binding, the resin is washed extensively (at least 5 ×15ml) or until no [^{32}P] is detectable in the washes with binding buffer. The resin is transferred into small eppendorf tubes and all liquid removed. For analysing hJAK-M6H by mass spectrometry the resin is boiled in SDS-loading buffer. For co-purifications, the proteins are eluted with 200μl Myc-CoPB in which Tris-HCl is replaced with 100mM glycine pH 2.5. The eluate is immediately neutralised wit 16μl 1M Tris-base and precipitated

with 1.75ml acetone (pre-cooled at –20°C). Precipitates are washed with 80% acetone (pre-cooled at –20°C), dried and separated on SDS-PAGE.

Table 3.5 Purification Buffers

	Purification for mass spectrometry (Myc-PB)	**Co-Purification of proteins (Myc-CoPB)**
Tris-HCl pH 8.0	50 mM	---
Tris-HCl pH 7.5	---	50mM
NaCl	300mM	---
KCl	---	150mM
$MgCl_2$	---	2mM
Na_3VO_4	0.5mM	0.5mM
NaF	25mM	25mM
EDTA	1mM	1mM
DTT	1mM	---
β-Mercaptoethanol	---	5mM
glycerol	10%	5%
NP-40	1%	0.5%
Protein A	100μl (50% slurry)	---
9E10	14μg	---
9E10-Protein A resin	---	100μl (50% slurry)

3.4.2 Far-Western with [^{35}S]-methionine labelled hJAK1-M6H

The target proteins for Far-Western analysis are separated by SDS-PAGE and blotted on PVDF (see Material and Methods). All the following steps are done at 4°C. The PVDF-membrane is incubated 5 times for 1h in the appropriate interaction buffer (table 3.6), followed by an o./n. incubation in the same buffer.

hJAK1-M6H or other probes and controls are *in vitro* translated (see Material and Methods) for 90min in 100μl reticulocyte lysate. The lysate is then diluted 1:20 with interaction buffer. The PVDF membrane is incubated on a roller for 2h with the different *in vitro* translated probes. The membrane is washed several times with the interaction buffer. The membrane is then washed several times for 20min with interaction buffer without BSA or milk protein, until no further radioactivity can be removed from the membranes. The last wash is in interaction buffer without detergents. The PVDF membrane is then wrapped in cling film and developed by autoradiography.

Table 3.6 Far-Western Buffers

	Far-Western with I.P.ed target FW-IP	**Far-Western with complex target mixtures FW-CM**
Tris-HCL pH 7.8	50mM	50mM
KCl	150mM	150mM
$MgCl_2$	1mM	1mM
Na_3VO_4	0.2mM	0.2mM
NaF	10mM	10mM
glycerol	5%	5%
NP-40	0.5%	0.5%
BSA	1%	---
Boiled milk powder	---	2.5%

Fig. 3.1 Schematic representation of the hJAK1 constructs used

hJAK1-Myc-6×His/pcDNA31 (also Pet30a, upper panel), hJAK1-Myc-12×His/pcDNA3.1 (also Pet30a, middle panel) and hJAK1-Myc-Tev-12×His/pcDNA3.1 (also Pet30a, lower panel). The hJAK1 cDNA without the stop codon is shown in red, the Myc-Tag is shown in blue, each stretch of 6 histidines is represented as a yellow box and the Tev-cleavage site is shown in pink. Linker regions are represented by a gap.

Figure 3.1

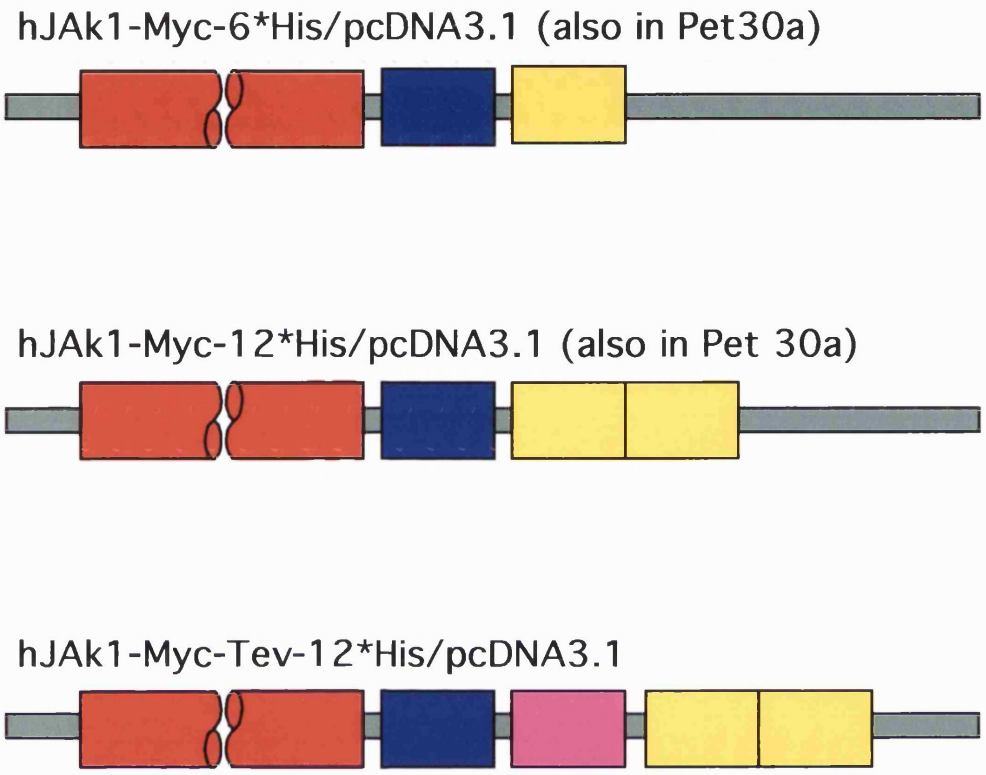
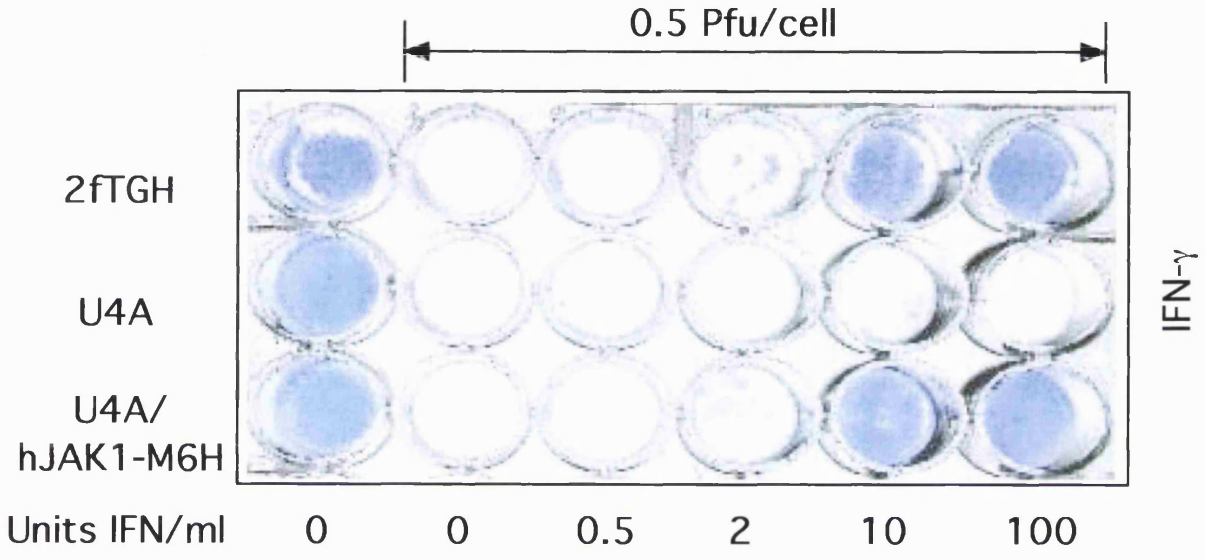

hJAk1-Myc-6*His/pcDNA3.1 (also in Pet30a)
hJAk1-Myc-12*His/pcDNA3.1 (also in Pet 30a)
hJAk1-Myc-Tev-12*His/pcDNA3.1
= hJAK1
= Myc-Tag (recognised by 9E10 antibody)
= proteolytic Tev cleavage site
= 6*histidine-Tag

Fig. 3.2 hJAK1-M6H rescues U4A cells in an antiviral assay

An antiviral assay with IFN-γ (upper panel) or IFN-α (lower panel) was performed for 2fTGH (top rows), U4A (middle rows) and U4A/hJAK1-M6H cells (bottom rows). Cells were incubated o./n. with different concentrations of IFN (four right columns) and then treated with EMC-virus (five right columns). After further 24h the surviving cells were stained and the survival compared to the untreated cells (left column). 2fTGH and U4A/hJAK1-M6H cells survive the virus infection, but U4A cells do not survive. (Experiment was performed once.)

Figure 3.2


0.5 Pfu/cell
2fTGH
U4A
U4A/
hJAK1-M6H
IFN-γ
Units IFN/ml
0
0
0.5
2
10
100

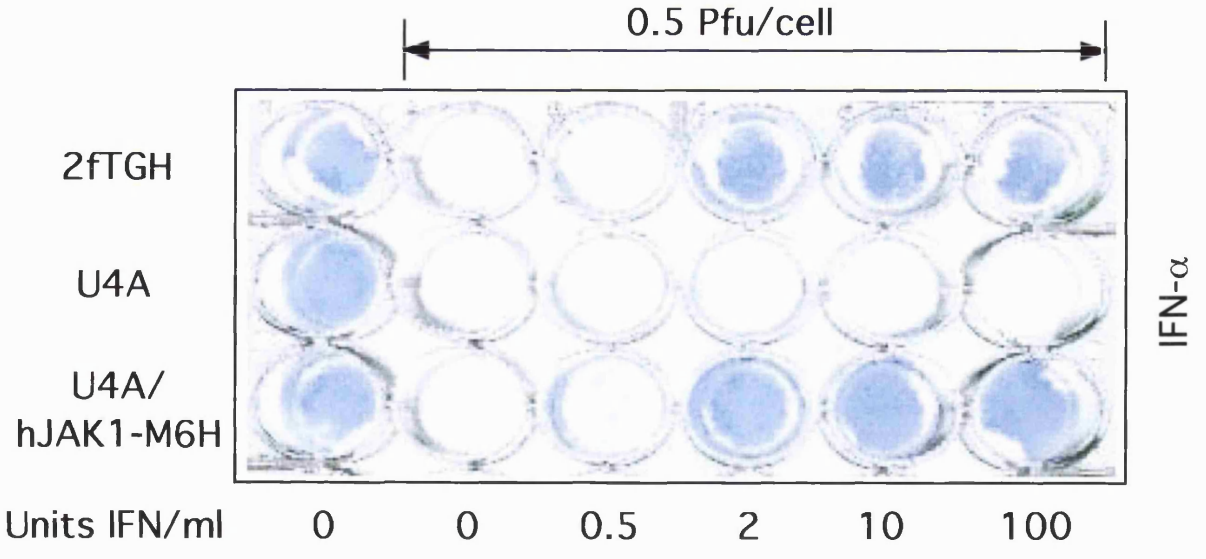

0.5 Pfu/cell
2fTGH
U4A
U4A/
hJAK1-M6H
IFN-α
Units IFN/ml
0
0
0.5
2
10
100

Fig. 3.3 hJAK1-M6H rescues MHC class II expression in U4A cells after IFN-γ treatment

2fFGH (top panel), U4A (middle panel) and U4A/hJAK1-M6H cells (bottom panel) were incubated with 10^3 IU/ml IFN-γ and analysed for MHC class II expression by FACS (see Material and Methods). The filled black graph represents the MHC class II expression on non-induced cells and the green graph is the induced MHC class II expression. In comparison to U4A cells, 2fTGH and U4A/hJAK1-M6H cells have inducible MHC class II expression. (Experiment was performed once.)

Figure 3.3

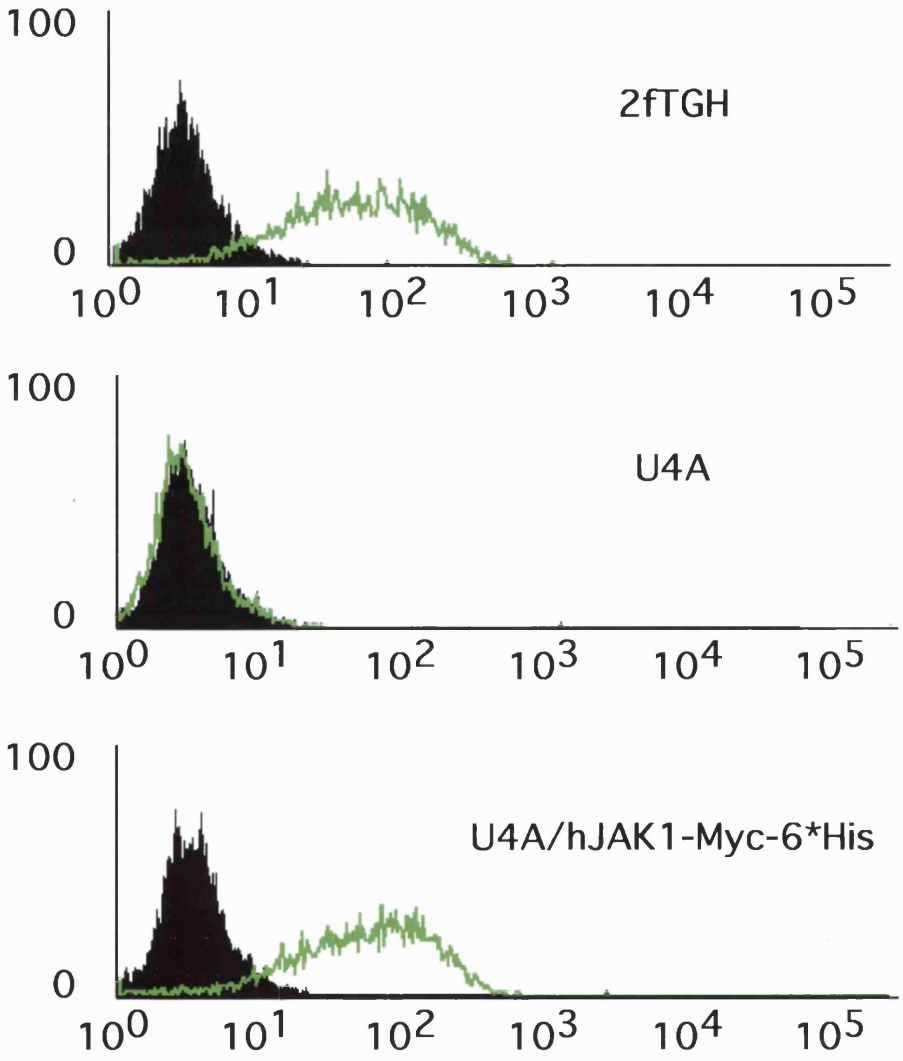

100
0
2fTGH
10^0 10^1 10^2 10^3 10^4 10^5
100
0
U4A
10^0 10^1 10^2 10^3 10^4 10^5
100
0
U4A/hJAK1-Myc-6*His
10^0 10^1 10^2 10^3 10^4 10^5

Fig. 3.4 Geneinduction in U4A/hJAK1-M6H cells after IFN-γ and IFN-α treatment

2fTGH (left lanes), U4A (middle lanes) and U4A/hJAK1-M6H cells (right lanes) were treated with either 10^3 IU/ml IFN-γ or IFN-α for 15h. Expression of inducible mRNAs (p48, 6-16, IRF-1, 9-27) was monitored by RNase protection.

(Experiment was performed once.)

Figure 3.4

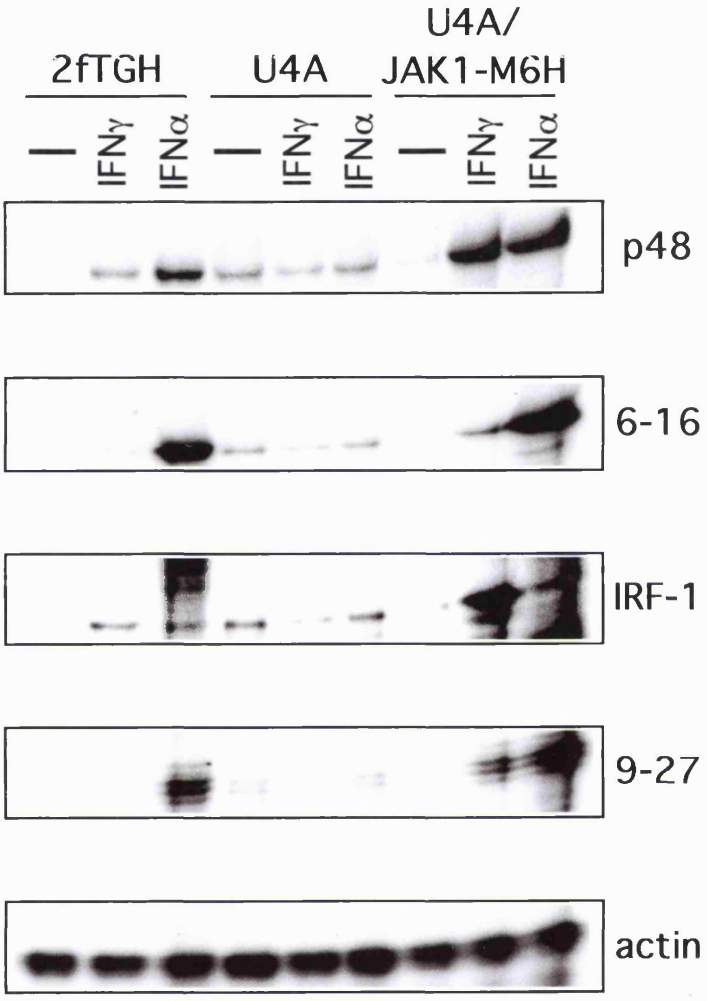

2fTGH
U4A
U4A/
JAK1-M6H
—
IFNγ
IFNα
—
IFNγ
IFNα
—
IFNγ
IFNα
p48
6-16
IRF-1
9-27
actin

Fig. 3.5 Phosphorylation analysis of the JAK/STAT pathway in response to IFNs in U4A/hJAK1-M6H cells

2fTGH (left lanes), U4A (middle lanes) and U4A/hJAK1-M6H cells (right lanes) were treated with either 10^3 IU/ml IFN-γ or IFN-α for 20min. JAK1, STAT1 and in the case of IFN-α treatment also STAT2 attached to STAT1 (top four panels), JAK2 and TYK2 (bottom 3 panels) were immunoprecipitated and analysed by Western-Blot. The blots were probed for tyrosine phosphorylation (1st and 5th panel) and protein level (2nd-4th, 6th and 7th panel). (Result is representative of three experiments.)

Figure 3.5

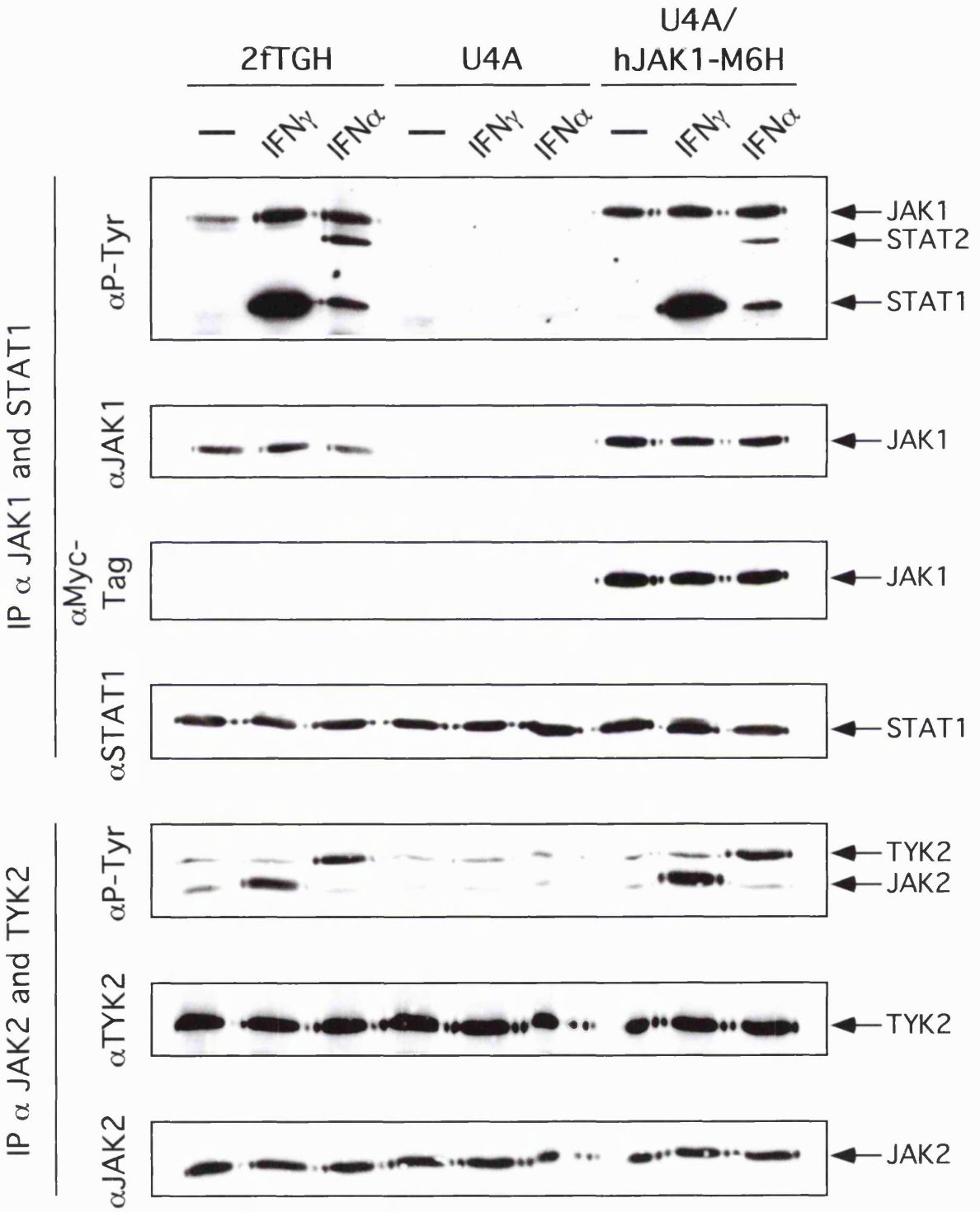

2fTGH
U4A
U4A/
hJAK1-M6H
—
IFNγ
IFNα
—
IFNγ
IFNα
—
IFNγ
IFNα
IP α JAK1 and STAT1
αP-Tyr
αJAK1
αMyc-
Tag
αSTAT1
JAK1
STAT2
STAT1
JAK1
JAK1
STAT1
IP α JAK2 and TYK2
αP-Tyr
αTYK2
αJAK2
TYK2
JAK2
TYK2
JAK2

Fig. 3.6 Phosphorylation analysis of the JAK/STAT pathway in response to IFNs in HeLa-S3/hJAK1-M6H cells

HeLa-S3/hJAK1-M6H cells were treated with 10^3 IU/ml IFN-γ or IFN-α for 20min. JAK1 and STAT1 (left lanes) as well as JAK2 and TYK2 (right lanes) were immunoprecipitated and analysed by Western-Blot. The blots were probed for tyrosine phosphorylation (top panel) and protein levels (bottom 4 panels).

