SIGNALING THROUGH THE JAK/STAT PATHWAY: REGULATION OF TYROSINE KINASE ACTIVITY

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ACADEMIC DISSERTATION

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1 CONTENTS

2 ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to by their Roman numerals in the text.

I. Saharinen, P., Takaluoma, K. and Silvennoinen, O.
Regulation of the Jak2 tyrosine kinase by its pseudokinase domain.
Molecular and Cellular Biology, **20**:3387-3395, 2000

II. Saharinen, P. and Silvennoinen, O.
The pseudokinase domain is required for suppression of basal activity of Jak2 and Jak3 tyrosine kinases and for cytokine-inducible activation of signal transduction.
Journal of Biological Chemistry, **277**, 2002, in press

III. Saharinen, P., Vihinen, M. and Silvennoinen, O.
Autoinhibition of the Jak2 tyrosine kinase is dependent on specific regions in its pseudokinase domain.
Molecular Biology of the Cell, in press

IV. Saharinen, P., Ekman, N., Sarvas, K., Parker, P., Alitalo, K. and Silvennoinen, O.
The Bmx tyrosine kinase induces activation of the Stat signaling pathway, which is specifically inhibited by protein kinase Cδ.
Blood, **90**:4341-4353, 1997

3 ABBREVIATIONS

Bmx	bone marrow tyrosine kinase gene in chromosome X
EGF	epidermal growth factor
EMSA	electrophoretic gel-mobility shift assay
EPO	erythropoietin
ERK	extracellular-signal regulated kinase
GH	growth hormone
GM-CSF	granulocyte-macrophage colony stimulating factor
IFN	interferon
IL	interleukin
JH	Jak homology
Jak	Janus kinase
JNK	c-Jun NH_2-terminal kinase
kDa	kilodalton
MAPK	mitogen-activated protein kinase
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PH	pleckstrin homology
PI3K	phosphatidylinositol-3-kinase
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol myristate ester
PTK	protein tyrosine kinase
PTPase	protein tyrosine phosphatase
PRL	prolactin
PtdIns3,4,5	phosphatidylinositol-3,4,5-triphosphate
RTK	receptor tyrosine kinase
SH	Src homology
STAT	signal transducer and activator of transcription
SCID	severe combined immunodeficiency
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
TPO	thrombopoietin
VEGF	vascular endothelial growth factor
Xid	X-linked immunodeficiency
XLA	X-linked agammaglobulinemia

4 SUMMARY

Extracellular signals regulate the functions of cells of multicellular organisms, such as proliferation, differentiation and apoptosis. Many of these signals are transduced into cells through transmembrane receptors that link ligand binding to activation of intracellular signaling pathways. One of the central mechanisms used in transmembrane signaling is protein tyrosine phosphorylation. In mammals, approximately 100 different tyrosine kinases mediate this function. Given the central role of tyrosine kinases in cellular responses, understanding the regulation and normal function of tyrosine kinases is of critical importance. In the current work, signaling by members from the Jak and Btk/Tec non-receptor tyrosine kinase families was analyzed. Aberrant function of kinases from both families has been implicated in malignant growth of cells.

The Jak kinases are critical mediators of cytokine-induced signaling. The binding of cytokine to its receptor results in activation of cytokine receptor-associated Jak kinases, and initiation of a number of different signaling pathways. In one such pathway, a group of transcription factors termed Signal Transducers and Activators of Transcription, Stats, are activated. Stats have been found essential in regulating cytokine-induced responses in the target cells. In addition, Btk/Tec tyrosine kinases are activated through cytokine receptors. In the current work, the Bmx tyrosine kinase, a member of the Btk/Tec family, was found to activate Stat transcription factors. The identification of Stats as potential substrates for Bmx was the first signaling event characterized for the Bmx kinase.

The Jak kinases have a characteristic domain structure consisting of a tyrosine kinase domain and a catalytically inactive pseudokinase domain, whose function has been unknown. In the current work, the function of the pseudokinase domain was analyzed in Jak2 and Jak3 kinases. The pseudokinase domain was found to suppress the basal activity of Jak2 and Jak3 in the absence of cytokine stimulation. The pseudokinase domain-mediated inhibition of Jak2 was independent of additional regulatory proteins, indicating autoinhibition of Jak2 by its pseudokinase domain. The pseudokinase domain inhibited the catalytic activity of Jak2 by lowering its maximal velocity (Vmax). Distinct regions in the pseudokinase domain were identified that mediate the inhibition, and using molecular modeling the inhibitory regions were located mainly to the C-terminal lobe of the pseudokinase domain.

The pseudokinase domain was also required for cytokine-inducible Jak activation, and in the absence of a pseudokinase domain, Jaks were unresponsive to cytokine stimulation. The results support a model, where the pseudokinase domain is part of both the uninduced and cytokine-induced Jak-receptor complex and acts as a cytokine-inducible switch to link cytokine binding to Jak activation. During ligand-induced receptor dimerization, the inhibitory kinase-pseudokinase domain interaction is relieved resulting in a new interaction between two kinase domains and increased Jak activity. The results from the current work identify a novel way of regulation of Jak activity, which adds to the understanding of the autoregulatory mechanisms of tyrosine kinases.

5 INTRODUCTION

5.1 Type I and type II cytokines

The cellular functions of multicellular organisms, such as growth, differentiation and apoptosis, are regulated by signals from the extracellular environment. In mammals, direct cell-cell contacts, cell-matrix contacts or locally or systemically acting soluble mediators provide the essential signals for the cells. One important group of signals for many cell types is provided by cytokines.

Cytokines are a large and diverse group of secreted, usually locally acting, glycoproteins, which mediate intercellular communication by binding to their specific receptors on the surface of a target cell. The term "cytokine" is loosely defined. One way to classify cytokines is based on their structural and functional properties and the receptor molecules they bind to. Growth factors, such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), utilize receptors with intrinsic tyrosine kinase activity, termed receptor tyrosine kinases (RTKs), while members of the transforming growth factor-β (TGF-β) superfamily utilize receptors with serine/threonine kinase activity. Other subsets of cytokines include the tumor necrosis factor (TNF) family and the chemokine family. Sometimes the term "cytokine" is used only for factors, whose main targets are the hematopoietic cells, and are thus called the hematopoietic cytokines. The hematopoietic cytokines, together with structurally related cytokines acting for example in the neural system, are collectively called type I cytokines and include interleukins (IL) -2, -3, -4, -5, -6, -7, -9, -11, -12, -13, 15, -21, -23, erythropoietin (EPO), thrombopoietin (Tpo), prolactin (PRL), growth hormone (GH), thymic stromal lymphopoietin (TSLP), ciliary neurotrophic factor (CNTF), oncostatin M (OSM), leukaemia inhibitory factor (LIF), cardiotorphin-1 (CT-1), neurotrophin-1 (NNT-1)/B-cell-stimulating factor-3 (BSF-3), and colony-stimulating factors for granulocytes (G-CSF) and granulocyte-macrophages (GM-CSF) (Bazan, 1991). Interferons (IFN) and IL-10 constitute an additional family related to type I cytokines termed type II cytokine family. IL-19, -20, -22, and -24 have been recently identified as IL-10-related cytokines.

The type I and type II cytokines share an α-helical three-dimensional structure. The type I cytokines typically consist of four α-helices, designated from A to D and connected by short loops (Bazan, 1991; Boulay and Paul, 1992; Rozwarski *et al.*, 1994). The type II cytokines, IFN-γ and IL-10, contain 6 α-helices instead of four (Ealick *et al.*, 1991; Zdanov *et al.*, 1995). The helical cytokines function usually as monomers, but IFN-γ and IL-10 are dimers (Walter *et al.*, 1995; Josephson *et al.*, 2001). The receptors utilized by type I and II cytokines are structurally related and form the hematopoietic receptor superfamily (Bazan, 1990). Importantly, receptors of the hematopoietic receptor superfamily share common mechanisms for signal transduction, which have been the subject of the current work. From now on, the term "cytokine" is used to refer to factors, which signal through the hematopoietic cytokine receptors, and these receptors, in turn, are referred to as "cytokine receptors".

5.1.1 Biological effects of cytokines

Practically all cells of the body are influenced by cytokines, but the main function of cytokines is in the hematopoietic system. The hematopoietic cells critically require cytokines for proliferation and development from the hematopoietic stem cell (hematopoiesis, Figure 1) (reviewed in Arai *et al.*, 1990; Watowich *et al.*, 1996). In addition to hematopoiesis, the cytokines regulate immune functions of mature hematopoietic cells, such as mediation of immune responses to viral, bacterial, fungal and parasitic infections, inflammatory response and antibody production. Outside the hematopoietic system, for example IFNs regulate the responses of nearly all cells towards viral and bacterial infections. CNTF exclusively functions in the nervous system by supporting the survival of ciliary and motor neurons (reviewed in Taga and Kishimoto, 1997). LIF functions as a cholinenergic differentiation factor in the nervous system as well, but can also maintain the undifferentiated state of embryonic stem (ES) cells (reviewed in Kishimoto *et al.*, 1994).

A characteristic feature of cytokines is their functional pleiotropy and redundancy. It was initially thought that each cytokine mediates a specific effect on its target cells, but now it is known that most cytokines exert a variety of biological effects on multiple tissue and cell types (Kishimoto *et al.*, 1994). One cytokine can induce growth on some cell types and differentiation on other cells, while a single cell type can respond for example by proliferating to multiple different cytokines. The functional pleiotropy and redundancy of the cytokines can now be partially explained by the utilization of shared receptors chains (Kishimoto *et al.*, 1994; Taga and Kishimoto, 1997). Most cytokine receptors consist of a ligand binding chain that is specific to each cytokine, and a common signal transducing chain utilized by many different cytokines.

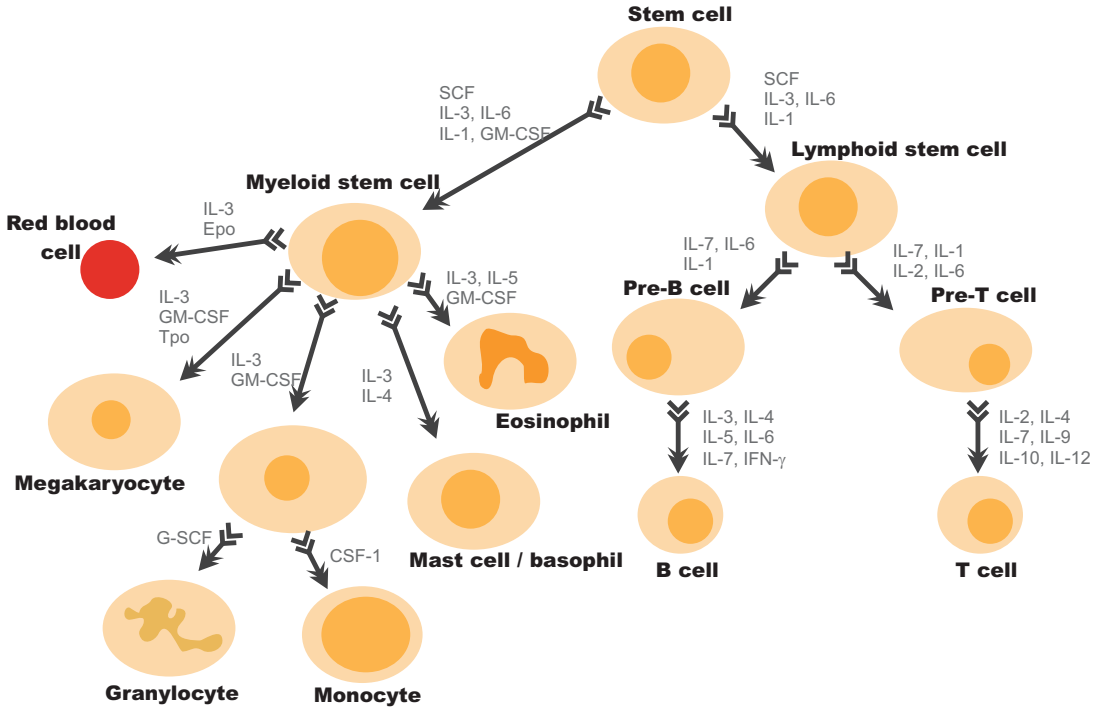

Figure 1. Hematopoiesis

5.2 The cytokine receptor superfamily

5.2.1 Cytokine receptors are composed of multiple chains

Cytokine receptors are membrane glycoproteins that have an extracellular domain of variable length, a single transmembrane domain and an intracellular region (Figure 2). The extracellular domains of cytokine receptors show structural similarity, which is the basis for their classification into the cytokine receptor superfamily (Bazan, 1989; D'Andrea *et al.*, 1989; Gearing *et al.*, 1989; Fukunaga *et al.*, 1990; Goodwin *et al.*, 1990; Idzerda *et al.*, 1990; Itoh *et al.*, 1990). The intracellular regions of cytokine receptors do not contain intrinsic catalytic activity, but link ligand binding to activation of signal transduction by associating with a number of signaling proteins. The mechanisms of signal transduction are shared between the receptors, and thus, in addition to structural similarity, the cytokine receptors are also functionally related (reviewed in Ihle *et al.*, 1995).

The cytokine receptors can be organized into subgroups by the number of receptor subunits and the use of common signaling chains (Figure 2, Table 1). One cytokine receptor subfamily is composed of single chain receptors (EPO, GH, PRL, TPO) (Yoshimura and Misawa, 1998). Other subfamilies utilize common signal transducing chains, which associate with ligand-specific receptor chains to form functional, high-affinity receptor complexes. One subfamily signals through a common gp130 or gp130-like signaling chain in combination with a ligand-specific subunit (reviewed in Taga and Kishimoto, 1997). IL-3, IL-5 and GM-CSF bind to their ligand-specific α-chains and by associating with a common β-chain form a functional, high-affinity receptor (reviewed in Miyajima *et al.*, 1993). Another subfamily forms a complex with a common γ-chain, γ_c. In addition to γ_c, the IL-2 receptor consists of a

Cytokine receptor superfamily	**Cytokines**
Type I cytokine receptors	
Cytokines whose receptors share γ_c, as the signaling chain	IL-2, IL-4, IL-7, IL-9, IL-13$^+$, IL-15, IL-21, TSLP^^
Cytokines whose receptors share β_c, as the signaling chain	IL-3, IL-5, GM-CSF
Cytokines whose receptors share gp130 or a related protein, as the signaling chain	IL-6, IL-11, IL-12*, IL-23, LIF, OSM, CNTF, CT-1, G-CSF, Leptin*, ^NNT-1/BSF-3
Cytokines utilizing single chain receptors, which bind ligand and function as the signaling chain	GH, EPO, PRL, TPO
Type II cytokine receptors	
Cytokines utilizing two or more distinct subunits	IFN-γ, IFN-αs/β/ω/Limitin, IL-10, IL-19, IL-20, IL-22, mda-7/IL-24, AK155/IL-26, FISP

Table 1. The cytokine receptor superfamily. $^+$IL-13 does not share γ_c but uses IL-4Rα, ^^TSLP binds to a related but γ_c independent receptor, *IL-12 and leptin do not share gp130, but their receptors are related to gp130, ^NNT-1/BSF-3 binds to IL-12Rβ1, mda-7 (melanoma differentiation-associated gene), NNT-1 (neurotrophin-1), BSF-3 (B-cell-stimulating factor-3), TSLP (thymic stromal lymphopoietin) (modified from Ihle *et al.*, 1995; Schindler, 2002).

β-chain unique to IL-2 and a ligand-binding α-chain required for formation of the high-affinity IL-2 receptor (reviewed in Leonard *et al.*, 1995). The biological functions within the receptor subfamilies are very similar, and the utilization of common signaling chains explains for the functional redundancy characteristic for cytokines. Type II receptors are related to the above-described type I cytokine receptors and include the receptors for IFNs, IL-10 and a newly identified group of IL-10-related cytokines, IL-19, IL-20, IL-22, IL-24 and IL-26. These receptors consist of at least two different subunits, both of which are used for signaling, but only one binds the cytokine (reviewed in Kotenko and Pestka, 2000).

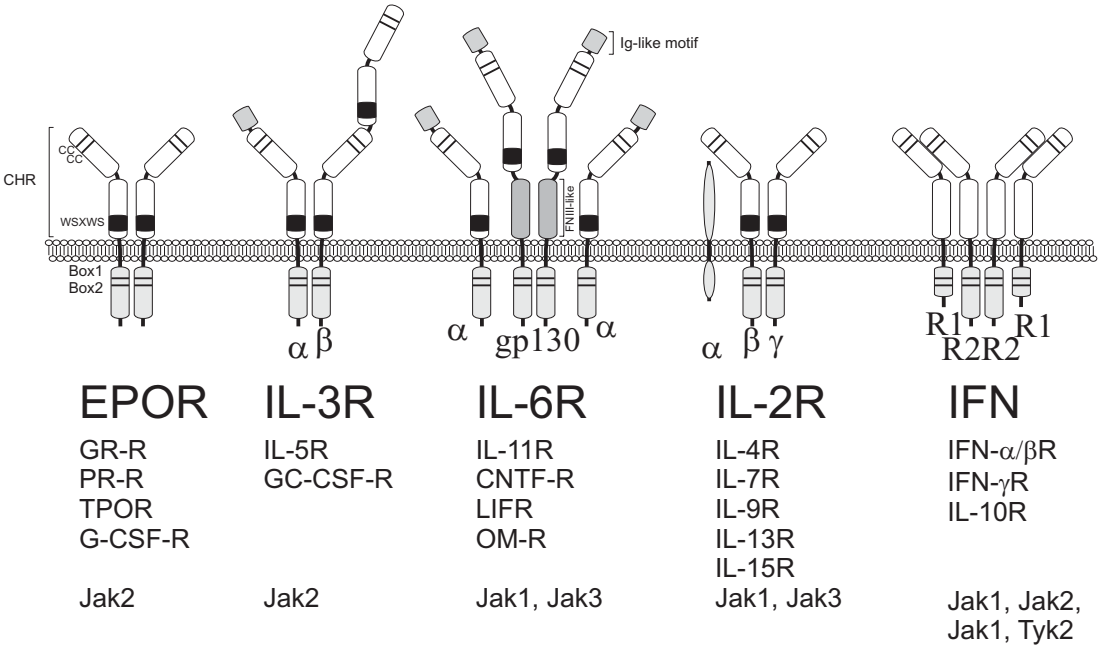

Figure 2. Schematic presentation of cytokine receptor families.

5.2.2 The extracellular domains of cytokine receptors are structurally related

The extracellular domains of type I cytokine receptors are characterized by an approximately 200 amino acids long homologous region with sequence identity between 14 to 25% (reviewed in Cosman *et al.*, 1990). This region is called the cytokine or hematopoietic receptor homology (CHR) domain, and it is composed of two domains, both approximately 90 amino acids long (Bazan, 1990). These 90 amino acid domains are related to fibronectin (FBN) type III domains, often found in cell surface proteins with adhesive functions (Bazan, 1990). The FBN type III domains consist of seven β-strands forming a β-sandwich motif. The N-terminal domain usually contains two pairs of conserved cysteines, which contribute to the tertiary structure of the domain by forming disulfide bridges (Bazan, 1990). The C-terminal

domain contains a conserved WSXWS motif. The three-dimensional structures of the extracellular domains of various cytokine receptors with ligands have shown that the hinge region between the two motifs of a CHR domain forms the binding interface for cytokines (de Vos *et al.*, 1992; Walter *et al.*, 1995; Livnah *et al.*, 1996Chow *et al.*, 2001). The WSXWS motif is located near the hinge region, but does not make contact with the cytokine, but instead interacts with the N-terminal CHR domain stabilizing the overall CHR structure (Quelle *et al.*, 1992; Yoshimura *et al.*, 1992; Hilton *et al.*, 1996; Syed *et al.*, 1998). The type II cytokine receptors have the similar extracellular domain structure as type I receptors, but differ by a unique cysteine distribution and lack of the WSXWS motif. The receptors may contain additional protein domains, such as FBN III or Immunoglobulin (Ig) domains.

5.2.3 The intracellular domains of cytokine receptors mediate signaling functions

The cytokine receptors do not contain any catalytic domains. The cytoplasmic regions of the cytokine receptors are less conserved than the extracellular regions, but the membrane-proximal parts show limited sequence similarity (Murakami *et al.*, 1991). Two conserved regions are referred to as box1 and box2 motifs (Murakami *et al.*, 1991). The consensus for the box1 motif is Al-Ar-Pro-X-Al-Pro-X-Pro or Ar-X-X-X-Al-Pro-X-Pro, where Al= aliphatic, Ar=aromatic and X is any amino acid (reviewed in Ihle *et al.*, 1995). Box1 is located within the first 20 amino acids of the cytoplasmic domain. The box2 motif is characterized by a stretch of hydrophobic amino acids followed by several charged amino acids. A conserved tryptophane residue is located between box1 and 2. Mutations of the box1 proline residues, of the conserved tryptophane and of residues in the box2 motif have been found to inactivate the receptors examined (Murakami *et al.*, 1991; Barge *et al.*, 1996; Haan *et al.*, 2000). Thus, the membrane-proximal regions are critical for overall receptor function. Especially, the membrane proximal regions have been found to contribute to cytokine-induced growth responses of the cells (He *et al.*, 1994; Howard *et al.*, 1995; Jiang *et al.*, 1996).

The membrane distal regions of cytokine receptors also mediate important signaling functions of receptors. The membrane distal part of the G-CSFR has been found to mediate induction of neutrophil-specific genes, and contribute to granulocytic differentiation as well as G-CSF-induced proliferation (Fukunaga *et al.*, 1993; Dong *et al.*, 1994; McLemore *et al.*, 2001). The membrane distal regions contain tyrosine residues that become phosphorylated upon cytokine stimulation, and serve as docking sites for Src homology 2 (SH2) containing signaling proteins. The membrane distal region of the EPOR contains a binding site for the SH2 containing protein tyrosine phosphatase (SHP-1) essential for termination of EPOR signaling (Yoshimura *et al.*, 1990; D'Andrea *et al.*, 1991; Klingmuller *et al.*, 1995). In addition, binding sites for many other signaling proteins have been located within the cytoplasmic domains of cytokine receptors. Jak tyrosine kinases associate with the membrane-proximal box1/box2 motifs (Frank *et al.*, 1994; Quelle *et al.*, 1994; Hackett *et al.*, 1995; Howard *et al.*, 1995Kirken *et al.*, 1995; Lebrun *et al.*, 1995b; Malabarba *et al.*, 1995; Nicholson *et al.*, 1995; Tanner *et al.*, 1995; Jiang *et al.*, 1996; Yan *et al.*, 1996a), while the binding sites for Stat transcription factors

are located in the membrane-distal regions (Greenlund *et al.*, 1994; Fujii *et al.*, 1995; Kirken *et al.*, 1995; Lebrun *et al.*, 1995a; Nicholson *et al.*, 1995; Stahl *et al.*, 1995; Hansen *et al.*, 1996; Quelle *et al.*, 1996; Weber-Nordt *et al.*, 1996; Yi *et al*., 1996; Pezet *et al.*, 1997).

5.2.4 Cytokine-induced receptor dimerization/oligomerization

In receptor activation, the function of the ligand is to induce dimerization/oligomerization of receptor subunits, which enables efficient interaction of the cytoplasmic domains of the receptor chains and of the receptor-associated signaling proteins (reviewed in Heldin, 1995). Each cytokine contains at least two binding sites for its receptor, or alternatively, the cytokine can bind the receptor as a homodimer, as in the case of IFN-γ. The three-dimensional structures of cytokines bound to the extracellular domains of their receptors, have revealed mechanisms how cytokines bind to and mediate activation of their specific receptors (reviewed in Mott and Campbell, 1995; Wells, 1996).

The crystal structure of the complex between the human GH and the extracellular domain of its receptor (GHbp) showed that GH induces homodimerization of its receptor by simultaneously binding two identical receptor subunits (de Vos *et al.*, 1992). Two distinct sites in one GH molecule bind identical CHR sites in both of the two receptors, although the two binding sites on GH have no structural similarity (de Vos *et al.*, 1992). The binding of GH to the GHbp occurs sequentially; one GHbp molecule forms first a complex with GH, followed by binding of the second GHbp (Cunningham *et al.*, 1991).

In certain other receptors other domains, in addition to CHR, make contacts with the cytokine. For example, gp130 ligands contain three distinct epitopes interacting with different receptors (reviewed in Bravo and Heath, 2000). One site is involved in interaction with a non-signaling receptor subunit, a second site is engaged with CHR of gp130, and a third site with a second signaling subunit (gp130, LIF-R or OSM-R). When the ligand makes two contacts with gp130, one contact is mediated via an Ig-like domain (IGD) located on the C-terminal side of CHR. Thus, ligands with two binding sites for gp130 will form hexameric signaling complexes (2:2:2 gp130: ligand: nonsignaling chain), and ligands binding just one copy of gp130 signal through trimeric complexes.

IFN-γ interacts as a homodimer with the ligand-binding IFNγRI chain, and induces dimerization of the receptor subunits (Walter *et al.*, 1995). However, the two IFNγRI chains in the dimer do not interact with each other, as shown by the crystal structure of the complex between IFN-γ and its soluble high-affinity receptor (Walter *et al.*, 1995). This makes the IFNγRI dimer inactive, although the cytoplasmic domains bind signaling proteins (Kotenko *et al.*, 1995). Activation of IFNγ receptor requires association of a second chain, IFNγRII, to form a tetrameric complex (Kotenko *et al.*, 1995).

