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Post-translational Modifications in Regulation of JAK-STAT Pathway

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ACADEMIC DISSERTATION

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CONTENT

1. ABSTRACT

Cytokines regulate the growth and differentiation of hematopoietic cells through activation of the JAK-STAT signaling pathway. Ligand binding to specific cell-surface receptor results in activation of the receptor-associated JAK tyrosine kinases. Activated JAKs phosphorylate specific tyrosine residues on the receptor cytoplasmic tail providing docking sites for the latent cytoplasmic STAT (signal transducer and activator of transcription) transcription factors. STATs are activated through JAK-mediated phosphorylation of a single C-terminal tyrosine residue causing their dimerization and translocation to the nucleus and finally modulation of transcription of their specific target genes. Regulation of the activity, function and localization of JAKs and STATs involves interaction with regulatory proteins such as SOCS (suppressors of cytokine signaling), PIAS (protein inhibitors of activated STAT) and PTPs (protein tyrosine phosphatases), as well as various other post-translational modifications. Post-translational modifications are a central mechanism to regulate the functions of cellular proteins. The ubiquitin-proteasome pathway degrades a wide range of protein substrates with high specificity through two discrete and successive steps: firstly, substrate recognition involving the ubiquitin conjugation cascade, and secondly the degradation process mediated by the proteasomes. Sumoylation of target proteins involves a reaction mechanistically similar to ubiquitination. However, sumoylation does not lead to protein degradation, but is involved in regulation of many cellular processes such as DNA replication and repair, transcription activation, nuclear transport and signal transduction.

The aim of this study was to investigate the regulatory mechanisms of JAK-STAT pathway. Ubiquitination was found to regulate JAK2 activation *in vivo* and *in vitro*, and tyrosine phosphorylation was a requirement for efficient polyubiquitination and proteasomal degradation of JAK2. The interaction between SOCS1 and Elongins B/C, which were implicated in the ubiquitination reaction, led us to investigate the role of SOCS proteins in the regulation of JAK2 protein turnover. SOCS1 but not SOCS3 enhanced the degradation of activated JAK2 in a SOCS-box dependent manner. These studies identified ubiquitination of JAK2 as a physiological regulatory mechanism in cytokine signaling, that appears to function as a final fail-proof mechanism to maintain cellular homeostasis in case of hyperactivation of JAK2.

The interaction between STAT1 and SUMO E3 ligase PIAS1 led us to investigate if STAT1 was subject to sumoylation. STAT1 was found to be a substrate for SUMO-1 conjugation at a single Lys703 residue in the conserved TAD domain (transactivation domain) in IFN-γ signaling. PIAS proteins were shown to act as SUMO E3 ligases and enhance the sumoylation of STAT1 and this function was dependent on the PIAS RING finger-like domain. Sumoylation had a negative regulatory role in STAT1-mediated transcription activation, and selectively inhibited the induction of genes with weak affinity promoter to STAT1. Sumoylation defective STAT1 mutant showed a prolonged DNA-binding activity and nuclear localization in response to IFN-γ stimulation. Removal of conjugated SUMO-1 from STAT1 was found to be mediated by SUMO-specific protease SENP1. De-conjugation of SUMO-1 correlated with increased STAT1-mediated transcription activation, confirming the negative regulatory role of sumoylation in STAT1-dependent transcription.

The results from the current work have identified two previously uncharacterized mechanisms for regulation of JAK-STAT signaling, which provide new insights into the molecular mechanisms involved in modulation and fine-tuning of cytokine responses.

2. LIST OF ORIGINAL COMMUNICATIONS

The thesis is based on the following original publications:

I Daniela Ungureanu, Pipsa Saharinen, Ilkka Junttila, Douglas Hilton and Olli Silvennoinen. Regulation of Jak2 tyrosine kinase activation through the ubiquitin-proteasome pathway involves phosphorylation of Jak2 on Y1007 and interaction with SOCS1. *Molecular and Cellular Biology*. 2002. May, 22; 10: 3316-3326.

II Daniela Ungureanu, Sari Vanhatupa, Juha Gronholm, Jorma Palvimo and Olli Silvennoinen. SUMO-1 conjugation selectively modulates STAT1-mediated gene responses. *Blood*. 2005, July 1; 106:224-226.

III Daniela Ungureanu, Sari Vanhatupa, Juha Gronholm, Jorma Palvimo and Olli Silvennoinen. Regulation of STAT1-dependent transcription by SUMO-specific protease SENP1. (*manuscript submitted*)

IV Daniela Ungureanu and Olli Silvennoinen. SLIM trims STATs: Ubiquitin E3 ligases provide insights for specificity in regulation of cytokine signaling. *Science*.STKE Oct. 7, 2005; 304; pe49.

3. ABBREVIATIONS

AR	Androgen receptor
ARIP3	Androgen receptor interacting protein-3
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
C	Carboxy
CBP	CREB binding protein
COS-7	SV40 transformed monkey kidney cell line
DBD	DNA-binding domain
cDNA	Complementary DNA
CREB	Cyclic AMP response element binding protein
DMEM	Dulbecco's modified Eagle's medium
EMSA	Electrophoretic mobility shift assay
EPO	Erythropoietin
ER	Estrogen receptor
ES	Embryonic stem
FCS	Fetal calf serum
FERM	Band four point one, ezrin, radixin and moesin
GAS	Gamma activated sequence
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GR	Glucocorticoid receptor
HA	Hemagglutinin
HeLa	Human cervix carcinoma cell line
ICSBP	Interferon consensus binding protein
IFN	Interferon
IL	Interleukin
IRF	Interferon regulatory factor
ISG	Interferon-stimulated gene
ISGF3	Interferon-stimulated gene factor 3
ISRE	Interferon-stimulated response element
JAK	Janus kinase
JH	Jak homology
kDa	Kilodalton
LBD	Ligand-binding domain
LPS	Lipopolysaccharide
Luc	Luciferase
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony-stimulating factor
N	Amino
NES	Nuclear export signal
NLS	Nuclear localization signal
PBS	Phosphate-buffered saline
PIAS	Protein inhibitor of signal transducer and activator of transcription
PI3K	Phosphatidylinositol-3-kinase
PKA	Protein kinase A
PKC	Protein kinase C

PML	Promyelocytic leukemia gene product
PMSF	Phenylmethylsulfonyl fluoride
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcriptase polymerase chain reaction
SAP	SAF-A/B, Acinus and PIAS motif
SCID	Severe combined immunodeficiency
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH2	Src homology-2
SIM	SUMO interacting motif
SOCS	Suppressors of cytokine signaling
STAT	Signal transducer and activator of transcription
SUMO	Small ubiquitin-like modifier
TAD	Transactivation domain
TNF	Tumor necrosis factor
Ub	Ubiquitin
UBA	Ubiquitin-associated domain
Ubc9	Ubiquitin conjugating enzyme 9
UIM	Ubiquitin interacting motif

4. INTRODUCTION

The JAK-STAT signaling pathway was discovered over a decade ago. It is now clear that cytokines as well as several growth factors utilize the JAK-STAT pathways to elicit a wide variety of responses. The specificity is conferred by highly dynamic protein-protein interaction, protein-DNA interactions and post-translational modifications. The JAK-STAT pathway has been shown to be regulated by various mechanisms involving interaction with transacting proteins such as SOCS (suppressor of cytokine signaling), PIAS (protein inhibitor of activated STAT) proteins and protein tyrosine phosphatases. Importantly, these regulatory proteins have evolved specific functions in modulating various post-translational modifications such as phoshorylation by PTPs, sumoylation by PIASs and ubiquitination for SOCS proteins.

Ubiquitin-mediated proteasomal degradation has been implicated in the regulation of cytokine signaling at all levels, including regulation of cytokine receptor expression, regulation of the half-life of JAKs and finally the turnover of STATs. The key determinants for specificity are the E3 ligases, which represent the largest and most diverse group of enzymes in the conjugation process. JAKs and STATs are regulated by the ubiquitin-proteasome pathway. Proteasome inhibitors can prolong the activation of JAK-STAT pathway and regulate the turnover of several cytokine receptors. Activated JAKs and STATs were shown to be substrates for ubiquitination and proteasomal degradation. However, the modification occurs with high specificity, indicating that there must be other factors that determine the kinetics of the reaction.

Recent studies have provided evidence about ubiquitination as a mechanism for immune system evasion utilized by paramyxoviruses to escape antiviral activities of interferons. More recently, the newly identified SLIM (STAT-interacting SLIM protein) was found to have ubiquitin E3 ligase activity and to promote both the degradation and the ubiquitination of activated STATs. SLIM may be the first ubiquitin ligase with specificity toward STAT proteins in mammalian cells and opens a new chapter for STAT-specific degradation.

Sumoylation is a recently identified post-translational modification involved in the regulation of STAT1 activation. Interestingly, PIAS proteins were found to function as SUMO E3 ligase and enhance the sumoylation of STAT1. The reversible reaction is mediated by SUMO-specific proteases SENPs. The underlying mechanism of the selective regulatory role of STAT1 sumoylation in IFN-γ induced gene responses is at present unknown. Promoter specific effects of post-translational modifications on gene regulation appear to be a rather common mechanism. The functional importance of STAT1 sumoylation is not completely understood, but SUMO conjugation appears to have a selective inhibitory role in STAT1-mediated transcription activation.

This study was set up to investigate the molecular mechanisms involved in the negative regulation of the JAK-STAT pathway.

5. REVIEW OF THE LITERATURE

5.1 Overview of cytokine signaling

Cytokines, a large family of secreted glycoproteins produced by numerous cell types, play a crucial role in immune responses, hematopoiesis and inflammation (Schindler 1999, 2002, Kisseleva et al. 2001). Most cytokines function through transmembrane receptors belonging to the cytokine receptor superfamily, and initiate downstream signaling through tyrosine phosphorylation eventually leading to modulation of gene responses. The cytokine receptor superfamily can be divided into two structurally related groups. Type I cytokine receptors include receptors for most interleukins and cytokines such as erythropoietin, prolactin, growth hormone, ciliary neutrophic factor, GM-CSF and cardiotrophin. Type II cytokine receptors mediate the actions for IFNs and IL-10 family (Leonard and O'Shea 1998). Type I and type II cytokine receptors lack intrinsic kinase activity and function by activating JAK kinases (Janus kinases) and their downstream effectors STAT (signal transducers and activators of transcription) proteins (Ihle 1995, 1996). The JAK-STAT pathway represents an extremely rapid mechanism to transduce the extracellular signals into the nucleus and can provide signal specificity through selected activation of four JAK kinases and seven STATs.

The JAK-STAT pathway first appeared during evolution in invertebrates and has evolved complexity in vertebrates and mammals. Characterization of the IFN-induced immediate gene responses provided the first evidence of the JAK-STAT pathway (Schindler and Darnell 1995, Ihle 1995).