Figure 3.6

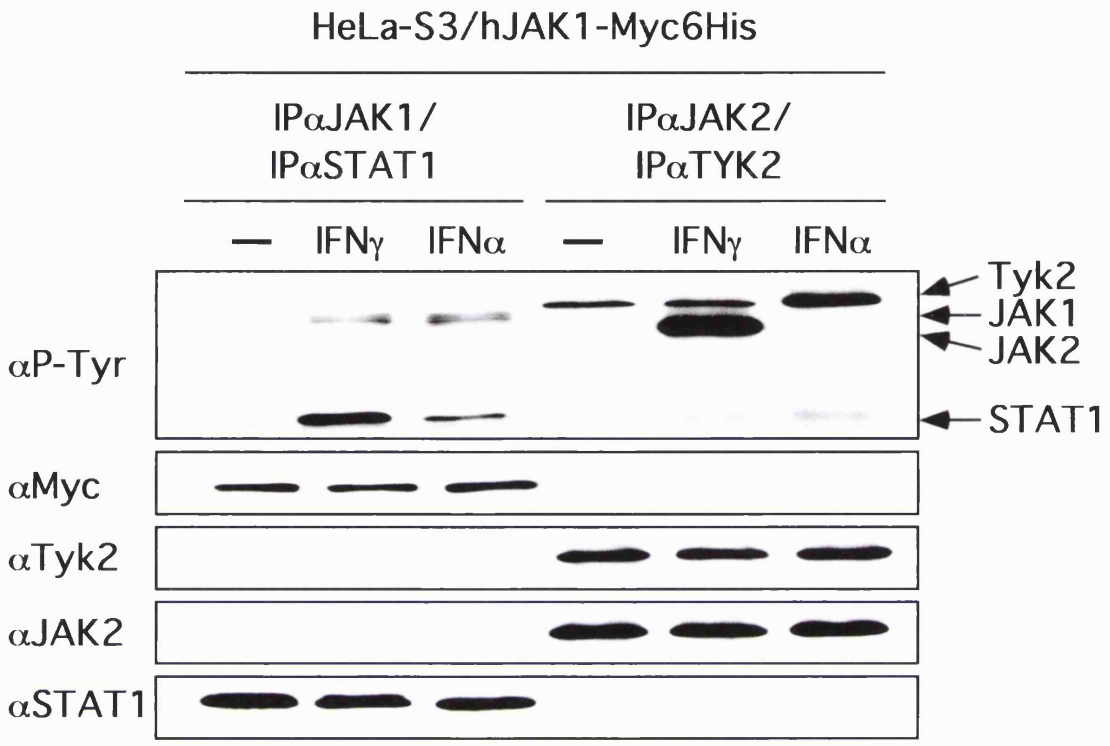

HeLa-S3/hJAK1-Myc6His
IPαJAK1/
IPαSTAT1
IPαJAK2/
IPαTYK2
—
IFNγ
IFNα
—
IFNγ
IFNα
Tyk2
JAK1
JAK2
STAT1
αP-Tyr
αMyc
αTyk2
αJAK2
αSTAT1

Fig. 3.7 hJAK1-M6H expression in bacteria

Bacteria from the strain FB810 or FB810 pLys transformed with hJAK1-M6H/Pet30a were grown to an OD_{600} of 0.4 and induced with 1mM IPTG. 50μl of culture were taken at different times after induction, the bacteria were harvested by centrifugation and lysed in 1.5×SDS-sample buffer. The protein was separated by SDS-PAGE, stained with Coomassie Brilliant Blue and controlled for hJAK1-M6H induction.

Figure 3.7

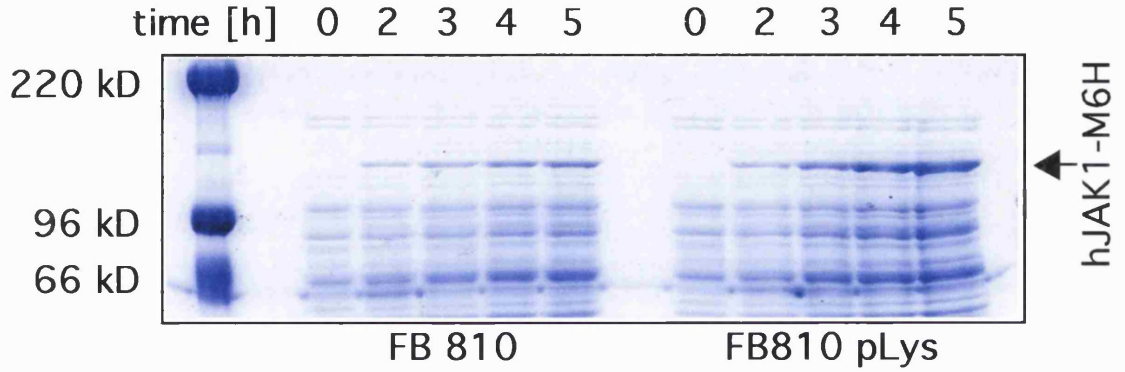
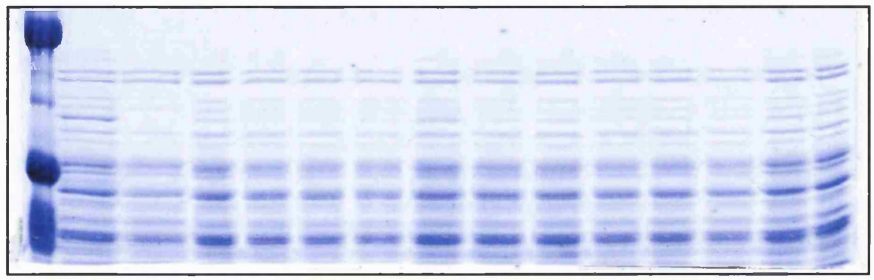

time [h] 0 2 3 4 5 0 2 3 4 5
220 kD
96 kD
66 kD
hJAK1-M6H
FB 810
FB810 pLys

Fig. 3.8 Examples of the solubility of bacterial expressed hJAK1-M6H in different lysis buffers

The bacterial strain FB810 transformed with hJAK1-M6H/Pet30a was induced with IPTG and 5ml aliquots were lysed under different buffer conditions. The samples were separated by SDS-PAGE and analysed by staining with Coomassie Brilliant Blue. The first two lanes of each gel contain a uninduced and an induced control of total protein from approximately the same amount of bacteria as the lanes with lysates. In this example, all buffer contained Tris-HCl pH 7.4, 5% glycerol and the protease inhibitor PMSF.

Figure 3.8

NaCl [mM]	50	150	50	150	50	150	50	150	50	150	50	150
0.3 mM EDTA	√	√	√	√	√	√						
0.5 mM DTT	√	√			√	√	√	√			√	√
5 mM β-ME			√	√					√	√		
1 % NP-40					√	√					√	√



10 mM β-ME

NaCl [mM]	10	25	50	100					50	50	50	50
KCl [mM]					10	25	50	100				
DTT [mM]	1	1	1	1	1	1	1	1	1	0.5	0.2	
NP-40 [%]	1	1	1	1	1	1	1	1	2	1	1	1

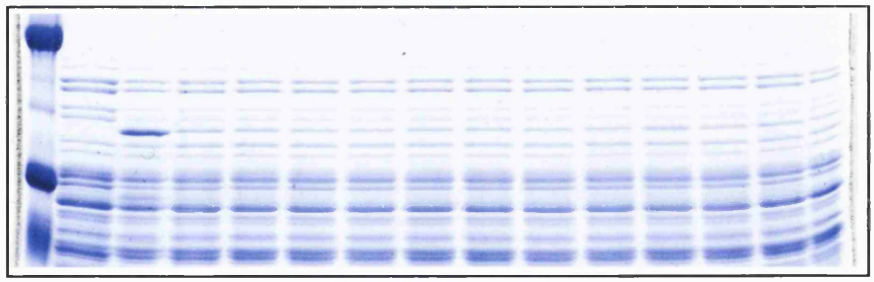


Fig. 3.9 Elution of wildtype hJAK1 from a polyclonal M8 antibody affinity column with high pH

HeLa-S3 cells were lysed in Schindler-Buffer and wildtype hJAK was bound to a M8 affinity column. The column was washed with Schindler-Buffer and bound protein was eluted with 25mM Na_2CO_3 pH 11.0 Schindler-Buffer. 500μl fractions were collected and neutralised. The fractions were analysed by Western-Blot and hJAK1 was detected with a specific antibody.

Fig. 3.10 Elution of wildtype hJAK1 from a polyclonal M8 antibody affinity column with KSCN

Wildtype hJAK1 was bound to an affinity column as described for figure 3.9. The bound protein was eluted with a gradient of 0M – 1M KSCN in Schindler-Buffer. 500μl fraction were collected and hJAK1 was detected by Western-Blot analysis.

Figure 3.9

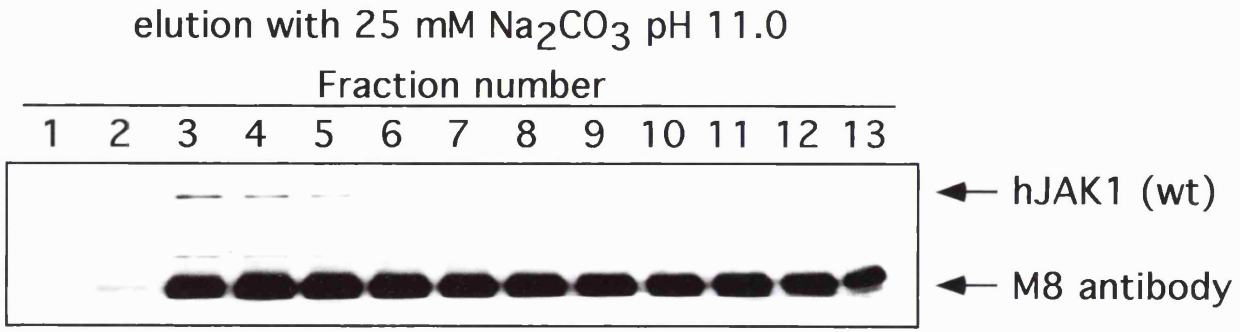


Figure 3.10

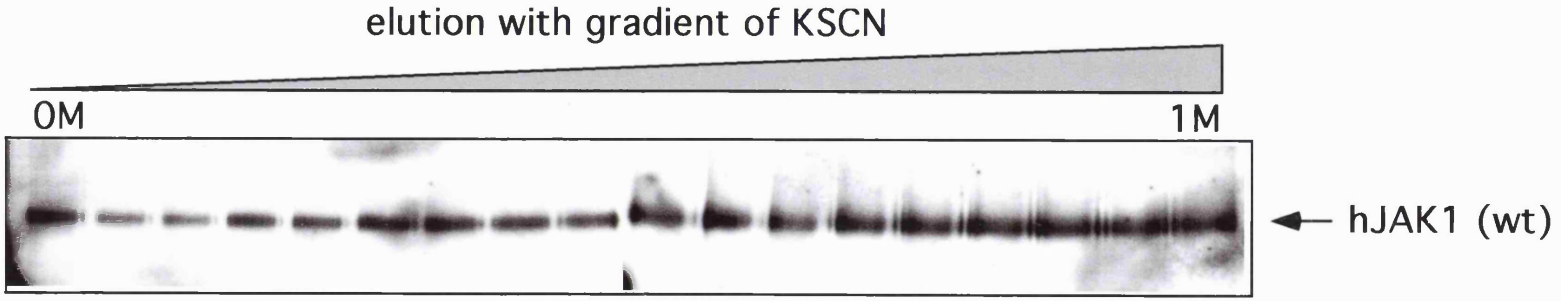


Fig. 3.11 An example of a purification of hJAK1-M12H with metal-affinity resin

2fTGH/hJAK1-M12H cells were lysed in 50mM Tris-HCl pH 7.8, 300mM NaCl, 10% glycerol, 5mM β-mercaptoethanol, 0.5% NP-40 and 10mM imidazole. The nuclei were removed by centrifugation and the lysate was applied to Ni-NTA resin. The resin was aliquoted and each aliquot was extensively washed with lysis buffer containing different imidazole concentrations (10 – 100mM). Protein bound to the resin was eluted by boiling in 1×SDS-sample buffer and analysed by Western-Blotting with the 9E10 antibody against the Myc-Tag.

Fig. 3.12 An example of a purification of hJAK1-MT12H with metal-affinity resin

U4A/hJAK1-MT12H cells were lysed and washed in the same buffer than for figure 3.11. The hJAK1-MT12H protein was bound to either Ni-NTA (left lanes) or TALON resin (right lanes). After washing in lysis buffer protein from half of the resin was eluted with 1×SDS sample buffer (lane 1 and 4). The other half was incubated with TEV-protease (see Material & Methods) and the supernatant was removed (lane 2 and 5). Protein which remained on the resin was eluted in SDS-sample buffer (lane 3 and 6). All samples were analysed by Western-Blot with the 9E10 antibody against the Myc-Tag.

Figure 3.11

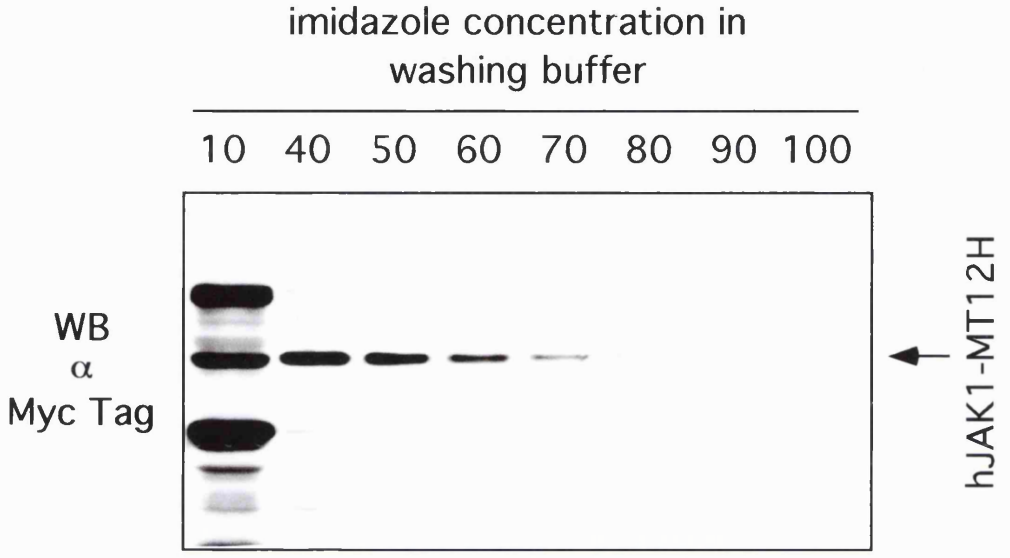


Figure 3.12

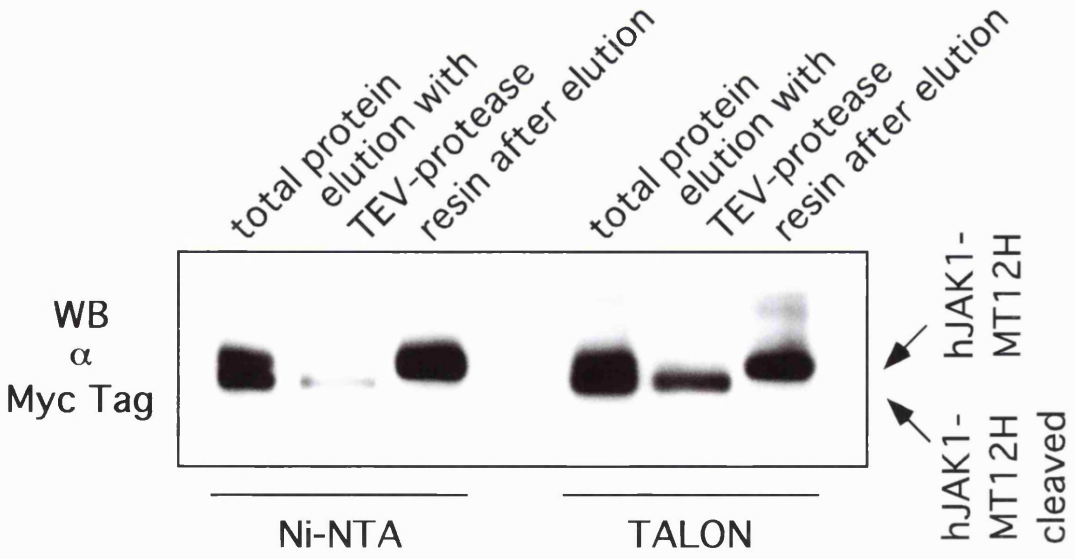


Fig. 3.13 An example of purification of hJAK1-M12H from bacteria

Bacteria were lysed in 50mM Tris-HCl pH 7.8, 300mM NaCl, 10% glycerol, 1% NP-40, 10mM β-mercaptoethanol and 10mM imidazole. The lysate was bound to Ni-NTA resin and washed with the same buffer containing 40mM imidazole. Bound protein was eluted with 200mM imidazole and the % of total yield indicated in the figure was loaded on SDS-PAGE. The gel was analysed for hJAK1-M6H by staining with Coomassie Brilliant Blue.

Figure 3.13

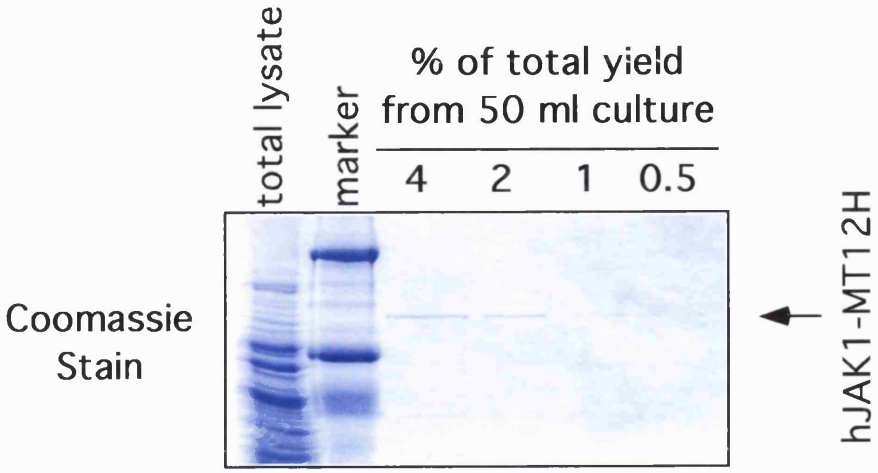

total lysate
marker
% of total yield
from 50 ml culture
4
2
1
0.5
Coomassie
Stain
hJAK1-MT12H

Fig. 3.14 Purification of hJAK1-M6H with a 9E10 antibody column

HJAK1-M6H was purified from HeLa-S3/hJAK1-M6H cells with purification buffer (section 3.4.1) containing either 150mM or 300mM NaCl. The protein was separated by SDS-PAGE and transferred onto a PVDF-membrane. The membrane was stained with Ponceau (left panel). Furthermore, the membrane was analysed for hJAK1-M6H by Western-Blot analysis with the 9E10αMyc antibody (right panel).