The structures for different cytokine-receptor complexes have shown that although the ligand-receptor interfaces are large, a cluster of few amino acids forms an energetic “hot spot” in the center of the binding interface responsible for the binding affinity (reviewed in Wells, 1996; Bravo and Heath, 2000). The most significant contacts are hydrophobic, and contribute to stabilization of the interaction mainly by decreasing the dissociation rate of the complex.

These findings have suggested that it might be possible to design small molecule agonists/antagonists that would interact with the few functional receptor epitopes that mediate cytokine-receptor interactions.

5.2.5 Cytokine-induced conformational change leads to receptor activation

Even though cytokine-induced dimerization/oligomerization of receptor chains is required for receptor activation, dimerization as such is not sufficient for activation. There is evidence that receptor dimers can adopt also inactive conformations (reviewed in Ballinger and Wells, 1998). The crystal structure of the extracellular domain of the EPO receptor (EPObp) in complex with EPO or two different EPO-mimetic peptides exhibiting either agonist or antagonist activity showed that all three ligands formed receptor homodimers. However, the relative orientation of receptor chains in dimers with EPO mimetics differed from that with EPO (Livnah *et al.*, 1998; Syed *et al.*, 1998). This interdomain orientation was found critical for optimal signaling, most likely affecting the relative orientation of the cytoplasmic domains as well. Thus, dimerization of receptor subunits may result in different types of receptor complexes, either competent or incompetent to connect to intracellular signaling.

The crystal structure of the unliganded extracellular domain of EPObp further showed that two EPObp molecules formed a dimer in the absence of ligand, but this dimer was inactive (Livnah *et al.*, 1999). The C-terminal ends of EPObp molecules at the predicted start site of the transmembrane region were 73 Å apart, compared to 39 Å in the EPObp-EPO agonist complex (Livnah *et al.*, 1999). In addition, *in vivo* data has confirmed that distinct dimers exist for unliganded and ligand-bound EPO receptors, and that ligand binding modulates the conformation of the cytoplasmic regions in a receptor dimer to allow signal transduction (Remy *et al.*, 1999). Thus, the binding of a cytokine induces dimerization or oligomerization of receptor chains, with a concomitant change in receptor conformation, thereby resulting in receptor activation and initiation of downstream signaling pathways (Figure 3).

5.3 Tyrosine phosphorylation as a mechanism of cellular signaling

Protein tyrosine phosphorylation, discovered in 1979, is the major mechanism of transmembrane signaling mediated by virtually all types of transmembrane receptors (reviewed in Hunter, 1998, 2000). The overall level of tyrosine phosphorylated proteins in vertebrate cells is very low, approximately 0.05%,

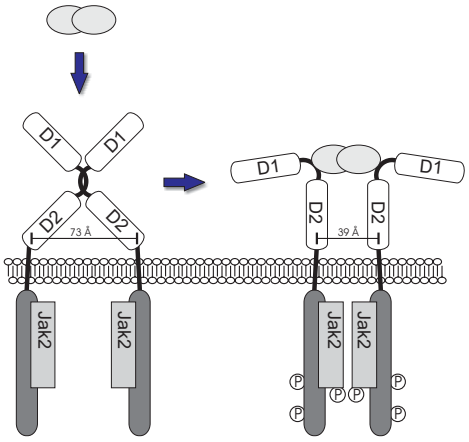

Figure 3. Schematic presentation of EPOR dimers (modified from Livnah *et al.*, 1999).

compared to phosphoserine in 90% of cellular proteins (reviewed in Hunter, 1998). Tyrosine phosphorylation as a signaling mechanism is only present in multicellular organisms from *Dictyostelium* to mammals, but not in yeast or lower organisms.

Tyrosine phosphorylation is mediated by protein tyrosine kinases (PTKs), of either receptor (RTKs) or non-receptor type (NRTKs) (reviewed in Hubbard and Till, 2000). RTKs are single spanning transmembrane proteins containing intrinsic tyrosine kinase domains in their cytoplasmic domains. NRTKs contain various protein domains, which recognize, for example, a phosphorylated tyrosine (SH2, PTB) or polyproline (SH3) in the context of a short linear peptide or for example phospholipid (PH). NRTKs can associate through their protein domains or via lipid modification to plasma membrane or interact with transmembrane receptors linking ligand binding to downstream signaling. Mammals contain 32 NRTKs, forming at least ten distinct families (Figure 4).

Ligand binding induces receptor dimerization and a conformational change of the receptor chains resulting in activation of intrinsic or associated PTKs. The receptor becomes phosphorylated on its tyrosine residues, and these residues recruit phosphotyrosine binding proteins, such as PTKs, adapter proteins, protein tyrosine phosphatases (PTPases), transcription factors etc. to the transmembrane receptor. These proteins form larger complexes through interaction of their modular signaling domains. Thus, the ability of certain protein domains to specifically recognize phosphotyrosine formed upon ligand binding results in formation of a high local concentration of signaling proteins at the plasma membrane, which enables activation of multiple signaling pathways. These signaling pathways are attenuated by the action of PTPases or regulatory proteins, through receptor endocytosis, feedback serine phosphorylation or phosphorylation-dependent ubiquitination and degradation.

The catalytic domains in PTKs are approximately 300 amino acids long and contain considerable sequence conservation of catalytically important residues (reviewed in Hanks *et al.*, 1988). The three-dimensional structures of kinase domains are highly conserved being composed of two lobes (reviewed in Hubbard and Till, 2000). The N-terminal (N) lobe is smaller and consists of β-sheets except for one α-helix, αC, which has important regulatory functions (reviewed in Huse and Kuriyan, 2002). The C-terminal (C) lobe is predominantly α-helical. ATP binds in a cleft formed between the two lobes, and the substrate binds across the nucleotide-binding pocket.

A sequence called activation loop (A-loop) in PTK domains contains tyrosine residues with a regulatory function. The PTK domain can exist in two distinct conformations. The active conformations of PTK domains are extremely similar; the A-loop tyrosines are phosphorylated and the A-loop lies out from the catalytic cleft. In the inactive conformation, the unphosphorylated A-loop may occupy the catalytic cleft preventing ATP or substrate binding. The inactive conformations of PTK domains differ considerably, and the knowledge of the inactive conformation has turned out to be important in the design of kinase inhibitors (Schindler *et al.*, 2000). Several distinct mechanisms are utilized to keep the kinase domains inactive (reviewed in Huse and Kuriyan, 2002). These may involve interactions with other protein domains in the molecule, which often directly or indirectly induce conformational

changes in the αC helix in the N lobe, which in turn modulates the A-loop conformation. Regulatory mechanisms of tyrosine kinases are discussed more extensively in chapter 9.6 in Discussion.

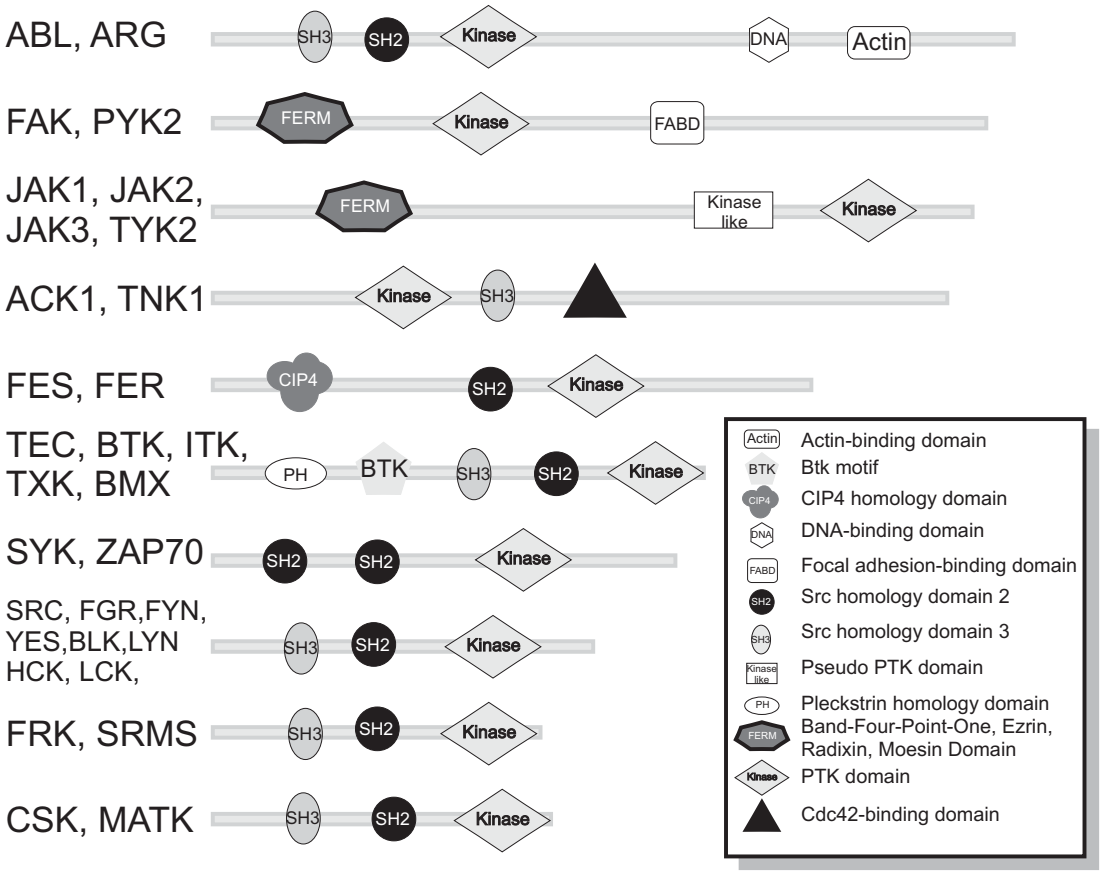

Figure 4. Mammalian non-receptor tyrosine kinase families.

5.4 Signaling through cytokine receptors, the Jak/Stat pathway

5.4.1 Background

Cytokine receptors lack intrinsic tyrosine kinase activity, but cytokine binding triggers rapid tyrosine phosphorylation of cytoplasmic signaling proteins. In the beginning of the 1990s, rapid progress was made in elucidating the events leading to altered gene expression from a cytokine-bound receptor. Tyrosine kinases belonging to a new tyrosine kinase family were cloned using low-stringency hybridization and degenerate PCR, and named as Jak1, Jak2, Jak3 and Tyk2 (for Just another kinase or Janus) kinases (Wilks, 1989; Firmbach-Kraft *et al.*, 1990; Krolewski *et al.*, 1990; Wilks *et al.*, 1991; Harpur *et al.*, 1992; Silvennoinen *et al.*, 1993c; Kawamura *et al.*, 1994; Rane and Reddy, 1994; Takahashi and Shirasawa, 1994; Witthuhn *et al.*, 1994). Using cell lines defective in IFN responses, Jaks were identified as mediators of IFN signaling (Velazquez *et al.*, 1992; Muller *et al.*, 1993; Silvennoinen *et al.*, 1993a; Watling *et al.*, 1993), and subsequently, found to be activated by a vast number of other cytokines as well (Table 2) (Argetsinger *et al.*, 1993; Witthuhn *et al.*, 1993; Johnston *et al.*, 1994; Lutticken *et al.*,

1994; Miyazaki *et al.*, 1994Russell *et al.*, 1994; Stahl *et al.*, 1994; Witthuhn *et al.*, 1994; Johnston *et al.*, 1995a). The Signal Transducers and Activators of Transcripion, Stats, were first identified as DNA-binding proteins in IFN-regulated gene expression (Fu, 1992; Fu *et al.*, 1992; Schindler *et al.*, 1992a; Schindler *et al.*, 1992b; Shuai *et al.*, 1992; Veals *et al.*, 1992; Shuai *et al.*, 1993, reviewed in Darnell *et al.*, 1994). Following cloning of the IFN-activated Stat1 and Stat2, five additional members of the Stat transcription factor family were cloned and found to be activated by a different set of cytokines (Table 2) (Akira *et al.*, 1994Hou *et al.*, 1994; Tian *et al.*, 1994; Wakao *et al.*, 1994; Yamamoto *et al.*, 1994; Zhong *et al.*, 1994a, b; Azam *et al.*, 1995; Gouilleux *et al.*, 1995; Hou *et al.*, 1995; Liu *et al.*, 1995; Mui *et al.*, 1995a; Pallard *et al*., 1995; Quelle *et al.*, 1995a; Wakao *et al.*, 1995; Lin *et al.*, 1996).

The finding of Jaks and Stats as mediators of cytokine signaling led to the concept of the Jak-Stat signaling pathway, formed by Jak tyrosine kinases and their substrates, Stats, which at the same time are signal transducer proteins and transcriptional activators, hence the name. The Jak-Stat pathway turned out to be unique being formed of only two components. Animal models defective in individual Jaks and Stats have revealed essential and non-redundant roles for Jak and Stat proteins in cytokine signaling, in rather well agreement with the specific association of different Jaks and different Stats with only a subset of cytokine receptors (reviewed in Leonard and O'Shea, 1998). In addition to cytokine receptors, Jaks and Stats have been found to be activated by a variety of other receptors, such as growth factor receptors for EGF (Silvennoinen *et al.*, 1993b), PDGF (Vignais *et al.*, 1996), HGF (Boccaccio *et al.*, 1998) and VEGF (Korpelainen *et al.*, 1999; Bartoli *et al.*, 2000), and through a seven transmembrane domain receptor AT1 (Marrero *et al.*, 1995) (Table 2), but the role of Jak/Stat activation in these receptors is not well-defined. The Jak/Stat pathway exists also in lower organisms, such as *Drosophila melanogaster* and *Coenorhabditis elegans (*Zeidler *et al.*, 2000). In *Dictyostelium discoiduem,* a Stat molecule has been characterized, which is activated via a seven transmembrane domain receptor (Araki *et al.*, 1998, reviewed in Aubry and Firtel, 1999).

5.4.2 Overview of signaling by the Jak/STAT pathway

The sequence of events leading to altered gene expression from a cytokine-bound receptor begins with activation of the receptor-associated Jaks, which is critical for further signal transduction (reviewed in Ihle *et al.*, 1995; Schindler, 1999; Imada and Leonard, 2000; Rane and Reddy, 2000; Candotti *et al.*, 2002). Cytokine receptors composed of two or more different chains activate at least two different Jak kinases, while single chain receptors activate typically Jak2.

The activated Jak kinases phosphorylate tyrosine residues in the intracellular domains of receptor chains, thus creating docking sites for SH2 domain-containing signaling proteins, such as Stats, which reside latent in the cytoplasm in the absence of cytokine stimulation (reviewed in Darnell *et al.*, 1994; Horvath and Darnell, 1997). Jaks phosphorylate the receptor-associated Stats, which subsequently dissociate from the receptors, dimerize through their SH2 domains and translocate to nucleus (Shuai *et al.*, 1994; Greenlund *et al.*, 1995). The Stat dimers bind to specific elements in the promoter regions of their target genes to induce transcription together with a number of other transcriptional co-activators (Figure 5). This simplified version of Jak/Stat signaling is becoming more complex as regulators of this

pathway are identified (reviewed in Yasukawa *et al.*, 2000). Also, there is evidence for cross-talk between different signaling pathways. For example, GH induces EGFR signaling by activating Jak2, which phosphorylates the Grb2 binding site in EGFR (Yamauchi *et al.*, 1997).

Cytokine /growth factor	**Jak kinase activated**	**Stats activated**
Type I cytokines		
Cytokines whose receptors share γ_c		
IL-2, IL-7, IL-9, IL-15	**Jak1, Jak3**	**Stat5a, Stat5b**, Stat3
IL-4	**Jak1, Jak3**	**Stat6**, Stat5a, Stat5b
IL-13	Jak1, Jak2, Tyk2	Stat3, Stat6
IL-21	Jak1, Jak3	Stat1, Stat3, Stat5
TSLP	none	Stat5
Cytokines whose receptors share β_c		
IL-3, IL-5, GM-CSF	**Jak2**	**Stat5a, Stat5b**
Cytokines whose receptors share gp130		
IL-6, IL-11, LIF, OSM, CNTF, CT-1	**Jak1**, Jak2	Stat1, **Stat3**
IL-12	**Tyk2**, Jak2	**Stat4**
Leptin	Jak2	Stat3
IL-23	?	Stat4
NNT-1/BSF-3	Jak1, Jak2	Stat1, Stat3
G-CSF	Jak1, Jak2	Stat3
Cytokines with homodimeric receptors		
GH	**Jak2**	Stat5a, **Stat5b**, Stat3
PRL	**Jak2**	**Stat5a**, Stat5b
EPO, TPO	**Jak2**	**Stat5a, Stat5b**
Type II Cytokines		
IFN-γ	**Jak1, Jak2**	**Stat1**, Stat2
IFN-αs/β/ω/Limitin	**Jak1**, Tyk2	**Stat1, Stat2**, Stats3-6
IL-10	Jak1, Tyk2	Stat3
IL-19, IL-20, IL-24	?	Stat1,Stat3
IL-22	Jak1, Tyk2	Stat1, Stat3, Stat5
Growth factors		
EGF	Jak1, Jak2	Stat1, Stat3, Stat5
PDGF	Jak1, Jak2	Stat1, Stat3, Stat5, Stat6
VEGF	?	Stat1, Stat3, Stat5, Stat6
HGF	?	Stat1, Stat3
CSF-1	Jak1, Tyk2	Stat1, Stat3, Stat5
Seven transmembrane domain receptors		
Angiotensin II AT1	Jak2, Tyk2	Stat1, Stat2

Table 2. Activation of Jaks and Stats. In certain receptors, the Jaks and Stats with essential signaling functions discovered to date are indicated in bold (modified from Leonard and O'Shea, 1998; Kisseleva *et al.*, 2002; Schindler, 2002).

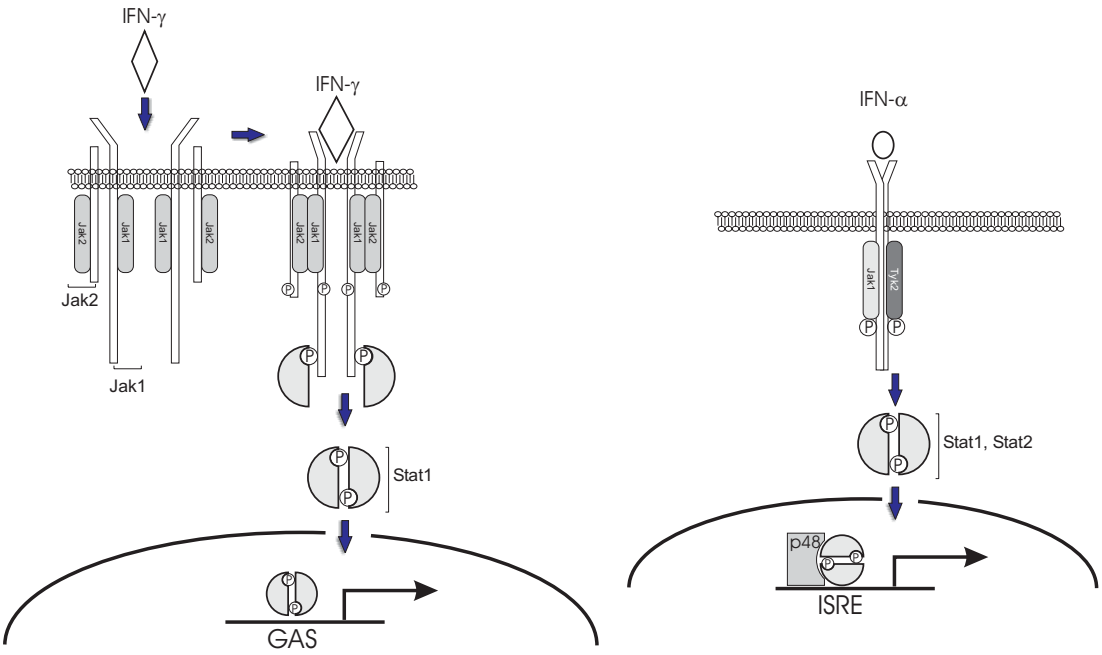

Figure 5. Jak/Stat signaling through the IFNγ and IFNα receptors.

5.4.3 Type I and type II interferons and two different types of Stat signaling

Signaling by IFNs represents two different concepts found in Stat signaling, (Figure 5) (reviewed in Kotenko and Pestka, 2000). The interferons can be divided into two groups, type I (IFN-αs, IFN-β, IFN-ω and IFN-τ) and type II interferons (IFN-γ). IFN-αRI associates with Tyk2 and IFN-αRII with Jak1, whereas IFN-γRI binds Jak1 and IFN-γRII Jak2. Ligand binding induces activation of associated Jaks, and subsequent tyrosine phosphorylation and activation of Stats (Velazquez *et al.*, 1992; Muller *et al.*, 1993; Silvennoinen *et al.*, 1993a; Watling *et al.*, 1993). IFN-γ predominantly activates Stat1, which forms homodimers and binds to a so-called GAS (IFN-γ activated sequence) element in the target promoters (Decker *et al.*, 1991; Lew *et al.*, 1991; Shuai *et al.*, 1992). Type I IFNs activate Stat1 and Stat2, which form heterodimers, and the dimers further associate with a third protein, IRF-9 (p48), to form the ISGF3 complex (Dale *et al.*, 1989a; Fu *et al.*, 1992Schindler *et al.*, 1992b). The DNA binding specificity of ISGF3 differs from that of other Stats, and it specifically interacts with promoters containing ISRE (IFN-stimulated response element) elements (Dale *et al.*, 1989b; Kessler *et al.*, 1990). The Stat1, Stat3, Stat4, Stat5a/b or Stat6 homodimers, or heterodimers, which are formed between Stat1 and Stat3, or between Stat5a and Stat5b, bind DNA sequences that are very similar to the GAS sequence, and most of the above-mentioned dimers can bind to GAS (reviewed in Decker *et al.*, 1997). However, the ISGF3 complex containing the Stat1-Stat2 heterodimer binds only ISRE sequences. Thus, ISRE elements appear to be a unique feature of type I IFN-induced signaling.

5.5 Jak tyrosine kinases

5.5.1 Expression of Jak kinases

The Jak family of non-receptor protein tyrosine kinases comprises of four mammalian members: Jak1, Jak2, Jak3 and Tyk2. In humans, the gene for Jak1 is located in chromosome 1p31.3, the Jak2 gene in chromosome 9p24, the Jak3 gene in chromosome 19p13.1 and the gene for Tyk2 in chromosome 19p13.2 (Firmbach-Kraft *et al.*, 1990; Pritchard *et al.*, 1992; Kumar *et al.*, 1996; Riedy *et al.*, 1996). The murine genes are located on chromosomes 4 (Jak1), 19 (Jak2) and 8 (Jak3 and Tyk2). Jak2 has two transcripts, and multiple alternatively spliced forms of Jak3 have been detected (Rane and Reddy, 1994; Ihle *et al.*, 1995; Lai *et al.*, 1995; Gurniak and Berg, 1996), one of which lacks kinase activity (Lai *et al.*, 1995), but the functional significance of these variant transcripts is not known. In addition, in *Drosophila melanogaster*, the *hopscotch* (*hop*) locus encodes a Jak homologue, Hop (Binari and Perrimon, 1994). In slime mold *Dictyostelium discoiduem* kinases (DPYK3 and DPYK4) with tandem kinase domain structure have been cloned, but overall they are not highly homologous to Jaks (Adler *et al.*, 1996).

The Jak kinases range in molecular mass between 110-140 kDa. Jak1, Jak2 and Tyk2 are ubiquitously expressed, but expression of Jak3 is more restricted. The expression of Jak3 is constitutive in NK cells and thymocytes and inducible in T cells, B cells and myeloid cells (Kawamura *et al.*, 1994; Rane and Reddy, 1994; Musso *et al.*, 1995; Tortolani *et al.*, 1995; Gurniak and Berg, 1996; Sharfe *et al.*, 1997). In addition to hematopoietic cells, Jak3 is also expressed in vascular smooth muscle cells and endothelium (Verbsky *et al.*, 1996).

5.5.2 The tandem kinase domain motif is a unique feature in Jak kinases

The domain structure of mammalian Jaks and *Drosophila* Hop is conserved (Figure 6). Based on sequence conservation, the Jak kinases are divided into seven Jak homology (JH) domains starting from the tyrosine kinase domain located at the C-terminus of Jaks and designated as JH1 (reviewed in Ziemiecki *et al.*, 1994). The hallmark in Jak kinases is the presence of a tandem kinase domain structure, which is unique among tyrosine kinases (Hubbard and Till, 2000). The tandem kinase domain structure is composed of the tyrosine kinase domain, JH1, and a pseudokinase or kinase-like domain, JH2, on the N-terminal side of JH1. Only the JH1 domain is catalytically active. The tandem kinase domain comprises approximately 50 % of the entire Jak molecule.

The JH1 domain has all the characteristics of a typical tyrosine kinase domain (Hanks *et al.*, 1988). JH1 is approximately 300 amino acids long, corresponding to 30 kDa. Mutation of the conserved lysine directly involved in ATP binding in kinase subdomain II, abrogates kinase activity (Briscoe *et al.*, 1996; Gurniak and Berg, 1996). The A-loop of Jaks contains a conserved double tyrosine motif, which is phosphorylated in Jaks in response to cytokine stimulation. Phosphorylation of A-loop tyrosines in Jaks regulates their catalytic activity, but the role of these two tyrosines varies between Jak family members (Gauzzi *et al.*, 1996; Feng *et al.*, 1997; Liu *et al.*, 1997; Zhou *et al.*, 1997). Phosphorylation of the first tyrosine in the double tyrosine motif is critical for the catalytic activity of Jak1 and Jak2, but it is less important for

Jak3 activity (Feng *et al.*, 1997; Liu *et al.*, 1997; Zhou *et al.*, 1997). In Jak3, mutation of the second tyrosine increases catalytic activity, but a similar mutation in Jak1 or Jak2 has no effect (Feng *et al.*, 1997; Liu *et al.*, 1997; Zhou *et al.*, 1997).