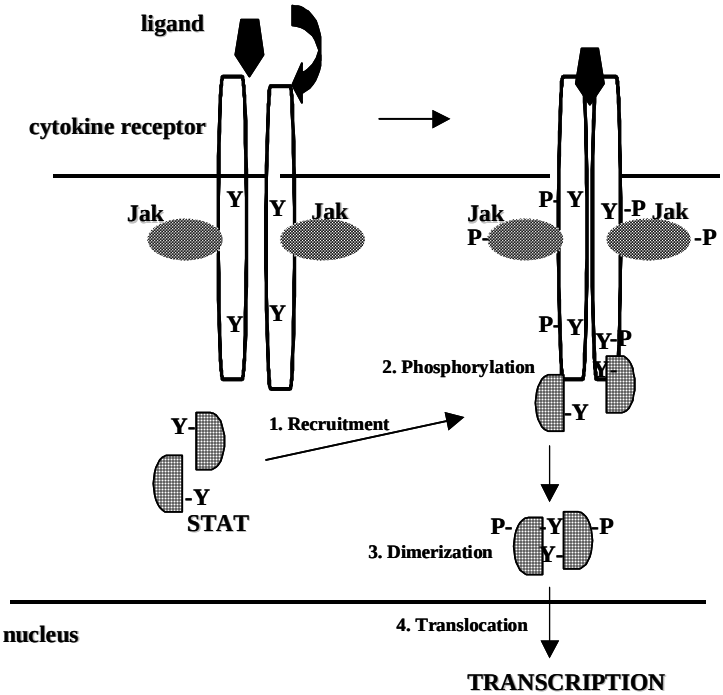

Figure 1. Schematic representation of the JAK-STAT signaling pathway. Binding of a cytokine to its specific receptor leads to the activation of JAK kinases and subsequent tyrosine phosphorylation of STATs. Activated STATs form dimers and translocate into the nucleus, where they regulate transcription of target genes.

Binding of a cytokine to its cognate receptor results in receptor homo- or heterodimerization and subsequent activation of receptor associated JAK tyrosine kinases. Activated JAKs mediate phosphorylation of specific receptors tyrosine residues that provide docking sites for the STATs, a family of latent cytoplasmic transcription factors. STATs are then recruited to the receptor complex and become activated by a JAK-dependent tyrosine phosphorylation event. Phosphorylated STATs dimerize and translocate to the nucleus to modulate the expression of target genes. Each of the JAK and STAT family members can be activated by one or more cytokines. Functionally related subclasses of cytokines use common receptor chains, which in turn transduce signals through the same sets of JAKs and STATs (Horvath and Darnell 1997, Leonard and O'Shea 1998). Studies using the knockout model have demonstrated highly specialized functions for individual JAKs and STATs in the control of various immune responses, and deregulation of the JAK-STAT signaling has been associated with several immune disorders and other diseases (Leonard and O'Shea 1998, Ihle 2001, Schindler 2002).

5.2. JAK family of tyrosine kinases

5.2.1 Overview of JAK kinases

Molecular cloning of cytokine receptors revealed that the receptors lacked an intrinsic catalytic domain, thus suggesting the existence of receptor-associated cytoplasmic kinases. Biochemical studies of IFN-mediated immediate gene responses led to the discovery of the JAK kinases, and directly coupled JAKs to STAT transcription factors activation (Shuai et al. 1993, Schindler and Darnell 1995, Ihle et al. 1995). The JAK family consists of four mammalian members, JAK1, JAK2, JAK3 and Tyk2. JAK homologs have also been identified in carp and zebrafish, and the *Drosophila* JAK homologue was identified in mutant flies with defects in *hopscotch* gene (Binari et al. 1994, Chang et al. 1996, Conway et al. 1997, Yin et al. 2000). Also, a tyrosine kinase with a tandem of kinase domain, a characteristic of JAK family, has been identified in *Dictyostelium* (Alder et al. 1996).

JAKs are relatively large kinases with an apparent molecular weight of about 120-135 kDa. Their expression pattern reflects their functional role, JAK1, JAK2 and Tyk2 are ubiquitously expressed. On the other hand, JAK3 expression is predominant in NK cells and thymocytes and it can be induced in T cells, B cells and myeloid cells (Kanamura et al. 1994, Rane et al. 1994, Musso et al. 1995, Tortolani et al. 1995, Gurniak et al. 1996, Sharfe et al. 1997). In addition, JAK3 expression can also be detected in vascular smooth muscle cells and endothelium (Verbsky et al. 1996). In humans JAK1 gene is located on chromosome 1p31.3, JAK2 on chromosome 9p24 and JAK3 together with Tyk2 are clustered on chromosome 19p13.1 and 19p13.2, respectively (Firmbach et al. 1990, Pritchard et al. 1992, Kumar et al. 1996, Riedy et al. 1996). JAK2 has two transcripts and JAK3 has multiple alternatively spliced forms, but their functional significance is not yet clear.

5.2.2 JAK deficient mouse models

Mouse knockout models have demonstrated the physiological roles of individual JAK kinases in cytokine signaling and pointed out that the non-redundant functions of JAKs are restricted to signaling through receptors of the cytokine receptor superfamily.

JAK1 deficiency results in perinatal lethality, and the mice are small at birth and fail to nurse. They have impaired lymphopoiesis with reduced numbers of T and B lymphocytes. JAK1 deficient cells fail to respond to IFNs, γ_c cytokines (IL-2, IL-4, IL-7, IL-9) and cytokines using gp130 or gp130-

like protein (IL-6, LIF, CT-1, CNTF, OSM, IL-11). The neurons from JAK1 deficient mice do not respond to LIF, CT-1 and CNTF, which is likely the cause for perinatal lethality and cell death due to apoptosis (Rodig et al. 1998).

JAK2 deficient mice die during embryonic development due to the absence of complete erythropoiesis. The phenotype is similar to the phenotype of mice deficient in EPO or EPOR, suggesting an essential role for JAK2 in EPO signaling. Also, JAK2-deficient fibroblasts do not respond to IFN-γ and JAK2 deficient fetal liver myeloid progenitor cells fail to respond to TPO and β_c cytokines (IL-3, IL-5 and GM-CSF) (Neubauer et al. 1998, Parganas et al. 1998).

JAK3 deficient mice have severely impaired T and NK cells development and defective B cells. JAK3 deficiency was first identified in humans with autosomal recessive severe combined immunodeficiency (SCID), resulting in the total absence of T lymphocytes and non-functional B lymphocytes (Macchi et al. 1995, Russell et al. 1995). JAK3 binds to γ_c, therefore deficiency of either JAK3 or γ_c abrogates signaling by the family of cytokines using this receptor subunit. The phenotype of JAK3-SCID disease is similar with the X-linked severe combined immunodeficiency (X-SCID) resulting from mutations in γ_c, demonstrating an essential role for JAK3 in γ_c-dependent lymphoid development (Park et al. 1995, Nosaka et al. 1995, Thomis et al. 1995). JAK3 deficient mice and γ_c deficient mice have similar phenotypes (Leonard 2001).

Tyk2 deficient mice show impairment in innate and adaptive immune responses, slightly increased susceptibility to certain virus infections. Moreover, their macrophages are defective in IFN-α- and LPS-induced nitric oxide (NO) production. Tyk2 deficient mice have impaired IFN-γ response, though Tyk2 is not activated by IFN-γ. This effect could be explained by reduced levels of Stat1 protein expression in Tyk2 deficient mice (Karaghiosoff et al. 2000, Shimoda et al. 2000).

5.2.3 Domain structure of JAK kinases

The three-dimensional structure of JAK proteins has not yet been solved, but recently the crystal structure of JAK3 tyrosine kinase domain was characterized (Boggon et al. 2005). JAKs have a characteristic domain structure, which is conserved between mammalian JAKs and the *Drosophila* Hop. The hallmark of JAK kinases is the tandem kinase domain structure. Like the Roman god of gates and doorways, the "Janus" kinases are "two-faced" consisting of a tyrosine kinase and pseudokinase domains. Using nomenclature analogous to that of the Src homology (SH) domain, the JAKs are divided into seven homology domains (JH) (Hubbard and Till 2000), consisting of the kinase domain (JH1) at the C-terminus of JAKs, preceded by a catalytically inactive pseudokinase or kinase-like domain (JH2), and a FERM domain, which encompass the N-terminal half of JAKs.

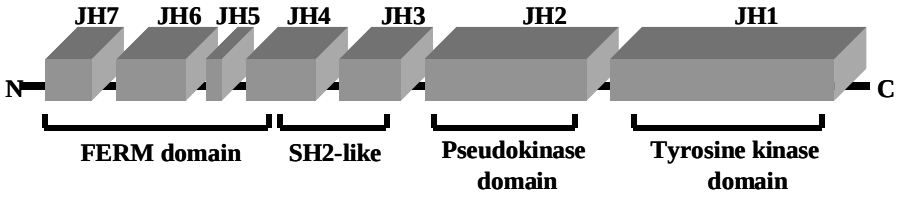

Figure 2. Schematic representation of JAK structure. The seven JAK homology domains (JH) start from the tyrosine kinase domain (JH1) located in the C-terminal part, followed by a catalytically inactive pseudokinase domain (JH2). The N-terminal half of JAK contains the FERM domain. (The abbreviations are explained in the text)

JH1 domain is a typical eukaryotic tyrosine kinase domain. The activation loop (A-loop) contains a conserved double tyrosine motif which is phosphorylated in response to cytokine stimulation: Y1038/Y1039 in JAK1, Y1007/Y1008 in JAK2, Y980/Y981 in JAK3 and Y1054/Y1055 in Tyk2 (Leonard and O'Shea 1998). JAK1 and JAK2 activation requires phosphorylation of the first Tyr in the A-loop motif, but not for JAK3 activation. JAK3 mutation of the second Tyr residue increases its catalytic activity, but a similar mutation in JAK1 and JAK2 has no effect (Feng et al. 1997, Liu et al. 1997, Zhou et al. 1997). The differences in the catalytic activities of different JH1 domains between individual JAK family members suggest that each JAK kinase has a specific function and role in cytokine signaling.

Mutation of the conserved Lys residue directly involved in ATP binding abrogates JAK activity (Briscoe et al. 1996). Structural studies support a model in which prior to phosphorylation, the A-loop blocks the access of the substrate to the active site of enzyme, whereas phosphorylation of the tyrosine residue (s) within the loop facilitates substrate accessibility. Recently, the crystal structure of the JAK3 kinase domain in complex with a staurosporine-based inhibitor AFN941 has been characterized (Boggon et al. 2005). The kinase is in an active, double-phosphorylated state. The phosphate group on Tyr981 in the activation loop is in part coordinated by an Arg residue in the regulatory C-helix, suggesting a direct mechanism by which the active position of the C-helix is induced by phosphorylation of the activation loop. This direct coupling has not been observed previously in any of the tyrosine kinases, suggesting a unique structure for JAK kinases (Boggon et al. 2005).