Figure 3.14

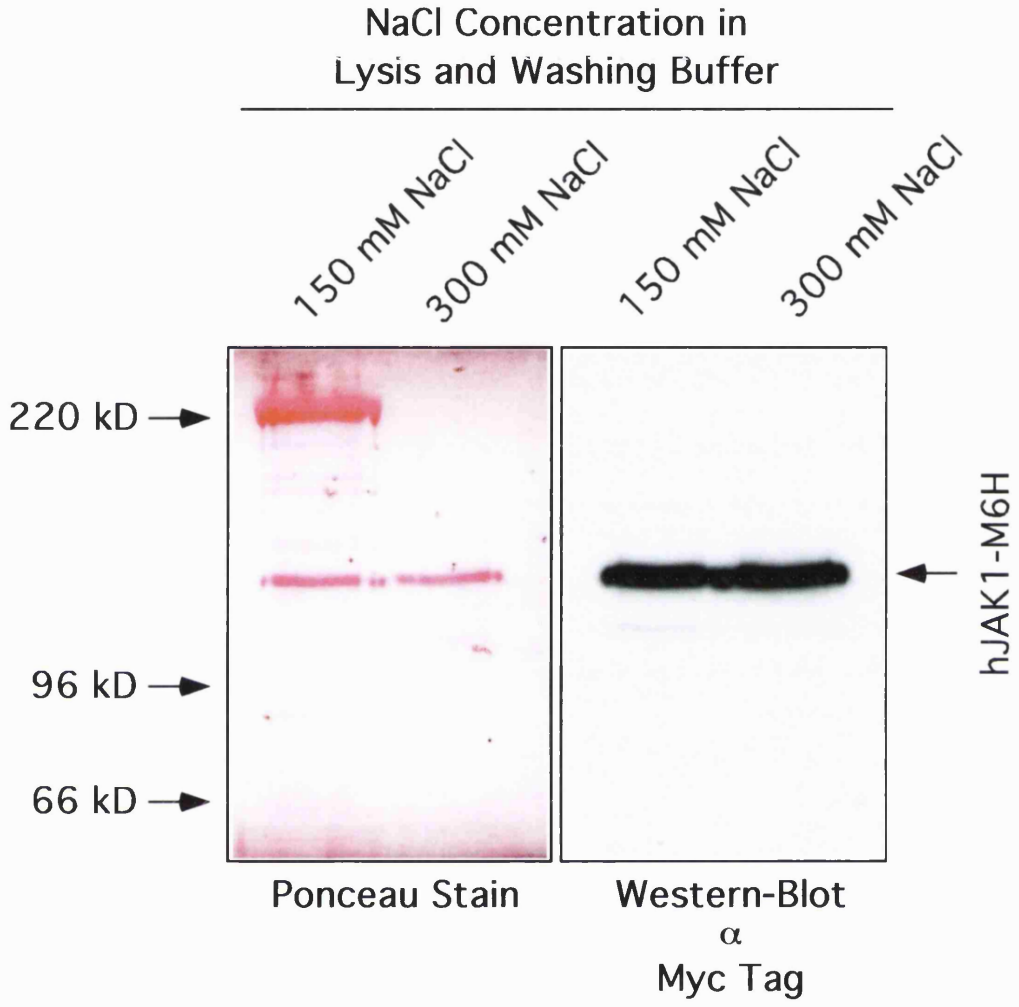

NaCl Concentration in
Lysis and Washing Buffer
150 mM NaCl
300 mM NaCl
150 mM NaCl
300 mM NaCl
220 kD
96 kD
66 kD
hJAK1-M6H
Ponceau Stain
Western-Blot
α
Myc Tag

Fig. 3.15 Indication of a hJAK1 associated proteins

HeLa-S3 cells were either not treated (1st lane) or treated with IFN-α (2nd lane), IFN-γ (3rd lane) or OSM (4th lane) for 20min. JAK1 was immunoprecipitated and analysed by Western-Blot for tyrosine phosphorylation. In the IFN-γ treated sample (3rd lane) an additional tyrosine phosphorytlated band of ~100kD (?) was detected.

Fig. 3.16 Further indication of a hJAK1 associated proteins

2fTGH cells were either not treated (1st lane) or treated with OSM (2nd lane), IFN-α (3rd lane) or IFN-γ (4th lane) for 20min. JAK1 was immunoprecipitated and analysed by Western-Blot for threonine phosphorylation. In all stimulated samples (2nd – 4th lane) an extra threonine phosphorylated band of ~100kD (?) was detected.

Figure 3.15

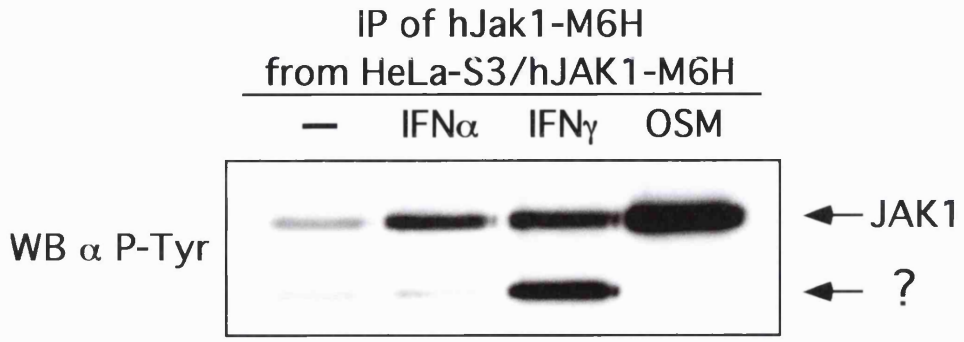


Figure 3.16

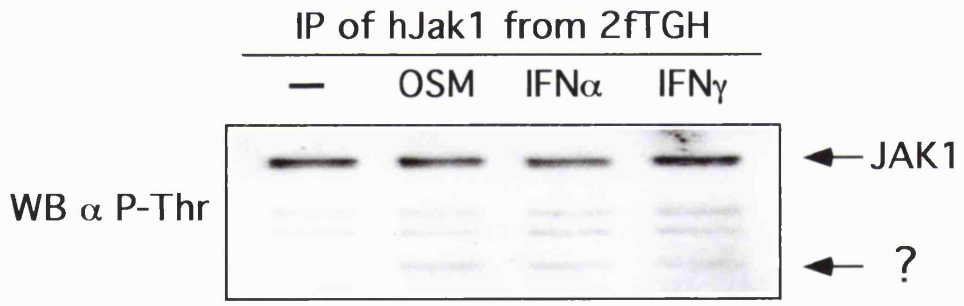


Fig. 3.17 A minimal model for Far-Western analysis

1. A mixture of target protein has to be separated by SDS-PAGE, blotted on PVDF-membrane and renatured. A complex mixture of target protein can prove difficult and it is advisable to use enriched target proteins. The protein is interacting with the membrane and only unbound parts of it will be able to renature. Due to the fixation to the membrane it is likely that every conformation of a protein is represented by the protein bound to the membrane.

2. The target protein is detected, using the interacting protein as a probe. The probe can be a recombinant protein, peptides or can be obtained from any other source.

3. The interaction is reconstituted on the PVDF membrane in an appropriate interaction buffer. The interaction buffer is very crucial and has to be stringent enough to inhibit all non-specific binding, but mild enough to enable the proteins to interact.

4. After extensive washes, the probe can be detected directly. For example, it can be detected by labelling it with [^{35}S]-methionine or fluorescent groups. Another possibility is indirect detection using an antibody against the probe or streptavidin in case of a biotinylated probe.

Figure 3.17

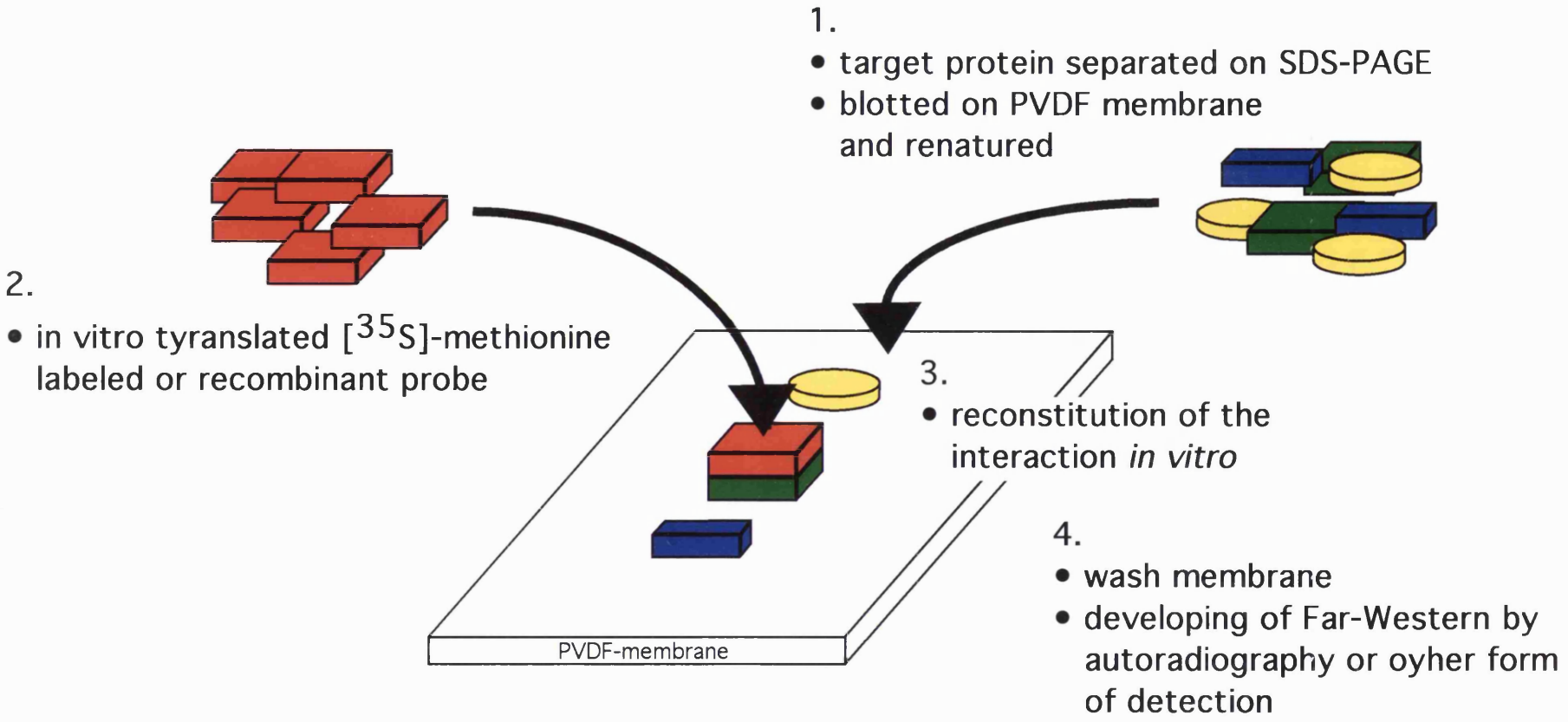

1.
• target protein separated on SDS-PAGE
• blotted on PVDF membrane and renatured
2.
• in vitro tyranslated [35S]-methionine labeled or recombinant probe
3.
• reconstitution of the interaction *in vitro*
4.
• wash membrane
• developing of Far-Western by autoradiography or oyher form of detection
PVDF-membrane

Fig. 3.18 Interactions of hJAK1-M6H with other JAKs in a Far-Western

2fTGH cells were not treated (left lanes), treated with IFN-γ (middle lanes) or IFN-α for 20min. JAK1 (lane 1, 4 and 7), JAK2 (lane 2, 5 and 8) and TYK2 (lane 3, 6 and 9) were immunprecipitated, seperated by SDS-PAGE and blotted onto PVFD-membrane. The membrane was used in a Far-Western analysis with [^{35}S]-methionine labelled hJAK1-M6H as a probe. (Result is representative of three experiments.)

Fig. 3.19 Phosphorylation dependent interaction of hJAK1-M6H with JAK1 and Tyk2

2fTGH cells were not treated (lane 1, 2, 4 and 5) or treated with IFN-γ (lane 3 and 6). JAK1 (lane 1-3) and TYK2 (lane 3-6) were immunoprecipitated. Furthermore, JAK1 (lane 1) and TYK2 (lane 4) were dephosphorylate with alkaline phosphatase when bound to the antibody/protein A sepharose beads. The proteins were separated by SDS-PAGE, blotted onto PVDF and used in Far-Western analysis with [^{35}S]-methionine labelled hJAK1-M6H as a probe. (Result is representative of three experiments.)

Figure 3.18

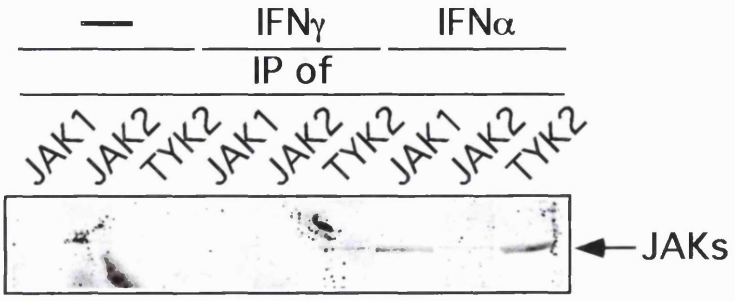


Figure 3.19

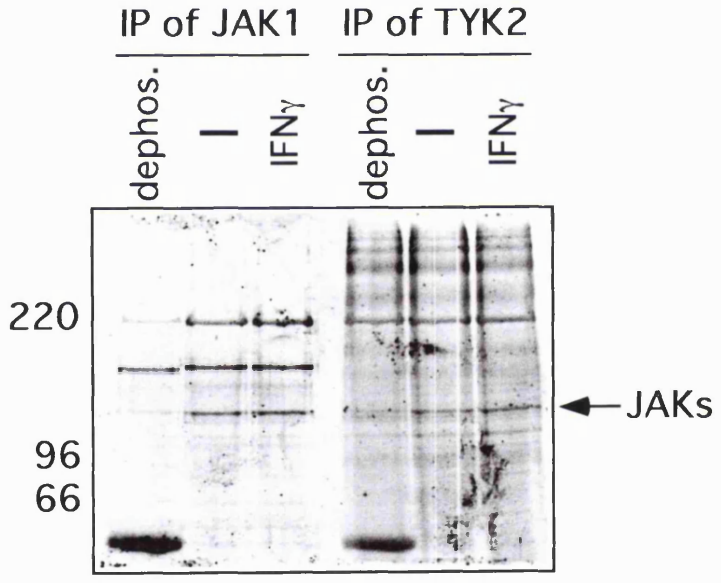


Fig. 3.20 Interactions of hJAK1-M6H with other unknown membrane associated proteins in a Far-Western analysis

Membrane associated proteins were purified from HeLa-S3 (lane 2, 4 and 6) and HeLa-S3/hJAK1-M6H cells (lane 1, 3 and 5). The proteins were separated by SDS-PAGE, blotted onto PVDF and used in Far-Western analysis with [^{35}S]-methionine labelled hJAK1-M6H as a probe. Coupling of the probe was inhibited with membrane associated proteins from HeLa-S3 (middle panel) or U4A cells (right panel).

Figure 3.20

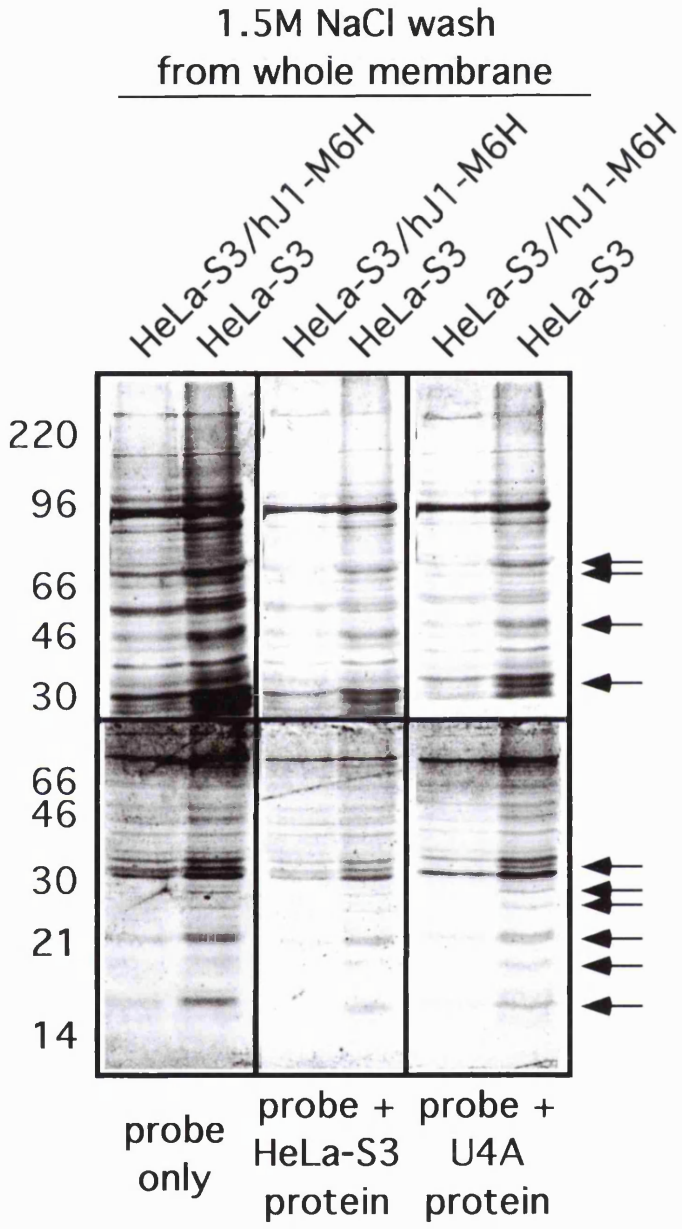

1.5M NaCl wash
from whole membrane
HeLa-S3/hJ1-M6H
HeLa-S3
HeLa-S3/hJ1-M6H
HeLa-S3
HeLa-S3/hJ1-M6H
HeLa-S3
220
96
66
46
30
66
46
30
21
14
probe only
probe + HeLa-S3 protein
probe + U4A protein

Fig. 3.21 Co-Purification of proteins associated with hJAK1-M6H

HeLa-S3 cells as a control (left lanes) or HeLa-S3/hJAK1-M6H cells (right lanes) were either not treated (lane 1 and 4) or treated with 10^3 IU/ml IFN-α (lane 2 and 5) or 10^3 IU/ml IFN-γ (lane 3 and 6) for 20min. HJAK1-M6H was purified using Myc-CoPB-buffer (section 3.4.1), eluted from the resin with low pH 2.5, neutralised and acetone precipitated. Precipitates were separated on a 6.5% - 15% gradient SDS-PAGE and the gel silver stained to visualise associated proteins. HJAK1-M6H is indicated with an arrow. (Result is representative of three experiments.)

Figure 3.21

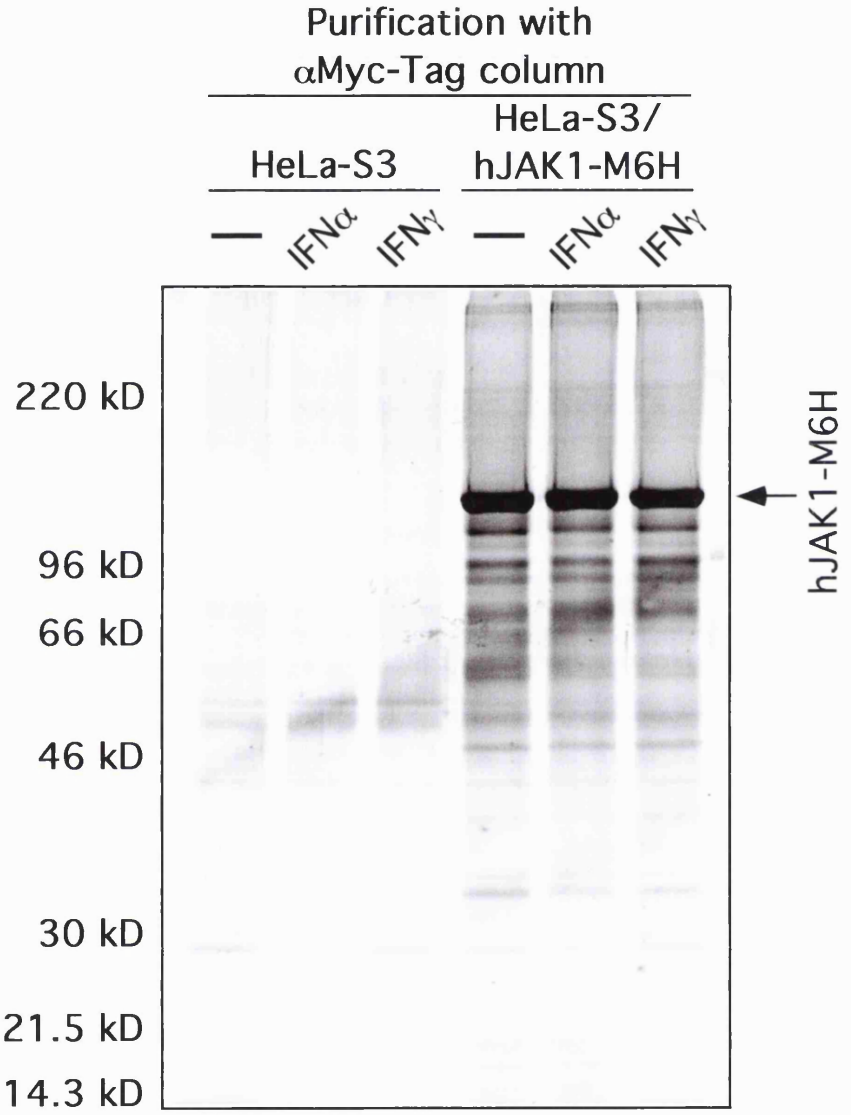

Purification with
αMyc-Tag column
HeLa-S3
HeLa-S3/
hJAK1-M6H
—
IFNα
IFNγ
—
IFNα
IFNγ
220 kD
96 kD
66 kD
46 kD
30 kD
21.5 kD
14.3 kD
hJAK1-M6H

Fig. 3.22 Phosphorylation of JAK1-M12H in 2fTGH/hJAK1-M12H cells

2fTGH cells were either not treated (1st lane) or treated with OSM (2nd lane), IFN-α (3rd lane) or IFN-γ (4th lane). HJAK1-M12H was immunoprecipitated, separated by SDS-PAGE and blotted onto PVDF. The blot was probed for tyrosine (top panel), threonine (upper middle panel), serine phosphorylation (lower middle panel) and protein (bottom panel). The probing for serine phosphorylation was weak and the bands only appeared after 1h exposure, compared to seconds for the other probings.