The JH2 domain has sequence similarity to kinase domains, but several of the residues required for phosphotransferase activity are altered from the canonical motifs, and consequently, JH2 has been considered catalytically inactive, and this has been confirmed experimentally (Wilks *et al.*, 1991; Frank *et al.*, 1995; Velazquez *et al.*, 1995). Thus, the JH2 domain is called the pseudokinase or the kinase-like domain. For example, the JH2 domains lack the third glycine in the conserved GXGXXG motif in kinase subdomain I, which normally forms the nucleotide binding loop (Hanks *et al.*, 1988). JH2 also lacks the invariant aspartic acid, functioning as the proton acceptor in the phosphotransfer reaction and located in the catalytic loop in subdomain VIb (Hanks *et al.*, 1988). Furthermore, JH2 lacks a conserved phenylalanine in the PEG motif in subdomain VII that is important for binding of the adenine ring of ATP (Hanks *et al.*, 1988).

At the beginning of the current work, the function of the JH2 domain was not known. Two reports had been published leaving the role of the JH2 domain unclear. In Tyk2, the JH2 domain had been deleted, resulting in inactivation of the kinase and inability of Tyk2 to mediate IFN-α signaling (Velazquez *et al.*, 1995), but the deletion of the Jak2 JH2 domain did not show a similar effect in GH signaling (Frank *et al.*, 1995). During the current work, a number of investigations were conducted to analyze the function of the JH2 domain, including those presented in the current work, and are reviewed in chapters 9.3-9.7 in the Discussion.

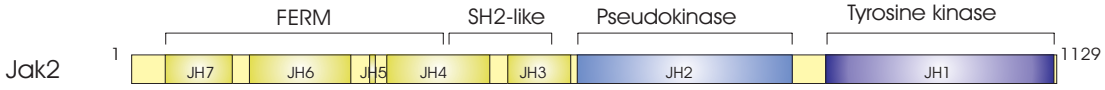

Figure 6. Schematic domain structure of Jak kinases.

5.5.3 The N-terminal half of Jak kinases contains a FERM domain

For many years, it was acknowledged that the N-terminal half of Jaks, containing JH domains 3-7, is unique among tyrosine kinases and does not contain any homology to known protein domains. However, recent studies based on computational methods indicated the presence of a SH2-like domain and a band-four-point-one, ezrin, radixin, moesin (FERM) homology domain in JH3-JH4 and JH4-JH7 regions of Jaks, respectively (Higgins *et al.*, 1996; Girault *et al.*, 1999; Kampa and Burnside, 2000; Al-Lazikani *et al.*, 2001).

In addition to Jaks, FERM domains are found only in one tyrosine kinase family, the FAK tyrosine kinases, although the FERM domains are widespread in other proteins (Chishti *et al.*, 1998). The FERM domain is a 300 amino acid domain, composed of three subdomains that form a compact clover-shaped structure (Turunen *et al.*, 1998; Hamada *et al.*, 2000; Pearson *et al.*, 2000). Subdomain A is a ubiquitin-like fold, subdomain B is an acyl-coenzyme A binding

protein-like fold and subdomain C is a PTB or PH fold. The linker between subdomains A and B, which is at the center of a hydrophobic core, has a critical role in stabilizing the structure of the FERM domain.

The FERM domains mediate often interactions with cytoplasmic domains of membrane proteins (Chishti *et al.*, 1998). Also, in Jaks the region required for binding to cytokine receptors is located within the FERM domain. For Jak3, the minimal region required for binding to γ_c is within JH7-JH6 (Chen *et al.*, 1997; Cacalano *et al.*, 1999). The JH7-JH6 region in Jak2 is crucial and sufficient for binding to GM-CSF and GHR (Frank *et al.*, 1995; Zhao *et al.*, 1995). The JH6-7 domains mediate also specific binding of Jak2 to IFNγRII and EPOR, but in these receptors the JH3-5 region contributes to binding as well (Kohlhuber *et al.*, 1997; Huang *et al.*, 2001). Similarly, the Tyk2 region required for binding to IFNαRI has been mapped to JH7-JH6, but regions within JH3-5 contribute to formation of the receptor-Tyk2 complex (Richter *et al.*, 1998; Yan *et al.*, 1998). Jak1 binds to gp130 through its FERM domain, and within the FERM domain, the binding site has been located in the loop 4 of the first subunit, the ubiquitin-like or β-grasp fold (Haan *et al.*, 2001; Hilkens *et al.*, 2001). This region is located within the JH7 domain in Jak1, and shows considerable sequence differences between Jak family members, in accordance with differential binding specificity generated by the JH7 domains of Jaks (Haan *et al.*, 2001). In line with the role of the Jak FERM domain in cytokine receptor interactions, the FERM domain in FAK has been found to mediate binding to EGFR (Sieg *et al.*, 2000).

The Jak FERM domain has been found to enhance the cell surface expression of certain cytokine receptors. The Tyk2 JH3-7 region was first found to be required for expression of the IFNαRI and recently, Jak2 and Jak1 were found to be required for proper folding and/or processing of EPOR and OSMR, respectively, by associating with the cytoplasmic domains of the cytokine receptors already in the endoplastic reticulum (ER) (Gauzzi *et al.*, 1997; Huang *et al.*, 2001; Radtke *et al.*, 2002). The kinase activity of Jaks is not required for promoting receptor expression, indicating that Jaks are essential structural components of the cytokine receptors. However, in this respect the cytokine receptors may differ, since Jak3 is not required for expression of γ_c (Suzuki *et al.*, 2000).

5.5.4 A SH2-like domain is present in Jak kinases

SH2 domains are common in NRTKs, like in Src and Btk families (Hubbard and Till, 2000). The presence of a SH2 domain in the JH3-4 region of Jaks is supported by several data including sequence-based predictions of secondary structures as well as multiple alignments of several SH2 domains from different proteins (Kampa and Burnside, 2000; Al-Lazikani *et al.*, 2001). However, the SH2 domain of Tyk2 does not contain a key arginine residue required for phosphotyrosine binding, but instead contains a histidine residue, which is estimated to be neutral at pH 7 (Al-Lazikani *et al.*, 2001). Thus, the SH2 domain of Tyk2 may have a different binding specificity, and be unable to bind phosphotyrosine (Al-Lazikani *et al.*, 2001). The function of the SH2 domain in Jaks is not known, and studies in cultured cells failed to show any effect upon mutation of the conserved arginine in Jak2 SH2 (Kohlhuber *et al.*, 1997).

5.5.5 The role of Jak kinases in the initiation of signal transduction

The key step in signaling through cytokine receptors is the activation of Jak kinases. Jaks are constitutively associated with the membrane-proximal regions of cytokine receptors, although in some cases Jak-receptor interaction is increased upon ligand binding. In the absence of ligand, Jaks remain inactive, but ligand-induced conformational change in the receptor complex leads to Jak activation through reciprocal interaction of two juxta-positioned Jak kinases (Remy *et al.*, 1999). Jak activation involves auto/transphosphorylation of their A-loop tyrosines (Gauzzi *et al.*, 1996; Feng *et al.*, 1997; Liu *et al.*, 1997; Zhou *et al.*, 1997). The Jak kinases do not need other tyrosine kinases for their activation, and supporting this, homodimerization of two Jak2 kinases has been found to be sufficient for induction of signaling (Sakai *et al.*, 1995; Nakamura *et al.*, 1996).

In single-chain receptors dimerization of two Jak2 kinases results in induction of Jak2 activity. In receptors composed of multiple chains, Jak activation is a result of interplay between two different Jak kinases, which has been elucidated to some extent using cell lines defective in IFN-α and IFN-γ signaling (Muller *et al.*, 1993; Watling *et al.*, 1993). In cells lacking Jak1, expression of a kinase-inactive Jak1 can sustain substantial IFN-γ-inducible gene expression, suggesting a structural as well as an enzymatic role for Jak1 in IFN-γ signaling (Briscoe *et al.*, 1996). On the other hand, kinase-negative Jak2 cannot sustain IFN-γ-inducible gene expression in cells lacking endogenous Jak2, indicating that catalytic activity of Jak2 is essential for IFN-γ signaling (Briscoe *et al.*, 1996). In the absence of Jak2, however, Jak1 is able to induce low level of Stat1 activation (Briscoe *et al.*, 1996).

A kinase domain deletion mutant of Tyk2 can sustain a very low level of IFN-α-inducible gene expression, while kinase-inactive Jak1 does not, suggesting a major role for Jak1 in IFN-α signaling (Velazquez *et al.*, 1995). Kinase-inactive form of Tyk2 is also phosporylated upon IFN-α stimulation in Tyk2 negative cells reconstituted with this Tyk2 mutant (Gauzzi *et al.*, 1996). A role for Tyk2 as an amplifier of cytokine signaling, rather than as an initiator of signaling, is also suggested by results from Tyk2-deficient mice (Karaghiosoff *et al.*, 2000; Shimoda *et al.*, 2000). In the cells from $Tyk2^{-/-}$ mice, the immediate signaling events following IFN-α treatment were present, although reduced, while IFN-α-mediated responses were totally lacking in $Jak1^{-/-}$ mice (Rodig *et al.*, 1998; Karaghiosoff *et al.*, 2000; Shimoda *et al.*, 2000). Studies using the Tyk2 negative cell line, 11.1, indicated a structural role for Tyk2 in mediating formation of the functional IFN-α receptor complex at the cell surface. The Tyk2 knockout mice, however, did not show a similar result (Gauzzi *et al.*, 1997; Richter *et al.*, 1998; Yeh *et al.*, 2000). Altogether, these results suggest that Jaks are, to some extent, interdependent in their activation, but are not functionally symmetrical. Furthermore, Jaks have also a structural role, in addition to an enzymatic one, in cytokine receptors. One such a role is the requirement of Jaks for cell surface expression of certain cytokine receptors (Gauzzi *et al.*, 1997; Yeh *et al.*, 2000; Huang *et al.*, 2001; Radtke *et al.*, 2002).

5.6 Stats, the signal transducers and activators of transcription

5.6.1 Seven Stat transcription factors are widely expressed

The Stat family of transcription factors contains 7 mammalian members, Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b and Stat6 (reviewed in Bromberg and Darnell, 2000). Stats range in length between 750-900 amino acids. The Stats are clustered in the human genome; Stat1 and Stat4 are located on human chromosome 2, Stat2 and Stat6 on chromosome 12 and Stat3 together with the two Stat5 genes, Stat5a and Stat5b, on human chromosome 17. Stat5a and Stat5b are over 90% identical at the amino acid level (Mui *et al.*, 1995b). Mammalian Stats are all ubiquitously expressed. A unique set of Stats is associated with each cytokine receptor (Table 2), which is important for the specificity of the induced biological responses. Stats have also been identified in *Drosophila melanogaster* (D-Stat) (Hou *et al.*, 1996; Yan *et al.*, 1996c), *Anopheles gambiae* (Ag-Stat) (Barillas-Mury *et al.*, 1999), *Dictyostelium discoideum* (Dd-Stat) (Kawata *et al.*, 1997) and *Coenorhabditis Elegans* (Liu *et al.*, 1999). These Stat proteins show highest similary with Stat3 and Stat5 of mammalian Stats. The existence of a Stat molecule in slime mold *Dictyostelium* is of general interest, since it argues for the presence of tyrosine phosphorylation also in *Dictyostelium* (reviewed in Darnell, 1997; Kay, 1997).

5.6.2 The domain structure of Stats

The Stats can be divided into several domains based on sequence comparison and the three-dimensional structures of the DNA-bound dimers of the core regions of Stats 1 and 3 (Becker *et al.*, 1998; Chen *et al.*, 1998). The domains include an amino terminal domain, coiled-coil, DNA-binding, linker, SH2 and transcriptional activation domain (Figure 7).

The SH2 domain is conserved in Stats and it is central to the function of Stats in converting Stats from latent cytoplasmic proteins into their active forms. Activation of Stats requires phosphorylation of the conserved tyrosine residue located directly on the C-terminal side of the SH2 domain. During this process, the Stats are recruited to tyrosine phosphorylated cytokine receptors through their SH2 domains (Greenlund *et al.*, 1994; Stahl *et al.*, 1995), and following phosphorylation of the conserved tyrosine, the Stats undergo dimerization through a reciprocal SH2-phosphotyrosine interaction (Shuai *et al.*, 1994; Greenlund *et al.*, 1995). Only the Stat dimer is transcriptionally active, and Stat monomers are unable to bind DNA, and importantly, only the dimer translocates to nucleus from the cytoplasm, where the Stats are activated. Furthermore, during activation of Stats, the SH2 domain has been found to be required for interaction with and subsequent phosphorylation by Jaks (Gupta *et al.*, 1996). Mutation of either the conserved, phosphotyrosine-binding arginine of the SH2 domain or the conserved tyrosine following the SH2 domain abrogates Stat activity. Differences in the SH2 domains of Stats determine, at least partially, the specificity of Stat binding to various cytokine receptors (Heim *et al.*, 1995).

The DNA binding domain is located in the middle of Stat molecules and is highly conserved between Stats. It forms a β-barrel with an immunoglobulin fold, showing similarity to DNA binding domains of NF-κB and p53 (Becker *et al.*, 1998; Chen *et al.*, 1998). All Stats, except for Stat2, bind to symmetrical DNA sequences called GAS, and the DNA binding

domain determines the specificity in the recognition of DNA sequences between Stats (Horvath *et al.*, 1995; Ehret *et al.*, 2001). Phosphorylated Stat2 only weakly binds DNA, suggesting it may function only in a heterotrimeric complex with Stat1 and IRF-9 in binding to the ISRE elements (Qureshi *et al.*, 1995). The linker domain is critical for the correct structure of the DNA binding domain. The highly conserved N-terminal domain has been reported to mediate oligomerization of Stat dimers to form tetramers or higher oligomers and thus, promote co-operativity between Stats in DNA binding (Vinkemeier *et al.*, 1996; Xu *et al.*, 1996; John *et al.*, 1999). Co-operative binding may be important, since the Stat-DNA interaction is defined by only few contacts (Becker *et al.*, 1998; Chen *et al.*, 1998).

The coiled-coil domain consists of a large, four-helix bundle forming an exposed hydrophilic area on the surface of Stats. The coiled-coil domain mediates interactions with several regulatory proteins such as IRF9, c-Jun, PIAS, StIP1 and N-myc interacting protein (Nmi) (Horvath *et al.*, 1996; Zhang *et al.*, 1999b; Zhu *et al.*, 1999; Collum *et al.*, 2000). This domain is also implicated in receptor binding, tyrosine phosphorylation and nuclear export of Stats (Begitt *et al.*, 2000; Zhang *et al.*, 2000a).

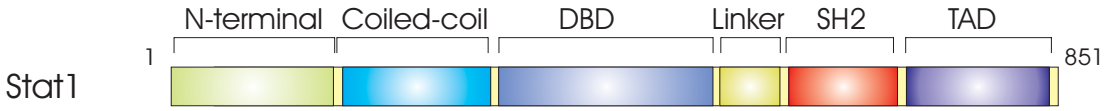

Figure 7. Schematic domain structure of Stat transcription factors.

5.6.3 The C-terminus of Stats is involved in transcriptional activation

The C-terminus encodes a transcriptional activation domain (TAD), and it is the most divergent part between Stats ranging from 50 to 150 amino acids in length. Stat isoforms missing TADs have been identified, and they are defined by the Creek alphabet β, to distinguish from the longer α isoforms. Although the β isoforms can function as dominant negatives when overexpressed, *in vivo* they may promote transcription by binding with other transcriptional coactivators (Schaefer *et al.*, 1995; Bromberg *et al.*, 1996; Caldenhoven *et al.*, 1996; Schaefer *et al.*, 1997; Ivanov *et al.*, 2001). The Stat3$\beta^{-/-}$ mice that have been generated suffer from delayed recovery from endotoxin shock and show hyperresponsiveness of a set of LPS-inducible genes in liver, indicating important functions for the Stat3β isoform *in vivo* (Yoo *et al.*, 2002).

Serine phosphorylation of the TADs of Stat 1, 3, 4 and Stat5a/b has been reported (Wen *et al.*, 1995; Zhang *et al.*, 1995). In general, serine phosphorylation of Stat1, Stat3 and Stat4 increases their transcriptional potential (Wen *et al.*, 1995). However, the effect of serine phosphorylation on transcription varies depending on the promoter, and in some promoters serine phosphorylation is not required and even in some cases decreased transcriptional activity of Stat3 has been detected (Chung *et al.*, 1997b; Kovarik *et al.*, 2001, reviewed in Decker and Kovarik, 2000). Phosphorylation of Ser 727 in Stat1 is required for association with MCM5, a member of the mini-chromosome maintenance (MCM) family proteins involved in DNA

replication (Zhang *et al.*, 1998). On the other hand, in the ISGF3 complex, where Stat1 heterodimerizes with Stat2, serine phosphorylation of Stat1 is not required, and the Stat1β is able to form the transcriptionally active ISGF complex (Bromberg *et al.*, 1996). Cytokines and growth factors, which induce Stat tyrosine phosphorylation, induce also serine phosphorylation, while a number of stimuli including LPS, UV and TNFα induce only Stat serine phosphorylation. The kinases responsible for serine phosphorylation of Stats vary depending on the stimuli, and the same stimuli can induce serine phosphorylation of distinct Stats via different routes. To summarize somewhat controversial data, p38 has been found to phosphorylate Stat1 in response to stress, such as UV, while it is not considered as the Stat1 kinase in IFN-γ signaling (Kovarik *et al.*, 1999; Kovarik *et al.*, 2001). Following growth factor and cytokine stimulation, Stat3 is a target for ERKs, and in stressed cells JNK may be responsible for Stat3 phosphorylation (Lim and Cao, 2001). In Src-transformed fibroblasts, p38 and JNK inhibitors interfere with the transcriptional activity of Stat3 (Turkson *et al.*, 1999). In addition, serine phosphorylation of Stat6 by p38 has been reported to enhance its transcriptional potential (Pesu *et al.*, 2000; Pesu *et al.*, 2002). All in all, the importance and mechanisms of serine phosphorylation-mediated regulation of Stats are not well understood.

Co-activators found to interact with Stats to date and modulate their transcriptional potential include Sp1 for Stat1 (Look *et al.*, 1995), c-Jun for Stat3β (Schaefer *et al.*, 1995), the glucocorticoid receptor (GR) for Stat3, Stat5a and Stat5b (Stocklin *et al.*, 1996; Zhang *et al.*, 1997; Cella *et al.*, 1998), CBP and p300 for Stat1, Stat2, Stat5a, Stat5b and Stat6 (Bhattacharya *et al.*, 1996; Zhang *et al.*, 1996a; Pfitzner *et al.*, 1998; Gingras *et al.*, 1999; McDonald and Reich, 1999), Nmi for Stat1 and Stat5 (Zhu *et al.*, 1999), and p100 for Stat6 (Yang *et al.*, 2002).

5.7 Regulation of the Jak/Stat pathway

Given the central role of Jak kinases in the activation of cytokine-mediated signaling as well as the crucial role of cytokines in determining cell fate, for example during hematopoiesis, it is clear that mechanisms negatively regulating the Jak-initiated Stat pathway must exist (reviewed in Yasukawa *et al.*, 2000). Much of the research of negative regulation of the Jak/Stat pathway was first focused on protein tyrosine phosphatases, but recently, new protein families regulating Jak/Stat pathway have been identified, those called as protein inhibitors of Stats, PIAS, and suppressors of cytokine signaling, SOCS, families. Some aspects of downregulation of Jak/Stat signaling are presented in this chapter.

5.7.1 Protein tyrosine phosphatases, PTPases

Tyrosine phosphorylation is a rapid and reversible process, and thus the obvious candidates for regulation of this phosphorylation process are protein tyrosine phosphatases (PTPases). Regulation of both Jak kinases and Stat proteins by PTPases has been reported, but only Jak regulation is discussed here.

The SH2-containing protein tyrosine phosphatase-1, SHP-1, has been found to suppress the effects of EPO, IL-3, Steel factor, IFN-γ, GM-CSF and CSF-1 (Yi *et al.*, 1993; David *et al.*, 1995a; Klingmuller *et al.*, 1995; Paulson *et al.*, 1996). SHP-1 exerts its effects by negatively regulating Jak2. In EPO signaling, SHP-1 binds to the tyrosine-phosphorylated

C-terminal region in the EPOR and dephosphorylates Jak2 (Klingmuller *et al.*, 1995; Yi *et al.*, 1995). The importance of SHP-1 in cytokine signaling is evident from the mutant *motheaten* mouse strain harbouring an inactivating mutation in SHP-1 (Shultz *et al.*, 1993; Tsui *et al.*, 1993). These mice suffer from loss of hair, lymphoid and myeloid abnormalities, immunodeficiency and autoimmune diseases. In humans, a truncated EPOR, where the binding site for SHP-1 is missing, results in increased hematocrit levels due to enhanced EPO signaling (de la Chapelle *et al.*, 1993).

CD45 is a transmembrane phosphatase expressed in all hematopoietic lineages during all stages of development. In lymphoid cells CD45 regulates T and B cell antigen receptor signaling, mainly through Src family kinases (Alexander, 2000). Recently, CD45 was found to negatively regulate signaling through IL-3, EPO and IFN-γ receptors as well. The cytokine-mediated activation of JAKs and STATs is enhanced in mice lacking the *cd45* gene and *in vitro*, CD45 directly dephosphorylates and binds to JAK (Irie-Sasaki *et al.*, 2001).

PTP1B is a cytoplasmic tyrosine phosphatase, which downregulates Jak2 and Tyk2 kinases by directly recognizing and dephosphorylating their A-loop sequences, which contain the consensus substrate motif (E/D-pY-pY-R/K) of PTP1B (Myers *et al.*, 2001). In contrast, PTP1B does not regulate Jak1 or Jak3, whose A-loops do not fit the consensus sequence. PTP1B-deficient mice show enhanced leptin signaling with increased tyrosine phosphorylation of Jak2 and Stat3, but not of Jak1, indicating that that Jak2 is a physiological substrate for PTP1B (Myers *et al.*, 2001; Cheng *et al.*, 2002).

While Jak2 and Tyk2 have been identified as substrates for PTP1B, the T cell PTPase (TCPTP) specifically interacts and dephosphorylates Jak1 and Jak3 (Simoncic *et al.*, 2002). TCPTP-deficient mice have defects in the hematopoietic system. T cells from these mice show increased Stat5 tyrosine phosphorylation by IL-2 stimulation, and IFN-treatment results in elevated Stat1 and Jak1 activation. The nuclear isoform of TCPTP, TC45, was recently identified as a nuclear PTPase responsible for dephosphorylation of Stat1 (ten Hoeve *et al*., 2002).

5.7.2 SH2b-β

SH2b-β was identified as an activator of Jak2 following GH stimulation (Rui and Carter-Su, 1999). SH2b-β belongs to a family of adapter proteins with two other members, APS and Lnk (Huang *et al.*, 1995; Yokouchi *et al.*, 1997). These proteins have a C-terminal SH2 domain and more N-terminal PH domain. Upon GH stimulation SH2b-β associates through its SH2 domain with Jak2 leading to Jak2 activation, but does not activate other Jak kinases (O'Brien *et al.*, 2001). On the other hand, APS inhibits Jak2 following GH stimulation, and can also inhibit Jak1. Both APS and SH2b-β bind to Jak1, Jak2 and Jak3 despite their differential effect on kinase activity, and may function as adapter proteins for Jaks.

5.7.3 Protein inhibitors of Stats, PIAS

The mammalian PIAS family consists of six members, PIAS1, PIAS3, PIASy, PIASxα and PIASxβ, and GBP (Valdez *et al.*, 1997, reviewed in Shuai, 2000). PIAS1 was identified as a Stat-interacting protein in a yeast two-hybrid screen (Liu *et al.*, 1998). PIAS1 and PIAS3 inhibit DNA binding of Stat1 and Stat3, respectively, and thereby Stat-mediated transcription

(Chung *et al.*, 1997a; Liu *et al.*, 1998). PIAS-mediated inhibition has been reported to be regulated by methylation of Stats, which was found to inhibit PIAS from binding to Stats, thus increasing the DNA binding ability of Stats (Mowen *et al.*, 2001). Interestingly, reduced methylation in certain malignancies may explain their unresponsiveness to IFN-γ treatment (Mowen *et al.*, 2001). PIASy suppresses Stat1-mediated transcription in IFNγ signaling, but does not affect Stat DNA binding, thus acting as a transcriptional co-repressor (Liu *et al.*, 2001). In agreement with the negative regulation of Stats by PIAS proteins in mammals, the *Drosophila* PIAS homolog dPIAS has been found to negatively regulate Stat92E (D-Stat) in the fly (Betz *et al.*, 2001). The PIAS proteins have been reported to function as SUMO E3 ligases (Kahyo *et al.*, 2001; Kotaja *et al.*, 2002), but the possible sumoylation of Stats remains to be resolved.