The JH2 domain is conserved in all JAK kinases, suggesting that it may have an important role for JAK function. The genomic organization of the JH2 domain is different from the JH1 domain, indicating that the two domains have distinct origins. The JH2 domain is catalytically inactive due to alteration in several residues required for phosphotransferase activity (Wilks et al. 1991). Molecular modeling suggests that the JH2 domain in JAK1 and JAK2 forms a kinase fold (Briscoe et al. 1996, Vihinen et al. 2000). Mutations or deletions of the JH2 domain have different effects, and either inhibit or enhance the catalytic function. Mutations in JH2 domain of JAK3 in SCID patients demonstrated its critical function in JAK3 regulation. Similar findings have been observed in JH2 mutants in Tyk2, which show hyperphosphorylated phenotype, but inability to mediate IFN-α signaling (Candotti et al. 1997, Yeh et al. 2001). Fine-mapping of the JH2 domain from JAK2 led to the identification of three inhibitory regions important for the negative regulation of JAK2, indicating that JH2 domain plays a role in autoregulation of JAK2 (Saharinen et al. 2003). Studies of the interaction between JH1 and JH2 domain in JAK2 have provided a model for the regulation of JAK activation in cytokine receptor signaling (Saharinen et al. 2003). Recently, a point mutation identified in the JH2 domain of JAK2, V617P, was associated with polycythemia vera and other myeloproliferative disorders and confirmed the role of JH2 domain in the regulation of JAK2 activation. Val-to-Phe substitution at position 617 disrupts the autoinhibitory interaction between the JH2 and JH1 domain, resulting in dysregulated JAK2 activation and pathogenic phenotype in patients carrying this mutation (Baxter et al. 2005, Levine et al. 2005, James et al. 2005).

The JH3-JH7 domains consist of an 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 et al. 2000, Al-Lazikani et al. 2001). The FERM domain has been shown to confer binding of JAK kinases to the box1 region in cytokine receptor, (Leonard and O'Shea 1998, Chishti et al.1998, Ihle 2001). The minimal receptor-binding region is JH6-JH7 in JAK2, JAK3 and Tyk2. The JH3-JH5 region can also contribute to the binding of JAK2 and Tyk2 to many receptors (Leonard and O'Shea 1998, Saharinen and Silvennoinen 2003).

Mutations in the FERM domain can abrogate binding of JAKs to their specific receptors. For example, naturally occurring mutations in JAK3 FERM domain abrogate binding of JAK3 to γ_c and result in SCID (Cacalano and Johnston 1999, Zhou et al. 2001). Natural and artificial FERM domain mutations abolished the *in vitro* catalytic activity of JAK3, indicating that the FERM domain provides structural support for a functional kinase domain (Zhou et al. 2001). FERM domain can also enhance cell surface expression of certain cytokine receptors. The FERM domain of Tyk2 was required for the expression of the IFNαRI, whereas JAK2 and JAK1 were found to be required for proper folding and/or processing of EPOR and OSMR (Gauzzi et al. 1997, Huang et al. 2001, Radtke et al. 2002).

Using secondary structure prediction and sequence alignment, it has been found that the JH3-JH4 region of JAKs has an SH2-like domain, although its function is at present unknown (Kampa and Burnside 2000, Al-Lazikani et al. 2001). Mutation of the conserved "Flavor" Arg residue in JAK2 SH2-like domain did not show any functional effect, and in Tyk2 this Arg residue is substituted with a His residue (Al-Lazikani et al. 2001).

5.3 Regulation of JAK activation

JAK kinases are constitutively associated with the membrane-proximal region of cytokine receptor. Ligand binding and conformational changes in the receptor complex are the initial steps required for JAKs activation. In the absence of ligand binding, JAKs remain inactive. Activation of JAKs occurs through reciprocal interaction of two juxtapositioned JAK kinases and auto/transphosphorylation of their A-loop tyrosines (Remy et al. 1999). In receptor complexes composed of heterodimers, JAKs are activated through the interplay between two different JAK kinases, whereas in single-chain receptor (homodimers) two JAK2 kinases can dimerize and induce JAK activation.

JAK kinases function in defined signaling cascades, and the role of individual JAKs in cytokine receptor signaling has been demonstrated using cell-lines deficient in various JAKs. In cells lacking JAK1, no phosphorylation of Tyk2 or JAK2 was observed upon stimulation with IFN-α or IFN-γ respectively. Similarly, no phosphorylation of JAK1 was seen in cells lacking Tyk2 or JAK2. In the absence of JAK3, no phosphorylation of JAK1 occurred in response to IL-2 stimulation (Briscoe et al. 1996, Oakes et al. 1996). In cells lacking JAK1, expression of a JAK1 kinase negative can sustain IFN-γ-inducible gene expression, low-level receptor phosphorylation and low-level STAT1 activation, suggesting that JAK1 has both a functional and a structural role in IFN-γ signaling. However, expression of a JAK2 kinase negative in cells lacking endogenous JAK2 could not sustain IFN-γ-inducible gene expression. This indicates that the catalytic activity of JAK2 is required for proper IFN-γ signaling (Briscoe et al. 1996). Therefore, in IFN-γ signaling it has been proposed that JAK1 is mainly responsible for receptor phosphorylation, and once STAT1 is recruited to the receptor complex, phosphorylation of STAT1 is mediated by the JAK2.

Recent studies of EPOR-JAK2 interaction suggest that binding of JAK2 to box1 motif of EPOR is necessary but not sufficient for JAK2 activation. Mutant EPOR lacking the hydrophobic residues in the juxtamembrane region cannot activate JAK2, although binding of JAK2 to EPOR is not impaired. This hydrophobic juxtamembrane region of EPOR is predicted to form a continuous α-helix with the transmembrane domain and this α-helix should be correctly positioned in order to allow JAK2 activation (Huang et al. 2001, Constantinescu et al. 2001). These studies have indicated that JAKs can interact with the cytokine receptors in two ways: one interaction occurs before the ligand binding and promotes cell surface expression of the receptor, while another interaction is involved in JAK activation.

5.3.1 Protein tyrosine phosphatases

Activation of JAKs requires tyrosine phosphorylation and this process is reversed by protein tyrosine phosphatases (PTPs). Several PTPs have been shown to regulate JAKs, including SHP1, SHP2, CD45, PTP1B and T-cell PTP (TCPTP) (Shuai and Liu 2003). PTP1B specifically recognizes and dephosphorylates the double tyrosine motif in JAK2 and Tyk2, and it has been implicated in the negative regulation of leptin signaling, possibly by targeting JAK2. Increased JAK2 phosphorylation has been observed in *Ptp1b*$^{-/-}$ mouse embryo fibroblasts (Myers et al. 2001). T-cell phosphatase (TCPTP), closely related to PTP1B, can dephosphorylate JAK1 and JAK3. In TCPTP$^{-/-}$ macrophages IFN-γ-induced tyrosine phopshorylation of JAK1 but not JAK2 is enhanced (Simoncic et al. 2002).

SHP-1 is mainly expressed in hematopoietic cells and it was shown to directly associate with the IL-3 receptor β-chain, c-KIT receptor and EPOR. EPO-induced phosphorylation of JAK2 is enhanced in cells expressing a mutant EPOR that is defective in SHP-1 binding (Klingmuller et al. 1995). SHP-1 is also implicated in the dephosphorylation of JAK1, as SHP1$^{-/-}$ macrophages show prolonged tyrosine phosphorylation of JAK1 in response to IFN-α-stimulation (David et al. 1995). SHP-2 has also been involved in the negative regulation of JAK activity. SHP-2$^{-/-}$ fibroblasts have increased level of JAK1 tyrosine phosphorylation after IFN-γ stimulation, suggesting that SHP-2 negatively regulates JAK1 activation (You et al. 1999). Cytokines that bind to gp130 receptor subunit appear to have impaired activity with regard to the activation of JAKs when SHP-2 is activated. These results have been also confirmed in mice where the SHP-2 binding site on gp130 has been disrupted (Ohtani et al. 2000, Tebbutt et al. 2002). However, SHP-2 can play a positive regulatory role in IL-2 signaling where it is constitutively associated with JAK1 and JAK3, and expression of a SHP-2 dominant negative form inhibits the activation of the JAK-STAT pathway (Gadina et al. 1998). Similar reports have demonstrated that expression of a dominant negative form of SHP-2 also disrupts IFNα, prolactin and growth hormone activation of the JAK-STAT pathway (David et al. 1996, Kim et al. 1998). However, other studies using SHP-2 $^{-/-}$ mice suggested that SHP-2 is a negative regulator of IFNα and growth hormone activation of the JAK-STAT pathway (Sofega et al. 2000, You et al. 1999). CD45 is a receptor PTP with a crucial role in antigen receptor signaling in T and B cells. CD45 can bind directly and dephosphorylate all JAKs. The expression of CD45 is required for the antiproliferative but not the antiviral effects of IFN-α (Petricion et al. 1997). In CD45$^{-/-}$ bone marrow derived mast cells IL-3-induced activation of JAK2 is enhanced compared with the wild type cells. Also, in CD45$^{-/-}$ Jurkat cells IFN-α-induced JAK1 phosphorylation is enhanced (Irie-Sasaki et al. 2001).

5.3.2 Suppressors of cytokine signaling (SOCS) proteins

Suppressors of cytokine signaling (SOCS) proteins were identified as negative regulators of cytokine receptor signaling and the JAK-STAT pathway (Alexander 2002, Alexander and Hilton 2004). The SOCS family consists of eight proteins: CIS (cytokine-inducible SH2 domain containing protein) and SOCS1 through SOCS7 (Yoshimura et al. 1995, Starr et al. 1997, Endo et al. 1997, Naka et al. 1997, Hilton et al. 1998). All SOCS proteins share a common modular organization of a central SH2 domain, an N-terminal region of variable length and a C-terminal 40-amino acid module called SOCS-box. The SOCS-box is also found in three other families: ankyrin repeat-containing proteins with a SOCS-box (ABSs), SPRY domain-containing proteins with a SOCS-bx (SSBs) and WD40 repeat-containing proteins with a SOCS box (WSBs) (Starr et al. 1997, Hilton et al. 1998, Kile et al. 2002). In addition, SOCS1 and SOCS3 contain a similar kinase-inhibitory region (KIR) in the N-terminal that is essential for JAK inhibition.

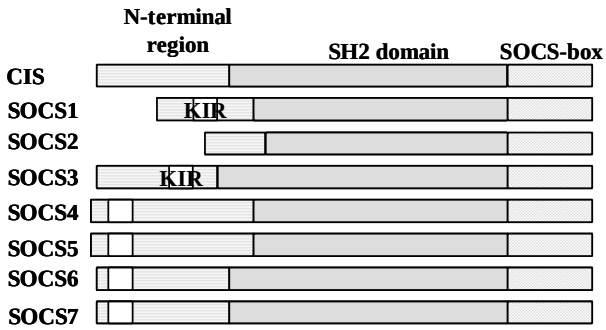

Figure 3. Schematic representation of the structure of SOCS proteins. All SOCS proteins contain a central SH2 domain flanked by an N-terminal region of variable length and sequence and a highly conserved C-terminal domain termed the SOCS-box. SOCS1 and SOCS3 have a similar KIR (kinase inhibitory region) important for JAK inhibition.