Figure 3.22

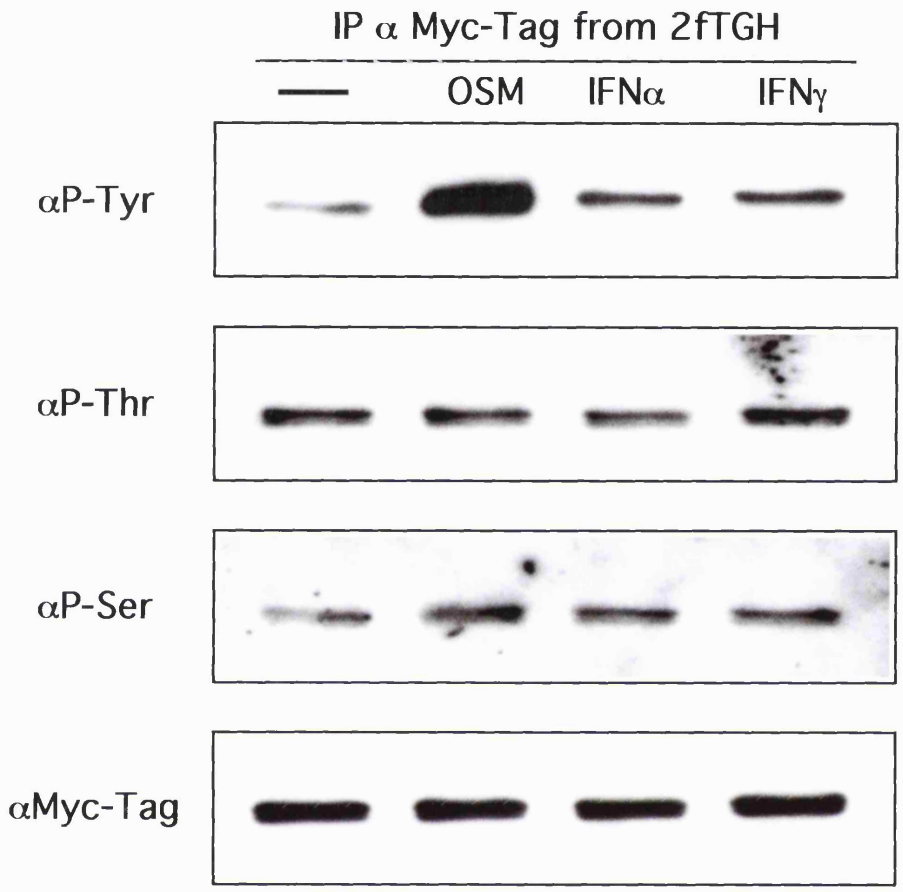
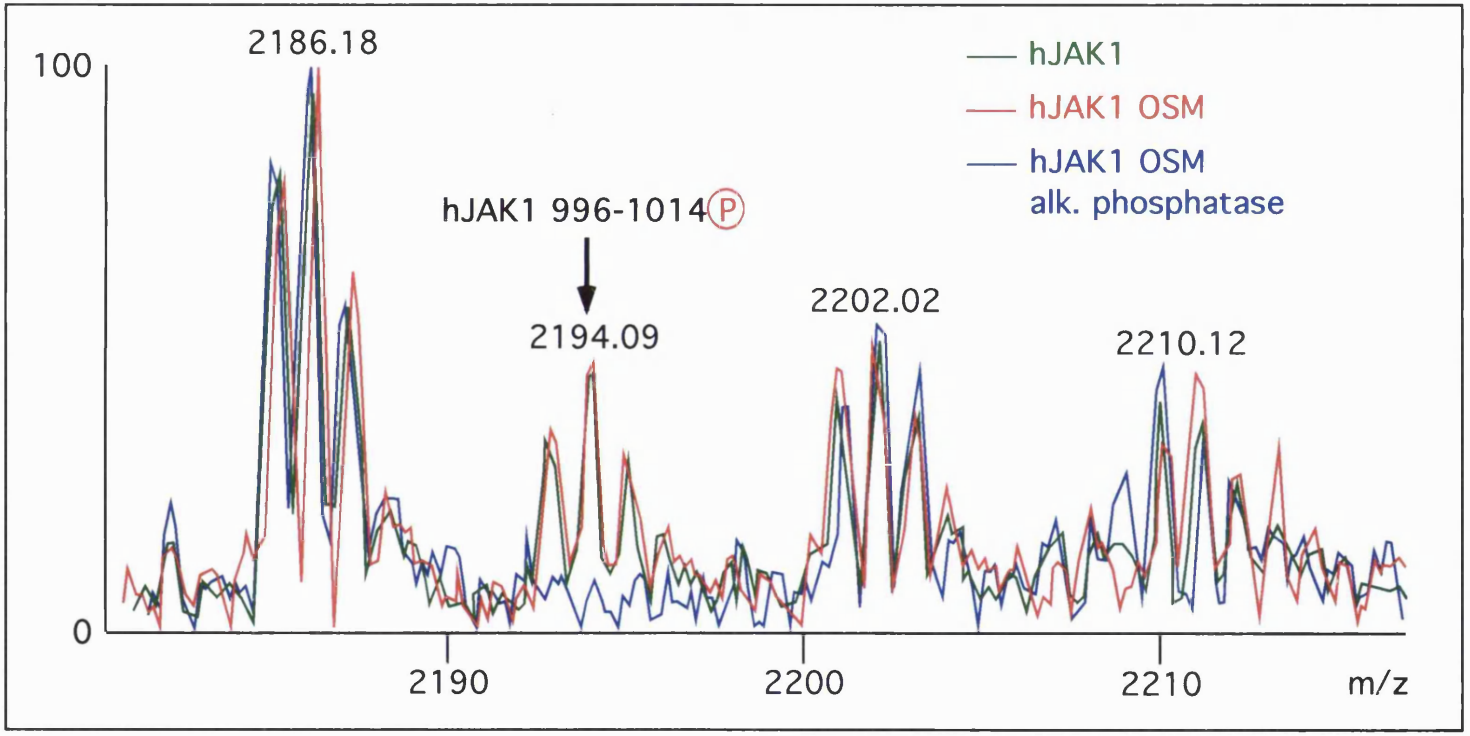

IP α Myc-Tag from 2fTGH
—
OSM
IFNα
IFNγ
αP-Tyr
αP-Thr
αP-Ser
αMyc-Tag

Fig. 3.23 Detection of threonine 1013 as a possible phosphorylation site in hJAK1

HeLa-S3/HJAK1-M6H cells were either not treated (green line) or treated with OSM for 20min (red and blue lines). HJAK1-M6H was purified using Myc-PB-buffer (Chapter 3.4.1) and one sample from OSM treated cells was dephosphorylated with alkaline phosphatase (blue line). The hJAK1-M6H protein band from SDS-PAGE was digested with trypsin and the tryptic fragments were analysed by mass spectrometry. The graph shows signals for peptides with a mass to charge ratio of 2180-2220. A differential peak (indicated with an arrow) was detected at 2194.09. The sequence of the correlating tryptic fragment (hJAK1 996-1014) is printed below the graph. Possible phosphorylation sites are indicated in red.

Figure 3.23



hJAK1 996-1014 = NVLVESEHQVKIGDFGLTK

Fig. 3.24 Phosphorylation of STAT1 in U4A/hJAK1-M12H (T1013>A) cells after IFN-γ treatment

U4A/hJAK1-M12H (odd lanes) or U4A/hJAK1-M12H (T1013>A) cells (even lanes) were treated with 10^3 IU/ml IFN-γ for 0min, 20min, 1h, 3h, 8h, 16, or 24h. Equalised amounts of protein were analysed by Western-Blot with an antibody either against the phosphorylated form tyrosine 701 in STAT1 (upper panel) or against STAT1 protein (lower panel).

Fig. 3.25 DNA binding of STAT1 in U4A/hJAK1-M12H (T1013>A) cells after IFN-α, IFN-γ or OSM treatment

U4A/hJAK1-M12H (odd lanes) or U4A/hJAK1-M12H (T1013>A) cells (even lanes) were either not treated (lanes 1, 2, 7 and 8) or treated with IFN-α (lanes 3 and 4), IFN-γ (lanes 5 and 6) or OSM (lanes 9 and 10) for 20min. DNA binding of STAT1 was analysed by EMSA using either a SIE-probe (lane 1 – 6) or a β-casein probe (lane 7 – 10).

Figure 3.24

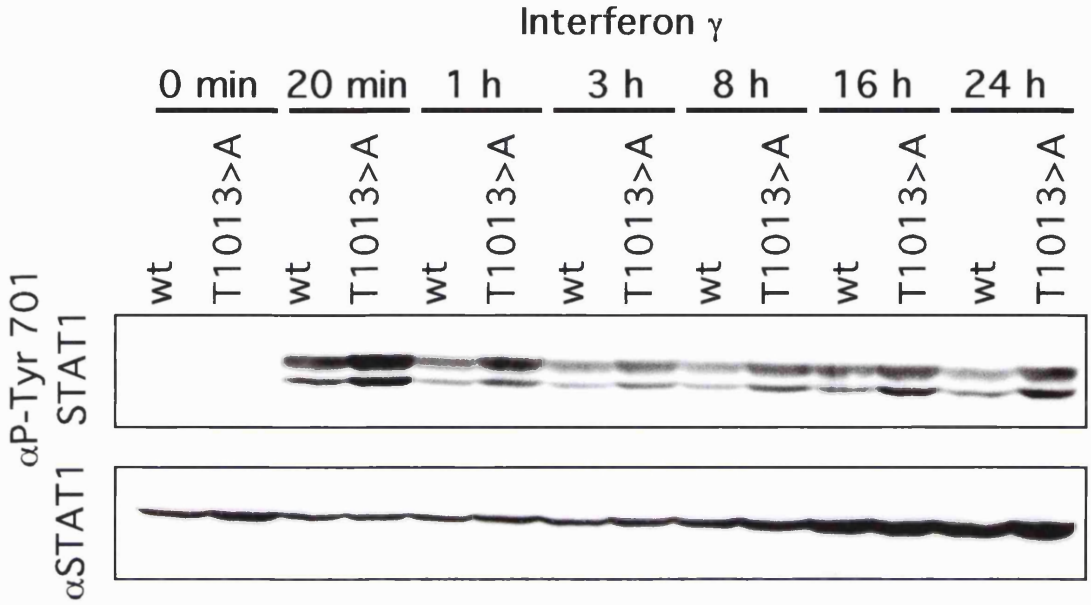


Figure 3.25

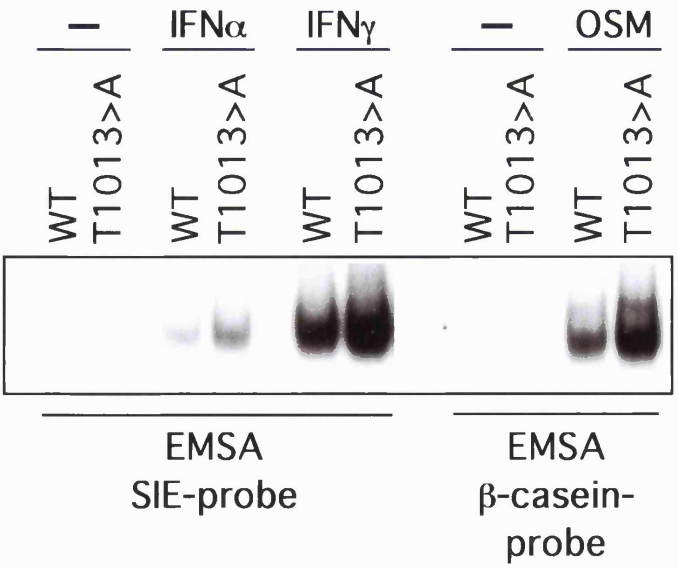


Fig. 3.26 An antiviral response in U4A/hJAK1-M12H (T1013>A) cells after IFN treatment

U4A/hJAK1-M12H (top panel) or U4A/hJAK1-M12H (T1013>A) cells (bottom panel) were analysed in an antiviral assay (see Material & Methods). Cells in the four right columns were treated o./n. with different concentrations of IFN-α (top rows), IFN-β (middle rows) or IFN-γ (bottom rows). Following this, the five right columns were infected with EMC-virus for 24h. Cells were stained and the survival levels were compared to the untreated cells (left column). There was no significant difference between the two cell lines.

Figure 3.26

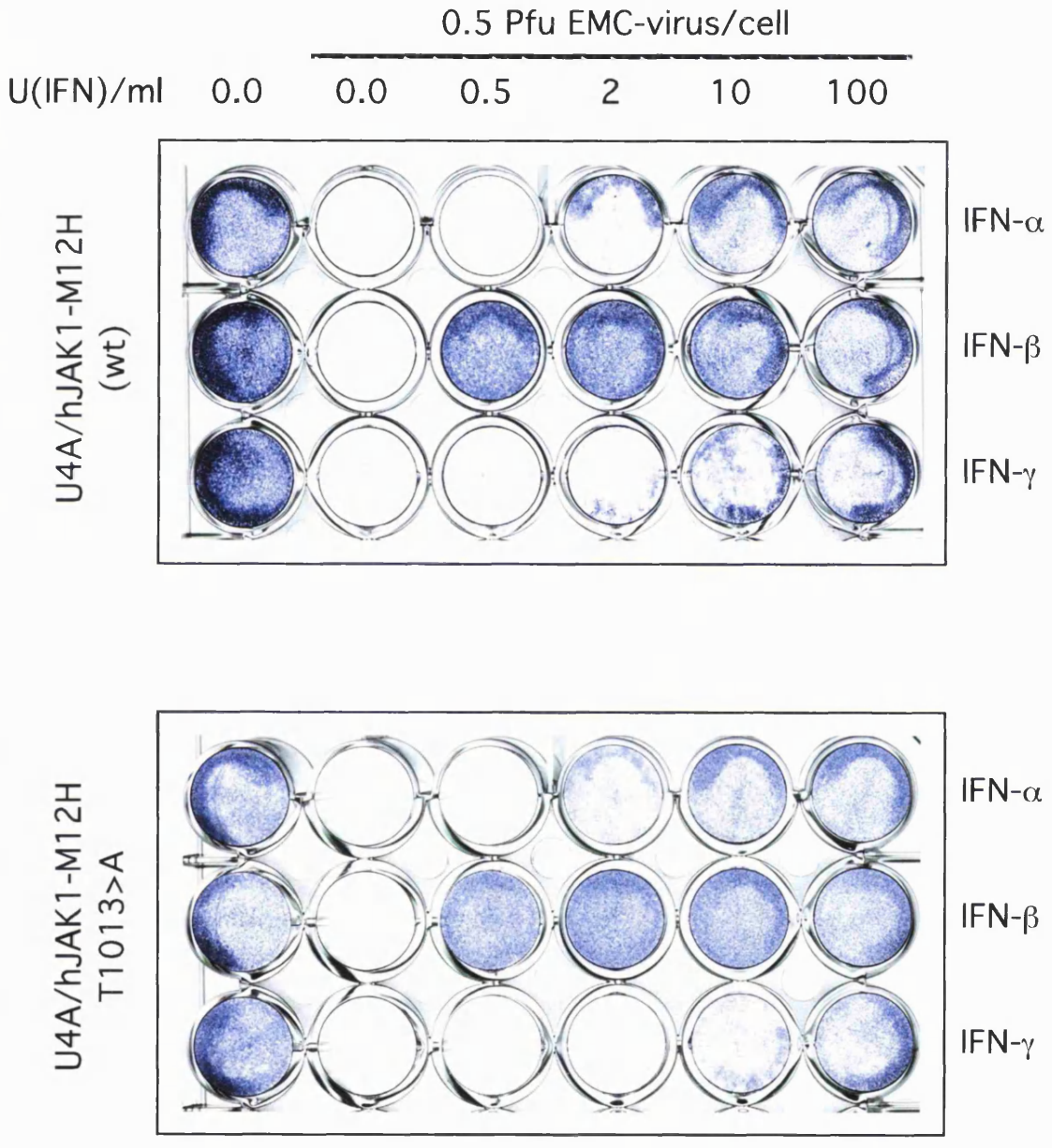

0.5 Pfu EMC-virus/cell
U(IFN)/ml
0.0
0.0
0.5
2
10
100
U4A/hJAK1-M12H (wt)
IFN-α
IFN-β
IFN-γ
U4A/hJAK1-M12H T1013>A
IFN-α
IFN-β
IFN-γ

Fig. 3.27 A minimal model of how to identify phosphorylation sites by [^{32}P] labelling peptides

Cells are labelled with [^{32}P]-phosphate, which incorporates into ATP. Cells are then treated with ligand to phosphorylate the protein of interest (in this work hJAK1-M6H). Following this, the protein is purified and separated by SDS-PAGE. The single protein band is digested with trypsin and the tryptic fragments are separated by reverse phase HPLC. Fraction containing [^{32}P] labelled peptides are identified and analysed by Edman degradation to determine the position of the phosphorylated amino acid in relation to the N-terminus of the peptide. The radioactive fraction and the following fraction, which may contain the unphosphorylated form of the peptide, are analysed for peptides by mass spectrometry. In the case of hJAK1-M6H, only the unphosphorylated form of a peptide could be detected. Comparison of the two sets of data can suggest phosphorylation sites in the protein of interest.

Figure 3.27

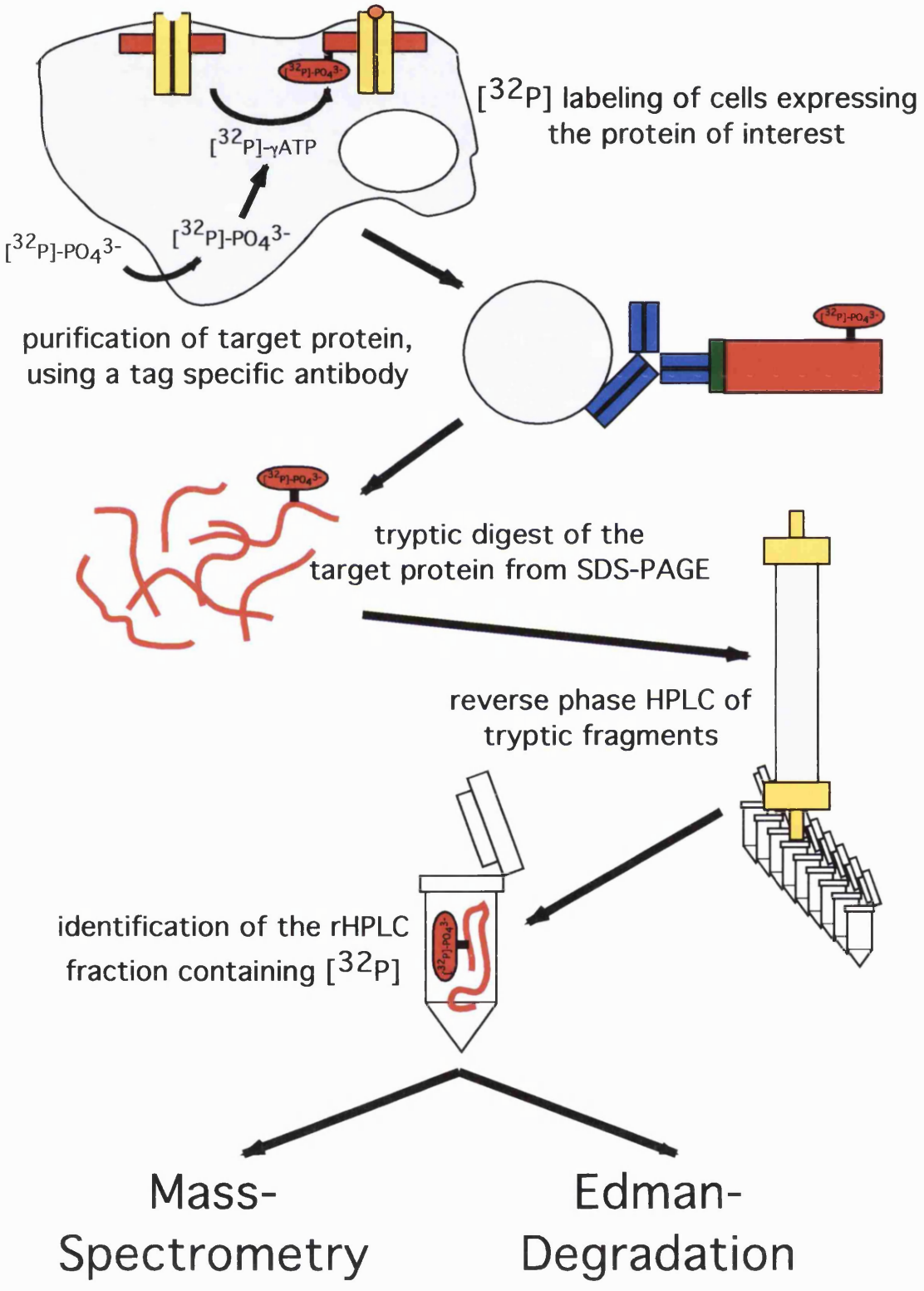

[32P] labeling of cells expressing the protein of interest
[32P]-γATP
[32P]-PO43-
[32P]-PO43-
purification of target protein, using a tag specific antibody
tryptic digest of the target protein from SDS-PAGE
reverse phase HPLC of tryptic fragments
identification of the rHPLC fraction containing [32P]
Mass-Spectrometry
Edman-Degradation

Fig. 3.28 Reverse phase HPLC of tryptic fragments from *in vivo* [^{32}P]-phosphate labelled hJAK1-M6H after OSM treatment

The graph shows the absorption profile at 280nm from a reverse phase HPLC of [^{32}P]-phosphate labelled tryptic fragments from hJAK1-M6H. The x-axis shows the number of the fractions. The fractions containing [^{32}P]-phosphate labelled peptide are marked with their fraction number in red. Fraction 24 contained the hJAK1 882-896 peptide, which contains threonine 889. This data was obtained from Nick Totti (Mass Spectrometry Department, ICRF).