5.7.4 The suppressors of cytokine signaling, the SOCS family

In contrast to the constitutively expressed phosphatases, the SOCS proteins are induced by a number of cytokines, summarized in table 3. The SOCS proteins are found in most hematopoietic tissues but the expression pattern varies between SOCS family members. Since the SOCS proteins negatively regulate cytokine signaling, they act as classical negative feedback regulators. The SOCS proteins are characterized by an N-terminal, relatively divergent region followed by a SH2 domain and a C-terminal SOCS box homology region. The

SOCS protein	Induced by	Action
CIS	IL-1, IL-6, IL-12, IL-13, LIF, G-CSF, GM-CSF, TPO, IFN-γ, IL-2, IL-3, GH, leptin, PRL, EPO, LPS, TSLP, CNTF	Suppresses EPO, IL-3, IL-2, GH and PRL signaling
SOCS-1	IL-2, IL-3, IL-6, IL-13, EPO, GH, PRL, GM-CSF, thyropropin, CNTF	Suppresses IFN-γ, IFN-α, IL-2, IL-3, EPO, IL-4, IL-6, LIF, GH, PRL, TNF-α, TSLP signaling
SOCS-2	IL-1, IL-2, IL-3, IL-4, IL-6, G-CSF, GM-CSF, EPO, IL-9, GH, PRL, IFN-γ, CNTF, IL-10	Partial suppression of GH, IL-6, LIF and IGF-1 signaling
SOCS-3	IL-1, IL-2, IL-3, IL-4, IL-6, IL-7, IL-12, IL-13, M-CSF, G-CSF, EPO, TPO, TNF-α, LIF, IL-1β, IL-9, IL-10, IL-11, GH, leptin, PRL, GM-CSF, LPS, insulin, CNTF, thyropropin	Suppresses EPO, IL-2, IL-3, IL-4, IL-6, IL-9 IL-11, LIF, IFN-γ, GH, PRL, leptin, insulin and IGF-1 signaling
SOCS-4	Unknown	Unknown
SOCS-5	Unknown	Partial suppression of IL-6 and LIF signaling
SOCS-6	Unknown	Unknown
SOCS-7	Unknown	Binds to Nck

Table 3. SOCS proteins in cytokine signaling, modified from (Greenhalgh and Hilton, 2001).

SOCS proteins regulate both Jaks and Stats, and despite conserved domain structure, the mechanisms utilized by the SOCS proteins in regulating cytokine signaling differ (reviewed in Greenhalgh and Hilton, 2001; Krebs and Hilton, 2001).

The first identified inhibitor, CIS (cytokine-inducible Src homology -2 containing protein), was cloned as an immediate early gene induced by IL-2, IL-3 and EPO (Yoshimura *et al.*, 1995). CIS binds phosphotyrosine residues in the EPOR and IL-3Rβ_c, and prevents activation of Stat5, possibly by sequestering phosphotyrosine motifs required for Stat5 association.

Certain other members of the SOCS family, bind directly to Jak kinases. SOCS-1 (JAB or SSI-1) can bind all Jak kinases, and inhibit their activity (Endo *et al.*, 1997; Naka *et al.*, 1997; Starr *et al.*, 1997). The SH2 domain of SOCS-1 has been shown to interact with Y1007 in the A-loop of Jak2, in a phosphorylation-dependent manner (Yasukawa *et al.*, 1999). It has been suggested that SOCS-1 inhibits Jak activity through its kinase inhibitory region, which presumably interacts with the catalytic cleft of Jak kinase domains (Yasukawa *et al.*, 1999). The kinase inhibitory region possesses sequence similarity to the A-loop double tyrosine motifs of Jaks, and it is suggested to mimic the activation loop by acting as a pseudosubstrate inhibitor for Jaks. In addition, SOCS-1 has been found to inhibit Tec, a member of the Tec/Btk family of non-receptor tyrosine kinases (Ohya *et al.*, 1997).

Eight SOCS proteins have been cloned today (CIS and SOCS1-7). Database searches have revealed a variety of cellular proteins containing the SOCS box, which are not otherwise related to SOCS family, and have not been found to regulate cytokine signaling (Hilton *et al.*, 1998). These proteins can be grouped by domains located in the N-terminal regions: WD-40 repeats (WSB-1 and –2), ankyrin repeats (ASB-1-9), SPRY (SSB-1-3), and small GTPase domains. A possible function for the SOCS box in these proteins comes from interaction studies, where the SOCS box has been found to interact with elongin B and elongin C, which are components of the ubiquitin proteasome pathway (Kamura *et al.*, 1998; Zhang *et al.*, 1999a). This interaction has been suggested to regulate the activity of SOCS proteins, or SOCS binding proteins, by directing them to degradation. Evidence for degradation-based regulation is emerging also in cytokine signaling. In GHR signaling, inhibition by CIS involves an active proteosomal pathway (Ram and Waxman, 2000), and the active, tyrosine phosphorylated Jak2 was recently found to be regulated by the ubiquitin-proteasome pathway in a SOCS-1-dependent manner (Ungureanu *et al.*, 2002). Thus, SOCS-mediated negative regulation may involve two steps: first, SOCS proteins inhibit signaling by binding to active signaling complexes, and second, SOCS bound signaling components are targeted to degradation via SOCS proteins (reviewed in Kile *et al.*, 2002).

The complex network of cytokines and their multiple effects on various cell types have been partly suggested to depend on SOCS proteins. The SOCS proteins are believed to mediate crosstalk inhibition between cytokine signaling pathways. For example, IL-10 attenuates a variety of inflammatory responses in immune cells, and has negative effects on IFN signaling. IL-10 induces expression of SOCS-3, which is supposed to inhibit INF-γ-induced Stat1 activation and gene expression in monocytes (Ito *et al.*, 1999).

While inhibiting the Jak/Stat pathway, SOCS-3 has been found to sustain activation of the Ras pathway (Cacalano *et al.*, 2001). Tyrosine phosphorylation of SOCS-3, which has been found to occur following cytokine and growth factor stimulation, results in interaction of SOCS-3 with RasGAP and sustained ERK activity (Cohney *et al.*, 1999; Cacalano *et al.*, 2001).

5.8 Biological role of the Jak/Stat pathway

In cytokine signaling, one cytokine can often induce the activation of more than one Jak kinase, and in addition, activate tyrosine kinases other than Jaks as well. Therefore, it has been difficult to find out, which of these kinases have unique or redundant roles in cytokine signaling. To clarify the *in vivo* function of the Jak/Stat pathway, mice lacking individual Jak and Stat family members have been generated, as well as a double knockout of Stat5a and Stat5b (reviewed in Leonard and O'Shea, 1998). These mice demonstrate nonredundant functions for Jaks and Stats in cytokine signaling. In addition, analysis of hematopoietic disorders and malignancies have revealed biological processes involving the Jak/Stat pathway.

5.8.1 Jak deficiency

Jak1-deficient mice show perinatal lethality (Rodig *et al.*, 1998). They are small at birth and fail to nurse. Lymphopoiesis, but not myelopoiesis, is impaired with reduced numbers of T and B lymphocytes, and Jak1-deficient cells fail to respond to IFNs, γ_c utilizing cytokines (IL-2, IL-4, IL-7, IL-9, IL-15) and cytokines using gp130 or gp130 like protein (IL-6, LIF, CT-1, CNTF, OSM, IL-11). The cause for the perinatal death is suggested to result from a neuronal defect; neurons from Jak1 deficient mice do not respond to LIF, CT-1 and CNTF, and die apoptotically.

Jak2-deficient mice are embryonically lethal, and die at embryonic day 12.5, due to absence of definitive erythropoiesis (Neubauer *et al.*, 1998; Parganas *et al.*, 1998). The similarities in phenotypes between mice deficient in Jak2, EPO or EPOR suggest a specific role for Jak2 in EPO signaling. Jak2-deficient fetal liver myeloid progenitors fail to respond to EPO, TPO and β_c utilizing cytokines (IL-3, IL-5, GM-CSF), while Jak2-deficient fibroblasts are unresponsive to IFN-γ.

Jak3-deficient mice exhibit phenotypes indistinguishable from those of γ_c-deficient mice in having severely impaired T and B cell development (Nosaka *et al.*, 1995; Park *et al.*, 1995; Thomis *et al.*, 1995), since signaling through γ_c-containing receptors is missing. These mice have smaller thymi, spleens and lymph nodes as compared to wild-type mice. They have defective B cell development and reduced numbers of thymocytes, and the residual T cells are functionally deficient. Mutations in Jak3 have been characterized in humans, and these patients suffer from severe combined immunodeficiency, SCID, with complete absence of T-lymphocytes and non-functional B-lymphocytes (Macchi *et al.*, 1995; Russell *et al.*, 1995). The phenotype of Jak3-SCID is very similar to the X-linked severe combined immunodeficiency (X-SCID) resulting from mutations in γ_c (reviewed in Leonard, 1996). Thus, Jak3 has an essential role in γ_c-dependent lymphoid development. At least 27 unique mutations affecting all JH domains have been characterized in Jak3, resulting in SCID, and the

mutations have been collected in a Jak3 database (http://www.uta.fi/Imt/bioinfo/Jak3base/) (Notarangelo *et al.*, 2001). These mutations have been especially interesting in aiming to understand the function and regulation of Jak3 by its JH domains (described in Discussion).

Tyk2-deficient mice do not show any obvious abnormalities or any difference with regard to lymphoid, monocyte, or myeloid cell development when compared to wild-type mice, but show impaired innate and adaptive immunological responses (Karaghiosoff *et al.*, 2000; Shimoda *et al.*, 2000). For example, IFN-α + LPS fail to induce nitric oxide (NO) production in peritoneal macrophages of Tyk2-deficient mouse, and the $Tyk2^{-/-}$ mice show selective susceptibility to viruses. $Tyk2^{-/-}$ mice were found to have clear, but reduced responses to type I IFNs, indicating a redundant role for Tyk2 in the activation of cytokine receptor signaling and suggesting a role for Tyk2 as an amplifier of cytokine signaling. This was surprising, since Tyk2 was originally cloned as an essential kinase for IFNα signaling using cell lines defective in type I IFN responses, and, for example, Jak1 deficiency results in total lack of type I IFN responses in mice (Velazquez *et al.*, 1992). This is also different from the previously found obligatory roles of other Jak kinases in cytokine receptors. It is possible that Tyk2 has a different role in mice and in humans, which might explain the different results from knockout mice and human cell lines. Tyk2 deficiency resulted also in impaired IFN-γ responses in mice, although Tyk2 is not activated by IFN-γ. This may be explained by reduced levels of Stat1 protein expression in $Tyk2^{-/-}$ mice compared to wild-type mice. Tyk2 was found to be required for IL-12-induced IFN-γ production by T cells, and thus for TH1 differentiation, while having a redundant role in IL-12-induced T cell proliferation. A selective, essential role for Tyk2 was found in type I IFN- and IL-12-induced activation of Stat3, which, however, is not considered essential for the major biological functions of these cytokines.

5.8.2 Stat deficiency

Stat1-deficient mice show increased susceptibility to infection by viruses and microbial pathogens (Durbin *et al.*, 1996; Meraz *et al.*, 1996). Although Stat1 is activated by a number of cytokines in cell lines, the major defect in Stat1-deficient mice is in IFN-α and IFN-γ signaling. Thus, Stat1 is primarily, if not entirely, important for signaling by IFNs. Recently, a L706S substitution in the Stat1 gene was reported from a patient, who suffered from a mycobacterial disease (Dupuis *et al.*, 2001). The Stat1 mutation was found to interfere with tyrosine phosphorylation of Stat1 resulting in impaired IFN-γ signaling. Stat2-deficient mice are primarily defective in signaling by type I IFNs, and are sensitive to viral infections (Park *et al.*, 2000).

The targeted disruption of the Stat3 gene in mice has been found to result in embryonic lethality, at embryonic day 6.5-7.5 (Takeda *et al.*, 1997). Stat3 has been found essential for maintaining the undifferentiated state of embryonic stem (ES) cells, but the function of Stat3 in early embryonic development remains unresolved (Matsuda *et al.*, 1999; Raz *et al.*, 1999). Using a Stat3 conditional knockout approach, Stat3 was found to be essential for IL-6-induced suppression of apoptosis of T cells (Takeda *et al.*, 1998), and IL-2-induced T cell proliferation by regulating expression of IL-2Rα (Akaishi *et al.*, 1998, reviewed in Akira, 2000; Levy and Lee, 2002). In macrophages Stat3 was found to be required for IL-10-mediated anti-inflammatory responses (Takeda *et al.*, 1999). The *in vitro* motility of epidermal cells

lacking Stat3 was impaired affecting, for example, wound healing in the Stat3$^{-/-}$ mice (Sano *et al.*, 1999). In mammary gland, Stat3 is specifically activated at the start of involution. In the Stat3$^{-/-}$ mice, the apoptosis of mammary epithelial cells was decreased and involution delayed (Chapman *et al.*, 1999). Stat3 deficiency in the brain resulted in perinatal lethality due to neuronal defects (Alonzi *et al.*, 2001). In liver, Stat3 was required for the acute phase response by IL-6, in accordance with the identification of Stat3 first as a factor binding to the acute phase response DNA element (Akira *et al.*, 1994; Lutticken *et al.*, 1994; Raz *et al.*, 1994; Zhong *et al.*, 1994b). In conclusion, many of the functions of Stat3 are linked to signaling through gp130.

Mice lacking Stat4 show a differentiation defect of T_H1 cells and enhanced differentiation towards T_H2 phenotype, due to defective IL-12 signaling (Kaplan *et al.*, 1996b; Thierfelder *et al.*, 1996). NK cells and T cells from these mice are unresponsive to IL-12. Thus, the effects of Stat4 are restricted to IL-12 signaling, in accordance with the findings that Stat4 is activated only by IL-12.

Stat5a-deficient mice show impaired lactation and lobuloalveolar proliferation of the mammary epithelium during pregnancy, indicating defective prolactin signaling (Teglund *et al.*, 1998). Bone marrow-derived macrophages from these mice exhibit defective GM-CSF-induced proliferation and gene expression. In contrast, Stat5b-deficient mice are impaired in GH signaling showing reduced body weight and loss of sexually dimorphic growth (Udy *et al.*, 1997). Analysis of the immune systems of Stat5a- and Stat5b-deficient mice suggests a role for both Stat5 proteins in signaling by IL-2. Stat5a was found to mediate its effects through maintaining the expression of IL-2Rα required for formation of the high affinity IL-2R. Stat5b-deficient mice showed impaired IL-2 signaling, which was not totally due to reduced level of the high affinity IL-2 receptor. Both of these mice showed a defect in NK cell development. Stat5a/5b double knockout mice do not have NK cells and show impaired T cell proliferation, due to loss of induction of cyclin D2, cyclin D3 and cdk6. Female mice lacking functional Stat5a and Stat5b are infertile (Teglund *et al.*, 1998; Moriggl *et al.*, 1999). Stat5a/5b knockout mice show fetal anemia due to impaired BclXL expression induced by EPO (Socolovsky *et al.*, 1999).

Stat6-deficient mice are defective in T_H1 differentiation, differentiation being biased towards T_H2 cells. The lymphoid cells from these mice do not respond to IL-4 or IL-13, and exhibit impaired IgE class switch (Takeda *et al.*, 1996aKaplan *et al.*, 1996a; Shimoda *et al.*, 1996; Takeda *et al.*, 1996b).

5.8.3 SOCS-deficient mice

The SOCS proteins are strongly linked to cytokine signaling and Jak/Stat pathway, and thus, the results obtained from mice deficient in SOCS-1, -2, -3 and CIS that have been generated, require consideration.

SOCS-1-deficient mice die at three weeks of age showing fatty degeneration of the liver, monocytic infiltration of organs, and lymphopaenia (Marine *et al.*, 1999b). The SOCS-1$^{-/-}$ phenotype is reminiscent of that induced by elevated IFN-γ levels in neonatal mice, and the SOCS-1$^{-/-}$ phenotype can be corrected by inactivating anti-IFN-γ antibodies or by crossing with IFN-γ$^{-/-}$ mice. Reconstitution of irradiated Jak3$^{-/-}$ mice with SOCS-1$^{-/-}$ bone marrow confers the lethal SOCS-1$^{-/-}$ phenotype, indicating an important role for lymphocytes in the SOCS-1$^{-/-}$

phenotype. Also, crossing SOCS-1-deficient mice with $RAG2^{-/-}$ mice rescues the $SOCS\text{-}1^{-/-}$ phenotype. These results support the model that SOCS-1 negatively controls IFN-γ signaling, but it may also have a role in signaling through other cytokine receptors.

The SOCS-2-deficient mice, especially males, grow significantly larger than their wild type counterparts, with a 40% increase in size by 12 weeks of age (Metcalf *et al.*, 2000). This suggests hyperresponsive GH signaling in SOCS-2-deficient mice. $SOCS\text{-}3^{-/-}$ mice die between embryonic days 12 and 14 due to increased erythrocytosis surrounding the fetal liver and abdominal region, whereas over-expression of SOCS-3 blocks fetal liver hematopoiesis (Marine *et al.*, 1999a). CIS-deficient mice show no abnormal phenotype (Marine *et al.*, 1999a). Transgenic animals over-expressing CIS have phenotypes similar to those resulting from Stat5a and Stat5b deficiency, suggesting that CIS negatively regulates Stat5 function in growth, mammary gland function and T cell development (Matsumoto *et al.*, 1999).

5.8.4 Jak/Stat pathway in oncogenesis

In contrast to normal signaling, where activation of Jaks and Stats is rapid yet transient, constitutive Jak/Stat signaling has been associated with malignant processes. Constitutive Jak and Stat activation has been detected in a number of transformed cells, induced either by viruses, such as HTLV-1, v-Abl and Epstein-Barr virus or by oncogene activation, such as Src (Table 4) (Danial *et al.*, 1995; Migone *et al.*, 1995; Yu *et al.*, 1995; Campbell *et al.*, 1997; Garcia *et al.*, 1997). In addition, constitutive Stat activation (Stat1, Stat3 and Stat5) has been detected in a number of primary tumors, such as breast cancer, lung cancer, prostate cancer, head and neck tumors, brain tumors, multiple myeloma, leukemias, lymphomas, renal cell carcinoma, melanoma, pancreatic cancer and ovarian cancer (reviewed in Bowman *et al.*, 2000; Bromberg, 2002).

There is growing evidence for direct participation of Stats in oncogenesis (Turkson *et al.*, 1998; Bromberg *et al.*, 1999). In one study, the dominant negative form of Stat3, Stat3β, was able to prevent Src-induced transformation of NIH3T3 cells, while it did not prevent Ras-induced transformation (Turkson *et al.*, 1998). In another study, ectopic expression of a genetically modified Stat3 capable for dimerization and DNA binding in the absence of tyrosine phosphorylation, induced transformation of NIH 3T3 fibroblasts (Bromberg *et al.*, 1999). This result demonstrated that the activated Stat3 is an oncogene, and that Stat3-driven gene transcription is sufficient to induce at least certain features of cell transformation in the absence of tyrosine kinase signaling (Bromberg *et al.*, 1999). The Stat3 target genes with a role in controlling cell proliferation and survival include Bcl-xl, Mcl-1, Bcl-2, cyclin D1, c-Myc, $p21^{WAF1/CIP1}$ and VEGF (Bromberg *et al.*, 1999; Catlett-Falcone *et al.*, 1999; Sinibaldi *et al.*, 2000; Bowman *et al.*, 2001; Epling-Burnette *et al.*, 2001; Niu *et al.*, 2002).

In addition to tyrosine phosphorylation, constitutive serine phosphorylation of Stat1 and Stat3 has been detected in human tumors (Frank *et al.*, 1997). Serine phosphorylation seems to have a role in transformation, since the S727A mutant Stat3 can prevent Src-induced transformation, and so can inhibition of the p38 serine kinase, which phosphorylates Stat3 (Bromberg *et al.*, 1998b; Turkson *et al.*, 1999).

Although Stat1 has been found activated in transformed cells, it unlikely contributes to oncogenesis. Instead, Stat1 has been found growth inhibitory, the apoptosis and growth arrest being mediated by induction of caspases and the cdk inhibitor $p21^{WAF1/CIP1}$ (Bromberg *et al.*, 1996; Chin *et al.*, 1996; Kumar *et al.*, 1997; Bromberg *et al.*, 1998a; Grimley *et al.*, 1998; Xu *et al.*, 1998). Stat1 also cooperates with the tumor suppressor BRCA1 in induction of certain IFN-γ-inducible genes, such as $p21^{WAF1/CIP1}$ (Ouchi *et al.*, 2000). Furthermore, Stat1-deficient mice gain tumors more frequently than their wild-type counterparts and Stat1 deficiency increases tumor development in $p53^{-/-}$ mice, suggesting a role for Stat1 as a tumor suppressor (Kaplan *et al.*, 1998).

Constitutive Jak activation has also been detected in certain malignancies. Jak3 is activated in Sezary's syndrome, and in human acute lymphocytic leukemia (ALL) Jak2 is directly involved in the malignant process (Zhang *et al.*, 1996b; Lacronique *et al.*, 1997; Ho *et al.*, 1999). In ALL, translocation of the short arm of chromosome 9, containing the kinase or the double kinase domain of Jak2, to the short arm of chromosome 12, containing the oligomerization domain of the TEL transcription factor, results in a constitutively active TEL-Jak2 fusion protein (Lacronique *et al.*, 1997; Ho *et al.*, 1999). Dimerization of the two Jak2 JH1 domains through the TEL fusion partner mimics cytokine-induced activation of Jak2.

Cell type	Oncogene	Activated Stats
Fibroblasts	v-Src	Stat3
	c-Src	Stat3
	v-Fps (Fes)	Stat3
	v-Sis (PDGF)	Stat3
	Polyoma virus middle T antigen	Stat3
	v-Ras	-
	v-Raf	-
	v-Ros	Stat3
	IGF-1 receptor	Stat3
	c-Eyk, v-Eyk	Stat1, Stat3
Myeloid	v-Src	Stat1, Stat3, Stat5
	v-Fgr	-
T-cell	Lck	Stat3, Stat5
Mammary/lung epithelial	v-Src	Stat3
	Etk/Bmx	Stat1, Stat3, Stat5
Gallbladder adenocarcinoma	v-Src	Stat3
Pre-B lymphocytes	v-Abl	Stat1, Stat5
Erythroleukemia/blast cells/basophils/mast cells	BCR-Abl	Stat1, Stat5
Primary bone marrow	BCR-Abl	Stat5

Table 4. Stat activation by oncogenes (modified from Bowman *et al.*, 2000).

TEL-Jak2, when introduced in a hematopoietic cell line, confers these cells factor-independent growth, which is dependent on TEL-Jak2-induced constitutive Stat5 activation (Schwaller *et al.*, 1998). The AG490 inhibitor of Jak2 can inhibit the leukaemia cells containing hyperactive TEL-Jak2, and also many other tumor-derived cell lines, suggesting that Jak2 is critical in many cancers (Meydan *et al.*, 1996; Turkson and Jove, 2000).

The SOCS proteins are important negative regulators of the Jak/Stat pathway. It has been discovered that in hepatocellular carcinomas (HCC) SOCS-1 levels are reduced due to abnormal methylation of the SOCS-1 gene leading to transcriptional silencing (Yoshikawa *et al.*, 2001). IL-6-driven Jak/Stat signaling is constitutive in HCC cell lines and primary HCCs, and can be inhibited by introducing SOCS-1 into these cells, or by inhibiting Jak2 activity with AG490 (Kishimoto and Kikutani, 2001; Yoshikawa *et al.*, 2001). Thus, SOCS-1 may be a tumor suppressor protein.

The first evidence for constitutive Jak/Stat activation in oncogenesis came from studies of *Drosophila* fruit fly. In *Drosophila*, the *unpaired* gene (*upd*) was found to encode a ligand that activates the *Drosophila* Jak/Stat pahway through the receptor mom (master of marelle), which is related to mammalian cytokine receptors, especially gp130 (Harrison *et al.*, 1998; Chen *et al.*, 2002). Lethal gain-of-function mutations, resulting in hyperactivation of the *Drosophila* Jak, Hop (hopscotch), were characterized and found to result in hematopoietic neoplasia resembling leukemia (Harrison *et al.*, 1995; Luo *et al.*, 1997). Dominant suppressors of this phenotype map to loss-of-function mutations in the *Drosophila* Stat (D-Stat) gene (*marelle*, *stat92E*) (Yan *et al.*, 1996bYan *et al.*, 1996b).

5.9 Other signaling pathways initiated by cytokines

In addition to the Jak/Stat pathway, a number of other signaling pathways are activated by cytokine receptors (Figure 8), including the Ras and PI3K pathways, as well as pathways involving tyrosine kinases from the Src and Tec/Btk families and protein kinase C family of serine/threonine kinases. However, Jaks have been found essential for activation of virtually all of the downstream signaling pathways initiating from cytokine receptors (Miura *et al.*, 1994a; Miura *et al.*, 1994b; Stancato *et al.*, 1997; Sakatsume *et al.*, 1998). One important function of Jak kinases, in addition to Stat activation, is the phosphorylation of the cytokine receptor itself. The phosphorylated tyrosines in the receptor serve as binding sites for many signaling proteins facilitating their activation, and progression of signaling downstream of the receptor.