SOCS mRNA and protein levels are generally present at low levels in unstimulated cells, and can be rapidly induced in response to cytokine stimulation (Alexander and Hilton 2003). Expression of SOCS proteins causes very rapid and active repression of cytokine signaling. It has been demonstrated that STAT proteins play an important role in regulation of SOCS gene transcription (Feldman et al. 1997, Verdier et al. 1998, Davey et al. 1999, Donnelly et al. 1999, Matsumoto et al. 1999, Schluter et al. 2000, Brender et al. 2001, Lee et al. 2002, He et al. 2003). SOCS proteins can inhibit signaling by a variety of cytokines that act through the JAK-STAT pathway via different mechanisms. SOCS1 interacts through its SH2 domain with Tyr1007 in the activation loop of JAK2 and inhibits its activation (Yasukawa et al. 1999). The KIR region can act as a pseudosubstrate and occupies the catalytic cleft of the kinase and prevents the access of the ATP and the substrate. CIS interacts with the phosphotyrosine residues in the cytoplasmic domain of cytokine receptors and competes for the binding with activated STAT (Matsumoto et al. 1999). SOCS3 can interact with both JAK and cytokine receptors, but binding of SOCS3 to cytokine receptors does not seem to interfere with STAT recruitment (Cohney et al. 1999, Sasaki et al. 1999, Nicholson et al. 2000). SOCS proteins have been implicated in the ubiquitin proteasome pathway through their interaction with elongin B and C, which is mediated by the SOCS-box. Elongin B and C form part of an E3 ubiquitin ligase complex involved in directing the substrates for proteasomal degradation (Kamura et al. 1998, Zhang et al. 1999). Consistent with this model, it has been shown that activation of STATs and JAKs can be prolonged in the presence of proteasome inhibitors (Yu et al. 1997, Verdier et al. 1998). Also, the interaction between CIS and EPOR or SOCS1 and Vav seems to promote proteasomal degradation of EPOR and Vav respectively (Verdier et al. 1998, De Spulveda et al. 2000).

Knockout models have demonstrated the unexpected specificity of SOCS proteins in the regulation of pro- and anti-inflammatory responses as well as normal physiology. SOCS1$^{-/-}$ mice die perinatally with a complex disease characterized by anomalously activated T cell, lymphopenia, macrophage infiltration of tissues and liver necrosis. This uncontrolled inflammation is caused by an inability to appropriately regulate production of and responses to IFN-γ in the absence of SOCS1 (Alexander et al. 1999, Marine et al. 1999). In mice engineered with a COOH-terminal gp130 "knock-in" mutation which deleted all STAT binding sites the inability of mutant gp130 to induce SOCS1 and to some extent SOCS3 was linked to the development of degenerative joint disease. Their synovial fibroblasts were hyperresponsive to the mitogenic effects of the LIF/IL-6 family of cytokines, and this effect could be corrected by ectopic expression of SOCS1 (Ernst et al. 2001).

5.3.3 Regulation of JAK activation by intramolecular interactions

Intramolecular interactions can modulate the rapid active-inactive conformational changes in the kinase domains. JH2 domain mediates important regulatory functions in JAK kinases. The first evidence came with the identification of a point mutation in JH2 domain of *Drosophila* Jak, Hop, which caused hematopoietic neoplasia. A similar mutation in mammalian JAK2 increases the kinase activity (Harrison et al. 1995, Luo et al. 1997). Mutations in the JH2 domain of JAK3 resulting in hyperphosphorylation have been found in patients with SCID. These JAK3 mutants failed to mediate IL-2 signaling in cells and are inactive in an *in vitro* kinase assay (Candotti et al. 1997). Similar mutations have been found in JH2 domain in Tyk2 showing hyperphosphorylated phenotype, but inability to mediate IFN-α signaling (Yeh et al. 2000).

Recently, results using chimeric constructs of JAK2 and JAK3 demonstrated that JH2 domain has a conserved function between the two kinases (Saharinen and Silvennoinen, 2003). Analysis of JH2-mediated regulation of JAK activity has revealed multiple mechanisms. JH2 deletion in JAK2 has a negative impact on the basal kinase activity in the absence of cytokine stimulation, but JH2 domain is absolutely required for cytokine-induced increase in JAK activity. In non-eukaryotic system, JH2-mediated negative regulation of JAK2 does not require additional regulatory proteins, indicating an intrinsic regulation of JAK activity by the JH2 domain (Saharinen et al. 2003).

More recently, JAK2 somatic mutation V617F has been associated with polycythemia vera (PV) and other myeloproliferative disorders such as essential thrombocythemia (ET) and chronic idiopathic myelofibrosis (CIMF) (Baxter et al. 2005, Levine et al. 2005, James et al. 2005). The mutation was present in different myeloid and erythroid cells and progenitors colonies grown from PV patients sample, but not in T lymphocytes. The mutation was also detected in a significant percentage of ET and CIMF specimens. It has been shown that mice transplanted with cells expressing V617F JAK2 developed erythrocytosis (James et al. 2005). V617P mutation results in constitutive tyrosine phosphorylation of JAK2 that promotes cytokine hypersensitivity. This functional event could be attributed to the fact that the mutation is located in the JAK2 pseudokinase domain, which negatively regulates the kinase domain. Both Val617 and Cys618 are important residues for maintenance of the kinase domain of JAK2 in an inactive conformation (Saharinen et al. 2000, Saharinen and Silvennoinen 2002, Lindauer et al. 2001).

In summary, a model for the regulation of JAK kinases by the JH2 domain in cytokine receptor signaling has been proposed. At first, in the absence of cytokine binding, JAK2 is maintained inactive through a JH1-JH2 interaction. Following cytokine binding, the receptor complex undergoes conformational change and brings the associated JAKs into close proximity. Stronger JH1-JH1 interaction releases the weak inhibitory JH1-JH2 interaction, resulting in increased JAK activity. Furthermore, JH2 takes part in another interaction, possibly with the receptor, that leads to formation of an active receptor complex and maximal JAK activity. In this model, JH2 domain functions as an inducible switch to regulate the change from an inhibited to fully active state of JAK2 in response to cytokine stimulation (Saharinen and Silvennoinen 2003)

5.4 STAT family of transcription factors

STAT (Signal Transducer and Activator of Transcription) proteins are a family of latent cytoplasmic transcription factors that are activated by cytokines, growth factors and other peptides that bind to their cell-surface receptors. Activated STATs translocate to the nucleus and bind to specific promoter elements of their target genes to initiate the transcription (Schindler et al. 1992, Darnell et al. 1994, Stark et al. 1998, Levy and Darnell 2002). In mammalian cells seven STAT

proteins exist: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6. The human STAT genes are localized in three clusters on three different chromosomes and each cluster represents a tandem duplication: STAT1 and STAT4 on chromosome 2 (2q32.2-2q32.3), STAT2 and STAT6 on chromosome 12 (12q13) and STAT3, STAT5a and STAT5b on chromosome 17 (17q11.2, 17q21). Identification of STAT genes in *Drosophila* and *Dyctiostelium discoideum* suggests that this family comes from a common ancestral gene. STAT homologues were also found in *Xenopus* and *Danio*, but not in yeasts (Pascal et al. 2001, Hou et al. 2002).

Based on sequence homologies human STAT family can be divided into two groups: STAT1, STAT2, STAT3 and STAT4 are in the first group, and STAT5a, STAT5b and STAT6 are in the second group. Alternative splicing at the 3' end of the gene transcript generates shorter isoforms (designated –β) of STAT1, STAT3, STAT4, STAT5a and STAT5b (the longer isoforms are designated –α) (Schindler et al. 1992, Wang et al. 1996). The short isoforms lack a functional transcriptional activation domain, but still have the capacity to occupy specific binding sites in the promoter of target genes. Those isoforms can inhibit transcription activation by competing with the full-length STATs for DNA binding. However, in multimeric complexes with other transcription factors they do not function as a negative regulators, but can cooperatively combine to initiate the transcription, as it has been shown with STAT1β, STAT2 and IRF9 in the process of ISGF3 complex formation (Schaefer et al. 1995, Wang et al. 1996). STAT3β and c-Jun cooperatively bind to an IL-6 responsive promoter element in α2-macroglobulin gene and activate its transcription (Schaefer et al. 1995, Zhang et al. 1999). Furthermore, the truncated STAT3β can rescue the embryonic lethality of a STAT3-null mutation and it can by itself induce the expression of specific STAT3 target genes. However, STAT3α retain its nonredundant roles such as modulation of cellular responses to IL-6 and mediation of IL-10 function in macrophages (Maritano et al. 2004).

5.4.1 STAT deficient mouse models

Generation of different Stat null mice has revealed the physiological role and importance of each of the STAT family members (Ortmann et al. 1999, Ihle 2001). Stat1 knockout mice have shown the importance of STAT1 in IFN signaling (Durbin et al. 1996, Meraz et al. 1996). The mice are viable with no developmental defects, but are defective in IFN-dependent immune response to viral and microbial pathogens. They fail to induce transcription of Stat1 target genes in response to both IFN-γ and IFN-α and to induce ISGF-3 target genes in response to IFN-α. Stat1 null mice also demonstrated the non-immune functions of Stat1. Stat1 deficient embryos have impaired FGF-dependent condrocyte proliferation (Sahni et al. 1999). Other studies have indicated that Stat1 may have a role in apoptosis by regulating the caspase pathway (Chin et al. 1997). It has been suggested that unphosphorylated Stat1 protects cells from apoptosis (Kumar et al. 1997) and Stat1 may cooperate with other transcription factors to regulate the basal expression of genes involved in apoptosis (Schindler and Strehlow 2000).

Stat2 knockout mice show normal development (Park et al. 2000). Similar to Stat1 deficient mice, Stat2 null mice were also susceptible to viral infection and their cells were unresponsive to IFN-α/β, suggesting that Stat2 plays an important role in ISGF3 complex induced by IFN-α. Stat1/Stat2 knockout mice are totally unresponsive to both classes of IFNs and are more susceptible to infection than single knockout model, suggesting that Stat2 may transduce some signals independent of Stat1, as demonstrated by the existence of Stat2/Stat2/p48 complex in Stat1 deficient cells (Bluyssen and Levy 1997).

Stat3 deficient mice have severe developmental defects resulting in early fetal death, making the studies difficult to conclude (Takeda et al. 1997). Embryos were able to implant and develop until

E6.5, when they started to degenerate and became re-absorbed before gastrulation. At this stage, Stat3 was highly expressed only in deciduas and visceral endoderm, suggesting that Stat3 could be involved in the process of implantation probably by mediating the function of LIF (Ernst et al. 2001). Recently, generation of Stat3 conditional knockout mice using tissue-specific targeting has shown that mice lacking Stat3 selectively have defects in proliferation to IL-2 and IL-6 (Takeda et al. 1998, Akaishi et al. 1998). Stat3 deficient macrophages from mice lacking Stat3 do not respond to IL-10 and have increased sensitivity to LPS (Takeda et al. 1999). Furthermore, Stat1/Stat3 double mutant mice developed chronic enterocolitis with increased production of TNF-α and IL-12p40 from macrophages (Kobayashi et al. 2003). TNF-α/Stat3 double mutant mice developed severe chronic enterocolitis with enhanced Th1 activity. In contrast, IL-12p40/Stat3 double mutant mice did not show any inflammation in the colon or Th1 activity. In the TLR/Stat3 double mutant mice, LPS-induced IL-12p40 production was abolished and the incidence of chronic enterocolitis was greatly reduced and delayed (Kobayashi et al. 2003). Taken together, Stat3 knockout models have demonstrated the vital role of Stat3 in signaling response to IL-2, IL-6 and IL-10.

Stat4 deficient mice are very similar to IL-12 deficient mice, demonstrating the essential but selective role of Stat4 in IL-12 signaling. Stat4 null mice showed severely impaired Th1 development (Kaplan et al. 1996a, Thierfelder et al. 1996). Also, Stat4-deficient NK cells do not enhance their function in response to IL-12 and Stat4-deficient T cells are unresponsive IL-12.