Figure 3.28

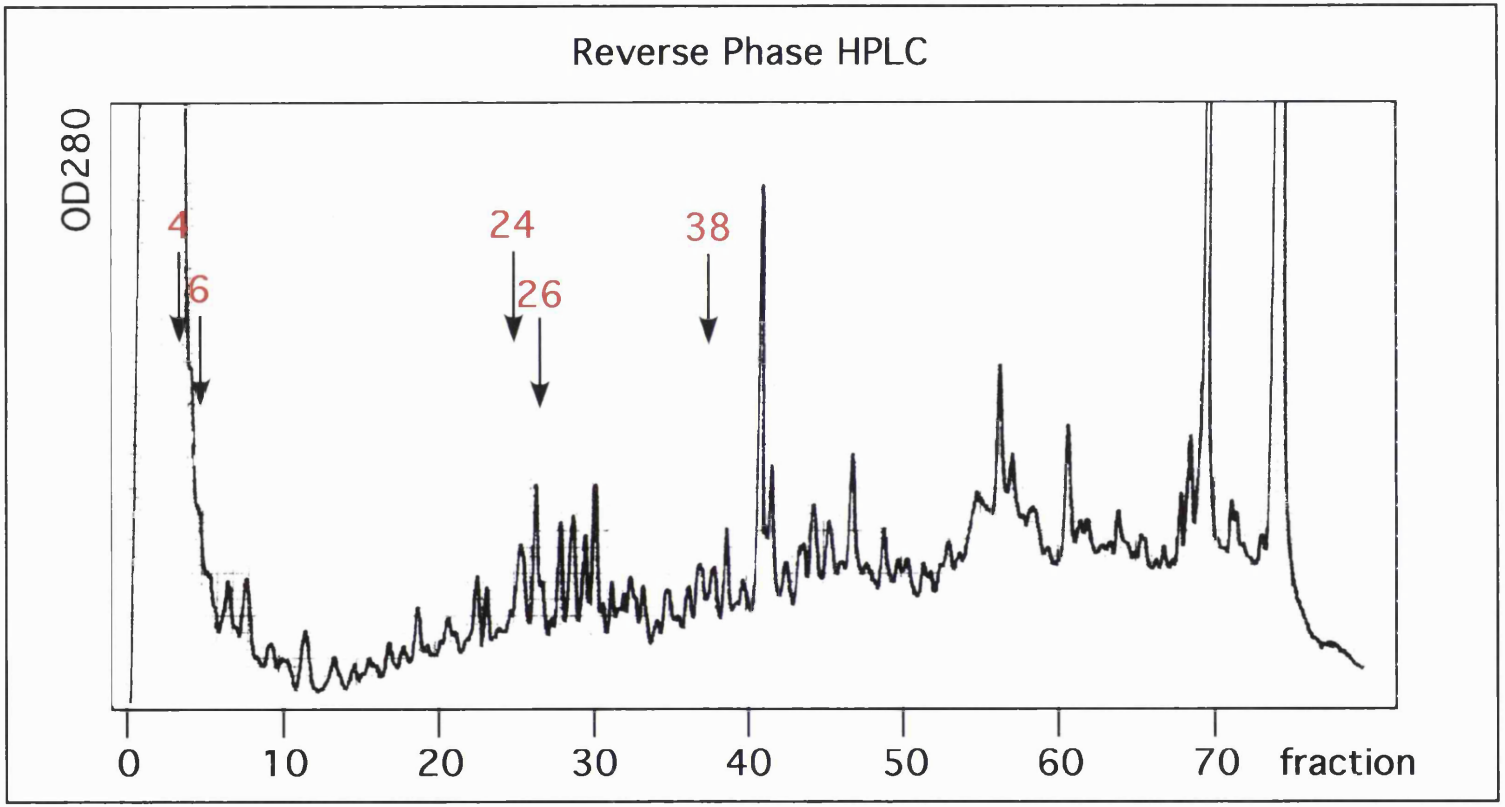

Reverse Phase HPLC
OD280
4
6
24
26
38
0
10
20
30
40
50
60
70
fraction

Fig. 3.29 Identification of peptide hJAK1 882-896 in fraction 25 from the reverse phase HPLC in Fig 3.28

Fraction 25 from the reverse phase HPLC analysis in Fig. 3.29 was analysed for containing peptides by mass spectrometry. The graph shows signals for peptides with a mass to charge ratio of 400-1000. A peak at 811.3 correlates the double charged tryptic fragment hJAK1 882-896. This data was obtained from Nick Totti (Mass Spectrometry Department, ICRF).

Fig. 3.30 Edman degradation of fraction 24 from the reverse phase HPLC in Fig. 3.28

The tryptic fragments from fraction 24 of the reverse phase HPLC in Fig. 3.29 were analysed by Edman degradation. The eluate of every cycle was analysed in a scintillation counter. The graph shows the radioactive counts in the eluates (X-axis) and the corresponding position in peptide hJAK1 882-896 (Y-axis). After 8 cycles a [^{32}P]-phosphate labelled amino acid was detected. This position correlates with the position of threonine 889 in peptide hJAK1 882-896. This data was obtained from Nick Totti (Mass Spectrometry Department, ICRF).

Figure 3.29

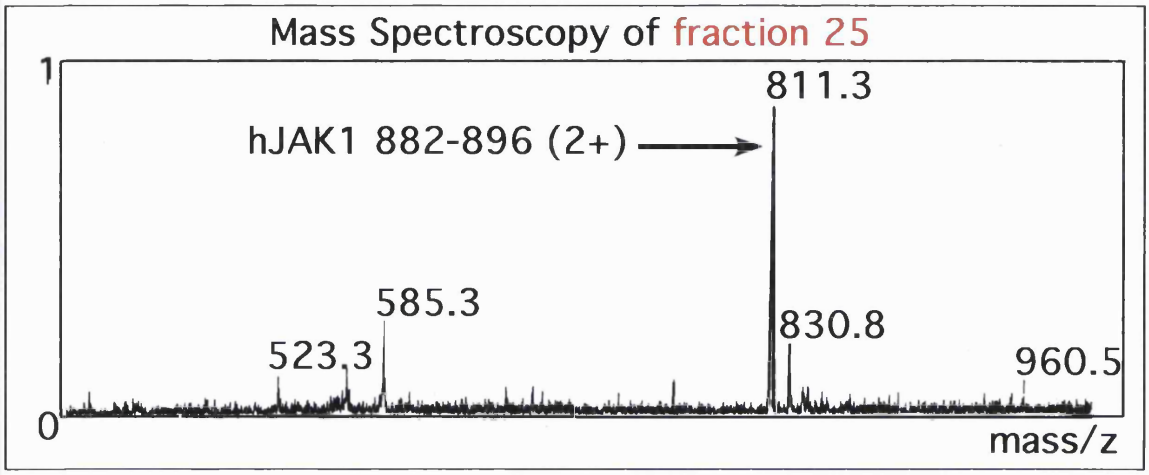
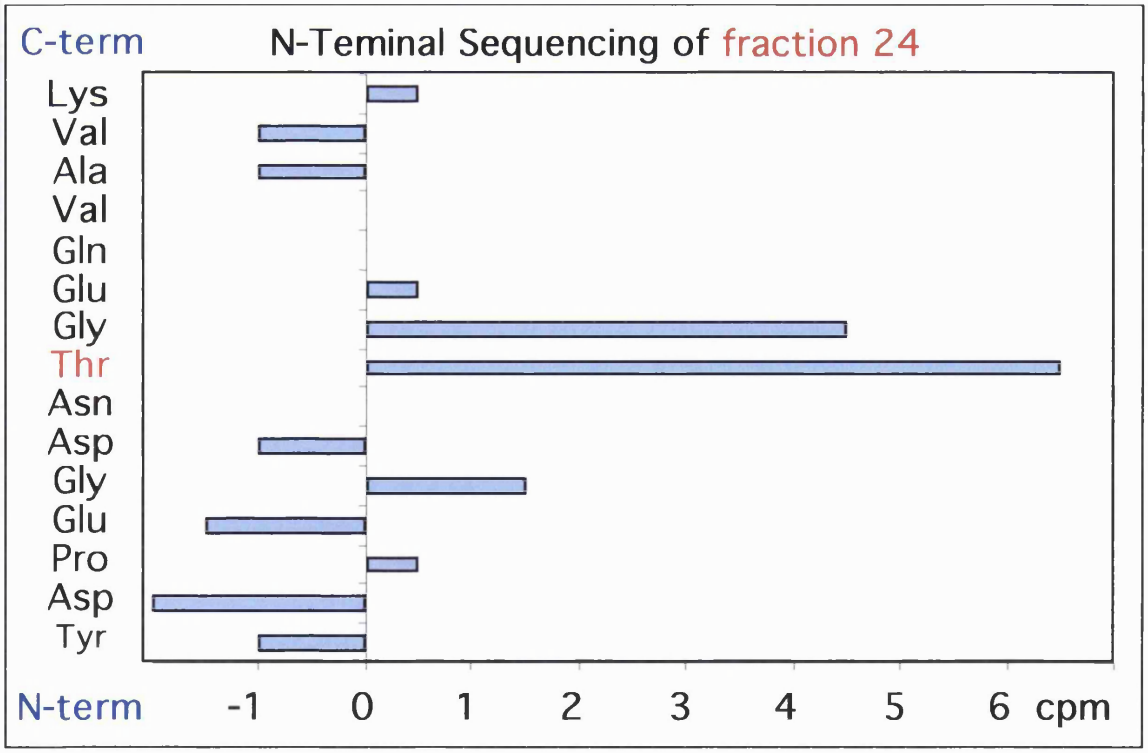


Figure 3.30



Chapter 4: STAT1 Movement in the Cell

4.1 Introduction

Appropriate localisation of proteins is crucial for their physiological function and regulation. Proteins transmit incoming signals by interaction with other molecules with or without movement into another location in the cell. Activation of transcription factors can occur in the cytoplasm or at the cell membrane. On activation they translocate to the nucleus through nuclear pores (reviewed in Kaffman and O'Shea, 1999). Little is known, however, about the mechanisms of transport to the nuclear pores. Movement through the cytoplasm could, a priori, be active along microtubules as observed for p53 (Giannakakou et al., 2000) or passive, by diffusion, as in a random walk.

In this chapter the dependence of IFN-α and -γ signalling on the cytoskeleton was investigated. Also, using a known biologically active STAT1-GFP (Koster and Hauser, 1999), we have examined for IFN-γ how STAT1 moves from the plasma membrane JAK/receptor complex to the nuclear pore and the nuclear mobility of STAT1.

4.2 Results

4.2.1 Interferon signalling is not dependent on an intact cytoskeleton

The inhibitors cytochalasin D, which prevents actin polymerisation, and nocodazole, which disrupts the formation of microtubules, were used to determine the influence of these structural elements on IFN signalling. 2fTGH fibrosarcoma cells were pre-incubated in the absence or presence of either drug for 90min, then stimulated with IFN-α or -γ for 15h under continued drug treatment. The efficacy of the drugs was confirmed by changes in cell morphology and the loss of cell adherence. Transcriptional activity upon IFN stimulation was measured by RNase protection assays using protection probes for the typical IFN-stimulated genes (ISGs) p48, 6-16, IRF1 and 9-27 (Fig. 4.1 top). The data were quantified by phosphoimaging corrected for actin levels (Fig. 4.1 bottom). The transcriptional response to IFN-α or -γ was not affected by either drug treatment in comparison to untreated controls. In additional experiments, neither the kinetics nor the dose responses were significantly affected (data not shown). Accepting this lack of dependence on the cytoskeleton the mobility of STAT1 was further investigated in live cells using a known functional STAT1-GFP (Koster and Hauser, 1999).

4.2.2 STAT1-GFP is a functional transcription factor

The construction and characterisation of a functional STAT1-GFP (Fig. 4.2), the behaviour of which is indistinguishable from native STAT1, has already been described (Koster and Hauser, 1999). Here the comparable restoration of IFN responses to STAT1 negative U3A cells by STAT1 and STAT1-GFP was confirmed by RNase protection assays monitoring the induction of representative sets of IFN-γ and IFN-α-inducible genes (Fig. 4.3 and Koster and Hauser, 1999). For the remainder of the experiments

wildtype 2C4 cells were used in preference to the multiply mutagenised U3A cells. The function of stably transfected STAT1-GFP in 2C4 cells again appeared indistinguishable from that of the, in this case, endogenous STAT1. The STAT1-GFP is comparably tyrosine phosphorylated /activated (Fig. 4.4), shows comparable DNA binding activity (Fig. 4.5) and is efficiently translocated to the nucleus in response to IFN-γ stimulation (Fig. 4.8, see Koster and Hauser, 1999). Comparison of endogenous STAT1 levels with those of the stably transfected STAT1-GFP by western blot shows an average threefold overexpression of the STAT1-GFP in the population (Fig. 4.6, top). The fluorescence of single cells (Fig. 4.6, bottom), together with the data from the western blot analyses, indicate that the majority of single cells express the STAT1-GFP to levels comparable with or up to tenfold higher than the endogenous STAT1. No significant difference was observed between high and low expressing cells in the single cell based fluorescence studies below.

4.2.3 High mobility of cytoplasmic STAT1-GFP: FLIP and FRAP analyses

Little is known about the mobility of STAT1 in the cytoplasm. In order to address these issues, FLIP and FRAP analyses were carried out to compare the mobilities of stably expressed STAT1-GFP, GFP and PKC-GFP in the cytoplasm of live 2C4 cells before and after treatment with IFN-γ or phorbol ester.

4.2.3.1 FLIP (fluorescence loss in photobleaching) analysis of cytoplasmic STAT1-GFP

In FLIP (Fig. 4.7) analysis a small region of the cytoplasm (white box, Fig. 4.8; the intensity of the fluorescence signal is indicated by a 'false colour' bar to the right of the images) was bleached by scanning for three consecutive periods of 90s with

maximum Laser intensity and the fluorescence of the whole cell monitored. To make sure there was no generalised bleaching effect due to the imaging, every bleached cell had an unbleached neighbouring cell in the same image, which maintained high fluorescence (Fig. 4.8). The behaviour of non-activated and IFN-γ activated STAT1 was compared with GFP, known to diffuse freely in cells, and free and membrane-bound (more slowly diffusing) PKC-GFP. Cells expressing GFP (Fig. 4.8, third row), STAT1-GFP, before and after treatment with IFN-γ (Fig. 4.8, rows 1 and 2) and free PKC-GFP without phorbol ester treatment (Fig. 4.8, row 4) lost most of their cytoplasmic fluorescence after the first 90s of bleaching. Further bleaching to totals of 180s and 270s seconds resulted in the entire loss of cytoplasmic fluorescence. For STAT1-GFP and PKC-GFP the nuclear envelope imposes a barrier to free diffusion. In these short time periods no STAT1-GFP is released from the nucleus (Fig. 4.8, rows 1 and 2). (There is a low level of constitutively activated STAT1-GFP in the nucleus of the control cells: Fig. 4.8, row 1). Conversely, PKC-GFP does not enter the nucleus of control cells (Fig. 4.8, row 4). In cells treated with phorbol ester PKC–GFP is activated and translocated to the membrane. The reduced mobility of membrane-associated PKC-GFP, visualised by focusing on the lower membrane, was obvious: even after three periods of bleaching, only the boxed area and the close surroundings showed a loss in fluorescence in these cells (Fig. 4.8, row 5). Depletion of ATP (see below) had no influence on the results of this type of FLIP analysis. The data indicate that most of the STAT1-GFP molecules passed through the region being bleached within 270s, moving rapidly throughout the cytoplasm in a random ATP-independent fashion. This is in contrast with the data for the more slowly diffusing, membrane-associated and activated PKC-GFP.

4.2.3.2 FRAP (Fluorescence recovery after photobleaching) analysis of cytoplasmic STAT1-GFP

In order to have a more quantitative measurement of mobility to permit the calculation of the amount of any immobile fraction of STAT1-GFP, FRAP (Fig. 4.7) analyses were performed on the same stably transfected cell lines as were used for the FLIP analysis A region in the cytoplasm was bleached for a period of 17.5s and the effect on the fluorescence intensity in this area was measured for a further 60s. In parallel, regions surrounding the bleached region were measured for the same period of time. To investigate whether the movement of the fusion proteins was an energy dependent process, a second set of cells were depleted of ATP by pre-treatment with sodium azide and 2-deoxyglucose prior to analyses (right hand panels, Fig. 4.9). This treatment is sufficient to inhibit ATP dependent fluid phase endocytosis of fluorescent Cy3 labelled antibodies (Wubbolts et al., 1996) and data not shown). With or without depletion of ATP the recovery rates in the bleached regions of the cytoplasm (empty squares, Fig. 4.9) for GFP (Fig. 4.9, third panels down), STAT1-GFP before and after IFN-γ treatment (Fig. 4.9, top and second panels) and PKC-GFP without phorbol ester treatment (Fig. 4.9, fourth panels down) were similar. In contrast, the recovery of PKC-GFP after phorbol ester treatment was slower and never complete. Again, this was not influenced by the depletion of ATP (Fig. 4.9, bottom panels).

Comparison of the bleached region (empty squares) versus the surrounding regions (filled triangles) did not show a detectable immobile fraction of GFP, STAT1-GFP before and after IFN-γ treatment and PKC-GFP without phorbol ester treatment. As little as one per cent of immobile GFP would have been detected in this type of analysis. In contrast to GFP per se (Fig. 4.9, row 3), very small differences directly after the bleaching were detectable in the cells expressing STAT1-GFP and PKC-GFP (Fig. 4.9, rows 1, 2 and 4). These most likely reflect differences in size and hence in mobility of

the fusion proteins versus free GFP. The comparison of the bleached region to the surrounding regions for PKC-GFP after phorbol ester treatment showed an immobile fraction of approximate 5 ± 2 % (n=10) for the membrane associated PKC-GFP (Fig. 4.9, bottom row).

4.2.4 Nuclear translocation of preactivated STAT1 does not depend on continued activity of JAK/receptor complexes

At any given instant in time a small percentage (below the 1% which would be detected by FRAP) of the STAT1-GFP might be associated with a 'hard-wired' directional transport mechanism linking active JAK/receptor complexes to nuclear pores. In the presence of the kinase inhibitor staurosporine (Haspel and Darnell, 1999) and hence the absence of continued JAK/receptor activity, preactivated STAT1-GFP is efficiently translocated into the nucleus. In initial EMSA analyses in 2C4 cells, adding staurosporine before stimulation with IFN-γ, showed that the drug is effective in inhibiting JAK activation of STAT1 in less than 2min under the conditions to be used (Fig. 4.10). In subsequent experiments, 2C4/STAT1-GFP cells were stimulated with IFN-γ and, after 15min, were incubated with or without staurosporine for a further 5 or 15min (to yield the 20 and 30min time points). The cells were fixed and imaged (Fig. 4.10). Up to 15min only a small amount of STAT1-GFP is translocated. Between 15 and 20min fluorescence intensities in the cytoplasm and nucleus are similar and after 30min most of the STAT1-GFP is translocated to the nucleus (Fig. 4.10). Translocation was comparable in the drug-treated and control cells. Accordingly, the inhibition of the activation of STAT1 at the membrane JAK/receptor complex by staurosporine was without effect on the translocation of randomly distributed, preactivated, cytoplasmic STAT1. It seems that a substantial portion of STAT1 molecules are activated within 15min of ligand stimulation and remain randomly distributed in the cytoplasm until

translocated through the nuclear pore. These data also confirm that at least 50% of the cytoplasmic STAT1-GFP molecules in the FRAP and FLIP experiments in IFN-γ-treated 2C4/STAT1-GFP cells were indeed preactivated at the time of analysis.

4.2.5 FLIP and FRAP analyses of nuclear STAT1-GFP

The mobility of STAT1-GFP in the nucleus of 2C4 cells after IFN-γ treatment was studied in comparison to 2C4 cells expressing GFP tagged with a nuclear localisation signal (GFPnls) rather than wild-type GFP. In the FLIP experiments (Fig 4.11), the boxed area was bleached for shorter periods of 30s, in accordance with the smaller volume of the nucleus. There was no detectable difference between STAT1-GFP and GFPnls with most of the fluorescence in the nucleus being bleached after 30s. A further two consecutive 30s bleach periods were required for a complete loss of fluorescence. No loss of fluorescence was observed in the cytoplasm of the 2C4/STAT1-GFP cells, reflecting the barrier presented to STAT1-GFP by the nuclear envelope (Fig 4.11, top row). FRAP analysis of STAT1-GFP and GFPnls in the nucleus showed comparable recovery rates for the two molecules with or without depletion of ATP (Fig. 4.12). Furthermore, no immobile fraction was detectable in either case. Movement of STAT1-GFP in the nucleoplasm appears, therefore, to be rapid and random.

4.2.6 Dynamic interactions exclude STAT1 from nucleoli

During the FLIP analyses it was noted, that in contrast to free GFP or GFPnls, STAT1-GFP was excluded from nucleoli (Fig. 4.11). Rapid association and dissociation of STAT1 with the DNA could account for both the retention and the high mobility of nucleoplasmic STAT1. The behaviour of two DNA binding mutants (hSTAT1 K336>A

and N460>A; Fig. 4.2) was therefore investigated. When expressed in STAT1 negative U3A cells the mutant STAT1s are phosphorylated/activated but do not bind DNA in response to IFN-γ (Fig. 4.13). The mutant STATs were translocated to the nucleus comparably to wild-type STAT1 (Fig. 4.14). Both the mutant and the wild-type STATs were, however, excluded from the nucleoli (Fig. 4.14). Nucleoplasmic GAS element-specific DNA binding alone cannot, therefore, account for the nucleolar exclusion of STAT1.

4.3 Discussion

It remains unclear to what extent signal transduction pathways are 'soft' versus 'hard' wired involving random diffusion versus directional movement on a scaffold or cytoskeletal element. Here we investigated, how STAT1 moves from the JAK/receptor complex at the cell membrane to the nuclear pore and from the nuclear pore to the DNA.

The induction of representative sets of IFN-α/β and IFN-γ inducible mRNAs was not affected by disruption of the actin cytoskeleton or microtubules (Fig. 4.1). Accordingly, for the cells used here, JAK/STAT signalling and hence the movement of STAT1 from the cell membrane to the nuclear pore in response to the IFNs is independent of the cytoskeleton. In contrast, translocation of p53 is dependent on interaction with microtubules and dynein, a minus-ended microtubule motor, that would move p53 along microtubules towards the nucleus in response to stress (Giannakakou et al., 2000).

In light of the absence of dependence on the cytoskeleton, STAT1-GFP was used to monitor the movement of STAT1 in live cells. The STAT1-GFP behaves indistinguishably from endogenous STAT1: it is activated, translocates and initiates gene expression with comparable efficiency to STAT1 (Koster and Hauser, 1999 and Fig. 4.3-4.5). It is not detectably cleaved to yield free GFP (Fig. 4.4). There was considerable cell to cell variation in the levels of expression of the stably transfected STAT1-GFP (Fig. 4.6). Throughout the photobleaching experiments similar results were obtained with high (>5 fold wild-type) and low (≈ wild-type) expressing cells. The results do not, therefore, reflect overexpression artefacts.