Jaks have been also found to directly participate in signaling pathways other than Stats. For example, Jak3 and Jak2 induce activation of Pyk2 through IL-2R and IFN-γR, respectively (Miyazaki *et al.*, 1998; Takaoka *et al.*, 1999). Activation of insulin receptor substrates IRS-1 and IRS-2 by IL-2, and subsequent activation of PI3-kinase, may depend on Jak1 and Jak3 (Yin *et al.*, 1994; Johnston *et al.*, 1995b). Jak2 is found in a complex with adapter proteins Grb2 and Shc, as well as nucleotide exchange factor SOS, which may activate Ras (Chauhan *et al.*, 1995; He *et al.*, 1995; Wang *et al.*, 1995; Giordano *et al.*, 1997). Jak1 and Jak2 have been found to interact with and phosphorylate Raf-1 leading to Raf-1 activation, and subsequent activation of the MAPK pathway (Winston and Hunter, 1995; Xia *et al.*, 1996; Stancato *et al.*, 1997; Sakatsume *et al.*, 1998). Activation of the MAPK pathway is required for transcriptional regulation of cytokine-inducible genes, and ERK kinases, as well as p38 and JNK have been

implicated in serine phosphorylation of Stat1, Stat3 and Stat6 (David *et al.*, 1995b, reviewed in Decker and Kovarik, 2000). Jak2 has been found to associate with Vav thereby possibly linking cytokine receptors to activation of the Rho family GTPases (Matsuguchi *et al.*, 1995). Jak1 and Jak2 have been implicated in IL-6 -induced activation of Btk and Tec, which also activate Rho proteins (Takahashi-Tezuka *et al.*, 1997).

There is also evidence that activation of Stats can occur independently of Jak kinases. CD40 engagement has been found to induce activation of Stat6, and BCR cross-linking has been found to induce activation of Stat5, but in both cases without detectable Jak activation (Karras *et al.*, 1996; Karras *et al.*, 1997). In IL-3 signaling Src has been found to activate Stat3 (Chaturvedi *et al.*, 1998). These data and the results from oncogene-mediated Stat activation suggest that other tyrosine kinases than Jaks are also involved in activation of Stats.

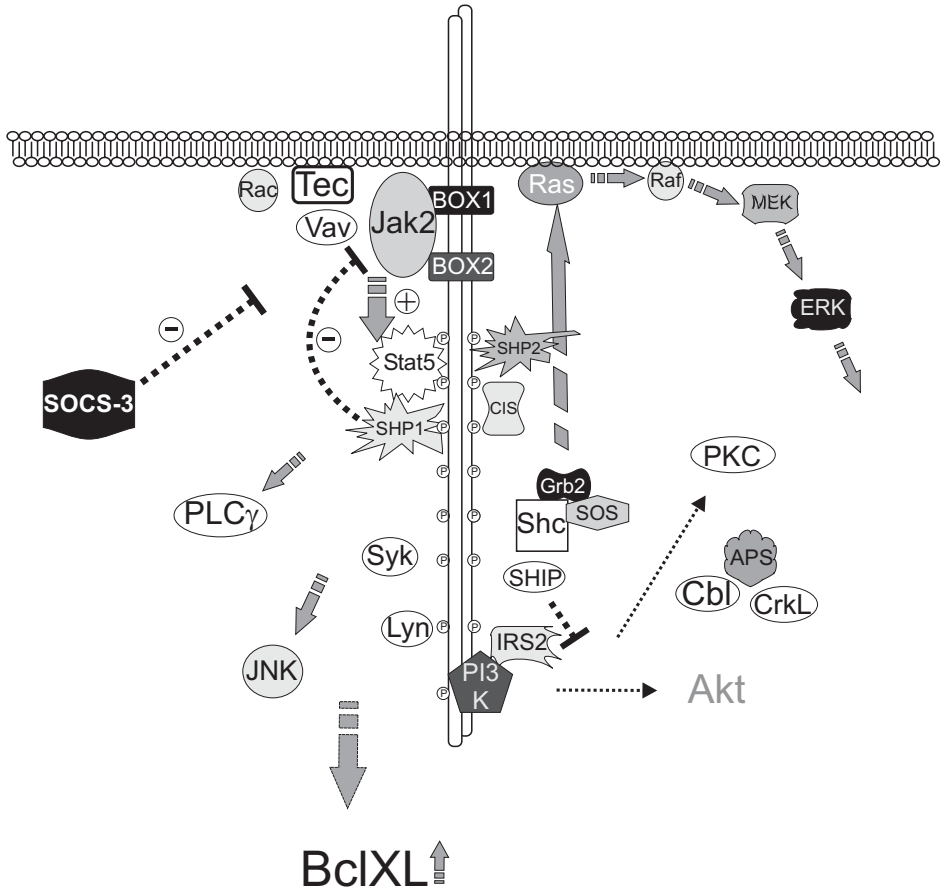

Figure 8. Schematic presentation of signaling proteins and activated pathways implicated in EPOR signaling (for references see reviews Klingmuller, 1997; Wojchowski *et al.*, 1999, and references therein).

5.9.1 Btk/Tec tyrosine kinase family

The Btk/Tec family of non-receptor tyrosine kinases includes five members, Tec, Btk/Atk, Itk/Emt/Tsk, Bmx/Etk, and Txk, which are characterized by the presence of a N-terminal PH domain, followed by TH, SH3, SH2 and tyrosine kinase domains (Figure 4). However, Txk does not contain PH and TH domains, and Bmx has a relatively poorly

conserved SH3 domain, and the proline-rich region in the TH domain is missing from Bmx. The Btk/Tec kinases are linked to a variety of different cellular processes, such as apoptosis, cell growth, differentiation and migration (reviewed in Qiu and Kung, 2000).

The Btk/Tec family kinases are predominantly expressed in cells of hematopoietic origin. Btk is expressed in B-lymphoid and myeloid lineages, and has a critical role in B-cell development. Mutations in Btk lead to immunodeficiency in human, called X-linked agammaglobulinemia (XLA), characterized by virtual absence of B-lymphocytes and plasma cells, and consequently, immunoglobulins (Tsukada *et al.*, 1993, reviewed in Mattsson *et al*., 1996). In mice, Btk mutations also lead to B-cell defects with a milder immunodeficiency disease (Xid) than in humans (Thomas *et al.*, 1993; Kerner *et al.*, 1995; Khan *et al.*, 1995). Itk is expressed predominantly in cells of T lymphoid lineage, and lack of Itk in mice results in the absence of mature thymocytes and defective signaling through the T cell receptor (Liao and Littman, 1995). Tec was originally characterized from hepatocarcinoma cells, but is also expressed in hematopoietic cells, and Txk is expressed in myeloid and T cells (Mano *et al.*, 1993; Qiu and Kung, 2000). Bmx (Bone Marrow tyrosine kinase gene in chromosome X) was identified in a bone marrow derived library, and later in prostate cancer cells (Tamagnone *et al.*, 1994; Robinson *et al.*, 1996). Bmx is expressed more widely than the other family members, including expression in cells of the myeloid lineage, in the endothelial cells of large arteries as well as in epithelial cells (Kaukonen *et al.*, 1996; Ekman *et al.*, 1997; Weil *et al.*, 1997; Bagheri-Yarmand *et al.*, 2001; Rajantie *et al.*, 2001).

The Btk/Tec kinases are involved in signaling through a large number of different transmembrane receptors, such as growth factor receptors, cytokine receptors, G-protein coupled receptors, antigen receptors and integrins. For example, Btk is activated through mast cell FcεR (Kawakami *et al.*, 1994; Hata *et al.*, 1998), BCR (Aoki *et al.*, 1994; de Weers *et al.*, 1994), IL-5R (Sato *et al.*, 1994) and gp130 (Matsuda *et al.*, 1995), Tec through gp130 (Matsuda *et al.*, 1995), c-kit (Tang *et al.*, 1994) and IL-3R (Mano *et al.*, 1995), and Itk through CD28 (August *et al.*, 1994), mast cell FcεR (Kawakami *et al.*, 1995) and TCR (Gibson *et al.*, 1996). In addition, the Btk PH domain binds Gαq both *in vivo* and *in vitro* and this leads to Btk activation (Bence *et al.*, 1997). By the time the current work was initiated, no receptor was known to activate the Bmx kinase.

The presence of at least four protein interaction domains in Btk/Tec kinases enables these kinases to participate in multiple different signaling complexes. The PH domain has a central role in Btk/Tec kinases, by mediating multiple protein-protein interactions. The PH domain has been found to interact with Gq proteins (Bence *et al.*, 1997), various protein kinase C isoforms (Yao *et al.*, 1994; Kawakami *et al.*, 1995; Yao *et al.*, 1997), F-actin (Yao *et al.*, 1999) and FAK (Chen *et al.*, 2001), and in some cases the interaction results in increased activity of the kinase.

The PH domain also binds phospholipids. The Btk PH domain preferentially binds phosphorylation products of PI3 kinase, such as phosphatidylinositol-3,4,5-triphosphate (PtdIns-3,4,5-P3) (Rameh *et al.*, 1997). Mutations in the Btk PH domain have been characterized that disrupt lipid binding by the PH domain and result in XLA or Xid (Rameh *et al.*, 1997; Vihinen *et al.*, 1998). In addition to Btk, PI3K can activate also other Btk/Tec kinases, except for Txk (Scharenberg *et al.*, 1998; Tomlinson *et al.*, 1999; Ekman *et al.*, 2000).

The Tec/Btk kinases are recruited to the plasma membrane, where they bind newly formed PtdIns-3,4,5-P3, and become activated. The importance of PI3K mediated activation of Tec/Btk kinases is strengthened by the finding that the p85α knockout mice have very similar phenotype as the Xid mice (Fruman *et al.*, 1999; Suzuki *et al.*, 1999).

Several non-receptor tyrosine kinases have been found to regulate Btk/Tec kinases. Src kinases have been found to phosphorylate a conserved tyrosine residue in the kinase domains of Btk, Itk and Bmx (Y551 in Btk and Y566 in Bmx) (Mahajan *et al.*, 1995; Park *et al.*, 1996a; Rawlings *et al.*, 1996; August *et al.*, 1997; Tsai *et al.*, 2000), resulting in increased activity of these kinases, and subsequent phosphorylation in the SH3 domain. The Itk TH domain can interact with its own SH3 domain, suggesting intramolecular inhibition of kinase activity by these domains (Andreotti *et al.*, 1997), and phosphorylation in the SH3 domain may disrupt the interaction leading to kinase activation. Thus, activation of Btk/Tec kinases involves two steps: first, membrane localization through the PH domain, and second, autophosphorylation induced by Src-mediated phosphorylation. In addition to Src kinases, also Jak kinases have been implicated in regulation of Btk/Tec kinases. Btk and Tec associate with the IL-6R through Jak1, which also directly phosphorylates Btk (Takahashi-Tezuka *et al.*, 1997).

At the beginning of this work, only few substrates were known for the Tec/Btk kinases, and no substrates had been characterized for Bmx. Btk was known to activate PLC-γ in BCR signaling (Takata and Kurosaki, 1996), leading to increase in intracellular calcium levels. Currently, the number of substrates has increased and now includes PKCβ1, TFII-I and the Wiskott-Aldrich protein (WASP) for Btk, Sab, a negative regulator of Btk, and Dok-1, vav, BRDG1, Akt and PI3K for Tec (references in Qiu and Kung, 2000).

6 AIMS OF THE PRESENT STUDY

The Jak tyrosine kinases and their best-known substrates, Stat transcription factors, were cloned in the beginning of the 1990s. Thereafter, an enormous amount of work has been done to understand the mechanisms utilized by the Jak-Stat pathway in mediating signaling from a vast number of cytokine receptors, as well as the biological role of this signaling pathway.

The aim of the current work has been to learn about the molecular mechanisms regulating immediate signaling events following cytokine binding to its cognate receptor. A critical event regulating cytokine signaling is activation of Jak kinases in cytokine receptors, and the regulation of Jak activity has been a major question in this study. The understanding of the mechanisms regulating Jak activation in Jak-receptor complexes as well as regulation of Stat activity may result in clinical applications in the form of new therapeutics targeted to modulate cytokine signaling.

The specific questions to be answered in this thesis study were:

1) How is the activity of the Jak2 tyrosine kinase regulated? What is the function of the different JH domains in regulation of Jak2?

2) What is the role of the JH2 domain in regulation of the Jak3 tyrosine kinase? What is the role of the JH2 domain in signaling through cytokine receptors?

3) How does the JH2 domain regulate Jak2? Can inhibitory regions be defined within the JH2 domain?

4) How is Stat signaling regulated? Are there kinases other than Jaks that can activate Stat proteins?

7 MATERIALS AND METHODS

7.0.1 Cell lines

Mammalian cell culturing was carried out in the medium mentioned in table 5, supplemented with 100U/ml penicillin and 50 μg/ml streptomycin. Before cytokine treatment, the cells were starved in serum-free medium. Stimulation of the cells with cytokines was carried out in serum-free medium. Insect cells were cultivated in a suspension culture at 28°C, and plated on cell culture dishes for experiments. Infections were carried on for 72 hours followed by cell lysis.

Name	Description	Used in	Culture medium
COS-7	African green monkey kidney epithelial cells, SV40 transformed, ATCC CRL-1651	I, II, IV	D-MEM + 10%FBS
293	Human kidney epithelial cells, ATCC CRL-1573	II	D-MEM + 10%FBS
293T	Human kidney epithelial cells, expressing the transforming gene of adenovirus 5, ATCC CRL-1573	I, II, III, IV	D-MEM + 10%FBS
γ2A	HT-1080, human fibrosarcoma cell line, ATCC CCL-121, derived Jak2 negative cell line, resistant to antibiotic geneticin (described in Watling *et al.*, 1993)	I, II, III	D-MEM + 10%FBS + G418
Sf-9	Insect cells (Smith *et al.*, 1985)	IV	Sf900 (Invitrogen) +10%FBS

Table 5. The cell lines used with their ATCC (American Type Culture Collection, Manassas, VA, USA) identification numbers.

7.0.2 Antibodies and cytokines

Name	Description	Reference or source	Used in
IL-2	human IL-2	R&D Systems	II
IFN-γ	human recombinant IFN-γ	R&D Systems	I, II, III
Anti-Jak2	polyclonal	Silvennoinen *et al.*, 1993c	I, II, IV
Anti-Jak1	monoclonal	Transduction Laboratories	IV
Anti-Jak3	polyclonal	Witthuhn *et al.*, 1994	II
Anti-Stat1	monoclonal, anti-Stat1(p91)	Transduction Laboratories	IV
Anti-Stat1	monoclonal, anti-ISGF-3(p91)	Transduction Laboratories	IV
Anti-Stat3	monoclonal	Transduction Laboratories	IV
Anti-Stat5	polyclonal	Santa Crutz Biotechnology	IV
Anti-Stat5	monoclonal, ST5a-2H2	Zymed Laboratories	I, II, III
Anti-HA	monoclonal ,12CA5/16B12	Berkeley Antibody Company /Covance	I, II ,III, IV
Anti-Phosphotyrosine	monoclonal, 4G10	Upstate Biotechnology	I, II, III, IV

Table 6. Antibodies and cytokines used.

7.0.3 DNA Constructs

Expression plasmids used are indicated in Table 7 and DNA constructs cloned in the current work are prsented in Figure 7.

Expression plasmid	Source / Reference	Used in
Bmx cDNA in Bluescript SK	Tamagnone *et al.*, 1994	IV
Bmx-HA-pCIneo	IV	IV
c-Src	Silvennoinen *et al.*, 1993b	IV
Fyn	Silvennoinen *et al.*, 1993b	IV
Syk	Dr. Tomas Mustelin, Couture *et al.*, 1994	IV
Stat1α-HA	Dr. Chris Schindler, Schindler *et al.*, 1992a	IV
Stat3	Dr. David Levy, Campbell *et al.*, 1995	IV
Stat5A, MGF	Dr. Berndt Groner, Wakao *et al.*, 1994	IV
Mouse Stat5A, and its SH2 and YF mutants	Dr. Timothy Wood	I, II, III
PKC-β1	Dr. Peter Parker, Coussens *et al.*, 1986	IV
PKC-γ	Dr. Peter Parker, Coussens *et al.*, 1986	IV
PKC-δ	Dr. Peter Parker, Olivier and Parker, 1991	IV
PKC-ε	Dr. Peter Parker, Schaap and Parker, 1990	IV
PKC-ζ	Dr. Peter Parker, Ways *et al.*, 1994	IV
Jak1 and its derivative Jak1-HA-pCIneo	Silvennoinen *et al.*, 1993a	II, IV
Jak2 and its derivative Jak2-HA-pCIneo	Silvennoinen *et al.*, 1993c, IV, Jak2-HA-pCIneo was used to clone the Jak2 deletion constructs used in I, II and III (Fig.7)	I, II, III, IV
Jak3 and its derivative Jak3-HA-pCIneo	Witthuhn *et al.*, 1994Jak3-HA-pCIneo was used to clone the Jak3 deletion constructs used in II (Fig.7)	II
pCI-Neo expression vector	Promega, Madison, WI	I, II, III, IV
GAS-Luc (WT)	Dr. Richard Pine, Pine *et al.*, 1994	I, II, II, IV
Mut-GAS-Luc	Dr. Richard Pine, Pine *et al.*, 1994	IV
Spi luciferase vector	Dr. Timothy Wood, Sliva *et al.*, 1994	IV
pEF-BOS	Dr. Kalle Saksela, Mizushima and Nagata, 1990	III
PGEX-4T-1	Amersham Pharmacia Biotech	III

Table 7. Expression plasmids used.

7.0.4 Sequencing and sequence analysis

DNA clones were sequenced using Amersham-Pharmacia's ALF Express (Amersham-Pharmacia Biotech, Uppsala, Sweden) and Perkin-Elmer's ABI 373, ABI 377 and ABI 310 automatic DNA-sequencers at the Haartman Institute and at Institute of Biotechnology, University of Helsinki. All sequence analysis was done using the GeneComposer sequence analysis package (http://www.GeneComposer.com). Contig assembly was done using Staden sequence

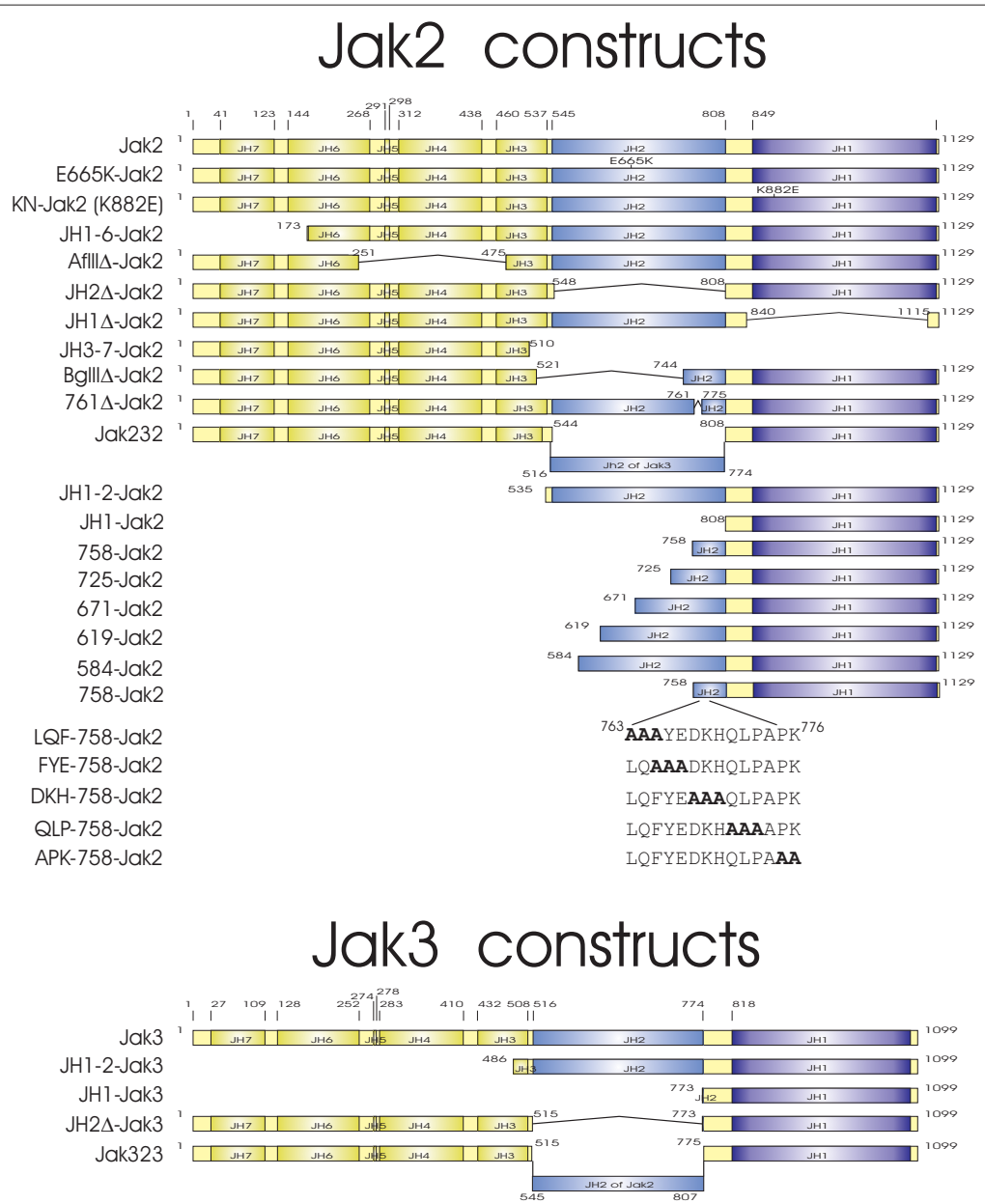

Figure 9. The Jak2 and Jak3 cDNA constructs cloned for use in I, II, III.

assembly package (Bonfield *et al.*, 1995). Domain prediction was done using the Smart program (Schultz *et al.*, 1998; http://smart.embl-heidelberg.de/). Multiple sequence alignment was done using Clustal W (Thompson *et al.*, 1994).

7.0.5 Molecular modeling

The Jak2 JH2 domain was modeled based on the structure of the activated insulin receptor kinase (Irk) at 1.9 Å resolution (Hubbard, 1997) (Protein Data Bank (PDB) (Abola *et al.*, 1997) entry 1ir3). The sequence alignment was performed with Clustal W and MULTICOMP (Vihinen *et al.*, 1992) program packages. The final alignment was obtained by manual combination of

information from multiple sequence analysis and secondary structural information from the three dimensional structures of tyrosine kinases. The model was built with the program InsightII (Accelrys, San Diego, USA), as described in III.

7.0.6 Transfection of cell lines

Cells were transfected prior to confluence using the Calcium-Phosphate Transfection kit (Invitrogen) or Fugene^{TM6} Transfection Reagent (Roche Diagnostics, Indianapolis, IN) according to manufacturer's instructions. The cells were harvested 72 h after transfection for immunoprecipitation and electrophoretic gel mobility shift assay and after 48 h (IV) or 24 h (I, II, III) for luciferase assay.

7.0.7 Immunoprecipitations

The buffers used for cell lysis and following immunoprecipitation are: Triton lysis buffer (50 mM Tris-HCl, pH 7.5, 10% glycerol, 150 mM NaCl, 1 mM EDTA, 1% TritonX100, 50 mM NaF, 1mM Na_3VO_4), kinase lysis buffer (10 mM Tris-HCl, pH 7.5, 1 % Triton X-100, 20 % glycerol, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 1 mM Na_3VO_4) supplemented with protease inhibitors PMSF, approtinin, leupeptin and pepstatin A and boiling SDS lysis buffer (0.5% SDS, 0.05 M Tris-HCl, pH 8, 1mM DTT, 50 mM NaF, 1mM Na_3VO_4). The SDS lysates were diluted with 1.5x RIPA buffer (1.25% NP-40, 1.25% sodium deoxycholate, 12.5 mM NaH_2PO_4, pH 7.2, 2 mM EDTA, 50mM NaF, 1mM Na_3VO_4 with protease inhibitors) before immunoprecipitation.

For co-immunoprecipitation cells were lysed in Brij-58 buffer (10 mM Tris-HCl, pH 7.5, 0.9% Brij-58, 0.1% NP-40, 150 mM NaCl, 50 mM NaF, 1mM Na_3VO_4) supplemented with protease inhibitors. Before immunoprecipitation bovine serum albumin was added to the precleared lysates (final concentration 1%). Immunoprecipitates were washed once with Brij-58 buffer, twice with Triton buffer, once with high salt buffer (Brij58 buffer with 350 mM NaCl) and twice with NP-40 buffer (50 mM Tris-HCl, pH 7.5, 10% glycerol, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 50 mM NaF, 1mM Na_3VO_4). Immunoprecipitations were carried out by incubating the lysates with specific antibodies at +4 C, following capture of the complexes using proteinA (Sigma)- or protein G-sepharose (Amersham Pharmacia Biotech).

7.0.8 Immunoblotting

Immunoprecipitates and cell lysates were separated in SDS-PAGE (Ready Gels, Bio Rad Laboratories) and transferred to nitrocellulose membrane. Immunodetection was performed using specific primary antibodies, biotinylated anti-mouse or anti-rabbit secondary antibodies (Dako A/S, Denmark) and streptavidin-biotin HRP-conjugate (Amersham Pharmacia Biotech) followed by ECL.

7.0.9 *In vitro* kinase assay

For kinase assay cells were lysed in kinase lysis buffer, the lysates were immunoprecipitated and the immunoprecipitates were washed four times with kinase lysis buffer and twice with kinase assay buffer (10 mM HEPES, pH 7.4, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM MnCl2, 50 mM NaF, 0.1 mM Na_3VO_4). The immunoprecipitates were suspended in kinase assay buffer containing DTT (1mM). The following peptides (1 mg/ml) were used as substrates: Stat5 (AKAADGYVKPQIKQVV), Stat1 (GPKGTGYIKTELISVS), Jak1YY (AIETDKEYYTVKDDRDS), Jak2YY (VLPQDKEYYKVKEPGES) or Jak3YY (LLPLDKDYYVVREPGQK). 10 µCi [γ-^{33}P] ATP or 10 µCi [γ-^{32}P] ATP (Amersham Pharmacia Biotech) was added to the reactions followed by 10 min incubation at room temperature and boiling in reducing Laemmli sample buffer. The reactions were separated in 20% SDS-PAGE followed by autoradiography or quantification of radioactivity using PhosphorImager (Fuji).