Stat5a deficient mice were generated earlier and showed defect in prolactin signaling (Liu et al. 1997). Stat5a-deficient bone marrow macrophages show defective GM-CSF-induced proliferation and gene expression (Feldman et al. 1997). In Stat5a-deficient T cells preactivated with mitogens, IL-2-induced expression of the IL-2Rα chain is reduced and the cells contain Stat5 dependent IL-2 response elements (Sperisen et al. 1995, John et al. 1996, Lecine et al. 1996). It has been shown that low levels of IL-2 do not induce proliferation in Stat5a-deficient T cells, but high concentration of IL-2 is sufficient to saturate intermediate type IL-2 receptors, suggesting that the effect of Stat5a in IL-2-induced cells growth is at least to maintain a high affinity IL-2 receptors. Furthermore, Stat5a deficient mice have approximately 50% reduction in NK cells compared with the wild type and also have impaired NK cells development (Imada et al. 1998).

Stat5b deficient mice show similar phenotype with growth hormone receptor deficient mice. The mice have more severe defects in NK cell development than Stat5a deficient mice, and this cannot be overcome by high concentration of IL-2 or IL-15. Stat5b-deficient NK cells have lower expression of IL-2Rβ on the cell surface and have defects in perforin expression (Lowin et al. 1994). Stat5b-deficient T cells showed a defect in proliferation even at higher concentrations of IL-2 (Imada et al. 1998).

Stat5a/5b double deficient mice showed a phenotype identical with prolactin receptor deficient mice, suggesting that all the physiological functions mediated by prolactin require Stat5a/b. The T cells from Stat5a/5b mice failed to develop after anti-CD3 stimulation (Moriggl et al. 1999). Those mice have no NK cells and show impairment in T-cell proliferation after activation, which was associated with a loss of induction of cyclin D2, cyclin D3 and cdk6 in response to anti-CD3 stimulation. Previous studies have shown that inactivation of the *Stat5* locus by using conventional gene targeting resulted in complete lack of mammary alveolar epithelium (Liu et al. 1997, Miyoshi et al. 2001). The activity pattern of two STAT proteins, STAT5a and STAT5b, in mammary tissue during pregnancy suggests an active role for these transcription factors in epithelial cell differentiation and milk protein gene expression. To investigate the function of STAT5a in mammopoesis and lactogenesis gene targeting strategies were employed. Stat5a deficient mice developed normally, and were indistinguishable from hemizygous and wild-type littermates in size,

weight and fertility. However, mammary lobuloalveolar outgrowth during pregnancy was curtailed, and females failed to lactate after parturition due to a failure of terminal differentiation. Although STAT5b has a 96% similarity with STAT5a and a robust expression pattern during mammary gland development it failed to counterbalance for the absence of STAT5a. These results suggest that STAT5a is the principal mediator of mammopoietic and lactogenic signaling (Liu et al. 1996, 1997, Hennighausen and Robinson 1998)

Stat6 deficient mice showed a defect in physiological functions associated with IL-4. T cell development toward Th2 cells is impaired (Takeda et al. 1996, Shimoda et al. 1996, Kaplan et al. 1996b). Stat6-deficient lymphoid cells do not respond to IL-4 and IL-13. The most obvious phenotype is the incapacity of B cells to undergo IgE class switch, demonstrating that IL-4-induced Stat6 activation promotes the transcription of IgE constant region of the heavy chain and makes it accessible for the switch recombinase system. Stat6 null mice also have defects in antigen-induced defective airway eosinophilia and airway reactivity (Akimoto et al. 1998, Kuperman et al. 1998).

5.4.2 STAT protein structure

The STAT protein consists of seven domains: N-terminus domain, coiled-coil domain, DNA-binding domain, linker domain, SH2 domain, phosphorylated tail segment and the transactivation domain.

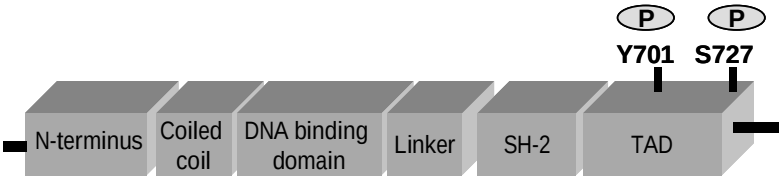

Figure 4. Schematic representation of STAT1 structure. The transactivation domain is located at the C-terminus and contains Tyr701 and Ser727 residues important for STAT-mediated transcription activation. The DNA binding domain is located in the middle of the molecule, followed by the linker and SH2 domain. The coiled-coil domain near the N-terminal end mediates interactions with various proteins.

The N-terminal domain of STAT1 is highly conserved among STAT family members. The crystal structure analysis of STAT4 N-terminal domain revealed that it consists of eight helices assembled into a hook-like structure. The N-terminal domain of STAT4 can form dimers and this dimerization could allow the formation of open-ended complexes of STAT dimers bound to DNA, as has been described for STAT1, STAT4 and STAT5 (Vinkemeier et al. 1996, Xu et al. 1996, Meyer et al. 1997). STAT1 N-terminal domain is required for tyrosine dephosphorylation, since N-terminal deletion mutant STAT1 showed constitutive phosphorylation on Tyr701 and enhanced antiproliferative activity for IFN-γ (Vinkemeier et al. 1996). Also, STAT1 N-terminal domain has been implicated in the interaction with the CREB-binding domain of CBP/p300, but how this interaction affects the transcriptional activation of STAT1 is not yet fully understood (Zhang et al. 1996). Replacement of the N-terminus domain of STAT1 with homologous regions of STAT2 or STAT5 did not change the functional properties of this region, but those chimeras have defects in nuclear translocation and deactivation, demonstrating that the N-terminal domain of STAT1 has a specific role in nuclear translocation (Strehlow and Schindler 1998).

STAT coiled-coil domain, initially predicted to be a part of STAT2 sequence, was confirmed by crystal structure analysis of STAT1 and STAT3 (Becker et al. 1998, Chen et al. 1998). This domain

consists of 4 long α-helices and its exposed position shows that it could easily interact with other proteins as well as with other domains of STAT protein. The coiled-coil domain of STAT1 interacts with the nuclear export machinery and contains two of the nuclear export signals (NES) (Begitt et al. 2000, Mowen and David 2000). Also, STAT1 coiled-coil domain interacts with IRF9, a component of the interferon-stimulated gene factor 3 (ISGF3) (Horvath et al. 1996). The coiled-coil domain of all STATs except STAT2 can interact with N-myc interactor (Nmi), and this interaction augments STAT1-mediated transcription in response to IFN-γ and STAT5-mediated transcription in response to IL-2, respectively (Zhu et al. 1999).

Crystal structure analysis of STAT1 and STAT3 homodimers showed that STAT DNA-binding domain has the general structure of an immunoglobulin variable fold similar to NF-κB/Rel transcription factors (Becker et al. 1998). STAT1 dimers bind to GAS motifs (gamma interferon activated sequence), a palindromic response element with general structure $TTCN_mGAA$. In the STAT-DNA binding complex there are few direct contacts between the STAT side chains and the DNA bases, and these contacts are mediated by amino acid residues largely conserved between the STAT family members. STAT1, STAT2 and IRF9 can form a heterotrimeric complex termed ISGF3 (interferon stimulated gene factor 3) that binds to ISRE elements (interferon-stimulated response elements: AGTTTNNNTTTCC) (Reich et al. 1987, Fu et al. 1990).

The linker domain connects the DNA-binding domain with the SH2 domain and is well conserved between the STAT family members. Experimental studies have shown that KE544-545AA point mutants in STAT1 abolished the transcriptional response to IFN-γ but not IFN-α, suggesting that the linker domain acts as a critical contact point during the formation of STAT1-transcriptional complex (Yang et al. 1999). The SH2 domain is very important for the specific contacts between STATs and the activated receptors. It is also important for dimerization of STATs, a process required for nuclear translocation and DNA binding. The SH2 domain binds to the phosphorylated tyrosine residue located in the intracellular domain of activated receptors (Greenlund et al. 1995). This interaction is highly specific and determines which STAT members are activated by different receptors (Heim et al. 1995, Stahl et al. 1995, Schindler et al. 1995). Crystal structure analysis of phosphotyrosine peptides bound to STAT SH2 domains has shown that the phosphate group of the phosphorylated tyrosine residue is bound in a deep pocket by a conserved Arg residue. Along with this, there are other four to five amino acids carboxyl-terminal to the phosphorylated tyrosine that make extensive contacts with other amino acids of the SH2 domain. These amino acids differ between STAT family members (except for STAT5a and STAT5b), and are responsible for the specificity of the phosphotyrosine-SH2 domain interaction (Becker et al. 1998, Chen et al. 1998). In the tyrosine phosphorylated tail segment, all STATs have a conserved Tyr residue that becomes phosphorylated upon activation at the receptor complex, and this residue is bound by the SH2 domain of the dimer partner. So far, only nine STAT dimers have been identified: STAT1:1, 1:2, 1:3, 3:3, 4:4; 5a:5a; 5b:5b and 6:6.

The transcriptional activation domain (TAD) located at the C-terminal of STATs is the most diverse domain among STAT family members. STAT1 TAD contains several important residues for regulation of STAT1-mediated transcription activation. Phosphorylation of Tyr701 is required for STAT1 activation and transcription regulation (Darnell et al. 1994). Phosphorylation of Ser727 in STAT1 TAD is required for maximal transcriptional activity of STAT1, but interestingly it selectively regulates gene response (Wen et al. 1995, Zhang et al. 1998). The molecular mechanism of STAT-mediated gene transcription is not yet clear, but the transactivation domain of STATs can interact with a number of proteins involved in gene transcription. The carboxyl-terminal part of STAT1 can interact with the E1A-binding domain of CBP/p300 and this interaction is required for transcriptional activation of STAT1 in response to IFN-γ activation. It has been proposed that C-

terminal STAT1 can compete with E1A protein for the binding to CPB/p300 and this competition could be responsible for the anti-viral effect of IFN-γ (Zhang et al. 1996). TAD from STAT1 directly interacts with MCM5 (minichromosome maintenance) in a Ser727-dependent phosphorylation manner and this interaction enhances STAT1-mediated transcription activation in response to IFN-γ (Zhang et al. 1998). MCM3 was also identified in the STAT1 TAD interacting complex, but does not interact directly with the TAD (DaFonseca et al. 2001). More studies are required in order to understand the molecular mechanism of STAT1-mediated transcription activation.