The FLIP and FRAP experiments provide insight into the nature of STAT1 movement. STAT1-GFP mobility, with or without ligand stimulation is comparable to that of freely diffusible GFP (Fig. 4.8). It is rapid, with all of the STAT1 molecules moving through all locations in the cytoplasm or nucleus within minutes, and

independent of ATP. An immobile fraction of STAT1-GFP was not detected and can be excluded down to a level of approximately 1% of the total STAT1-GFP (Fig. 4.9). A priori, at any given instant in time, a small percentage of the STAT1-GFP might be associated with a 'hard-wired' directional transport mechanism linking the JAK/receptor complex to the nuclear pore. However, in the presence of staurosporine and hence the absence of an enzymatically active JAK/receptor complex, a substantial fraction (about 50%) of total STAT1-GFP is translocated into the nucleus (Fig. 4.10). Thus, preactivated, randomly distributed STAT1-GFP is translocated to the nucleus as efficiently as in non staurosporine-treated cells (Fig. 4.10). One still cannot exclude the possibility that, at any given time, a small percentage of STAT1-GFP (too low to be detected by FLIP or FRAP) is transported directionally to the nuclear pore. Access to any such putative transport system would, however, have to be available to randomly distributed preactivated STAT1-GFP. In this model the putative translocation system would conceptually be an extension of the nuclear pore/importin complex, with random access through free diffusion after release from the receptor. In an alternative approach, STAT1 generated from a completely foreign receptor can sustain an IFN-γ like response. This favours modular signalling and 'soft' rather than 'hard-wiring' of the IFN-γ induced cytoplasmic signal transduction pathway(s) (Arulampalam, Strobl, Is'harc and Kerr, in preparation). Overall, the data are consistent with free diffusion of the STAT1 from the membrane JAK/receptor complex to the nuclear pore. Activated STAT1 forms a dimer, dimerisation being required for recognition by importin NPI-1 and translocation through the nuclear pore (Sekimoto et al., 1997). Larger STAT1 complexes have been reported (Lackmann et al., 1998; Ndubuisi et al., 1999) and highly dynamic interactions with randomly distributed cytoplasmic complexes, remain perfectly possible. Such interactions would not, however, confer directionality to the movement of STAT1.

The behaviour of STAT1-GFP in the nucleus is also of interest. Nucleoplasmic movement is again rapid, random, independent of ATP and comparable to that of freely diffusible GFP (Fig. 4.14). STAT1-GFP, in contrast to free GFP, is, however, excluded from nucleoli (Fig. 4.14), presumably through interaction with nucleoplasmic protein/DNA complexes. Such interactions would have to be highly dynamic with high rates of association and dissociation to sustain both the high mobility and localisation of the STAT1-GFP. Interactions of this type with similar effects on localisation have recently been described for a number of nuclear proteins (Phair and Misteli, 2000). More particularly, for the glucocorticoid receptor, association with transcriptional complexes is similarly highly dynamic, in accord with a 'hit and run' model rather than the formation of stable initiation complexes (McNally et al., 2000). As the loss of specific GAS site DNA binding function does not influence nucleoplasmic localisation, direct interaction with GAS elements in the DNA is not solely responsible for exclusion from the nucleoli (Fig. 4.13 & 4.14). The retention of the STAT1-GFP in the nucleoplasm may, therefore, reflect a 'scanning' process for specific sites in addition to the formation of a small percentage of specific transcriptional initiation complexes.

Although peripheral to the main argument, the contrast in the results obtained here with the K336A and N460A STAT1 DNA binding mutants with those of others for the EE428/429AS DNA binding mutant is also of potential interest. Nuclear localisation of the latter mutant in response to IFN-γ was only observable in the presence of leptomycin B an inhibitor of CRM1 interaction and nuclear export (McBride et al., 2000). Both K336A and N460A lie outside the STAT1 sequence, AA392-413, known to be required for CRM1 binding (McBride et al., 2000). Taken together, these data, like those for the retention of the STAT1 mutants in the nucleoplasm, suggest that factors besides GAS-element-specific DNA binding and interaction with CRM1 may contribute to retention of STAT 1 in the nucleus. A much more detailed analysis of the characteristics of the mutants, with respect, for example, to the kinetics of

dephosphorylation and interaction with transcriptional coactivators, will, be required to establish whether or not this is the case and, if so, reveal the nature of the factors involved.

Currently there is increasing evidence for both 'hard' and 'soft'-wiring of cytoplasmic signal transduction pathways (Teruel and Meyer, 2000; Vousden and Woude, 2000). Moreover, there is no a priori reason why both types of element should not be involved in a given pathway. Indeed arguably the nuclear pore represents a 'hard-wired' section to what appears to be an otherwise 'soft-wired' JAK/STAT1 pathway. The data presented here taken together with available data on nuclear import and export lead to a model in which for STAT1 there are a series of random walks interrupted by dynamic interactions with membrane JAK/receptor complexes, nuclear pores and nucleoplasmic protein/DNA complexes. More specifically, randomly diffusing cytoplasmic STAT1 is activated through dynamic interaction with activated JAK/receptor complexes at the cell membrane. The activated STAT1 dimerises and, with or without interaction with additional proteins diffuses to the nuclear pore where interaction with importin NPI-1 initiates translocation into the nucleus. On release from the nuclear pore the STAT1 diffuses freely through the nucleoplasm to which it is localised by dynamic interaction with protein/DNA. These interactions likely involve both the scanning of chromatin for potential transcriptional initiation sites and the formation of specific initiation complexes. Dephosphorylation of bound or unbound STAT1 leaves non-phosphorylated unbound STAT1 to diffuse freely to nuclear pores where interaction with CRM 1 mediates export to the cytoplasm, rendering inactive STAT1 available for further rounds of activation. Superimposed upon this basic cycle there will be additional controls. Although at first sight arguably unappealing, a model involving random walks interspersed with dynamic interactions has the advantage of potential flexibility, rapid modulation and fine tuning, which might be necessary for the maintenance of an appropriate balance in response to multiple stimuli.

Fig. 4.1 Interferon signalling is not dependent on an intact cytoskeleton

(Upper panel) 2fTGH cells were incubated for 90min with cytochalasin D or nocodazole, as indicated, then treated with IFN-α or γ for 15 h under continued drug treatment. Expression of inducible mRNAs (p48, 6-16, IRF-1, 9-27) was monitored by RNase protection (Materials and Methods). (Lower panel) Quantitation of the data in (upper panel) by Phosphoimager analysis. Fold induction was calculated after correction for the γ-actin loading control.

Figure 4.1

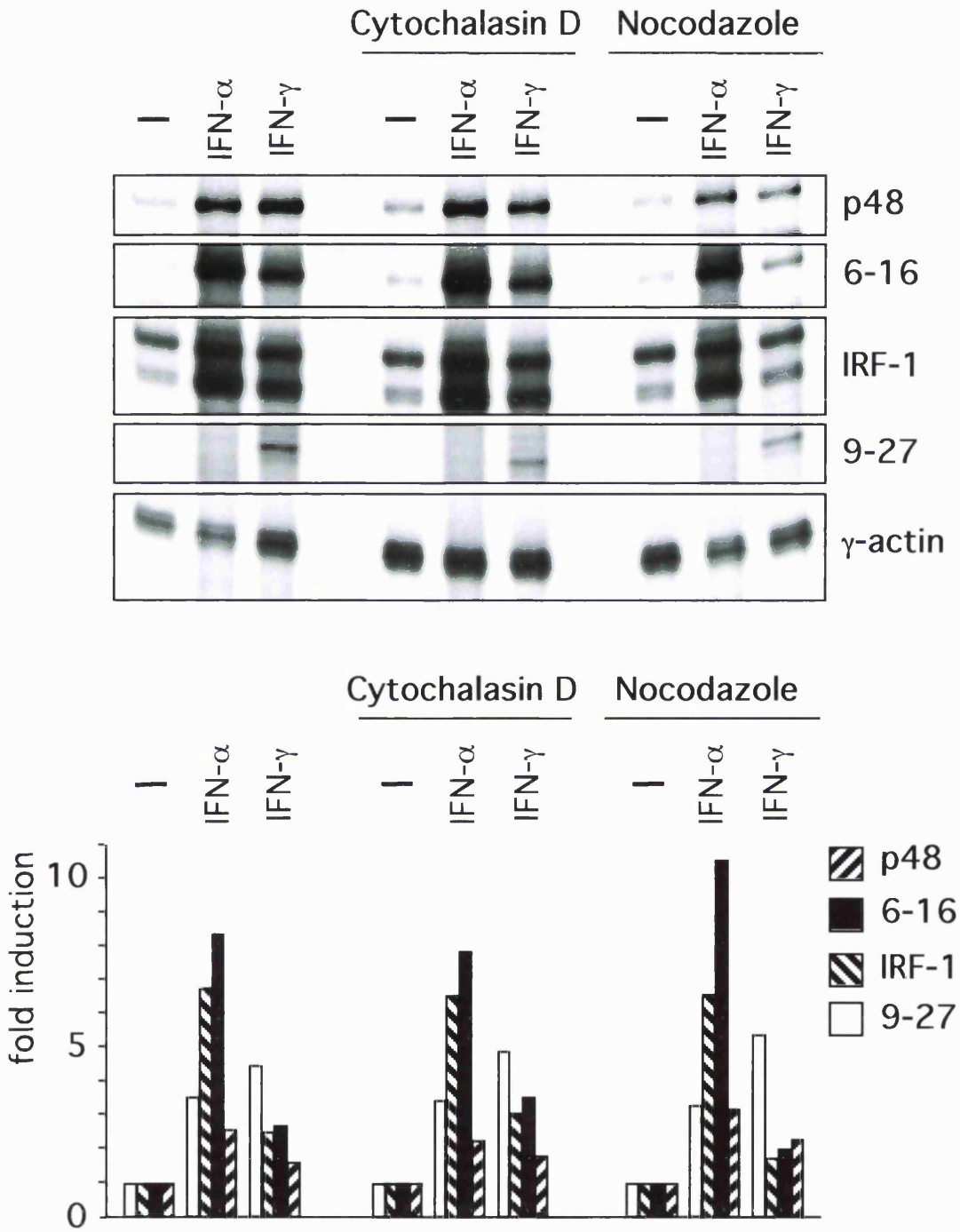

Cytochalasin D
Nocodazole
–
IFN-α
IFN-γ
p48
6-16
IRF-1
9-27
γ-actin
fold induction
10
5
0

Fig. 4.2 Schematic representation of used STAT1 constructs

STAT1-GFP fusion protein (upper panel, Koster and Hauser, 1999), STAT1 (K336>A) and STAT1 (N460>A) DNA binding mutant (gift of J. Darnel). The hSTAT1 cDNA is red with an orange DNA binding domain. The GFP is represented in green with the linker in-between GFP and STAT1 shown as a gap. The approximate position of the mutations is shown by arrows.

Figure 4.2

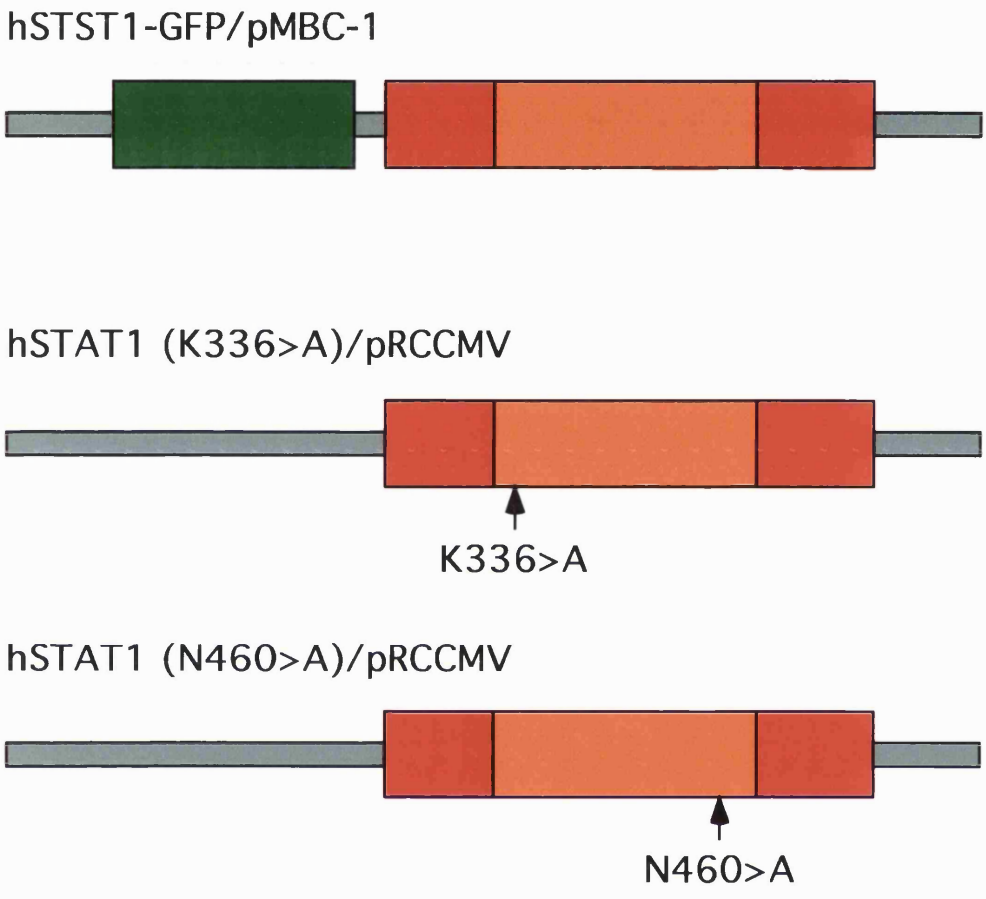

hSTST1-GFP/pMBC-1
hSTAT1 (K336>A)/pRCCMV
K336>A
hSTAT1 (N460>A)/pRCCMV
N460>A
= hSTAT1
= DNA- binding domain
= GFP

Fig. 4.3 STAT1-GFP function by RNase protection assay

U3A (left lanes), U3A/STAT1 (middle lanes) and U3A/Stat1-GFP cells were stimulated with 10^3 IU/ml IFN-α or γ for 15h. Expression of inducible mRNAs (p48, 6-16, IRF-1, 9-27) was monitored by RNase protection. (Experiment was performed once.)

Figure 4.3

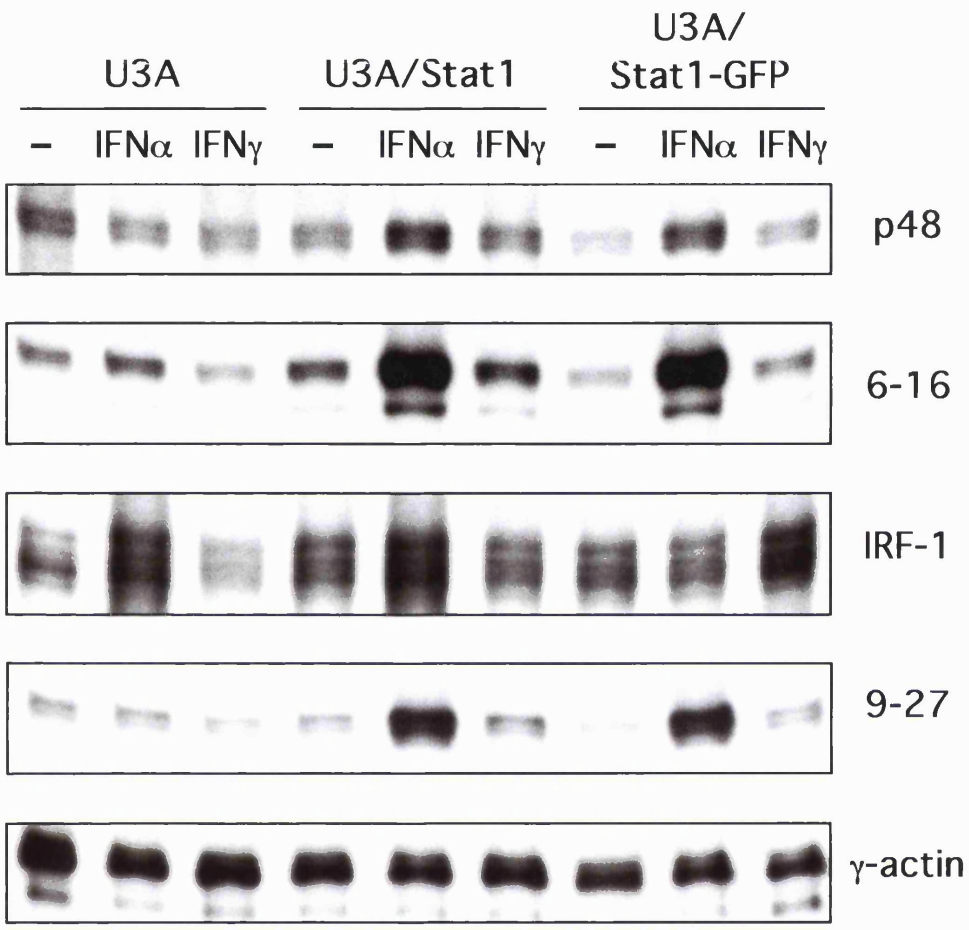

U3A
U3A/Stat1
U3A/
Stat1-GFP
–
IFNα
IFNγ
–
IFNα
IFNγ
–
IFNα
IFNγ
p48
6-16
IRF-1
9-27
γ-actin

Fig. 4.4 STAT1-GFP phosphorylation profile after IFN-α and IFN-γ

2C4 (left lanes) and 2C4/STAT1-GFP cells (right lanes) were stimulated with 10^3 IU/ml IFN-α or γ for 20min. Phosphorylation of STAT1-GFP. Western blot analysis of total cell lysates with an anti P-Tyr701 specific antibody (upper panel); an anti-STAT1 antibody as loading control (middle panel) and an anti-GFP antibody to monitor for any STAT1-GFP cleavage (lower panel). No evidence for free GFP was obtained even on prolonged exposure (data not shown).

Fig. 4.5 DNA binding of STAT1-GFP after IFN-α and IFN-γ

DNA binding of STAT1-GFP was analysed by EMSA of whole cell extracts with an SIE probe.

Figure 4.4

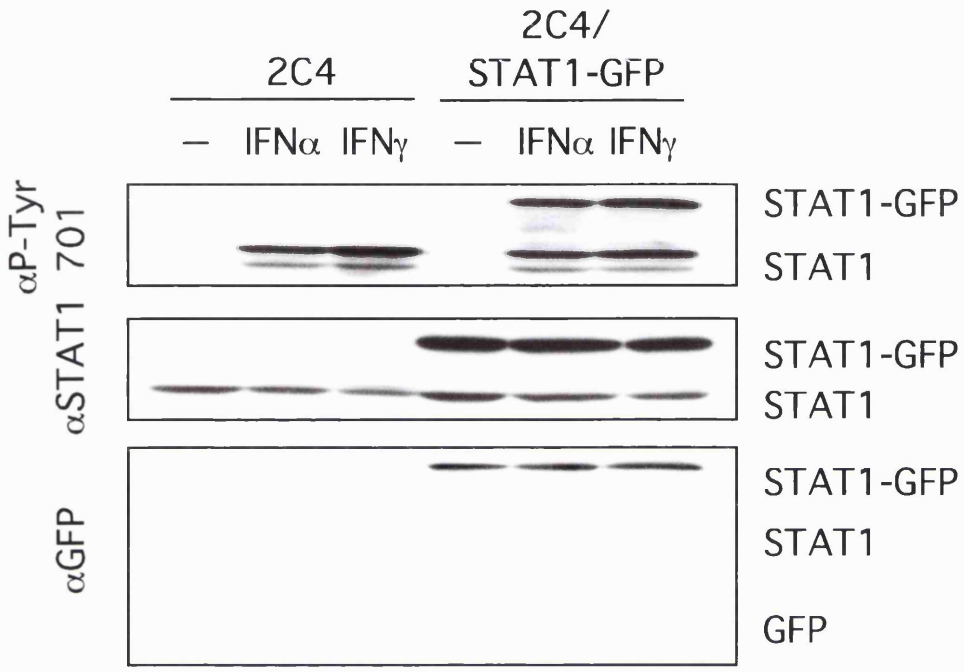


Figure 4.5

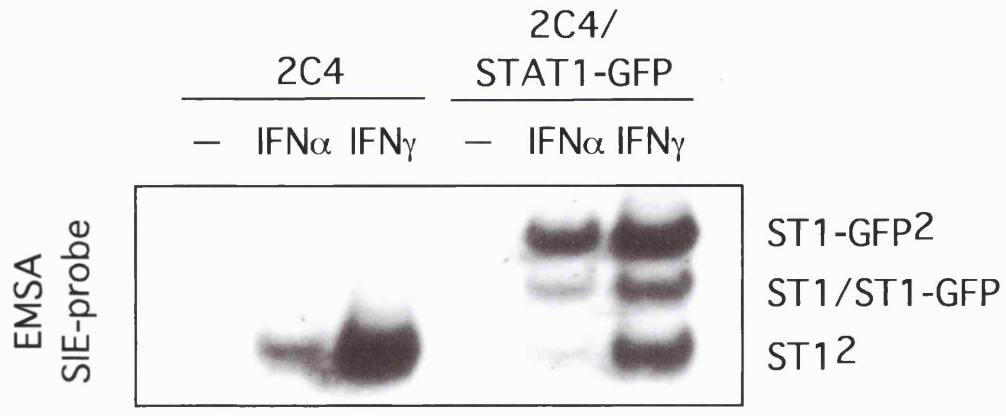


Fig. 4.6 Expression levels of 2C4/STAT1-GFP cells

The expression of STAT1 and STAT1-GFP in the 2C4/STAT1-GFP population was compared by analysing a series of dilutions of a total cell lysate by western-blot analysis with antibody to STAT1 (upper panel). The relative fluorescence intensity of 54 single cells was analysed by confocal microscopy (left ordinate, lower panel, Methods). The ratio of STAT1-GFP to endogenous STAT1 (right ordinate, lower panel) was calculated from the two data sets. The dotted line shows both, the average fluorescence (left scale) and the average expression relative to STAT1 (right scale) of STAT1-GFP in the population.