For kinetic analysis of catalytic activity, the assay was done as described above, but 6μCi of [γ-^{33}P] ATP was used and the final ATP concentration was adjusted to 250 μM with unlabeled ATP.

7.0.10 Baculovirus construction

Jak2-expressing baculovirus was constructed by cloning Jak2-HA cDNA into pVL1392 (Pharmingen) and transfecting it into Sf9 insect cells using the BaculoGold™ Transfection Kit (Pharmingen). Bmx-expressing baculovirus was constructed by cloning the HA-tagged Bmx cDNA into pBluebac4 (Invitrogen, San Diego, CA) and using the Bac-N-Blue Transfection Kit (Invitrogen) for creating the virus. The Stat1 and Stat3 baculoviruses were kind gifts from Dr. James N. Ihle (Quelle *et al.*, 1995b).

7.0.11 Luciferase assay

Luciferase activity was determined using Dual-Luciferase Reporter Assay System (Promega) according to manufacturer's instructions. The Stat-dependent luciferase activity of GAS-Luc and Spi-Luc was normalized to the activity of the co-transfected pRLTK plasmid constitutively expressing *Renilla* luciferase.

7.0.12 Purification of His-tagged proteins

Cells were lysed in kinase lysis buffer without EDTA. After clearing by centrifugation equal amounts of protein from the lysates were diluted tenfold with Urea binding buffer (8 M urea, 50 mM NaH_2PO_4, 10 mM Tris, pH 8, 150 mM NaCl) and incubated with Talon metal affinity resin (Clontech) for 30 min at room temperature. The resin was washed four times with Urea washing buffer (8 M urea, 50 mM NaH_2PO_4, pH 7, 150 mM NaCl) before elution with 100 mM EDTA and boiling in reducing Laemmli sample buffer.

7.0.13 Electrophoretic gel-mobility shift assay

For gel-mobility shift assay cells were lysed in WCE lysis buffer (50 mM Tris-HCl, pH 8, 0.5 % NP-40, 10 % glycerol, 0.1 mM EDTA, 150 mM NaCl, 50 mM NaF, 0.5 mM Na_3VO_4) supplemented with protease inhibitors. Annealed GAS oligonucleotide (GAS site of murine IRF-1 gene, 5'-CTA GAG CCT GAT TTC CCC GAA ATG ATG AG-3') or high affinity SIE oligonucleotide (5'-GAT CAG CAT TTC CCG TAA ATC CC-3') was end-labeled by T4 polynucleotide kinase using [γ-^{32}P] ATP or [γ-^{33}P] ATP (Amersham Pharmacia Biotech). Specifically, cell lysates and 200 ng poly-dI-dC (Amersham Pharmacia Biotech) were incubated on ice for 15' followed by additional 15' incubation after adding labeled GAS/SIE oligonucleotide. Reactions were analyzed in 4.5% TBE-PAGE (2.2x TBE concentration) followed by autoradiography. Stat5 gel-mobility shift assay was performed as described above except for that annealed oligonucleotide corresponding to the Stat5-binding site in the β-casein gene promoter (5'-AGATTTCTAGGAATTCAAATCC-3') was labeled by end-filling with Klenow and [α-^{32}P] dCTP (Amersham Pharmacia Biotech). The reactions were analyzed in 4.5% TBE-PAGE (0.25x TBE concentration).

Nuclear extracts were prepared as described earlier (Mui *et al*., 1995b). In brief, cells were lysed in buffer A (50 mM HEPES, pH 7.5, 100 mM NaF, 10 mM sodium pyrophosphate, 2 mM Na_3VO_4, 2 mM sodium molybdate, 2 mM EDTA (PSB buffer), 0.2 % NP-40, 10 mM magnesium chloride and protease inhibitors) and centrifuged. The nuclei were washed once with buffer B (same as A without NP-40), and finally lysed in buffer C (PSB with 0.1 % NP-40, 0.3 M NaCl, 10%

glycerol and protease inhibitors). Nuclear extracts (6 µg), poly-dI-dC (240 ng/µl), BSA (1.5mg/ml) and radioactively labeled GAS oligonucleotide (0.05 ng) were incubated 30' RT, and the reactions were resolved in 4.5% TBE (0.25X) PAGE, followed by autoradiography.

7.0.14 GST proteins

The JH1-domain and the JH1-2 domains of Jak2-HA were cloned into pGEx-4T-1, and transformed into XL-1 Blue Escherichia coli cell line (Stratagene). Overnight cultures were diluted 1:200 in Luria media, and incubated for 3 hours following induction with 1 mM IPTG (Sigma). Alternatively, the cells were directly lysed without induction, and used for immunoblotting.

8 RESULTS

8.1 The JH2 domain regulates Jak2 and Jak3 kinases (I, II)

At the beginning of this thesis study, Jak tyrosine kinases had been identified as linking cytokine receptor activation to changes in transcriptional regulation in the nucleus. Numerous other tyrosine kinases had been cloned a couple of years ago and found to form subfamilies, and Jak kinases were referred to as a family of "just another kinases". However, immediately following their cloning Jaks were found unique within other kinase subfamilies in containing two kinase domains forming half of the entire Jak molecule, while the other half of the molecule showed only little homology at sequence level to other kinases, or to protein domains characterized. Therefore, it was of interest to analyze what was the function of the second kinase domain as well as the non-homologous N-terminus in Jaks. Jak2 was first analyzed, since it had been cloned by Dr. Silvennoinen (Silvennoinen *et al.*, 1993c). As it became important to find out if the results concerning Jak2 were also applicable to other Jak kinases, Jak3 was analyzed, since it has the highest homology to Jak2 of the three other Jak kinases.

8.1.1 Deletion of JH2 increases the activity of Jak2 and Jak3 kinases (I, II)

In order to investigate the role of the JH domains in Jak2 and Jak3, a number of deletion constructs were cloned into a mammalian expression vector, and the constructs were transiently expressed in either 293T or COS cells. Over-expression of Jak kinases in these cell types results in activation of the kinase without the need for stimulation with cytokines or growth factors. The constructs used are listed in the Materials and Methods section (Figure 9).

Deletion of JH2 from Jak2 was found to result in increased tyrosine phosphorylation of Jak2 itself as well as co-expressed Stat5, while other deletions encompassing JH domains 3-4 and 6-7, had no such effects (I: Fig. 2B and 3). A Jak2 protein containing the double kinase domain motif (JH1-2-Jak2) was phosphorylated similarly to Jak2, and the two proteins induced equal level of Stat5 phosphorylation, indicating that the N-terminal JH3-7 domains had no effect on the activity of Jak2 (I: Fig. 2D and 3). However, the activity of the isolated JH1 domain was significantly increased when compared to Jak2, as detected by increased level of autophosphorylation and activation of co-expressed Stat5 (I: Fig. 2D and 3).

A similar approach with Jak3 demonstrated an identical situation; deletion of JH2 either from full-length Jak3 or from JH1-2-Jak3, containing the JH1 and JH2 domains, resulted in increased phosphorylation of Jak3 as well as co-expressed Stat5 (II: Fig. 3BCD). These results indicated that the JH2 domain negatively regulated the activity of Jak2 and Jak3 in mammalian cells. Furthermore, the isolated JH1 domains of Jak2 and Jak3 were functional kinases, when expressed alone, and the presence of JH2 reduced the activity of JH1 in mammalian cells.

8.1.2 The effect of JH2 on *in vitro* kinase activity of Jak2 and Jak3 (I, II, III)

The effect of JH2 on the activity of Jak2 and Jak3 was also studied using *in vitro* kinase assay. Upon deletion of JH2 the activity of Jak2 was increased 5-10 fold, while deletion of JH domains 3-4, 6-7 or 3-7 had no such effects (I: Fig. 2AB). Also, the isolated JH1 domain had

over 50-fold higher activity when compared to Jak2 (I: Fig. 2B). Thus, the results from mammalian cells correlated well with the *in vitro* kinase data indicating negative regulation of Jak2 by the JH2 domain.

The deletion of JH2 in Jak3 only slightly increased the kinase activity of Jak3, and the isolated JH1 domain had twofold activity when compared to Jak3 (II: Fig. 3E). Interestingly, JH1-2-Jak3, containing the double kinase domain motif, showed reduced activity when compared to JH1, indicating downregulation of JH1 activity by JH2 (II: Fig. 3E). Of note is that the activity of JH1-2-Jak3 was reduced by 30% when compared to Jak3. Thus, the results from *in vitro* kinase assay showed negative regulation of Jak3 JH1 by the JH2 domain. However, the effect of JH2 on Jak3 kinase activity was not as pronounced as in Jak2.

To gain insight into the mechanism by which JH2 negatively regulated the *in vitro* kinase activity of Jak2, a kinetic analysis of kinase activity was performed. The Jak2 JH1 domain or the double kinase domain of Jak2, JH1-2-Jak2, was expressed in 293T cells, and the activity of the immunoprecipitated proteins was analyzed in regard to time and varying substrate peptide concentration (III: Fig. 2). Both JH1-Jak2 and JH1-2-Jak2 showed a typical linear relationship between time and phosphorylation of the substrate. The activity of the kinases increased similarly with increasing substrate concentrations, and JH1-Jak2 and JH1-2-Jak2 reached their half-maximal activities in similar peptide concentrations. However, the highest activity of JH1 was approximately 5-10 times higher than that of JH1-2. Thus, the JH2 domain decreases the maximal velocity (Vmax) of Jak2, but does not significantly affect the Km for a peptide substrate.

8.1.3 The Stat5 SH2 domain is required for activation by Jak2, but not by JH2Δ-Jak2 (I)

The SH2 domains of Stats are critical for their activation by Jak kinases (Gupta *et al.*, 1996). In accordance with this, Jak2 was found to phosphorylate Stat5, but not its SH2 mutant carrying a R618L substitution (I: Fig. 4). However, the Jak2 deletion mutants lacking JH2 activated both Stat5 and its SH2 mutant (I: Fig. 4). Deletion of JH domains 3-7 in Jak2 did not change the requirement for the SH2 domain in activation of Stat5, although increasing amounts of JH1-2-Jak2 were used resulting in higher tyrosine phosphorylation of JH1-2-Jak2 than JH2Δ-Jak2 (I). Thus, the high catalytic activity of the JH2 deletion mutants was not the explanation for phosphorylation of the SH2 mutant Stat5.

Phosphorylation of tyrosine 694 in Stat5 is required for dimerization and activation of Stat5. To test if JH2Δ-Jak2 phosphorylated specifically this residue in Stat5, JH2Δ-Jak2 was co-expressed with Y694F-Stat5 in 293T cells. JH2Δ-Jak2, as well as Jak2, was unable to phosphorylate the Y694F mutant of Stat5 (I). This indicated that despite of its high activity, JH2Δ-Jak2 retains its specificity by phosphorylating only the critical tyrosine 694 in Stat5. Taken together, these results indicate interdependence between the Jak2 JH2 domain and the Stat5 SH2 domain in Jak2-mediated activation of Stat5, suggesting that the JH2 domain affects interactions of Jak2 with its target proteins.

8.2 JH2 is required for cytokine receptor signaling (I, II)

8.2.1 The JH2 domain inhibits of basal activity of Jak2 and Jak3 (I, II)

To analyze the effect of JH2 in cytokine receptor signal transduction, Jak2 and Jak3 kinases lacking JH2 domains were expressed in cells, where activation of Jaks is dependent on stimulation with IFN-γ or IL-2, respectively (I: Fig. 7, II: Fig. 6). Activation of Jaks was monitored using a Stat-dependent luciferase construct. Wild-type Jak kinases showed very little, if at all, Stat activation in the absence of stimulation, but responded with increased activity to cytokine treatment. However, both JH2 deletion mutants showed unregulated activity by inducing constitutively elevated Stat activity in the absence of cytokine stimulation. This indicated that the function of JH2 was to negatively regulate basal Jak activity in the absence of cytokine stimulation, where Jaks are supposed to be inactive. It is of note, however, that upon deletion of JH2 from Jak2, Stat1 activation was much more prominent than Stat5 activation resulting from deletion of JH2 in Jak3. Thus, it was concluded, together with the *in vitro* kinase assay data, that the negative regulatory role of JH2 might not be as important in Jak3 as it is in Jak2.

8.2.2 The JH2 domain is required for cytokine-inducible activation of signal transduction (II)

A second result from the analysis of the function of JH2 in cytokine receptor signaling was that Jak2 and Jak3 kinases lacking JH2 were unresponsive to cytokine stimulation (II: Fig. 6). Wild-type Jak2 and Jak3 showed low basal activity, and upon stimulation, a clear induction in Stat activation. However, the elevated level of basal signaling mediated by JH2Δ-Jak2 and JH2Δ-Jak3 was not further increased when the cells were stimulated with IFN-γ or IL-2, respectively. This indicated that the JH2 domains of Jak2 and Jak3 were required for cytokine-inducible activation of signal transduction.

8.2.3 The effect of JH2 in Jak activation in cytokine receptors (II)

To find out, if JH2 was required for cytokine-inducible activation of the Jak kinases themselves, in addition to being required for inducible Stat activation, activation of JH2Δ-Jak2 was analyzed in IFN-γ receptor signaling. In Jak2-negative cells transfected with JH2Δ-Jak2, IFN-γ had no effect on tyrosine phosphorylation of JH2Δ-Jak2, while in Jak2-transfected cells IFN-γ-induced increased tyrosine phosphorylation of wild-type Jak2 (II: Fig. 7A). Furthermore, JH2Δ-Jak2 showed significantly increased tyrosine phosphorylation when compared to that of Jak2. Thus, JH2 was found to be required for inhibition of basal Jak2 activity. In addition, JH2 was required for rendering Jak2 competent to respond to cytokine stimulation with increased activity.

Jak kinase activation in response to IFN-γ most likely requires the reciprocal interaction of Jak1 and Jak2. Thus, it was possible that in the absence of JH2, the Jak1 and Jak2 kinases were unable to interact, resulting in the lack of IFN-γ-inducible activation of JH2Δ-Jak2. Therefore, kinase-inactive forms of Jak2 and JH2Δ-Jak2 were co-expressed with Jak1 (II: Fig. 7B). The analysis of the tyrosine phosphorylation levels of Jak2 and JH2Δ-Jak2

indicated that Jak1 phosphorylated both Jak2 proteins equally well. Thus, JH2 was not required for interaction of Jak1 and Jak2 as such, suggesting that inducible activation of JH2Δ-Jak2 was specifically disturbed in the context of the IFN-γ receptor.

8.3 Comparison of JH2 and JH1 domains of Jak2 and Jak3 (II)

8.3.1 The JH2 domains of Jak2 and Jak3 are functionally similar, but not identical (II)

The results from the analysis of Jak2 and Jak3 indicated that JH2 negatively regulated both kinases, but the results also suggested that negative regulation of Jak3 was not as significant as that of Jak2. To directly compare the function of the JH2 domains in Jak2 and Jak3, chimeric constructs were cloned, in which the JH2 domain of Jak2 was transferred to Jak3 and vice versa. These chimeric constructs were analyzed in 293T or COS cells. Tyrosine phosphorylation of the chimeric proteins was comparable to that of wild-type Jak2 and Jak3, and similarly, the co-expressed Stat5 was equally phosphorylated by the chimeric and wild-type Jak kinases (II: Fig. 4 and 5). Thus, the JH2 domains were found to be interchangeable between Jak2 and Jak3.

The chimeric constructs were also analyzed in cytokine receptor signaling. Jak232, containing the JH2 domain of Jak3, behaved as wild-type Jak2 in IFN-γ signaling by having low basal activity and inducing significant increase in Stat activation following IFN-γ stimulation (II: Fig. 6B). Thus, the JH2 domain of Jak3 was able to restore the wild-type phenotype of Jak2.

Jak323, containing the JH2 domain of Jak2, had reduced basal activity when compared to JH2Δ-Jak3 and showed induction in Stat5 activity following IL-2 treatment, although the induction was significantly lower than that induced by wild-type Jak3 (II: Fig. 6A). This suggested that JH2 of Jak2 could not fully complement the Jak3 JH2 domain in IL-2 receptor signaling.

8.3.2 Comparison of kinase activity of Jak1, Jak2, and Jak3 kinases (II)

Having analyzed the effect of various JH deletions on the activity of Jak2 and Jak3, the activity of full-length Jak kinases was compared. Jak1, Jak2 and Jak3 were expressed in 293T cells, and the kinase activity as well as tyrosine phosphorylation of the expressed proteins were analyzed (II: Fig. 1). Jak2 had very low kinase activity and tyrosine phosphorylation when compared to those of either Jak1 or Jak3. Interestingly, Jak1 and Jak3 showed different substrate peptide preference. Jak1 was most efficient in phosphorylating a peptide containing the phosphorylation site of Stat5, while Jak3 preferred peptides containing the autophosphorylation site sequences of Jak1, Jak2 and Jak3.

The activity of the isolated tyrosine kinase domains of Jak2 and Jak3 was also compared, and this analysis showed that the JH1 domain of Jak2 had significantly lower activity than that of Jak3 (II: Fig. 2).

8.4 The mechanism of JH2-mediated inhibition of Jak2 (I, III)

8.4.1 PTPase inhibitors activate Jak2 independently of JH2 (I)

To elucidate the mechanism of JH2-mediated inhibition of Jak2 activity, several possibilities were considered. First, Jak2 might be a target for a protein tyrosine phosphatase, whose activity would be dependent on the presence of JH2. Therefore, the effect of PTPases was analyzed by treating cells expressing different Jak2 deletion mutants with phosphatase inhibitors (I: Fig. 5). In cells treated with pervanadate, tyrosine phosphorylation of Jak2 was increased. Similarly, pervanadate increased phosphorylation of JH1 and JH2Δ-Jak2, indicating that in the absence of JH2, Jak2 was regulated by PTPases. Thus, it was concluded, that PTPase-mediated regulation of Jak2 might not be abrogated by deletion of JH2, and that JH2 might regulate Jak2 in other ways than through the recruitment of a PTPase.

8.4.2 The JH2 domain inhibits the activity of co-expressed JH1 (I)

Another possible mechanism for JH2-mediated inhibition was that JH2 interacted with JH1, and thereby inhibited its kinase activity. To test this, co-immunoprecipitation studies were undertaken using 293T cells expressing JH1 and JH2. However, the expression of JH2 remained extremely low, and co-immunoprecipitation could not be analyzed, although numerous different JH2 expression plasmids were cloned for this purpose. Therefore, it was questioned, if JH1 was able to interact with Jak2, and if so, what JH regions affect this interaction. JH1 was expressed together with Jak2 or Jak2 lacking JH1 (JH1Δ-Jak2), and found to co-immunoprecipitate with both Jak2 and JH1Δ-Jak2 (I: Fig. 6A). However, co-immunoprecipitation of JH1 with JH1Δ-Jak2 was much weaker than with Jak2. This indicated that JH1 could interact with Jak2 in two different ways. First, JH1 interacted with another JH1, since deletion of JH1 reduced the association of JH1 with Jak2. This type of interaction is supposed to be important during activation of Jak2. Second, JH1 could associate with JH regions 2-7 in Jak2, but this association was weaker than the JH1-JH1 interaction. Deletion of JH domains 3-7 did not affect interaction of JH1 with Jak2 suggesting that JH1 did not interact with domains 3-7 in Jak2 (I).

To analyze the effect of the interaction of JH1Δ-Jak2 with JH1, JH1 was co-expressed with JH1Δ-Jak2, or with a kinase-inactive Jak2. The kinase inactive form of Jak2 is able to inhibit activation of Jak2 when co-expressed, most probably by competing in dimerization with wild-type Jak2, which is required for autophosphorylation and subsequent activation (Zhuang *et al.*, 1994). Co-expression of either JH1Δ-Jak2 or kinase-inactive Jak2 reduced the activity of JH1 as detected by lower level of autophosphorylation of JH1 as well as by reduced level of substrate peptide phosphorylation (I: Fig. 6BC). On the contrary, co-expression of JH3-7 domains with JH1 had no effect on autophosphorylation of JH1 (I: Fig. 6D). In conclusion, the results demonstrated that interaction of JH1Δ-Jak2 with JH1 was associated with inhibition of JH1 activity, while no inhibition was obtained if JH2 was further deleted. Thus, the results suggested that JH1 was inhibited by interaction with JH2.

8.4.3 Autoinhibition of Jak2 by the JH2 domain in *E. coli* (III)

The results from mammalian cells suggested that JH2 regulated JH1 by interacting with it. Again, several possibilities existed; JH2 might keep JH1 in a conformation where catalytic activity was autoinhibited or directly prevent the access of ATP or substrates to the catalytic cleft, or alternatively, recruit a regulatory protein to the vicinity of JH1, which might be responsible for inhibition of JH1 activity.

To distinguish between these possibilities, JH1-Jak2 and JH1-2-Jak2 were expressed as GST-fusion proteins in bacterial cells (III: Fig. 1). Prokaryotes do not have tyrosine kinases, and thus are not expected to have proteins regulating these kinases. JH1-2-GST and JH1-GST were both tyrosine phosphorylated, indicating that the expressed proteins were active in bacterial cells. However, phosphorylation of JH1-GST was much higher than that of JH1-2-GST, indicating that JH1-GST was more active than JH1-2-GST. Moreover, a number of bacterial proteins were tyrosine phosphorylated in JH1-GST expressing cells, but not in JH1-2-GST cells. Thus, JH2 regulated the activity of JH1 also in bacterial cells, indicating that JH2-mediated regulation of Jak2 was an intrinsic property of the Jak2 molecule and not dependent on additional regulatory proteins, strongly suggesting for JH2-mediated autoinhibition of Jak2.

8.5 Autoinhibitory regions in the JH2 domain (III)

To investigate the possible mechanism for JH2-mediated autoinhibition of Jak2, an attempt was started to identify possible regions in JH2 required for Jak2 regulation. The backbone construct JH1-2-Jak2 was sequentially deleted, starting from the N-terminus of JH2, to create five deletion constructs. The deletion constructs were co-expressed with Stat5 in 293T cells (III: Fig. 5BC). This analysis revealed three inhibitory regions (IR1, IR2 and IR3) that when deleted from the JH1-2-construct, resulted in increased phosphorylation of co-expressed Stat5. IR1 contained amino acids 618-670 located at the border of the N-terminal and C-terminal lobes of the JH2 domain. IR2 and IR3 were located in the C lobe and encompassed amino acids 725-757 and 758-807, respectively. Localization of the inhibitory regions was based on molecular modeling of the 3-dimensional structure of the JH2 domain of Jak2 (III: Fig. 3).

IR3 is a 50-amino acid region at the C-terminus of JH2 directly preceding the linker domain and JH1, and it was found to significantly inhibit JH1, suggesting direct interaction between IR3 and JH1 (III: Fig. 5C). The model structure of JH2 suggested that IR3 might fold properly as an independent unit, thereby supporting the finding that IR3 alone was able to inhibit JH1. The addition of IR2 and IR1 enhanced IR3-mediated inhibition of JH1 (III: Fig. 3B).

The presence of an inhibitory region in the C-terminus of Jak2 JH2 was further strengthened by the finding that two JH2 deletions differing by a 60-amino acid C-terminal JH2 region had different activities (III: Fig. 4BCD). Specifically, the entire JH2 domain was deleted in JH2Δ-Jak2, but the 60 residues from the C-terminus of JH2 were present in BglIIΔ-Jak2. BglIIΔ-Jak2 showed increased activity when compared to Jak2, indicating that the inhibitory regions present in JH2 were missing in BglIIΔ-Jak2. These included the previously identified

IR1 and IR2. However, the activity of BglIIΔ-Jak2 was lower than that of JH2Δ-Jak2, indicating that the C-terminal 60 amino acids in JH2, including IR3, contained an inhibitory region.

To further analyze the role of IR3 in JH2-mediated regulation of Jak2, alanine mutations were created in the 758-Jak2 construct. The activity of 758-Jak2 was increased when amino acids 763-765 (LQF), 765-767 (FYE) or 771-773 (QLP) were substituted with alanines (III: Fig. 6B). Molecular modeling suggested that residues 763L, 764Q, 765F, E767, 771Q and 773P in IR3 might be exposed on the surface of the JH2 domain, thus being able to interact with JH1. Y766, on the other hand, projects inward in the model and is not likely to interact with JH1, and consequently, may not be phosphorylated. The molecular model also showed that residues 763-767 are located in αG helix, and the helical structure might be an important structural feature and locally distorted by alanine mutations, thus causing deregulation of inhibition.

The sequence, where the alanine mutations were introduced was also deleted from full-length Jak2 (761Δ-Jak2), and this resulted in increased phosphorylation of Jak2 and elevated level of basal IFN-γ signaling (III: Fig. 7BC). However, 761Δ-Jak2 had lower activity than JH2Δ-Jak2, where the entire JH2 domain was deleted, in accordance with the finding that IR1 and IR2 also inhibited Jak2 activity. These results indicated that IR3 is involved in inhibition of basal Jak2 activity, but it is not solely responsible for inhibition. Rather, inhibition of Jak2 is dependent on multiple sites in JH2.