5.4.3 Mechanisms of STAT1 activation and deactivation

STAT1 has a major role in IFN-mediated biological responses (Leonard and O'Shea 1998, Stark et al. 1998). Latent STAT1 resides as a freely diffusible protein mainly in the cytoplasm, although a minor fraction is found in the nucleus and membrane rafts (Sehgal et al. 2002). Following IFN-γ stimulation, STAT1 is recruited through its SH2 domain to the phosphorylated tyrosine residue on the receptor and is phosphorylated on Tyr701 by JAK. Following tyrosine phosphorylation, STAT1 forms stable dimers that are recognized by importin α5 (Sekimoto et al. 1997). Importin α5 recognizes only tyrosine phosphorylated STAT1 dimers that have a functional NLS (nuclear localization signal). STAT1 dimers interact with the C-terminus of importin α5 containing Arms 8 to 10, whereas classical NLS sequences associate with Arms 2, 3, 4, 7 and 8, suggesting that STAT1 may have unconventional NLS (Conti et al. 1998, Fontes et al. 2000). Mutation of a small region in the DNA-binding domain of STAT1 disrupted nuclear translocation and this atypical NLS sequence has been identified as a Leu407 and Lys410 and 413 (McBride et al. 2002, Fagerlund et al. 2002). Other studies have proposed that the N-terminus of STAT1 is also required for nuclear translocation, indicating that additional residues in STAT1 dimers may also interact with importin α5 (Strehlow and Schindler 1998).

Nuclear translocation is transient and STAT1 relocates to the cytoplasm within a few hours after activation (Haspel et al. 1996, Koster et al. 1999). STAT1 dephosphorylation correlates with its nuclear export (McBride et al. 2000). The nuclear form of T cell PTP (protein tyrosine phosphatase) known as TC45 has been involved in dephosphorylation of STAT1 dimers *in vitro* (Simoncic et al. 2002). Experiments using site-directed mutations demonstrated that the CRM1-dependent NES (nuclear export signal) sequence in STAT1 is located within the residues 399-410 in the DNA-binding domain (DBD) (McBride et al. 2002). Using a STAT1 mutant that can be tyrosine phosphorylated but cannot bind DNA, a model of STAT1 nuclear export has been proposed (McBride et al. 2000). Location of NES in the DBD domain of STAT1 suggests that NES may be masked when STAT1 is bound to DNA and led to the hypothesis that the NES function is conditional. When STAT1 dimers are bound to DNA, CRM1 cannot access NES, but when STAT1 dimers are not bound to DNA, then CRM1 gains access to NES and targets the nuclear export of STAT1.

In conclusion, the mechanism of STAT1 intracellular trafficking involves a conditional NLS dependent on STAT1 dimerization through tyrosine phosphorylation and a conditional NES dependent on STAT1 dephosphorylation and dissociation from DNA. The location of these signals within the STAT1 molecules indicates that their function has co-evolved with the ability of STAT1 to bind DNA and regulate gene expression. However, more studies are needed to address the mechanism that regulates basal levels of STAT1 and the role of N-terminal export signals (McBride and Reich 2003).

Recent results obtained from crystal structure analysis of the unphosphorylated N-terminal domain of STAT1 revealed an anti-parallel configuration for the body of STAT1 dimers. The SH2 domains are at opposite ends of the dimmer and the coiled-coil domain of one monomer interacts with the DNA-binding domain of its partner. Furthermore, it has been proposed that STAT1 phosphodimer not bound to DNA changes conformation from parallel to anti-parallel in order to display more efficiently the phosphotyrosine for dephosphorylation (Zhong et al. 2005). Similar studies suggested that within STAT1 structure there are two flexible loops: one connects the N-terminal domain (residues 1-123) with the core fragments (residues 132-683), while the other connects the core fragments to the phosphotyrosine tail (residues 684-699). These tethers enable STAT to adopt different conformations before and after tyrosine phosphorylation and to achieve rapid and efficient transition for the functional diversity of STATs (Mao et al. 2005).

5.5 Regulation of STAT1-mediated transcription activation

Regulation of STAT1 activation involves post-translational modifications such as phosphorylation of Ser727 in the transactivation domain (TAD) and interaction with regulatory proteins such as protein tyrosine phosphatases (PTP), SOCS (suppressors of cytokine signaling), PIAS (protein inhibitor of activated STAT) proteins and SLIM (STAT-interacting LIM protein) (Chung et al. 1997, Liu et al. 1998, Decker and Kovarik 2000, Levy and Darnell Jr. 2002, O'Shea et al. 2002, Tanaka et al. 2005). PTPs, SLIM and SOCS1/SOCS3 regulate the tyrosine phosphorylation-mediated signaling events, while the mechanism of PIAS-mediated inhibition remains less well defined.

5.5.1 Serine phosphorylation of STAT

The first evidence for serine phosphorylation of STATs was provided using phospho-peptide mapping and phospho-amino acid analysis for STAT1 and STAT3. Mutation of Ser727 in the C-terminal tryptic peptide of STAT1 abrogated serine phosphorylation (Wen et al. 1995). Functional analysis of S727A mutation in STAT1 showed that the transcription activation in response to IFN-γ is reduced by approximately 80%. Induction of Ser727 phosphorylation in STAT1 can increase the expression of STAT1 target genes upon further treatment with IFN-γ. STAT1 Ser727 phosphorylation is not required for the transcriptional activity of ISGF3, even though evidence exists that a fraction of ISGF3 complex contains Ser727-phosphorylated STAT1 (Bromerg et al. 1996, Kovarik et al. 1998, 1999, Goh et al. 1999) Currently, there is no evidence that the DNA-binding or nuclear translocation of STAT1 could be affected by Ser727 phosphorylation, though some reports have demonstrated a correlation between STAT1 Ser727 phosphorylation and increased DNA-binding activity (Eilers et al. 1995). Reconstitution of U3A STAT1-deficient cells with STAT1 S727A mutant showed no effect on IFN-γ-mediated tyrosine phosphorylation, whereas reconstitution of U3A cells with STAT1 Y701A mutant concluded that constitutive or IFN-γ-mediated serine phosphorylation occurs independent of tyrosine phosphorylation (Zhu et al. 1997).

The significance of STAT1 Ser727 phosphorylation has been studied in U3A cells reconstituted with STAT1 S727A mutant. Serine phosphorylation of STAT1 can be induced through the activation of many signaling pathways that target the P(M)SP motif in STAT1. p38 MAPK has been shown to phosphorylate STAT1 on Ser727 and STAT3 *in vitro*. STAT1 Ser727 phosphorylation in response to cellular stress (UV irradiation) or inflammatory signals (LPS, TNA-α) is inhibited by treatment with SB203580, a specific p38MAPK inhibitor (Kovarik et al. 1999). Cell-type specificity may be an important factor for the outcome of the response. For example, in macrophages SB203580 inhibitor does not have any effect on IFN-γ-mediated Ser727 phosphorylation of STAT1, whereas in HeLa cells SB203580 treatment has a minor inhibitory

effect on STAT1 Ser727 phosphorylation, suggesting that p38MAPK could be upstream of STAT1 (Kovarik et al. 1999, Goh et al. 1999). IFN-α/β can also activate p38MAPK and SB203580 treatment inhibited STAT1 Ser727 phosphorylation in response to IFN-α and diminished the transcription activation of ISGF3 (Goh et al. 1999, Uddin et al. 1999). ERK2 was also shown to induce Ser727 phosphorylation in STAT1, and a dominant negative ERK decreased the transcription response to IFN-α (David et al. 1995).

5.5.2 Protein phosphatases

After activation and nuclear translocation, STAT1 is dephosphorylated and relocated to the cytoplasm, a cycle that each individual STAT1 molecule undergoes for several time during a full response to cytokine stimulation (Haspel and Darnell 1999). TC45, the nuclear isoform of T-cell PTP, was shown to dephosphorylate STAT1β *in vitro*, and it has been suggested that TC45 is largely responsible for dephosphorylation of STAT1 in the nucleus. Overexpression of TC45 inhibited IFN-induced STAT1 tyrosine phosphorylation and in TC-PTP null MEFS (mouse embryonic fibroblasts) the dephosphorylation of IFN-induced tyrosine-phosphorylated STAT1 is defective (ten Hoeve et al. 2002). Also, SHP-2 was shown to dephosphorylate nuclear STAT1 on Ser and Tyr residues (Wu et al. 2002).

5.5.3. SLIM (STAT-interacting LIM protein)

By using a cDNA library prepared from a mouse Th1 cell clone, Tanaka et al. recently identified a novel nuclear protein, SLIM, that specifically interacts with N-terminal STAT4 bait and contains one PDZ domain and one LIM domain (Tanaka et al. 2005). SLIM specifically inhibits STAT4 and STAT1-mediated gene expression, suggesting that SLIM is a negative regulator of STAT-mediated signal transduction. The LIM domain of SLIM forms a Zn finger structure related to the RING finger and PHD domain structure, which are known to be present in proteins with E3 ligase activity. SLIM was found to have ubiquitin E3 ligase activity and promote both the degradation and ubiquitination of STAT1 and STAT4. Furthermore, SLIM was shown to impair the tyrosine phosphorylation of STAT4 independent of proteasome-dependent degradation, suggesting that SLIM can act, through its LIM or PZD domains, as an adaptor molecule to recruit nuclear tyrosine phosphatases to dephosphorylate STAT1. Another mechanism could be that ubiquitination of STAT protein itself induces dephosphorylation through conformational changes that would allow interaction with a protein tyrosine phosphatases. Data from *SLIM*-deficient mice revealed high levels of STAT1 and STAT4 protein expression, along with higher production of IFN-γ by Th1 cells, suggesting that SLIM regulates both the level of STAT expression and the extent of its activation. These findings suggest that SLIM may be the first ubiquitin ligase with specificity toward STAT proteins in mammalian cells, opening a new chapter for STAT-specific degradation.

5.5.4 Protein inhibitor of activated STAT (PIAS) proteins

The family of PIAS proteins consists of six members (PIAS1, PIAS3, PIASy, PIASxα/ARIP3 and PIASxβ/Miz1) that have been implicated in the regulation of several nuclear proteins. PIAS proteins show significant sequence homology (40%) and share several highly conserved domains: a SAP domain (SAF-A/B Acinus and PIAS domain) that contains a LXXLL signature motif, a RING finger-zinc-binding motif (RING), an acidic domain (AD/SIM) and a serine-threonine rich region (S/T domain) that is not present in PIASy. The most conserved domain among PIAS proteins is the RING finger-zinc-binding domain, showing high similarity to the genuine RING domains, but lacks two of the conserved cysteine residues involved in zinc chelation. The SAP domain of SAF-A/B has been shown to interact with the scaffold attachment regions/matrix attachment regions

(SAR/MAR) resulting in anchoring independent chromatin loops to the nuclear scaffold (Kipp et al. 2000). The LXXLL motif has been shown to mediate the interaction between nuclear receptors and their co-regulators (Heery et al. 1997, Torchia et al. 1997).

PIAS1 and PIAS3 were identified as interaction partners for STAT1 and STAT3 respectively, and were found to inhibit the DNA-binding activity of activated STATs (Chung et al. 1997, Liu et al. 1998). PIASy can interact with activated STAT1 and represses STAT1-mediated gene responses without affecting the DNA binding activity of STAT1 (Liu et al. 1998, 2001, Liao et al. 2000). The STAT1-binding domain has been mapped at the C-terminal region (amino acids 392-541) in PIAS1 (Liao et al. 2000). PIAS1, PIASxα, PIASxβ, PIASy and yeast PIAS-like proteins Siz1 and Siz2 have been shown to function as E3-type ligase for SUMOs (small ubiquitin-like modifier), and this function requires the RING finger-like domain of PIAS (Takahashi et al. 2001, Johnson et al. 2001, Kotaja et al. 2002).