Figure 4.6

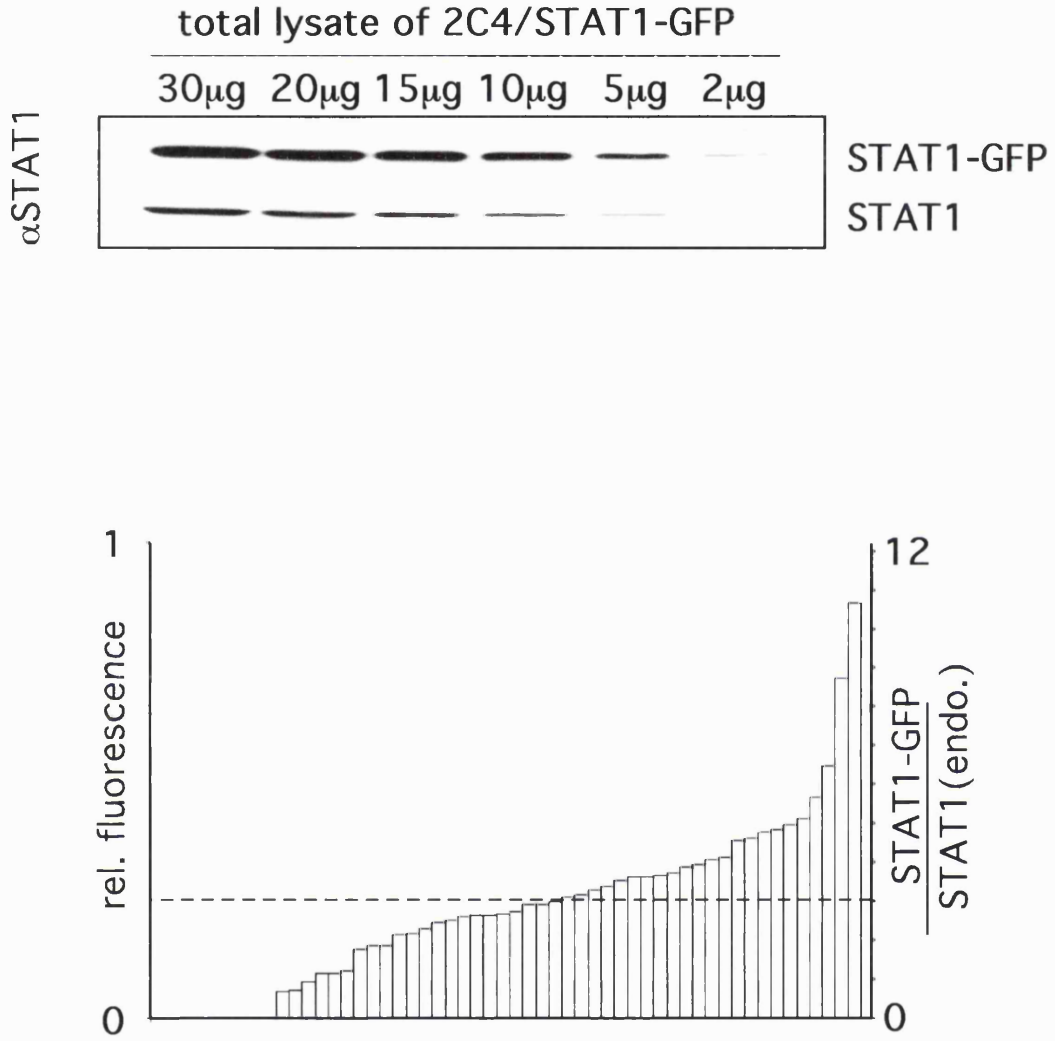

total lysate of 2C4/STAT1-GFP
30μg 20μg 15μg 10μg 5μg 2μg
αSTAT1
STAT1-GFP
STAT1
1
0
rel. fluorescence
12
0
STAT1-GFP / STAT1(endo.)

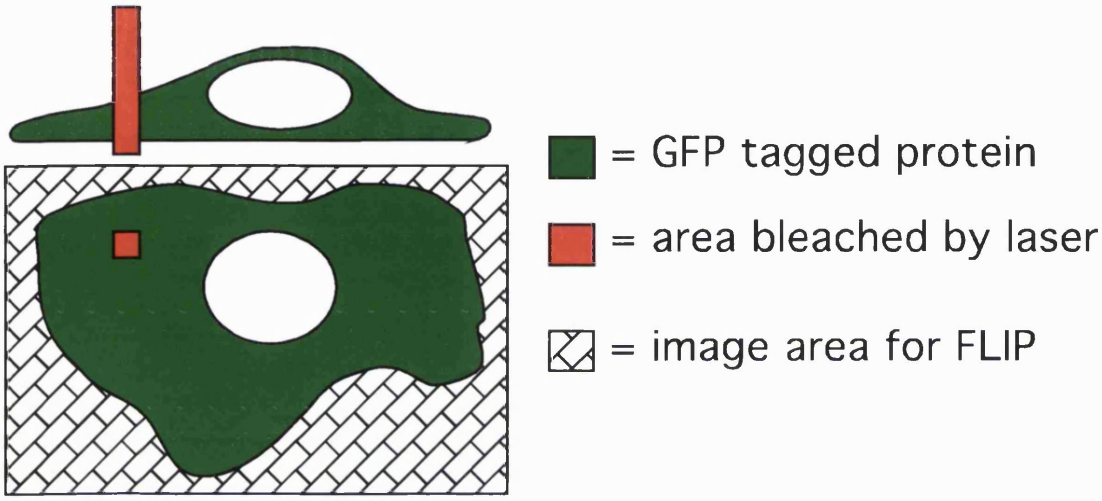
Fig. 4.7 Schematic model for fluorescence loss in photobleaching (FLIP) and fluorescence recovery after photobleaching (FRAP)

In FLIP experiments (upper panel), a GFP tagged protein (green colour) is expressed in cells. The living cells are imaged (image area is the blocked background) to analyse the localisation and concentrations of the GFP fusion protein. A small area of the cell is bleached for a certain period of time with maximum laser intensity (red square). Directly after the bleach period, the cell is imaged again to analyse changes in distribution of the fusion protein. This can be repeated until there are no further changes in the images of the cell or the fusion protein is completely bleached.

In FRAP analysis (lower panel) a small area (red square) in the living cells is bleached for a short period with maximum laser power. After the bleaching, the area is constantly quantified until no further increase of fluorescence. To measure the amount of immobile protein, the surrounding areas (yellow squares) are also measured and averaged to compensate for local variations.

Figure 4.7

FLIP
(fluorescence loss in photobleaching)



FRAP
(fluorescence recovery after photobleaching)

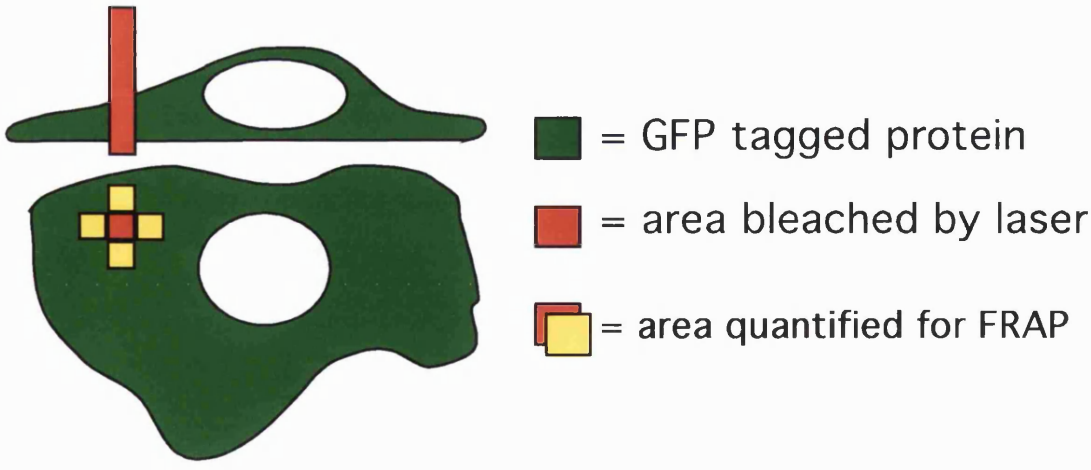


Fig. 4.8 FLIP analysis of STAT1-GFP, GFP and PKC-GFP in the cytosol

2C4 cells stably transfected with: STAT1-GFP without (row 1) and after 15min of IFN-γ treatment (10^3 IU/ml; row 2); GFP (row 3); PKC-GFP without (row 4) and after 5min of TPA treatment (Material and Methods, row 5). Every image in a row contains the same cells. The bleach region in the cytoplasm is indicated with a white square and fluorescence intensity is shown in false colour code (vertical bar, top right). Each row shows the fluorescence prior to bleaching (0s) and after three consecutive 90s bleach periods (90s, 180s and 270s). In the case of PKC-GFP after TPA the plane of focus was the membrane parallel to the cover slip (row 5).

Figure 4.8

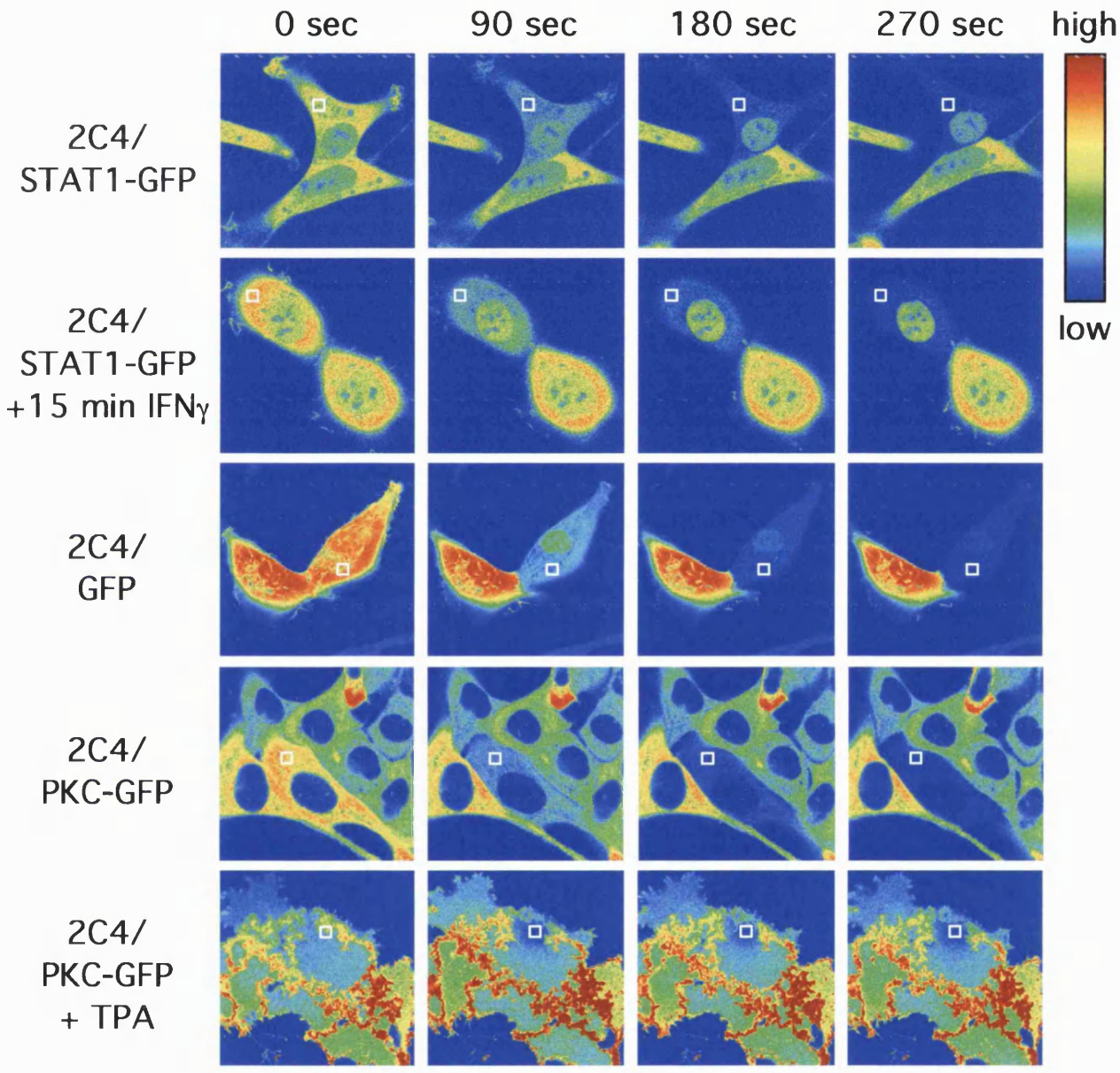

0 sec
90 sec
180 sec
270 sec
high
low
2C4/
STAT1-GFP
2C4/
STAT1-GFP
+15 min IFNγ
2C4/
GFP
2C4/
PKC-GFP
2C4/
PKC-GFP
+ TPA

Fig. 4.9 FRAP analysis of STAT1-GFP, GFP and PKC-GFP in the cytosol

2C4 cells stably transfected with: STAT1-GFP without (row 1) and after 15min of IFN-γ treatment at 10^3 IU/ml (row 2); GFP (row 3); PKC-GFP without (row 4) and after 5min of TPA treatment (Methods, row 5). Cells were bleached (Material and Methods) for 17.5s in an area comparable to the bleach area in the FLIP analysis (white squares, Fig. 4.8). The recovery of fluorescence in the bleach area (squares) and four surrounding areas (triangles) of the same size (averaged) was quantified over a period of further 60s in 1sec intervals. The fluorescence was normalised to the remaining fluorescence in the cell at the end of the experiment. The right hand panels show data for cells incubated for 20min with sodium azide and 2-deoxyglucose to deplete ATP, prior to bleaching. Each graph represents the average of data from 10 single cells. The apparently more extensive initial bleach level for membrane-associated PKC-GFP (row 5, squares) compared with that in all other rows reflects the slower movement of the membrane-associated PKC-GFP than the freely diffusing GFP and other GFP constructs during the switch of the laser from the bleach to the record modes.

Figure 4.9

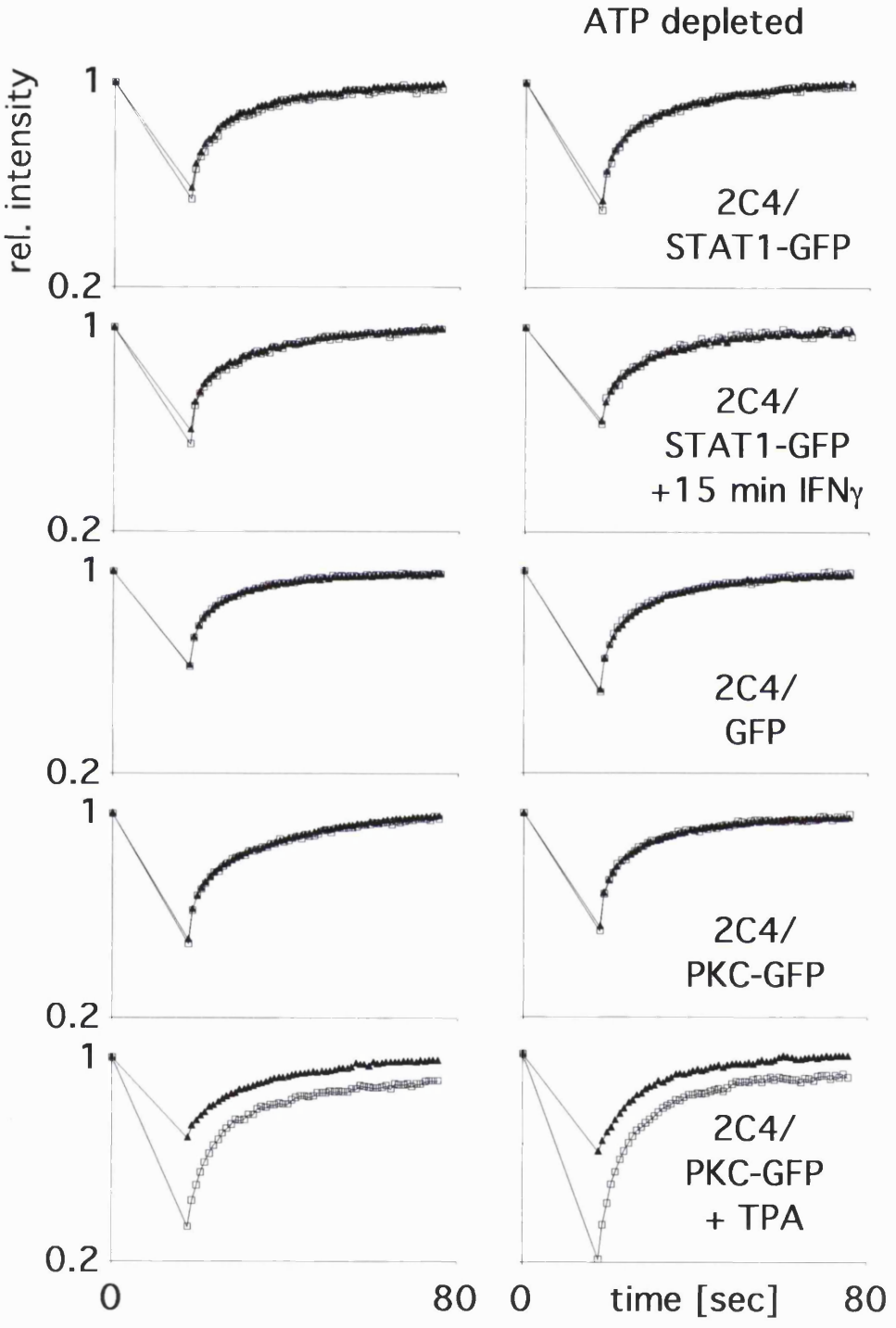

ATP depleted
rel. intensity
1
0.2
2C4/
STAT1-GFP
2C4/
STAT1-GFP
+15 min IFNγ
2C4/
GFP
2C4/
PKC-GFP
2C4/
PKC-GFP
+ TPA
0
80
time [sec]

Fig. 4.10 Nuclear translocation of STAT1-GFP is not dependent on active JAK/receptor complexes

(Upper panel) Inhibition of JAK-dependent STAT1 activation by staurosporine. 2C4 cells were pre-treated with 100nM or 500nM staurosporine for 2, 5, 15 and 30min and stimulated with IFNγ at 10^3 IU/ml for a further 20min in the continued presence of the drug. Activation of STAT1 was monitored by EMSA of whole cell extracts with a SIE probe in comparison with extracts from cells without drug treatment. (Similar results were obtained when the phosphorylation of STAT1 was monitored directly by (less sensitive) western-blot analysis.)

(Lower panel) Nuclear translocation of preactivated STAT1. 2C4/STAT1-GFP cells were incubated with 10^3 IU/ml of IFN-γ for 15min to activate the STAT1-GFP. Staurosporine (500nM) was added to half of the cells and both the staurosporine treated and non-staurosporine-treated cells incubated for a further 5 and 15min (to yield the 20 and 30min time points, respectively). At 0, 15, 20 and 30min samples were fixed in paraformaldehyde and the distribution of STAT1-GFP analysed by confocal imaging. Fluorescence intensities are shown in false colour code (vertical bar, top right). In each image the nucleus of one cell is indicated by a dotted line.

(Result is representative of three experiments.)

Figure 4.10

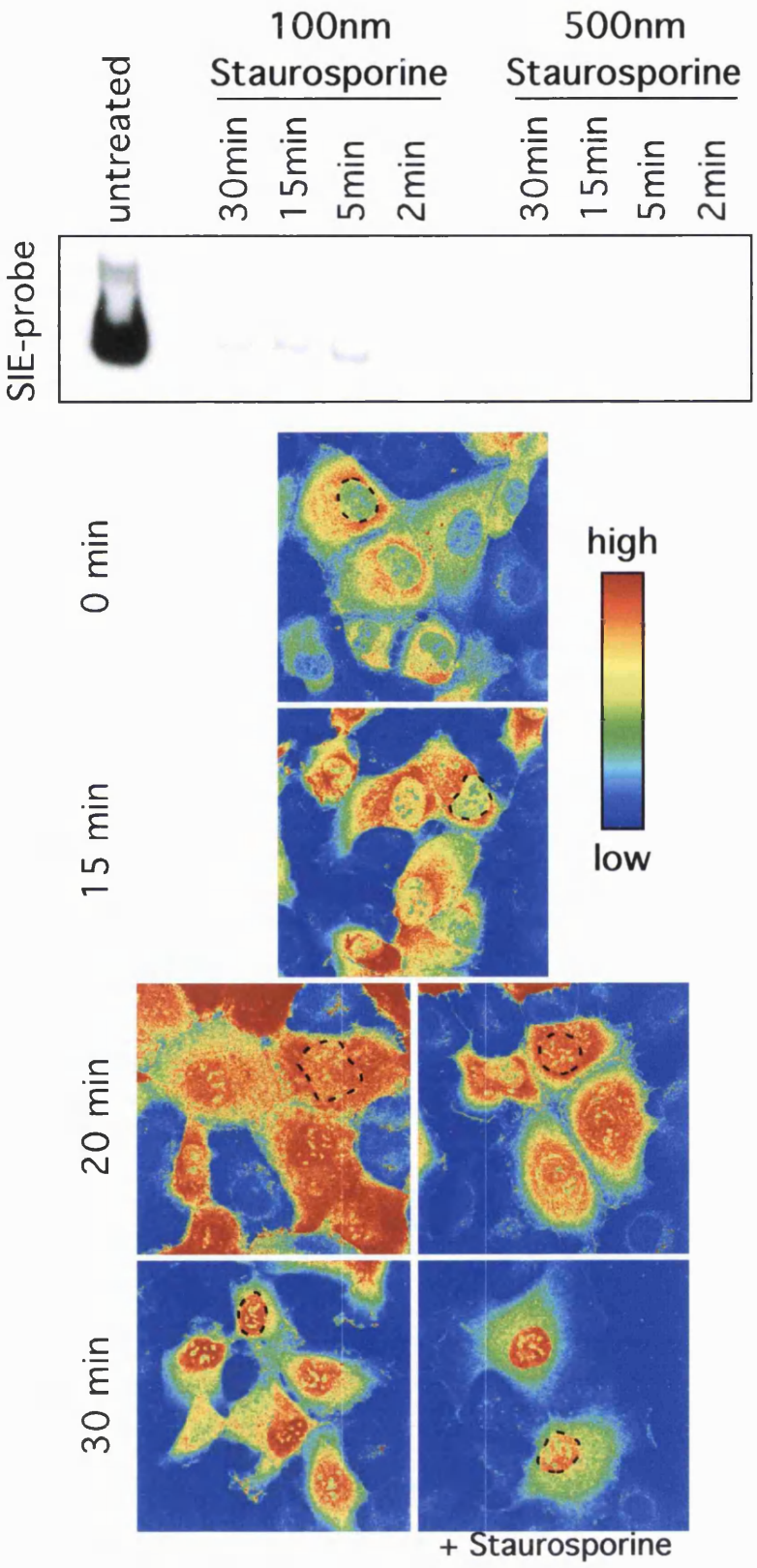

100nm
Staurosporine
500nm
Staurosporine
untreated
30min
15min
5min
2min
30min
15min
5min
2min
SIE-probe
0 min
15 min
20 min
30 min
high
low
+ Staurosporine

Fig. 4.11 FLIP analysis of nuclear STAT1-GFP and GFPnls

2C4 cells stably transfected with: (row 1) STAT1-GFP and treated with IFN-γ at 10^3 IU/ml for 25min; (row 2) GFPnls. Every image in a row contains the same cells. The bleach region in the cytoplasm is indicated with a white square and fluorescence intensity is shown in false colour code (vertical bar, top right). Each row shows the fluorescence prior to bleaching and after three consecutive 30s bleach periods (0s, 30s, 60s and 90s, respectively) of the nuclear area bounded by the white square.