8.6 The Bmx kinase induces activation of the Stat signaling pathway (IV)

The Bmx tyrosine kinase had been cloned at the Haartman Institute in 1994 (Tamagnone *et al.*, 1994) based on screening of a bone marrow derived cDNA library. In 1995, when the current work was started, not much was known about the Bmx kinase. For instance, it was not known, what cytokines or growth factors might activate Bmx or what substrate proteins were activated by the Bmx kinase. To study the signaling mechanisms of the Bmx kinase, a collaborative study was started with the laboratory of Prof. Kari Alitalo.

The possibility that Bmx might function in the newly identified Stat pathway was studied. Bmx was expressed in COS cells, and the activity of endogenous Stat proteins was analyzed. COS cells express significant amounts of Stat1, but not other Stats. Stat1 was found activated in Bmx-expressing cells as detected by the ability of Stat1 to bind DNA, and the level of Stat1 activation was comparable to that induced by Jak2, which was used as a control (IV: Fig. 5).

To analyze the ability of Bmx to activate also other Stat proteins, Bmx was co-expressed with Stat3 and Stat5, and also, as a control, Stat1 in COS cells. Bmx induced activation of all the Stat proteins analyzed at a similar level, as did Jak2 (IV: Fig. 1, 2, 3 and 4). In contrast, other cytoplasmic tyrosine kinases tested, namely c-Src, Syk and Fyn, showed only very little Stat activation (IV: Fig. 1 and 2). To rule out Jak kinase-mediated activation mechanism for Bmx-induced Stat activation, Bmx- and Jak2-expressing baculoviruses were created. In insect cells, Jak2 and Bmx induced activation of Stat1 and Stat3, but

phosphorylation of Stat3 by Bmx was much higher than that induced by Jak2 (IV: Fig. 7 and 8). Furthermore, endogenous Jak activation could not be detected in Bmx-expressing cells (IV: Fig. 6). Altogether, these results indicated that Bmx was able to induce activation of multiple Stat proteins, and thereby suggested that Bmx might function in signaling pathways leading to Stat activation.

Other Btk/Tec family kinases, such as Btk and Itk, had been found to be regulated by members of the PKC family of serine/threonine kinases. In the case of Btk, the PKC isoforms α, β1, β2, ε and ζ had been found to inhibit Btk activity, whereas the activity of Itk was enhanced by PKCα, PKCβ1 and PKCβ2 (Yao *et al.*, 1994; Kawakami *et al.*, 1995; Yao *et al.*, 1997).

Therefore, it was analyzed, if Bmx was also a target for regulation by PKC. Bmx was co-expressed with different PKC isoforms and Stat1, and the effect of PKC on Stat1 activation was analyzed (IV: Fig. 9 and 10). PKC-δ, but not other PKC isoforms, PKC-β1, PKC-ε or PKC-ζ, significantly inhibited activation of Stat1 by Bmx, suggesting that PKC-δ negatively regulated signaling by Bmx.

9 DISCUSSION

Protein tyrosine kinases are essential mediators of cellular signaling pathways. Reversible protein phosphorylation plays a key role in diverse cellular processes such as proliferation, differentiation and apoptosis. The activity of PTKs is normally tightly regulated. Abrogation of kinase regulation, for example due to mutations in PTKs themselves, has been found to result in hyperactivated kinases, and even cancer (reviewed in Blume-Jensen and Hunter, 2001). Many of the known oncogenes code for malfunctioning PTKs. Therefore, understanding the normal function and regulation of tyrosine kinases is of critical importance. The current work has focused on kinases from the Jak and Btk/Tec non-receptor PTK families, their regulation and functions in the Stat signaling pathway.

9.1 Bmx-induced Stat activation

In this work, activation of Stat transcription factors was identified as a potential downstream signaling event for the Bmx tyrosine kinase (IV). Expressed Bmx induced activation of endogenous Stat1, as well as co-expressed Stat1, Stat3 and Stat5 (IV). While these studies did not show *in vivo* activation of Stats by Bmx, subsequently Bmx has been shown to activate Stats in similar as well as in more physiological systems (Wen *et al.*, 1999; Jui *et al.*, 2000; Tsai *et al.*, 2000), supporting the results from the current work (IV). One possible explanation for Bmx-induced Stat activation is that Bmx activates endogenous Jak kinases. However, endogenous Jak activation in Bmx-transfected cells was not detected (IV). Furthermore, the transfected Bmx and Jak kinases were equally effective in induction of Stat activation (IV). This would be unlikely if Bmx works through activation of endogenous Jak kinases. In addition to Bmx, another family member, Btk, directly interacts and phosphorylates Stat5 *in vitro* (Mahajan *et al.*, 2001), but the prototype member of the Btk/Tec family, Tec, is unable to induce Stat activation (Quelle *et al.*, 1995b). Btk is crucial in activating Stat5 through BCR (Mahajan *et al.*, 2001), where Stat activation has been found to occur within minutes after activation of BCR, although Jaks are not activated (Karras *et al.*, 1996).

A number of receptor tyrosine kinases, such as EGFR and PDGFR have been found to directly phosphorylate Stat proteins (Quelle *et al.*, 1995b; David *et al.*, 1996; Leaman *et al.*, 1996; Park *et al.*, 1996b; Vignais *et al.*, 1996; Vignais and Gilman, 1999; Paukku *et al.*, 2000). In cancer cells, constitutive Stat activation is dependent on upstream tyrosine kinases, often other than Jaks. For example, NPM/ALK induces activation of Stat3 and Stat5, and BCR/ABL directly activates Stat5, although Stat5 may not be required for transformation by BCR/ABL (de Groot *et al.*, 1999; Nieborowska-Skorska *et al.*, 1999; Sexl *et al.*, 2000Nieborowska-Skorska *et al.*, 2001; Sonoyama *et al.*, 2002; Zhang *et al.*, 2002a). Activation of Stat3 is required for tumorigenesis induced by the Met receptor tyrosine kinase (Zhang *et al.*, 2002b). Overexpressed Syk, c-Src or Fyn showed much weaker ability to activate Stats as compared to Bmx or Jak2, illustrating clear differences in their substrate specificity (IV). c-Src has been found to activate Stat3 in NIH3T3 cells, but v-Src was able to induce much higher level of Stat3 activation (Yu *et al.*, 1995). Although v-Src has been found to directly activate Stats (Cao *et al.*, 1996; Chaturvedi *et al.*, 1998), it is not entirely clear if v-Src-mediated Stat activation is always direct. Overexpression of v-Src in a myeloid cell line

induces constitutive activation of Stat1, Stat3 and Stat5, but only Stat3 is activated directly by v-Src (Chaturvedi *et al.*, 1998). Thus, Src-induced Stat activation may depend on other kinases, such as Jaks, which show increased activation in v-Src transformed cells (Campbell *et al.*, 1997; Murakami *et al.*, 1998). In some cases Src and Jak are suggested to co-operate in Stat activation (Zhang *et al.*, 2000b). On the contrary, Bmx enhances v-Src-mediated Stat activation in fibroblast cells, which can be explained by a sequential activation pathway: v-Src positively regulates the activity of Bmx, which activates Stat3 more efficiently than v-Src, in agreement with the results in the current work (IV; Tsai *et al.*, 2000). Dominant negative Bmx also reduces v-Src -mediated transformation and, although Bmx itself does not induce transformation of NIH3T3 cells, the results indicate that the Src-Bmx-Stat3 pathway is important in cellular transformation (Tsai *et al.*, 2000). Thus, depending on the particular receptor and on the cellular contex other tyrosine kinases, in addition to Jaks, regulate the Stat pathway, and Tec/Btk kinases have been found to be among those (IV; Wen *et al.*, 1999; Tsai *et al.*, 2000; Mahajan *et al.*, 2001). Based on these and similar data, the Stat signaling pathway is sometimes suggested to be more appropriately called the "PTK-Stat" pathway (reviewed in Rane and Reddy, 2000; Reddy *et al.*, 2000; Rane and Reddy, 2002).

Bmx has been implicated in malignant processes of various cell types. In contrast to other Tec kinases, Bmx is expressed also outside the hematopoietic system in a variety of cells and tissues, including breast, lung and prostate tissues as well as epithelial and endothelial cells (Tamagnone *et al.*, 1994; Ekman *et al.*, 1997; Qiu *et al.*, 1998; Rajantie *et al.*, 2001). The expression of Bmx in mammary epithelial cells is regulated during mammary gland development (Bagheri-Yarmand *et al.*, 2001). Bmx is also highly expressed in human breast cancer cell lines, where expression of a dominant negative Bmx inhibits the tumorigenic phenotype of these cells (Bagheri-Yarmand *et al.*, 2001). Bmx can also enhance anchorage-independent growth of a non-invasive breast cancer cell line (Bagheri-Yarmand *et al.*, 2001). In this context, it is interesting to note that Stat5A is essential for mammary gland development, and that constitutive activation of Stat3 frequently occurs in breast cancers (Garcia *et al.*, 1997; Sartor *et al.*, 1997; Teglund *et al.*, 1998). Furthermore, constitutive Stat activation is detected in tumor-infiltrated lymph nodes, suggesting it may be a property of more invasive tumors (Bowman *et al.*, 2000). A role for Bmx in metastasis has been suggested by studies showing that a dominant negative Bmx inhibits the motility of metastatic mammary and prostate carcinoma cells, where Bmx is highly expressed (Chen *et al.*, 2001).

Bmx has been linked to Stat activation in malignant processes, but as to yet, evidence is missing for participation of Bmx in Stat activation in its normal cellular pathways (Tsai *et al.*, 2000). By the time the work (IV) was started, no upstream activator for Bmx was known, and the identification of Stat transcription factors as substrates for Bmx was the first downstream signaling event reported for Bmx, and among the first substrates identified for any of the Btk/Tec kinases. Currently, Bmx is known to be activated by IL-3, IL-6 and GM-CSF, through endothelium-specific receptors Tie-2/Tek and vascular endothelial growth factor receptor 1 (VEGFR-1) as well as heterotrimeric G proteins (Mao *et al.*, 1998; Qiu *et al.*, 1998; Ekman *et al.*, 2000; Rajantie *et al.*, 2001). All these upstream activators also activate Stats (Marrero *et al.*,

1995; Mui *et al.*, 1995b; Korpelainen *et al.*, 1999; Bartoli *et al.*, 2000). The phenotype of Bmx knockout mice is normal, however, and does not reveal any nonredundant functions for Bmx (Rajantie *et al.*, 2001).

Recently, Bmx was found to mediate integrin signaling and cell migration through FAK (Chen *et al.*, 2001). FAK binds, through its FERM domain, to the PH domain of Bmx, which results in activation of Bmx. Activation of Bmx has been found to result in reorganization of actin filaments (Hamm-Alvarez *et al.*, 2001). Disruption of the FAK-Bmx interaction inhibits migration of normal as well as metastatic prostate and mammary cancer cells (Chen *et al.*, 2001). Bmx has been found to activate RhoA, a small GTPase, involved in cytoskeletal reorganization, suggesting that Bmx enhances cell migration through activation of RhoA (Mao *et al.*, 1998Kim *et al.*, 2002). Interestingly, a role for Stat3 has been implicated in the motility of epidermal cells (Sano *et al.*, 1999).

FAK-mediated activation of Bmx does not require PI3K, which is now known to activate Bmx (Qiu *et al.*, 1998; Ekman *et al.*, 2000), nor lipid binding by the PH domain. This suggests that FAK activates Bmx by inducing a conformational change in Bmx, which disrupts inhibition mediated by the Bmx PH domain (Chen *et al.*, 2001). Similarly, a protein tyrosine phosphatase D1 (PTPD1) induces Bmx activation through binding to the PH domain, which results in increased activation of Stat3 in salivary Pa-4 cells (Jui *et al.*, 2000).

All in all, Bmx has been found to be involved in several different, and even opposing cellular processes, such as apoptosis (Ekman *et al.*, 2000; Wu *et al.*, 2001) and anti-apoptosis (Xue *et al.*, 1999), differentiation (Qiu *et al.*, 1998), cell migration (Chen *et al.*, 2001; Pan *et al.*, 2002) and cell transformation (Tsai *et al.*, 2000). Thus, the function of Bmx appears to be complex, and most likely is dependent on a particular cellular context, where Bmx is expressed.

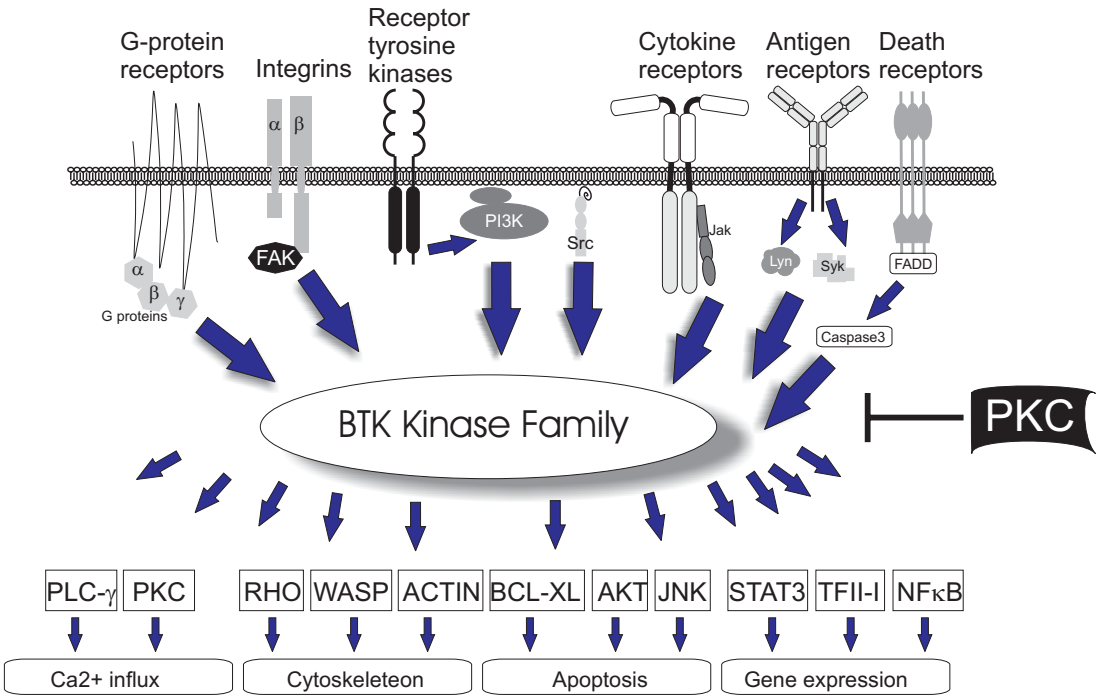

Figure 10. Schematic presentation of signaling by Btk/Tec kinases, modified from Qiu and Kung, 2000.

*9.2 Bmx signaling is regulated by PKC*δ

PKCδ, but not β1, ε or ζ isoforms, was found to regulate Bmx signaling by inhibiting Bmx-mediated tyrosine phosphorylation of Stat1 (IV). PKCδ belongs to the group of novel PKCs (PKCδ,, ε, η, θ), which are regulated by diacylglycerol, but not calsium, distinguishing them from classical (PKCα, β, γ) and atypical (ζ, λ) PKCs. Hematopoietic transmembrane receptors, such as cytokine, antigen and Fc receptors, commonly activate PKC. In PKCδ signaling, PKCδ often becomes tyrosine phosphorylated, which modulates its kinase activity. For example, substance P, carbacol, EGF-, IL-6- and IgE-induced signaling pathways have been found to induce tyrosine phosphorylation of PKCδ (Haleem-Smith *et al.*, 1995; Soltoff and Toker, 1995; Denning *et al.*, 1996; Jain *et al.*, 1999). Co-expression of Bmx with PKCδ also induced tyrosine phosphorylation of PKCδ indicating possible regulation of PKCδ by Bmx (IV).

The mechanism by which PKCδ inhibits Bmx-induced Stat activation is currently unknown. Recently, PKCδ has been found to participate in IL-6- and type I IFN-induced Stat pathways (Jain *et al.*, 1999; Schuringa *et al.*, 2001; Uddin *et al.*, 2002). IL-6 stimulation induced tyrosine phosphorylation and activation of PKCδ, and its concomitant association with Stat3 leading to serine phosphorylation of Stat3. This resulted in decreased tyrosine phosphorylation, DNA binding and transcriptional activation by Stat3 (Jain *et al.*, 1999), in agreement with the results found in the current work (IV). However, serine phosphorylation by PKC-δ has also been reported to increase transcriptional activation by Stat3 (Schuringa *et al.*, 2001). PKCδ also enhances transcriptional activity of Stat1 by α/β IFNs (Uddin *et al.*, 2002). Thus, PKCδ has been found to both inhibit and enhance Stat signaling. A similar phenomenon has been described for ERK-mediated regulation of Stat3. Stat3 tyrosine phosphorylation was found to be negatively regulated through the ERK pathway (Chung *et al.*, 1997b; Jain *et al.*, 1998), while ERK was also found to phosphorylate Stat3, resulting in increased transcriptional activity of Stat3 (Turkson *et al.*, 1999). The different results concerning the role of ERK in Stat3 signaling have been suggested to depend on the expression level of ERK, low amounts being inducing and high levels inhibiting to Stat3 (Turkson *et al.*, 1999). Whether this explains the different effects of PKCδ on Stat activation is not known.

Bmx itself might also be the target for negative regulation by PKCδ. Several PKC isoforms have been implicated in signaling by Btk/Tec kinases (Yao *et al.*, 1994; Kawakami *et al.*, 1995; Yao *et al.*, 1997Johannes *et al.*, 1999; Kawakami *et al.*, 2000; Kang *et al.*, 2001; Crosby and Poole, 2002). PKCβ negatively regulates Btk activity by directly phosphorylating a serine residue in the TH domain of Btk (Yao *et al.*, 1994; Kang *et al.*, 2001). Mutation of the serine residue results in enhanced tyrosine phosphorylation and membrane localization of Btk as well as increased signaling through BCR and FcεRI (Kang *et al.*, 2001). The PKCβ phosphorylation site is conserved in other Btk family members, except for Bmx, suggesting that the activity of Bmx is regulated differentially from other family members. Interestingly,

PKCδ and PKCα were found to inhibit Stat activation through the IL-3R, and this was found to result from direct inhibition of Jak2 activity by PKC (Kovanen *et al.*, 2000). However, regulation of Bmx by PKCδ could not be detected (IV).

PKCδ might also activate a phosphatase capable of dephosphorylating Stats or Bmx. In primary monocytes, IFN-α signaling has been found to be inhibited by phorbol esters through an as yet uncharacterized protein tyrosine phosphatase (Petricoin *et al.*, 1996). Phorbol esters have also been found to increase the catalytic activity and expression of the SH2-containing phosphatase, SHP-1, which is implicated in the downregulation of cytokine receptor signaling by dephosphorylating the Jak kinases (Uchida *et al.*, 1993). Yet, it is possible that PKCδ induces expression of proteins negatively regulating Stat activity, such as SOCS or PIAS proteins.

9.3 The JH2 domain regulates the basal activity of Jak2 and Jak3

The central finding in this thesis work is the identification of the pseudokinase domain, JH2, as a regulatory domain in Jak2 (I) and Jak3 (II) kinases. Deletion of JH2 from Jak2 and Jak3 resulted in increased Jak activity and constitutive Stat activation, indicating a negative regulatory role for the JH2 domain (I, II). Although negative regulation by JH2 was more evident in Jak2 than in Jak3, the chimeric constructs demonstrated that the Jak3 JH2 domain was also an inhibitory domain (II).

At the beginning of the current work, not much was known about the function of the JH2 domain in Jaks, but in the course of these studies a number of reports were published, stating that JH2 regulated Jak kinases (I, II, III, Duhe and Farrar, 1995; Frank *et al*., 1995; Velazquez *et al.*, 1995; Luo *et al.*, 1997; Sakai and Kraft, 1997; Barahmand-Pour *et al.*, 1998; Chen *et al.*, 2000; Yeh *et al.*, 2000). Consistent with the results from this study, a negative regulatory role has been assigned to Jak2 and *Drosophila* Jak, Hop, in studies by others (Luo *et al.*, 1997; Barahmand-Pour *et al.*, 1998). In yeast, deletion of JH2 increased Jak2-mediated activation of Stat5, but the isolated JH1 domain was less active than Jak2 (Barahmand-Pour *et al.*, 1998). A mutation in the *Drosophila* Hop JH2 domain produced a hyperactive kinase and induced hematopoietic neoplasia in the fly (Luo *et al.*, 1997). The corresponding mutation in Jak2 resulted also in increased Jak2 activity (Luo *et al.*, 1997; I), although deletion of the entire JH2 domain resulted in much higher activity (I). In Hop, however, deletion of JH2 resulted in inactivation of the kinase (Luo *et al.*, 1997). Similarly, in both Tyk2 and Jak3, deletion of JH2 resulted in loss of kinase activity, which suggested an essential role for JH2 in maintaining overall activity of these kinases (Velazquez *et al.*, 1995; Chen *et al.*, 2000).

The analysis of SCID patients revealed mutations in various Jak3 JH domains, and interestingly, also in the Jak3 JH2 domain (Candotti *et al.*, 1997). The SCID phenotype suggested that the JH2 mutations impaired Jak3 activity. Indeed, when the Jak3 mutants were analyzed, they did not mediate IL-2 signaling in cells, and were inactive in an *in vitro* kinase assay (Chen *et al.*, 2000). However, the Jak3 mutants were hyperphosphorylated in cells compared to wild-type Jak3. The increased tyrosine phosphorylation of Jak3 mutants was most likely dependent on their own catalytic activity, since mutation of K930 in the JH1 domain of

Jak3 abolished their *in vivo* phosphorylation (Chen *et al.*, 2000). Very similar results were also obtained with Tyk2. Artificially made mutations in the Tyk2 JH2 domain abolished the *in vitro* kinase activity of the Tyk2 mutants, with a concomitant loss of IFN-α signaling (Yeh *et al.*, 2000). However, tyrosine phosphorylation of the Tyk2 mutants was increased in cells compared to wild-type Tyk2, and again, mutation of the conserved lysine K855 in JH1 abolished *in vivo* tyrosine phosphorylation (Yeh *et al.*, 2000). Currently, it is not not known, why the Jak3 and Tyk2 mutants that show enhanced autophosphorylation *in vivo*, are catalytically inactive *in vitro*, but these results show that JH2 is in a critical position in Jaks by regulating the activity of the tyrosine kinase domain (Chen *et al.*, 2000; Yeh *et al.*, 2000).

The analysis of different Jaks with differential deletions or mutations in different assay systems produced a rather complex view of the JH2-mediated Jak regulation. Thus, it was not clear, if the JH2 domains had different functions in the four Jak kinases. One purpose of the current work was to compare the function of JH2 in the different Jak kinases in same experimental settings, and by so doing, obtain information that is not available when studying one Jak kinase at a time.

In the current work, the function of the JH2 domain was found to be conserved, to a high degree, between Jak2 and Jak3 (II). The result that Jak3 lacking JH2 was active in this work (II), whereas it was found inactive by others (Chen *et al.*, 2000), highlights the importance of critically specifying the domain boundaries used to create the deletion constructs. It is also possible that the different results are partially due to differences between mouse (II) and human Jak3 (Chen *et al.*, 2000). As described above, mutations and deletions in JH2 have, surprisingly often, produced Jak kinases with different activities. These findings collectively suggest that the JH2 domain has a complex function in Jak kinases. In addition to its negative regulatory role, the JH2 domain may be required for the JH1 domain to acquire its active conformation, and the two domains may form a co-fold being linked together tightly in terms of structure and function. The functions may be slightly different in the four Jaks, and in certain Jaks, the JH2 domain cannot be deleted without disturbing JH1.

Interestingly, despite similar inhibitory JH2 domains, the basal activity of Jak2 was much lower than that of Jak3, indicating that the two kinases are differentially regulated (II). The results indicate that the JH1 domains may have inherently different activities, which is in agreement with the varying role of A-loop tyrosines in different Jaks (Gauzzi *et al.*, 1996; Feng *et al.*, 1997; Liu *et al.*, 1997; Zhou *et al.*, 1997). These results also support the conclusion that the deletion of JH2 may have distinct effects in different Jaks due to differential activation requirements of the JH1 domains. The activity of the expressed Jak2 JH1 domain was also dependent on the length of the sequence on the N-terminal side of JH1 (I).

It is also possible that conditions in the *in vitro* kinase assay may affect the results obtained with Jak3 (II; Chen *et al.*, 2000). Interestingly, a recent report on c-Abl revealed a rather similar paradigm as found with the Tyk2 and Jak3 JH2 mutants (Chen *et al.*, 2000; Yeh *et al.*, 2000; Pluk *et al.*, 2002). Mutations in the Abl kinase had been found to result in increased signaling in cells, but when the *in vitro* kinase activity of these mutant proteins was analyzed, no difference was detected when compared to wild type c-Abl. However, when the composition of the *in vitro* kinase assay buffer was changed, the mutant proteins showed increased activity over c-Abl (Pluk *et al.*, 2002).

Although the results differ concerning the kinase activity of the Jak3 mutants, the role of the JH2 domain in both positive and negative regulation of Jak activity suggested by Yeh *et al.* and Chen *et al*. is in line with the results from the current work (II; Chen *et al.*, 2000; Yeh *et al.*, 2000). Furthermore, the results from the current study may give additional explanations for the phenotypes of the Jak3 and Tyk2 mutants (see chapters 9.4 and 9.5).