PIASy$^{-/-}$ mice are phenotypically normal, but have slight defects in transcriptional responses induced by IFN-γ. Analysis of PIAS1$^{-/-}$ mice showed that disruption of PIAS1 resulted in enhanced immune responses to viral or bacterial infections. PIAS1 was shown to selectively regulate a subset of IFN-γ and IFN-β responsive genes in macrophages by interfering with the recruitment of STAT1 to the promoters that show low affinity to STAT1 such as guanylate-binding protein1 (GBP1), but does not affect promoters with stronger affinity binding sites (IRF1) (Liu et al. 2004). PIAS proteins have been shown to modulate nuclear hormone receptor-dependent transcription, acting as co-activators or co-repressors, depending on the cell-type, receptor or promoter context (Kotaja et al. 2000, Tan et al. 2002).

5.6. Post-translational modifications of proteins

Post-translational modification is a common mechanism to regulate protein function, activity or localization. A specific amino-acid sequence within the target proteins will be chemically modified by various molecules such phosphate, acetate, lipids, sugars or small proteins. Ubiquitin and ubiquitin-like proteins form a distinct category of small molecules intensively involved in the post-translational modification of intracellular proteins.

Types of post-translational modifications include: phosphorylation, the addition of a phosphate group, usually to serine, tyrosine or threonine; acetylation, the addition of an acetyl group, usually at the N-terminus of the protein; methylation, the addition of a methyl group, usually at Lys or Arg residues; isoprenylation, the addition of an isoprenoid group (e.g. farnesol and geranylgeraniol); glycosylation, the addition of a glycosyl group to either Asp, hydroxyLys, Ser or Thr, resulting in a glycoprotein; ubiquitination, the covalent linkage of the protein ubiquitin to a target protein, typically interpreted by the cellular machinery as a degradation signal; sumoylation, the covalent linkage of the SUMO protein (Small Ubiquitin-like Modifier) to a target protein; ISGylation, the covalent linkage of the ISG15 protein (Interferon-Stimulated Gene 15) to a target protein.

5.7. The ubiquitin-proteasome pathway

The ubiquitin and ubiquitin-mediated conjugation pathway (ubiquitination) plays a key role in proteolysis (Hershko and Chiechanover 1998). Ubiquitin, discovered almost 30 years ago as a lymphocyte differentiation-promoting factor, is a small peptide of 76 amino acids, highly conserved during evolution, with a globular structure displaying a C-terminal glycine residues protruding from the main body of the protein (Golstein 1975).

Protein ubiquitination is involved in a vast array of cellular processes, including cell-cycle control, signal transduction, transcriptional regulation, DNA repair, receptor downregulation, antigen presentation and apoptosis (Hershko and Ciechanover 1998). Abnormalities in the ubiquitination process have been shown to cause pathological responses such as malignant transformation and many genetic diseases (Schwartz and Ciechanover 1999). Conjugation of ubiquitin chains to intracellular substrates serves as a marker that can be recognized by the 26S proteasome for degradation in order to remove excessive cellular molecules. Conjugation of ubiquitin to cell surface receptors causes their downmodulation through the endosomal-lysosomal pathway (Hershko and Ciechanover 1998, Hicke 2001). Recently, it has been demonstrated that ubiquitination represents an important post-transcriptional modification in a proteolysis-independent manner and can be involved in ubiquitin-dependent processing, protein-protein interaction, subcellular translocation, kinase activation and transcriptional activation (Liu Yu-Cai 2004).

Although protein degradation is the best-characterized function of ubiquitin, other regulatory functions have been discovered in recent years. Chain linkage of ubiquitin occurs upon conjugation of the C-terminal glycine of ubiquitin to a Lys residue within ubiquitin itself. Lys48 (K48)-linked polyubiquitination composed of a minimal unit of four ubiquitin moieties targets proteins for degradation by the 26S proteasomes and occurs mainly for cytosolic and nuclear proteins. In addition, K48-linked polyubiquitination is involved in the endoplasmic reticulum-associated degradation (ERAD) pathway that targets misfolded proteins for retranslocation from the ER and proteasomal degradation. In contrast, K29- and K63-linked ubiquitin chains mediate other non-proteasomal function (Marmor and Yarden 2004). It has been shown that monoubiquitination modulates the activity or localization of the protein, and is also a signal for receptor internalization, vesicle sorting, DNA repair and gene silencing (Mastrandrea et al. 1999, Johnson 2002, Marmor and Yarden 2004). Alternatively, a monoubiquitin moiety or a distinct polyubiquitin chain could serve as a specific protein-protein interaction domain, for example to recruit other proteins involved in the ubiquitination reaction.

5.7.1. The ubiquitin conjugation system

The conjugation of ubiquitin to a designated substrate, often called ubiquitination, uses a complex biochemical machinery and entails a cascade of enzymatic reactions: first, ubiquitin is activated by a ubiquitin activating enzyme or E1; second, the activated ubiquitin can be transferred to one of the ubiquitin conjugating enzymes (Ubc) or E2; third, the ubiquitin is transferred by the ubiquitin ligase or E3 to the ε-amino group of Lys residues in the substrate protein. Ultimately, most ubiquitinated proteins are recognized by the 26S proteasome and degraded in an ATP-dependent manner (Hershko and Chiechanover 1998). Different combinations of E2 and E3 can recognize each degradation signal in the substrate and offer high specificity for the ubiquitination reaction (Pickart 2001, Wiesmann 2001).

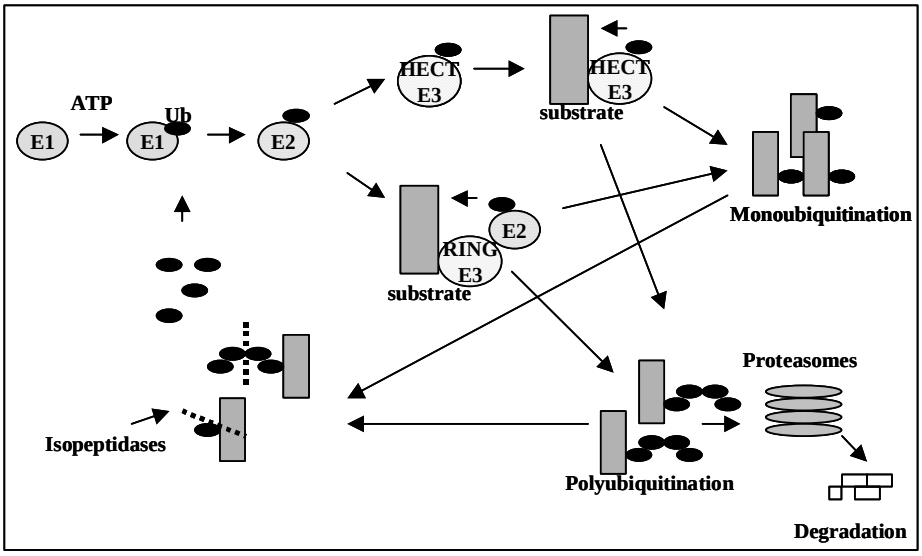

Figure 5. Schematic representation of the ubiquitin-proteasome pathway. The conjugation reaction involves three steps; the ubiquitin residues are first activated by ubiquitin activating enzyme (E1), and then transferred to ubiquitin conjugating enzyme (Ubc) (E2), and finally ubiquitin ligase (E3) catalyzes the transfer of ubiquitin to the Lys residues in the substrates. The polyubiquitinated proteins are recognized and degraded by the 26S proteasomes in an ATP-dependent manner.

Ubiquitin activating enzyme (E1)

Ubiquitin conjugation is initiated through the activation of ubiquitin at the C-terminal glycine (Gly76) residues in an ATP-dependent manner by the ubiquitin activating enzyme (E1) identified in humans and yeast (Hershko and Ciechanover 1998). Yeast E1 is essential for cell-viability, since deletion of E1 is lethal. In mammalian cells temperature-sensitive inactivation of E1 causes reduced antigen presentation, suggesting that the ubiquitin pathway is involved in immune system function (McGrath et al. 1991, Michalek et al. 1993).

Ubiquitin conjugation enzymes (E2)

In humans more than 20 ubiquitin conjugation enzymes (E2) have been identified and 13 isoforms have been identified in yeast. All E2 conjugation enzymes contain a common core structure of approximately 130 amino acids called the UBC domain, which has interfaces for the interaction with ubiquitin. UBC domain contains an active Cys residue that forms an intermediate thioester bond with the activated ubiquitin Gly residue transferred from E1 (Pickart 2001). In addition to the classical ubiquitin conjugation enzymes (UBC), there are noncannonical E2 variants (UEVs) resembling a classical UBC but not containing the catalytically active site to form a thioester bond with the activated ubiquitin. Such examples involve the yeast MMS2, the mammalian MMS2 homologue Uev1 and the tumor susceptibility gene 101 (Tsg101) (Li and Cohen 1996, Hofmann and Pickart 1999, Deng et al. 2000).

Ubiquitin E3 ligases

The E3 ubiquitin ligases play a critical role in the ubiquitination process by recruiting the ubiquitin-loaded E2, recognizing specific substrates and facilitating the transferring of ubiquitin from E2 to the Lys residue of the substrate. So far, hundreds of E3 ligases have been identified, either as a single protein or a multisubunit complex. E3 ligases can be generally divided into two large subgroups: HECT-domain containing proteins (the homology to the E6-associated protein carboxyl terminus) and the RING-domain containing E3, comprising single subunit E3s and multisubunit E3s. More recently, RING-like domain-containing E3 ligases have been identified, such as the

PIAS family of SUMO ligases, the plant homeodomain (PHD) domain-containing E3s and the U-box E3s (Liu 2004).

The HECT-type E3 ligases belong to a family of proteins with a highly conserved region of approximately 350 amino acids named HECT domain (Scheffner et al. 1993, 1995, Huibregtse et al. 1993, 1995). The C-terminus of HECT domain contains a conserved Cys residue, which forms a high-energy thioester bond with ubiquitin and facilitates the transfer of ubiquitin to the substrate. Viral E6-associated protein (E6-AP) was initially discovered by studies on human papillomaviruses, and identified as a complex with the oncogenic E6 that induces the degradation of p53 tumor suppressor. Interestingly, E6-AP appears not to be a physiological E3 ligase, because it does not induce degradation of p53 in HPV-uninfected cells (Talis et al. 1998). Other HECT domain-containing E3 ligases have an N-terminal Ca2+-binding domain, a protein kinase C-related C2 domain followed by multiple WW domains. The WW domains are found in proteins involved in cell signaling or regulation (Sudol 1996, Pirozzi et al. 1997). One example of a well-studied WW domain-containing HECT type E3s in mammalian cells is Nedd4, implicated in the regulation of Na+ channel in kidney and other tissues. Nedd4 deletion is associated with human Liddle's syndrome (Staub et al. 1996).

The RING-type E3 ligases have been identified relatively recently and contain a consensus sequence of C3HC4 (C, cysteine; H, histidine), which coordinates two cross-braced Zinc-binding sites and forms the RING finger (Freemont 2000). The RING-type E3s do not form a thioester bond with ubiquitin, but help to bring the ubiquitin-loaded E2s and the substrate into proximity and promote ubiquitin transfer directly from E2 to the substrate (Joazeiro and Weissman 2000).