Fig. 4.12 FRAP analysis of nuclear STAT1-GFP and GFPnls

FRAP analysis of the nuclear fluorescence of 2C4 cells stably transfected with: (row 1) STAT1-GFP and treated with 10^3 IU/ml of IFNγ for 25min; (row 2) GFPnls. Cells were bleached for 17.5s in an area comparable to the bleach area in the FLIP analysis (white squares, Fig. 4.11). The recovery of fluorescence in the bleach area (squares) and four surrounding areas (triangles) of the same size (averaged) was quantified over a period of further 60s in 1sec intervals. The fluorescence was normalised to the remaining fluorescence in the cell at the end of the experiment. The right hand panels show data for cells incubated for 20min with sodium azide and 2-deoxyglucose to deplete ATP, prior to bleaching. Each graph represents the average of data from 10 single cells.

Figure 4.11

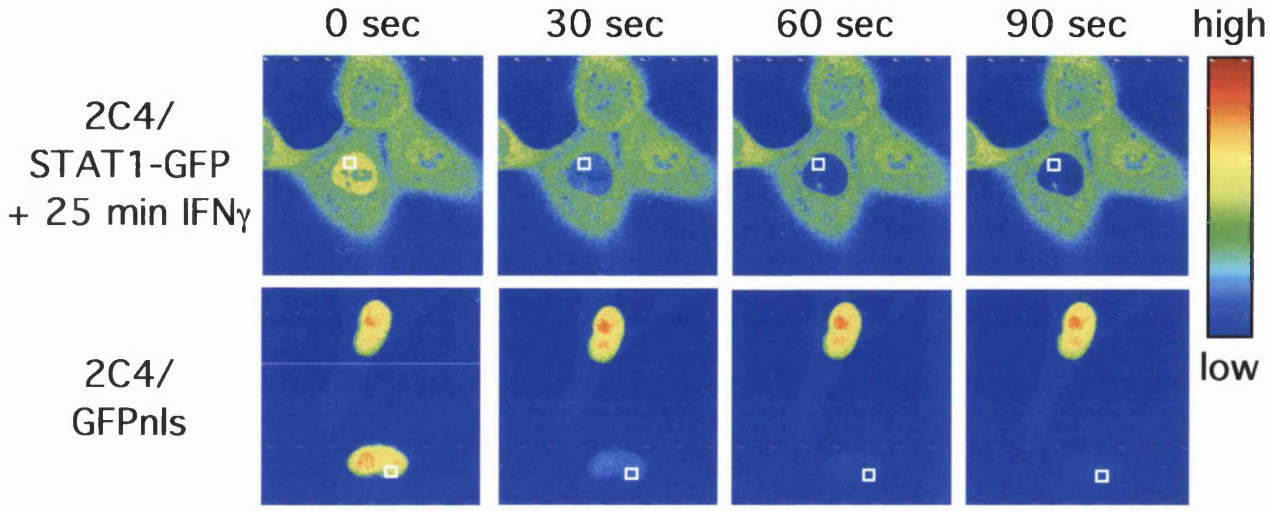


Figure 4.12

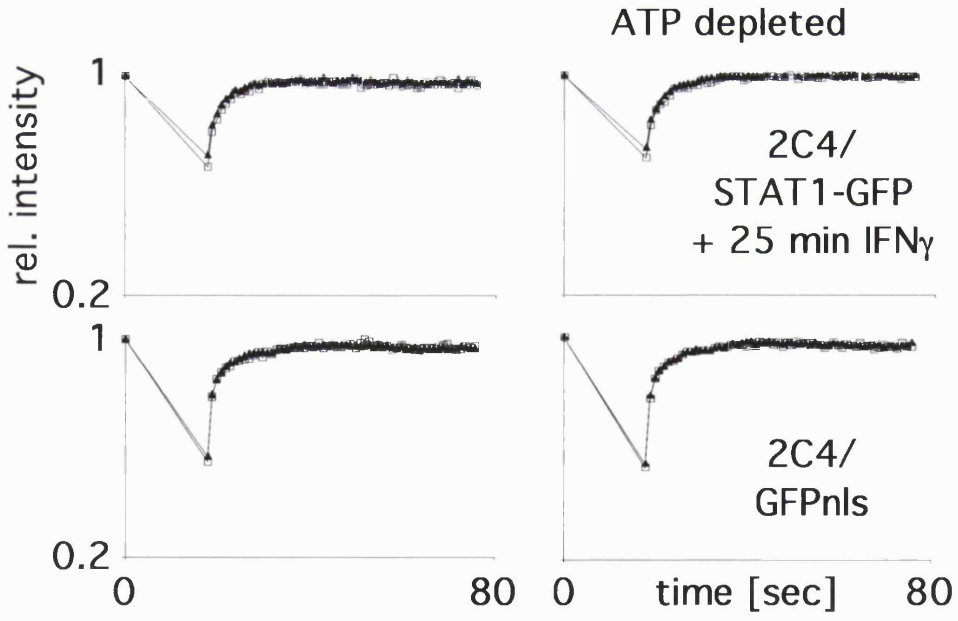


Fig. 4.13. Analysis of the STAT1 DNA-binding mutants

STAT1-negative U3A cells stably transfected with STAT1, STAT1-N460A or STAT1-K336A were stimulated for 20min with 10^3 IU/ml of IFN-γ. STAT Phosphorylation/activation: Proteins were immunoprecipitated with anti-STAT1 antibody and western blots performed with anti-STAT1-P-Tyr701-specific antibody (upper panel) and anti-STAT1 antibody (middle panel). STAT DNA binding: EMSAs of whole cell extracts with a SIE probe (lower panel).

(Result is representative of three experiments.)

Figure 4.13

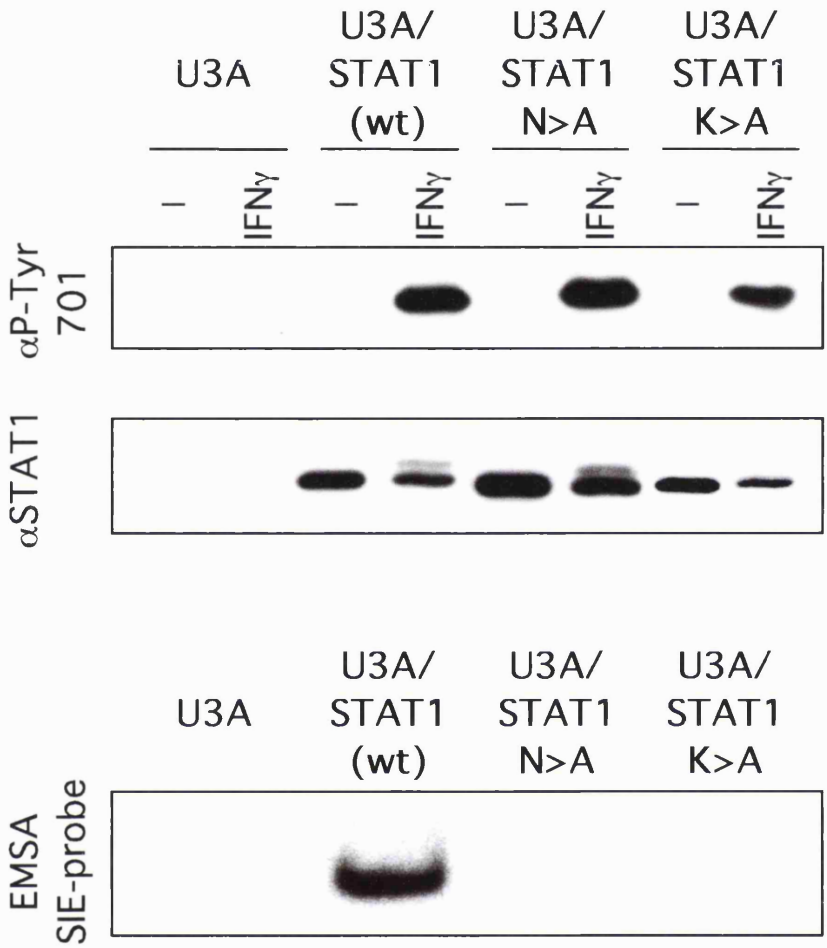
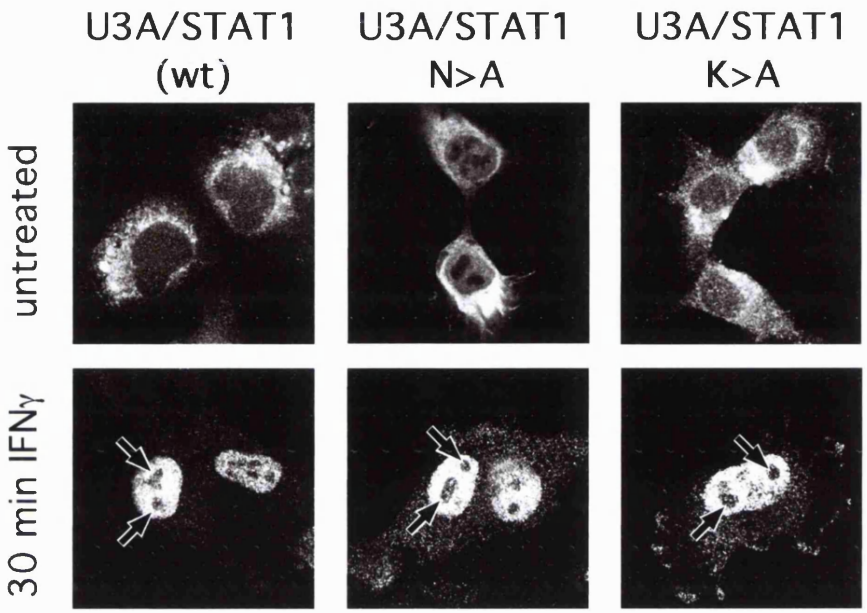

U3A
U3A/ STAT1 (wt)
U3A/ STAT1 N>A
U3A/ STAT1 K>A
–
IFNγ
αP-Tyr 701
αSTAT1
U3A
U3A/ STAT1 (wt)
U3A/ STAT1 N>A
U3A/ STAT1 K>A
EMSA SIE-probe

Fig. 4.14 Exclusion of STAT1 and STAT1 DNA-binding mutants from nucleoli.

STAT1-negative U3A cells stably transfected with STAT1, STAT1-N460A or STAT1-K336A were stimulated for 30min with 10^3IU/ml of IFN-γ. Nuclear translocation: Cells were fixed and stained with antibody to STAT1. Arrows indicate the nucleoli. (Result is representative of three experiments.)

Figure 4.14



Chapter 5: Future Directions

5.1 Expression, Purification and structural analysis of hJAK1

An initial objective was to establish a high level expression system for JAK1 to facilitate structural analysis and crystallisation of JAK1 or JAK1 domains. The hJAK1-M6H/HeLa-S3 expression system described in this thesis could yield enough protein to do a small number of partial digests to potentially identify JAK1-domains. Such domains might be more amenable to bacterial expression, purification, crystallisation and/or could be used to study interactions of JAK1 with other proteins *in vitro*. Additionally, the possible use of the denatured and purified hJAK1-M12H from the bacterial expression system as a substrate in *in gel* kinase assays should be investigated.

5.2 Proteins associated with hJAK1

15 proteins were co-purified with hJAK1-M6H from hJAK1-M6H/HeLa-S3 cells (section 1.2.3) and are being identified using mass spectrometry. Proteins identified can be divided into five groups:

1. Proteins already known to be involved in JAK1 signalling. STATs, receptor chains and molecules like Grb2 are examples for this class of proteins. To 're-identify' components of the JAK/STAT pathway will increase the probability that the additionally identified proteins are involved in JAK1 signalling and the association of the co-purified proteins is specific.

2. Proteins with fully characterised cDNAs, but not yet known to be involved in JAK1 signalling. For instance, in two-hybrid screens protein-arginine methyltransferases were found to be associated to either IFNAR1 (Abramovich et al., 1997) or JAK2 (Pollack et al., 1999) and recently it was demonstrated that arginine methylation of

STAT1 modulates IFN-α/β induced transcription (Mowen et al., 2001). If the molecules are already known, it will be easier to analyse their function in JAK1 signalling. Available reagents like antibodies, knockout mice and functional assays will be used to characterise the protein in context of cytokine signalling.

3. Novel ESTs (at least a partial cDNA encoding a protein with unknown functions). Incomplete ESTs will have to be re-cloned to obyain a full length cDNA and reagents will have to be generated to analyse the function. Therefore, the analysis of these molecules won't be straight forward and the significance of these proteins in cytokine signalling should be established before committing to their more in depth analyses (see below).

4. Completely unknown proteins will have to be cloned and as for group (3.) their importance should be established beforehand.

5. Proteins non-specifically associated with JAK1. Again, these proteins have to be eliminated by additional analyses.

As mentioned above, criteria for significance have to be applied to the identified proteins to increase the probability of a successful future analysis of these molecules. Inducibility of the association and/or phosphorylation is a general criteria for specificity. As described previously (section 3.3.2), the HeLa-S3/hJAK1-M6H expression system has higher constitutive phosphorylation and is therefore not ideal to assess inducibility. Alternatively, once a protein is identified the inducibility of the association and/or phosphorylation can be established in a wild-type non-overexpressing cell system. This would require reagents such as specific antibodies or activity assays, for the candidate protein. A further criteria for specificity is the selective interaction with one of the JAKs (in this thesis JAK1). JAK2-M12H/pcDNA3.1 is available for a differential analysis of JAK1 and JAK2 and could be a useful tool to identify JAK1 specific signalling pathways. The above would make the analysis of known molecules (group 2) most promising for meaningful future results and therefore a priority. Prime candidates after

the initial co-purification are the 100kD, 80kD and the 30kD proteins (table 3.3), which already showed some form of inducibility in the hJAK1-M6H/Hela-S3 cells.

Once the significance of a protein is established, a variety of downstream analyses are possible: For every interacting protein, it would be of interest to determine if the protein is directly interacting with JAK1 or if it is part of a multi-protein complex. This can be tested using *in vitro* interaction systems like the established Far-Western (section 3.2.3.2) or cross-linking analysis. Overexpression and anti-sense RNA analysis may help to characterise the general function of the proteins. For example, overexpression of an inhibitory protein will desensitise the pathway (as seen for the SOCS molecules, see section 3.4.8) and the inhibition of its expression by antisense RNA may prolong or sensitise the response to cytokines. If the identified molecules are kinases or phosphatases, enough is known about their general mechanisms to obtain dominant negative mutants, such as the kinase dead JAKs.

5.3 Phosphorylation sites in hJAK1

Two phosphorylation sites have been provisionally identified in this thesis, threonine1013 and threonine 889. Analysis of the U4A/hJAK1-M12H (T1013>A) cells did not yield conclusive data, but the quantitative difference between wildtype and mutant (T1013>A) JAK1 in STAT1 phosphorylation and STAT1 DNA binding could be of importance. As discussed earlier, the quantitative difference has to be confirmed or disproved by analysis of additional clones of both wildtype and mutant (T1013>A) cell lines. The polyclonal rabbit sera against the phosphorylated form of Thr1013 can be used to determine the phosphorylation status of this site. Several possibilities for future investigations have to be considered: (1) inducibility of phosphorylation by different ligands, (2) dependence of constitutive phosphorylation on components, like FCS, in the media, (3) cell type specificity and (4) influence of phosphorylation on the kinetics in

the signalling events. Additional micro arrays and new assays may help to identify the *in vivo* function of Thr1013. Examples for additional experiments are peptide-fishing with the Thr1013 phosphopetides, *in gel* kinase and phosphatase assays to identify the modifying enzymes.

Phosphorylation of Thr889 has yet to be confirmed and its functional analysis is in progress. Two mutant cell lines [U4A/hJAK1-M12H (T889>A) and (T889>D)] and polyclonal sera against the phosphorylated form of Thr889 have been generated for future analysis, as for Thr1013. As discussed previously, Thr889 is conserved in all JAKs and localised in the ATP binding loop (like tyrosine15 in CDK2). Therefore it is possible that the Thr889 to Ala889 or Asp889 mutants show some functional differences when expressed in U4A cells (section 3.3.4).

The *in vivo* labelling of hJAK1-M6H with [^{32}P]-phosphate and the detection of radioactive tryptic fragments showed that there may be additional phosphorylation sites. If confirmed, these will be analysed as done for Thr1013 and 889. Furthermore, this system may be used for the analysis of additional protein modifications, like methylation, in JAK1.

5.4 STAT1: dynamics and interactions

Daniel Ziecha (Microscopy, ICRF) has established a mathematical model for cytoplasmic diffusion to determine diffusion rates of protein complexes, as well as, theoretical results of FRAP experiments with GFP fusion proteins and their complexes. Comparing theoretical with experimental FRAP data may allow the 'measurement' of average sizes of GFP fusion proteins and their complexes. Further analysis (in collaboration with Daniel Ziecha) of GFP and STAT1-GFP may allow determination of the average size of complexes containing STAT1-GFP before and after activation of the JAK/STAT pathway, as to date it has not been determined whether STAT1 molecules

exist as monomers or in high molecular weight complexes ('statosomes') (Lackmann et al., 1998; Ndubuisi et al., 1999).

As discussed in section 4.3 the STAT1 DNA-binding mutants behave in different ways compared to wildtype STAT1. The STAT1 mutants described in chapter 4 [STAT1 (K336A) and (N460>A)] translocate and accumulate in the nucleus, but do not initiate transcription. The STAT1 (EE428/429AS) mutant (McBride et al., 2000) translocates to the nucleus but is immediately exported back into the cytosol. It has been proposed that such mutations affect the nature of interaction with DNA/protein complexes in the nucleus. Wildtype STAT1 is thought to be able to 'scan' chromosomal DNA for promoter sites with low affinity and then bind with higher affinity to these sites. The STAT1 (K336>A) and (N460>A) mutant is not able to bind specific promoter sites (section 4.2) but may still be able to 'scan' the DNA. STAT1 (EE428/429AS) is probably not able to interact with DNA at all. These differences could be used to establish the 'scanning and binding' model and to identify proteins involved in these differences. For example, in differential protein fishing with wildtype and mutant STAT1 molecules. Furthermore it could be possible to establish if the different interaction with DNA are involved in STAT1 regulation. Hence, is DNA binding stabilising the activated form of STAT1 and prolonging the response time post activation?

5.5 'All or None' JAK/STAT signalling

During the analysis of STAT1 dynamics (section 4.2), using the STAT1-GFP fusion protein, different concentrations of IFN were used to stimulate the cells. The results suggested that different concentrations of IFN were effecting the percentage of cells which translocate more than the amount of STAT1 translocated to the nucleus. If this is true this would indicates that for the JAK/STAT pathway activation is a 'switch'

and that for a given cell the signal is essentially 'on or off'. Even the FACS sorted population of 2C4/STAT1-GFP cells remains very heterogeneous and it is difficult to quantify these observations. It might be possible to use the cloned 2C4 cells and look for surface expression of CD2 (section 1.3.3) by FACS analysis after stimulation with different concentrations of IFN-γ. Provided the CD2 construct in the 2C4 cells is uniformly retained (not in high copy numbers with different activation thresholds and no additional processing or signalling is influencing the surface expression of CD2) it might be possible to detect CD2 in an early and direct response as an 'all or none' expression marker. This would mean for every given cell there are only two levels of CD2 surface expression, if JAK/STAT signalling is 'all or none'. In case of a gradual response it should be possible to detect intermediates.

In future work, priority will be given to the identification of the remaining phosphorylation sites and their characterisation. Hopefully, this will give new insights into the modulation of JAK1 dependent signalling, as well as, possible links between different signal transduction pathways. Once significance is established for any of the co-purified proteins, the characterisation of their interaction with JAK1 and their role in JAK1 signalling will also be of major interest. These two main objectives will be part of the future work in the 'Biochemical Regulatory Mechanisms' laboratory.

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