9.4 The JH2 domain is required for cytokine-dependent activation of Jak2 and Jak3

In addition to its role as a negative regulator of Jaks, the JH2 domain was found to positively influence Jak-mediated signaling (I, II). Specifically, while the Jak2 and Jak3 JH2 deletion mutants showed increased activity in the absence of cytokine, they failed to further induce Stat signaling following IFN-γ and IL-2 stimulations, respectively (I, II). The JH2 domain was required to render Jaks competent to respond to cytokine stimulation with increased activity, and thus, link cytokine-induced changes in receptor conformation to Jak activation. Interestingly, the deletion of 13 residues in the Jak2 JH2 domain (IR3) abolished IFN-γ-mediated induction of signaling (III). Thus, an intact JH2 domain was required for proper regulation of Jaks in response to cytokine stimulation. This result is in agreement with studies showing that mutations in the JH2 domains of Tyk2 and Jak3 resulted in abrogation of cytokine signaling (Chen *et al.*, 2000; Yeh *et al.*, 2000).

The exact mechanism by which the JH2 domain mediates induction of Jak activity in cytokine receptor signaling remains to be found out, but several possibilities can be considered. The Jak2 and Jak3 JH2 deletion mutants retained catalytic activity, indicating that JH2 was not required for kinase activity as such (I, II). JH2 could still be required for stabilizing the activated state of JH1. Given that the Jak2 JH1 domain is extremely active compared to full-length Jak2, this explanation may not be very likely, although it cannot be excluded.

Induction of negative feedback regulation by the constitutively active JH2 deletion mutants is one plausible explanation for the lack of cytokine-responsiveness. Expression of SOCS proteins, which negatively regulate cytokine signaling, is induced by many cytokines. However, co-expression of SOCS-proteins with JH2Δ-Jak2 in the Jak2-negative cell line failed to suppress IFN-γ signaling, although signaling induced by wild-type Jak2 was significantly reduced (unpublished observation by P. Saharinen). Furthermore, the relatively low activity of JH2Δ-Jak3 in the IL-2 receptor signaling is unlikely to result in induction of SOCS protein expression and prevention of further signaling by IL-2 (II).

The results from the current work (I, II) showing that 1) the Jak JH2 deletion mutants were catalytically active, 2) were able to activate Stat5 and interact with other Jaks, and 3) the results by others that JH2 is not required for coupling Jaks to cytokine receptors, collectively indicate that Jaks are able to interact with all these components of the signaling pathway in the absence of JH2, but the JH2 domain is required to connect cytokine receptor activation to Jak activation and induction of signaling. Thus, the functions of JH2 are important in the context of the Jak-receptor complex, and suggest that the JH2 domain has an active structural role in Jak-receptor signaling complexes during cytokine-induced activation of Jaks.

Recent data give insight into the organization of the receptor-Jak complexes, and indicate structural requirements in receptors for Jak activation, as outlined below. There is increasing evidence that binding of a Jak kinase to a box1 motif of a cytokine receptor, although required, is not sufficient for cytokine-inducible Jak activation. Instead, parts of the receptor appear to be involved in positioning Jaks correctly, so that ligand-induced change in the conformation of the receptor dimer allows mutual activation of Jaks. Specifically, substitution of hydrophobic residues in the juxtamembrane region of EPOR resulted in mutant receptors unable to activate Jak2, although binding of Jak2 to EPOR was not impaired (Constantinescu *et al.*, 2001; Huang *et al.*, 2001). The hydrophobic juxtamembrane region in EPOR is predicted to form a continuous α-helix with the transmembrane domain, and the orientation of this juxtamembrane helix affected Jak2-mediated signaling (Constantinescu *et al.*, 2001). Also, physical separation of the Jak2 binding site (box1) from the precisely oriented hydrophobic α-helix in the juxtamembrane segment abrogated activation of Jak2 signaling (Ketteler *et al.*, 2002). The JH7 domain in Jak2 mediates the binding to box1 of EPOR, and this interaction is not affected by juxtamembrane mutations. Interestingly, the juxtamembrane region of EPOR is suggested to interact with the JH1-2 domains in Jak2 during Jak activation, and consequently, mutations in EPOR might affect this interaction (Constantinescu *et al.*, 2001; Huang *et al.*, 2001).

Similarly, interaction of Jak1 with the gp130 signal transducing chain has been analyzed. It was found that mutation of W652A in the box1 region of gp130 abolished Jak1 activation, without affecting binding of Jak1 to the receptor (Haan *et al.*, 2002). In addition, the relative orientation of the juxtamembrane region of gp130 was critical for Jak1-mediated signaling (Greiser *et al.*, 2002). Furthermore, the structural requirements of a receptor cytoplasmic domain for Jak activation could be distinguished from those required for Jak-mediated Stat activation (Greiser *et al.*, 2002).

These results suggest that the organization of the ligand binding extracellular domain, the transmembrane domain, the membrane proximal region and the Jak2 binding site (box1) form an entity required for successful Jak activation (Ketteler *et al.*, 2002). The activation of Jaks is dependent on the structural determinants on cytokine receptors in a complex way, which influences Jak activation following cytokine-induced change in the receptor complex. Thus, it is likely that Jaks interact with cytokine receptors in at least two ways: one interaction takes place before ligand binding and promotes cell surface expression of the specific receptor and another interaction is involved in switching on Jak activity (Huang *et al.*, 2001).

In the light of the data presented above, the results of the current work are suggestive of a following model. In the resting state JH2 mediates inhibitory interaction with JH1, but ligand-induced conformational changes in the receptor may trigger interactions between JH2 and other protein domains in the receptor complex. The JH2-mediated interactions induced by cytokine are required for formation of the active Jak-receptor complex and progression of signal transduction. The hydrophobic motif in the juxtamembrane domain of the EPOR required for Jak activation, but not for initial binding to EPOR, is one potential interaction partner for JH2 (Constantinescu *et al.*, 2001; Huang *et al.*, 2001). In agreement with this model, a structural role for the Tyk2 JH2 domain has been found in the assembly of the IFNAR complex, where Tyk2 associates with the IFNαR1 chain (Yeh *et al.*, 2000). The Tyk2 JH2

domain was found to be required for restoration of high-affinity IFN-α binding at the cell surface, suggesting that Tyk2 binding in the cytoplasmic domain of IFNαR1 may affect the ability of the extracellular domain to bind ligand (Yeh *et al.*, 2000). However, the requirement for Tyk2 in the IFNAR cell surface expression was not evident from Tyk2-deficient mice (Karaghiosoff *et al.*, 2000; Shimoda *et al.*, 2000).

9.5 The JH2 domain in IFN-γ versus IL-2 signaling

Although the JH2 domains regulated Jak2 and Jak3 very similarly, the function of the JH2 domain was not completely identical in IFN-γ and IL-2 signaling. When Stat activation induced by JH2Δ-Jak2 was compared to that induced by JH2Δ-Jak3, a significant difference was detected (II). Deletion of JH2 resulted in constitutive Stat activation, but this activation was lower by JH2Δ-Jak3 than by JH2Δ-Jak2. Similarly, the Jak232 and Jak323 chimeras restored inducible Stat activation by IFN-γ and IL-2, respectively, but while the Jak232 chimera behaved as wild type Jak2 in IFN-γ signaling, the Jak323 chimera was not as potent in inducing Stat activation by IL-2 as wild-type Jak3. On the other hand, in a receptor-independent system, the Jak323 chimera was able to induce Stat activation similarly as wild-type Jak3. These results suggest that the JH2 domain is more critical in signaling through the IL-2 receptor than through the IFN-γ receptor, and that the JH2 domain of Jak3 may have a very specific role in the IL-2 receptor, which cannot be totally complemented by the Jak2 JH2 domain.

The outcome of SCID mutations (Chen *et al.*, 2000) and deletion of the Jak3 JH2 domain in the current work is rather similar (II). The SCID JH2 domains were found to be more potent inhibitors of the Jak3 JH1 domain than the wild type JH2 domain, thus explaining for the abrogation of signaling by the SCID mutations. However, in this work, the low level of signaling is not due to increased inhibition by JH2. As discussed above, lack of functional JH2 may abrogate signaling due to the potential role of JH2 in Jak-receptor complexes. This may also contribute to disease pathogenesis in the SCID patients by enforcing the effects of the SCID JH2 mutations. Furthermore, the structural role of the JH2 domain may be receptor-specific, thereby explaining for the differences found between IFN-γ and IL-2 signaling.

The result that Jak3 showed preference for Jak autophosphorylation peptides, while the Stat5-derived peptide was a better substrate for Jak1, support a cascade model for IL-2 signaling, where Jak3 activates Jak1, which in turn phosphorylates Stat5 (II). However, Jak3 lacking functional JH2 may be defective in activation of Jak1, which might perturb downstream signaling, thus also explaining for the critical role of JH2 in IL-2 signaling. In line with this notion, Stat5 has been previously found to be a better substrate for Jak1 than for Jak3 (Liu *et al.*, 1997).

Recently, Jak3 mutations occurring in the FERM domain (JH4-7) were characterized from SCID patients (Cacalano *et al.*, 1999; Zhou *et al.*, 2001). These mutations impaired the ability of Jak3 to associate with γ_c, and thus resulted in defective IL-2 signaling. Also, very surprisingly, the same mutations caused inactivation of catalytic activity of Jak3, and a number of other artificially made mutations in the FERM domain of Jak3 were found to have similar

effects. In only one case, a mutation in Jak3 JH7 (Y100F) was found to abrogate kinase activity without affecting binding to γ_c (Zhou *et al.*, 2001). This led to a conclusion that the N-terminal FERM domain had a role in positively regulating Jak3 activity (Zhou *et al.*, 2001). Using a chimeric approach, the N-terminus of Jak3 was shown to complement the inactivating Y1034F/Y1035F double mutation in the A-loop of Jak1, also suggesting that the N-terminal half of Jak3 could positively regulate kinase activity (Liu *et al.*, 1997). The results from the current work showing that JH1-2-Jak3 had lower activity than wild-type Jak3 also suggest a positive regulatory role for the Jak3 N-terminus (II). Previously, FERM domains have been found to mediate intra- and intermolecular protein interactions. For example, deletion of the FERM domain in FAK resulted in hyperphosphorylation of the kinase, suggesting regulation of FAK by the FERM domain, but somewhat differently from the regulation by that domain in Jak3 (Schlaepfer and Hunter, 1996).

9.6 Autoregulation of Jak kinases

Inhibition of Jak2 by its JH2 domain was found to be an intrinsic property of the Jak2 molecule and not dependent on additional regulatory proteins, thereby suggesting an autoinhibitory mechanism for JH2-mediated regulation of Jak2 (III). Autoinhibition most likely is dependent on molecular interactions between the JH2 and JH1 domains of Jak2, as suggested by inhibition of JH1 by co-expressed JH2 and by co-immunoprecipitation experiments (I). However, co-immunoprecipitation between various Jak2 JH domains was weak, therefore making it a less suitable technique for further studies. The JH2 domain of Jak3 has been also found to interact with JH1 and inhibit its activity in mammalian cells (Chen *et al.*, 2000).

A number of other non-receptor tyrosine kinases belonging to distinct kinase families are regulated through autoinhibitory mechanisms, where N-terminal protein domains often modulate the C-terminal kinase domain (reviewed in Hubbard *et al*., 1998). The crystal structure of the inactive form of Src kinases, solved in 1997, revealed regulatory protein-protein interactions, and confirmed the models of Src regulation that were based on previous biochemical data. In c-Src and Hck, the SH2 domain interacts with a C-terminal tyrosine residue and the SH3 domain associates with the linker between the SH2 and kinase domains, resulting in inactive conformation of the activation loop, which blocks the substrate-binding groove (Sicheri *et al.*, 1997; Xu *et al.*, 1997; Xu *et al*., 1999). In Btk/Tec family kinases, Btk and Itk, the SH3 domain interacts with the N-terminal proline rich region (Andreotti *et al.*, 1997; Hansson *et al.*, 2001). In c-Abl, the N-terminal region is responsible for the inhibition of the kinase domain through intramolecular interaction (Pluk *et al.*, 2002). The significance of the above-described intramolecular regulation is emphasized by mutations that abrogate the interactions resulting in ligand-independent activation of the kinases. For example, in v-Src, the C-terminal inhibitory tyrosine residue is mutated, and this results in cell transformation. Thus, regulation of kinase activity by intramolecular interactions appears to be a general mechanism for non-receptor tyrosine kinases. Based on the current work (III), Jak2 can be added to the list of tyrosine kinases with autoregulatory properties. Kinetic analysis of Jak2 activity indicated that JH2 reduced the maximal velocity (Vmax) of the kinase, but did not

affect the Km value of Jak2 (III). This is indicative of non-competitive inhibition by the JH2 domain, and suggests that the JH2 domain may regulate Jak2 via a conformational change, resulting in distortion of the structures essential for catalysis (III).

Deletion analysis of the Jak2 JH2 domain identified three inhibitory regions and those refer to amino acids 618-670 (IR1), 725-757 (IR2) and 758-807 (IR3) (III). IR2 and IR3 are located in the C lobe of JH2, while IR1 extends from the C lobe to the N lobe, as deduced from the molecular model of the JH2 domain (Figure 11). The finding that IR3 is able to inhibit the kinase domain alone suggests that this region may directly interact with the kinase domain. The model structure of JH2 also suggests that IR3 may fold as an independent unit (III). IR1 and IR2 increased IR3-mediated inhibition, and it is possible that IR1 and IR2 make additional contacts with JH1. Alternatively, IR1 and IR2 may stabilize the structure of IR3, thereby enforcing the inhibitory function of IR3.

The previously characterized mutations in the JH2 domains of Jak2, Jak3 and Tyk2 causing aberrant kinase function, localize quite well to the inhibitory regions found in the current work (III). The E695K substitution in the *Drosophila* Jak JH2 domain is hyperactivating, and the corresponding mutation (E665K) has a similar, but less pronounced effect in Jak2 (Luo *et al.*, 1997; I). In the model of Jak2 JH2, E695/E665 localizes to helix D in IR1. Two mutations in the Tyk2 JH2 domain result in constitutive Tyk2 tyrosine phosphorylation (Yeh *et al.*, 2000). H669P in Tyk2 corresponds to H606 in Jak2, located close to, but outside of the N-terminal start site of IR1. R856G in Tyk2 corresponds to R795 in Jak2 located within IR3. In Jak3, the mutations in JH2 appear to be concentrated in the C-terminal half of the domain (Notarangelo *et al.*, 2001). The C759R mutant Jak3 characterized from a SCID patient is interesting, because it is constitutively highly phosphorylated (Chen *et al.*, 2000). In Jak2, C759 corresponds to C787 in IR3, and in Jak3 this mutation has been predicted to disturb the structure of the C lobe of JH2 (Vihinen *et al.*, 2000).

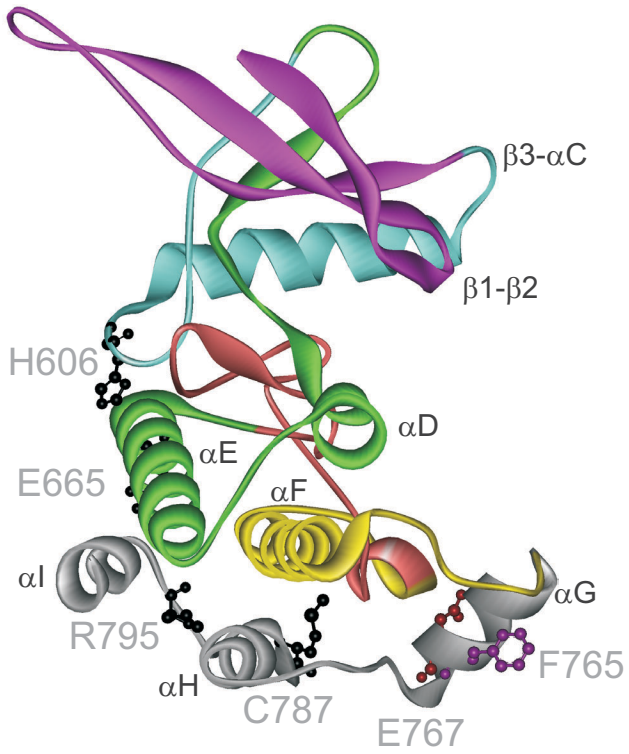

Figure 11. Model of the three-dimensional structure of the Jak2 JH2 domain (III).

The role of IR3 in regulation of Jak2 activity was further studied by mutational analysis. Certain, but not all, alanine mutations in IR3 resulted in increased Jak2 activity. Using molecular modeling, residues 763L, 764Q, 765F, E767, 771Q and 773P, showing inhibitory function are predicted to be exposed on the

surface of JH2, while Y766 projects inward in the model and is not likely to interact with JH1, and consequently, may not be phosphorylated. The alanine substitutions in IR3 may distort the α-helical structure (αG) required for inhibition, and this might affect, directly or indirectly, the conformation of JH1.

In the EphB2 receptor tyrosine kinase, autoregulation is dependent on the interaction between the kinase domain and a helical juxtamembrane (JM) domain on the N-terminal side of the kinase domain (Wybenga-Groot *et al.*, 2001). In EphB2, interaction of two juxtamembrane helices with the kinase domain results in distortion of the N lobe and prevents the A-loop from attaining its active conformation (Wybenga-Groot *et al*., 2001). The regulatory interactions in EphB2 are mediated via conformational change alone and do not involve conventional SH2/SH3 domain mediated interactions. The JH2-based regulation of Jak2 may rely on interactions alike to those found in the EphB2 receptor. Intramolecular regulation in tyrosine kinases often targets two key regulatory elements, helix C in the N lobe and the A-loop in the C lobe (reviewed in Huse and Kuriyan, 2002). The helix C contains an important catalytic residue (Glu 678 in the EphB2 receptor), which serves to orient the invariant lysine residue in PTKs (Lys 661). The Lys residue, in turn, is required for correct positioning of the bound ATP. In EphB2, the N-terminal JM domain interacts with the helix C, and induces a bend in the helix, which removes the catalytic Glu away from its correct position (Wybenga-Groot *et al*., 2001). The helix C also makes direct contacts with the A-loop, which undergoes large conformational changes between the active and inactive states of kinases. In EphB2, the A-loop structure is disrupted due to sterical constraints induced on the A-loop tyrosines by the JM domain. EphB2 can be activated through tyrosine phosphorylation of JM tyrosines, which disrupts the inhibitory interactions.

The inactive conformation of many kinases can be relieved also by binding of a substrate, which disrupts the intramolecular contacts. For example, Src family kinases can be activated by SH2/SH3 domain containing ligands that bind to the regulatory elements in the kinase (Moarefi *et al*., 1997). The requirement of the Stat SH2 domain for activation by Jak2 (Gupta *et al.*, 1996), but not by the JH2 deletion mutant (I), suggests that the SH2 domain may be essential for relieving the inhibited state of Jak2. Interestingly, an SH2-containing protein, SH2-Bβ, can bind to and activate Jak2 (Rui and Carter-Su, 1999). The mechanism for SH2-Bβ mediated activation of Jak2 is not known, but may involve the modulation of JH2 function (O'Brien *et al.*, 2001).

9.7 A model for the function of the JH2 domain in cytokine signaling

Based on the results from this study (I, II, III), a model for Jak2 activation in cytokine receptors can be envisioned (Figure 12). In the absence of cytokine stimulation, Jak2 is associated with a monomeric cytokine receptor chain or is present in an inactive cytokine receptor complex. In this resting state, JH2 interacts with JH1, resulting in conformational effects that keep JH1 in an inactive conformation. Ligand binding induces a conformational change in the receptor and brings the associated Jaks into close proximity. An activating JH1-JH1 interaction is formed between two Jak kinases and JH1 is released from the inhibitory

JH2 interaction, with a concomitant increase in Jak activity. At the same time, JH2 is engaged in new types of interactions, possibly with the receptor, to enable formation of an active receptor complex, which is required for maximal induction of Jak activity. Thus, the JH2 domain is part of both the uninduced and ligand-induced receptor complex, and may be viewed as an inducible switch in regulating change from an inhibited to fully active state of Jak2 in response to cytokine stimulation.

The results supporting the model are as follows. The JH2-mediated inhibition of Jak2 was found to be a property of the Jak2 kinase and independent of other regulatory proteins (III). Kinetic analysis revealed that JH2 does not compete with substrate binding, suggesting regulation via a conformational change (III). JH1 was found to interact more efficiently with another JH1 than with JH2, supporting the concept that interaction between two JH1 domains is important during activation of Jak2 (I). During juxta-positioning of Jaks upon receptor dimerization, the "activating" intermolecular JH1-JH1 interaction would thus be preferred over the weaker "inhibitory" intramolecular JH1-JH2 interaction, resulting in partial activation of Jaks (III). However, for maximal Jak activation removal of JH2-mediated inhibition is not sufficient, since catalytically active JH2 deletion mutants are unable to respond to cytokine stimulation (II). Instead, JH2 needs to build up an active Jak-receptor complex where Jak activation can be induced. The role of JH2 might be in proper construction of the receptor complex or to stabilize the activated state of Jaks. The first explanation is favoured, since JH1, lacking JH2, has significantly higher activity than Jak2 (I). Therefore, it is likely, that in addition to its interaction with JH1, JH2 interacts with other proteins, protein domains or even non-protein ligands to mediate its dual regulatory function in cytokine receptors. This interaction may be receptor-specific, since Jak2 and Jak3 JH2 domains showed preference over the two receptor models tested (II). The model presented here for the function of the pseudokinase domain is in line, and gives additional explanations for the results obtained by other groups using Tyk2 and Jak3 mutants derived from SCID patients (Chen *et al*., 2000; Yeh *et al.*, 2000).

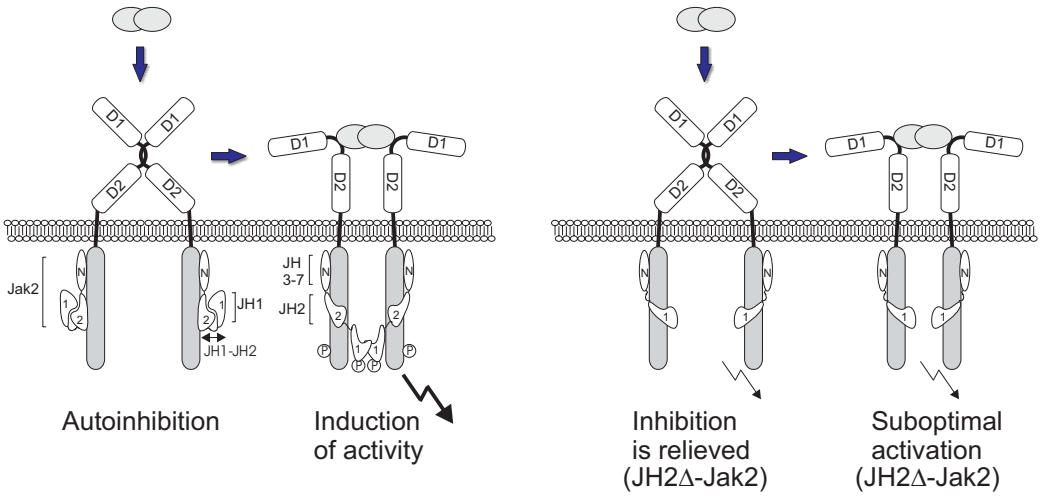

Figure 12. A schematic model for the function of the JH2 domain in cytokine signaling by Jak kinases, see text for details.

10 PERSPECTIVE

With the almost completion of the Human Genome Project, the total number of tyrosine kinases in the human genome is 90, of which 32 are of non-receptor type, including the four Jak kinases and five Tec kinases (Robinson *et al.*, 2000). During the last five years, the crystal structures for many tyrosine kinases have been solved. This has increased the understanding of tyrosine kinase function enormously, especially, how the activity of these central signal transduction proteins is regulated, with implications in drug development for the treatment of a variety of diseases.

Understanding the activation of cytokine signaling at a molecular level is of importance, since cytokines play a central role in so many physiological processes, and there is often interest to modulate these processes. The current study shows that Jak kinases are autoinhibited through their pseudokinase domains (I II, III), a unique feature of Jak kinases. This regulation takes place in a situation, where cells are deprived from cytokines (I, II). Upon cytokine stimulation, the cytokine receptor undergoes a change into an active conformation, and the Jak kinases are activated simultaneously. The results from this work (I, II) suggest that the pseudokinase domain links receptor activation to activation of Jak kinases.

The increased knowledge of the receptor epitopes required for Jak activation and the large number of different mutations in the JH2 domains of Jaks that abolish cytokine-inducible signaling, suggests that a Jak kinase forms a fine-tuned complex with the receptor, where small disturbances abrogate effective signaling. Results showing that Jaks enhance cell surface expression of certain of the cytokine receptors, and that the cytoplasmic reagion of EPOR does not fold properly in the absence of Jak2 (Remy *et al.*, 1999), also suggest a structural role for Jaks in cytokine receptors. Thus, the receptors and Jaks may be viewed as forming one functional entity much like receptor tyrosine kinases.

One of the major questions waiting to be solved concerning Jak kinases is their three-dimensional structure, which will very likely reveal the mechanism of their autoinhibition. Of high interest is also the three-dimensional structure of a cytoplasmic domain of a cytokine receptor, which has not been solved either. Finally, structural knowledge of the complex between a receptor and a Jak kinase will be of importance in understanding the tight connection between activation of a cytokine receptor and a Jak kinase.

The increasing data of constitutive Stat activation in a variety of cancers indicates Stats as potential targets for drug discovery. Since Stat activation is dependent on upstream tyrosine kinases, the kinases themselves appear as drugable targets as well, in cases, where a connection between a PTK and Stat is known. The Bmx and Jak2 tyrosine kinases are among the potential targets for future therapeutics.

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