Single protein RING-type E3s consist of a protein containing a RING finger domain. One of the most studied single protein RING-type E3 ligases is Cbl, a 120kDa proto-oncogene product containing an N-terminal tyrosine kinase-binding domain (TKB), a RING-finger, a C-terminal proline rich sequences and tyrosine phosphorylation residues (Joaziero et al. 1999, Levkowitz et al. 1999, Yokouchi et al. 1999). The RING finger domain of Cbl forms an E2-binding groove similar to that in E6-AP, and recruits the UbcH7 to promote ubiquitination of the receptor tyrosine kinases (Lupher et al. 1999).

The multisubunit RING-type E3 ligases consist of a superfamily including the SCF (Skp1-Cullin1-F-box protein), the APC (Anaphase-promoting-complex) and the VCB (VHL-elongin C/elongin B) (Pickart 2001, Tyers and Willems 1999). The best characterized E3 ligase complex is the APC complex (anaphase-promoting complex), comprising 12 subunits in yeast and at least 10 subunits in mammals (Nasmyth 1996, King et al. 1996, Zachariae et al. 1998, Yu et al. 1998). The basic components of APC complex include a distant cullin homolog APC2, a substrate-recognizing Cdc20 and a recently identified RING finger-containing APC11. The substrates for APC complex are mostly proteins important for mitotic progression such as mitotic cyclins, certain anaphase inhibitors and spindle-associated proteins (King et al. 1996). There is a similar basic architecture of the APC and SCF complexes, suggesting a common mechanism for substrate recognition and ubiquitination (Peters 1998, Skowyra et al. 1999).

The SCF complex is composed of two evolutionary conserved factors, Skp1 and a member of the Cullin/Cdc53 family of proteins. The complex contains also an F-box protein (Bai et al. 1996, Skowyra et al. 1997, 1999, Feldman et al. 1997). F-box proteins are involved in substrate recognition and contain WD40 repeats or leucine-rich domains responsible for selecting different SCF substrates in a phosphorylation-dependent manner (Zhang et al. 1995, Chu et al. 1998, Zhou et al. 1998). In addition, a small RING finger-containing protein Rbx1/ROC1/HRT1 was also

identified as an essential component of SCF complex (Kamura et al. 1999, Tan et al. 1999, Seol et al. 1999).

VCB E3 ligase complex consists of the von Hippel Lindau (VHL) tumor suppressor protein, Elongin C (a Skp1 homolog), the Ub-like Elongin B and Cul2 (Cdc53 homolog) (Jackson et al. 2000). The VHL contains two domains, the α domain, which binds to Elongin C that forms a complex with Cullin 2 and Roc1, and β domain that recognizes a substrate protein, hypoxia-inducible factor-α (HIF-α) and induces its ubiquitination and degradation (Maxwell et al. 1999, Ohh et al. 2000). In this complex, VHL proteins contain a SOCS-box, a distant homolog of the F-box and can be replaced with other proteins containing SOCS-box (Stebbins et al. 1999, Kamura et al. 1999). The function of the SOCS-box as a recruiting element of the E3 ligase complex suggested that the SOCS family of proteins is involved in the ubiquitin-mediated degradation pathway. The SOCS-box is required for SOCS1-mediated degradation of the oncogenic TEL-JAK2. The phenotype of SOCS-box-deficient *SOCS1* mice demonstrated that SOCS-box is also required for full SOCS1 activity (Kamizono et al. 2001, Frantsve et al. 2001, Zhang et al. 2001). Furthermore, it has been shown that the SOCS-box is important for the stabilization of SOCS1 and SOCS3 proteins (Kamura et al. 1998). Interaction between SOCS-box and Elongin C stabilizes SOCS3 protein expression, whereas phosphorylation of SOCS-box tyrosine residue disrupts the complex and enhances proteasome-mediated degradation of SOCS3 (Haan et al. 2003).

5.7.2. The proteolytic system: 20S and 26S proteasomes

As a central part of the proteolytic system, the 20S and 26S proteasomes have been intensively studied through structural and mutagenesis approaches. The structure of 20S proteasome is well conserved in virtually all organisms and consists of a hollow and a barrel shape of four stacked heptameric rings forming a central chamber. Each ring is composed of seven subunits that can be classified into two groups: α-subunits comprising the outer two rings and β-subunits for the inner two rings (Lowe et al. 1995, Groll et al. 1997, Kopp et al. 1997). The eukaryotic proteasome contains an additional 19S regulatory complex, in the form of a lid and a base and binds to the 20S particle to form the 26S proteasome holoenzyme (Glickman et al. 1998). Within this structure, the lid is responsible for recognizing the ubiquitinated proteins with high fidelity, whereas the base caps at the end of the 20S proteasome core, unfolds the protein substrates and threads it into the catalytic chamber in an ATP-dependent manner (Braun et al. 1999). The eukaryotic 20S proteasome alone has only modest proteolytic activities, and can be stimulated by association with 19S and 11S (PA26) regulatory complexes (Ma et al. 1992, Hoffman and Reichsteiner 1994, Yao et al. 1999, Groll et al. 2000).

The 20S proteasome possesses multiple proteolytic activities such as chymotrypsin-like (CT-L), trypsin-like (T-L) and post-glutamyl peptide hydrolyzing (PGPH), respectively, ensuring that virtually all peptide bonds within a protein substrate are susceptible to cleavage (Wilk and Orlowski 1983, Cardozo 1993). The catalytic β-subunits of the 20S proteasome are synthesized in an inactive form. The N-terminal prosequence is removed upon assembly into the 20S proteasome core, preventing proteolysis of cellular proteins by the newly synthesized β-subunits of 20S proteasome (Frentzel et al. 1994, Yang et al. 1995).

Proteasome inhibitors

Most inhibitors for the ubiquitin-proteasome pathway directly target and inhibit the 20S proteasome, the core of the proteolytic machinery, rather than upstream molecules involved in the ubiquitin conjugation system. The synthetic inhibitors are peptide-based compounds such as

benzamides, peptide α-ketoamides and peptide aldehydes. Upon binding to the active site of the 20S proteasome, a peptide aldehyde forms a covalent hemiacetal adduct reversible under physiological conditions and inhibits the CT-L and T-L activity of the 20S proteasome (Wilk and Orlowski 1983, Orlowski 1990, Figueiredo-Pereira et al. 1994). Natural proteasome inhibitors include linear peptide epoxyketones, peptide macrocycles, γ-lactam thiol ester and epipolythiodioxipiperazine toxin. One of the most studied natural products is lactacystin, a *Streptomyces lactacystinaeus* metabolite that targets the 20S proteasome by an irreversible modification of the N-terminal threonine of β-subunit. The active component of lactacystin is the *clasto*-lactacytin β-lactone, a rearrangement product of lactacystin in aqueous conditions (Omura et al. 1991, Dick et al. 1996). Examples of synthetic peptides include MG115 and MG132, which are all potent and CT-L selective inhibitors, cell-permeable and largely used in studying the role of proteasome in various cellular processes (Rock et al. 1994).

5.7.3. Deubiquitination

Deubiquitinating enzymes are involved in processing the product of ubiquitin genes and negatively regulate the function of ubiquitination. They are also involved in regenerating the free ubiquitin after proteasomal processing and salvaging the ubiquitin from adducts with small nucleophiles in the cells (Wing 2003).

Based on sequence homology, deubiquitination enzymes can be grouped into two defined classes: ubiquitin carboxy-terminal hydrolases (UCH) and ubiquitin processing proteases (UBPs) also referred to as ubiquitin specific proteases (USPs). UCH enzymes possess four conserved motifs involved in catalysis, two of which contain the cysteine, histidine and aspartic acid residues. The residues act together in a catalytic mechanism similar to that in cysteine proteases, and binding of ubiquitin substrate induces a conformational change in the active site giving the enzyme specificity for cleaving ubiquitin fusions (Cope et al. 2002, D'Andrea and Pellman 1998, Wilkinson 1997, Yao and Cohen, 2002). UBP/USP enzymes possess six conserved motifs, the two outer motifs contain the cysteine and histidine residues essential for activity (Gilchrist and Baker 2000). Many deubiquitination enzymes contain an extension on one or both sides of the core region, which is important for protein-protein interaction and in targeting subcellular structures (Baek et al. 1998).

Ubiquitin is encoded in the genome as a fusion protein, either as a copy of ubiquitin chains arranged in tandem or as a fusion with L40 or S27a ribosomal subunits (Finley et al. 1987). Deubiquitination enzymes have the function of processing the precursors into their mature forms, since linear polyubiquitin or the fusion proteins are not detectable in cellular lysates (Baker et al.1992).

Many UBPs/USPs have been demonstrated to have *in vitro* isopeptidase activity against short polyubiquitin chains linked via Lys48 (Falquet et al. 1995, Wilkinson et al. 1995). The isopeptidase activities can result in the reverse effect of ubiquitination (often called *editing*) or in regenerating the free ubiquitin once the function of ubiquitination has been completed (called *recycling*). However, these functions are not completely distinct, as editing releases ubiquitin that can be recycled. In the *editing* function, the isopeptidases negatively regulates the role of ubiquitination in proteolysis and even play a role in endocytosis and regulation of transcriptional activity (Hicke 2001, Salghetti et al. 2001). In the *recycling* function, the isopeptidases depolymerize the polyubiquitin chain to permit the return of the ubiquitin to the free pool. Deubiquitination enzymes are regulated by various mechanisms. Some have tissue restricted expression, some are developmentally regulated or hormonally regulated or induced upon environmental stimulation (Wilkinson et al. 1989, Zhu et al. 1996, 1997, Hegde et al. 1997, Liu et al. 1999, Lin et al. 2000). Recent studies have revealed that localization also plays a key role in regulating deubiquitinating

activity, since many UBPs have distinct subcellular localization (Borodovsky et al. 2001, Leggett et al. 2002).

5.8. Protein modification by sumoylation

The SUMO (*s*mall *u*biquitin-like *mo*difier) family of proteins belongs to the ubiquitin-related protein modifiers, collectively termed Ubls. They are post-translationally attached to substrate proteins by enzymatic reactions similar to ubiquitin conjugation (Jentsch and Melchior 2000, Schwartz et al. 2003). However, despite the similarities in their structure and the enzymatic reactions underlying their conjugation, SUMO and ubiquitin have distinct functions. SUMO substrates include many proteins that participate in diverse cellular processes, including chromosome segregation and cell division, DNA replication and repair, transcriptional regulation, nuclear transport and signal transduction. Sumoylation is also involved in the regulation of many processes such as inflammatory response in mammals and the regulation of flowering in plants (Dohmen 2004, Johnson 2004).

The ubiquitin-like protein SUMO-1 was discovered in 1996 by studies on RanGAP1 nuclear import in mammalian cells. SUMO-1 conjugation targets cytosolic RanGAP1 to the nuclear pore complex (NPC), where it participates in the nuclear import by activating the GTPase activity of the cytosol/nucleus shuttling factor Ran (Matunis et al. 1996, Mahajan et al. 1997, 1998). Four different SUMO isoforms were characterized in mammals: SUMO-1 (also known as sentrin, PIC1, GMP1, Unl1 and Smt3c), SUMO-2 (sentrin-3, Smt3a), SUMO-3 (sentrin-2, Smt3b) and SUMO-4. SUMO-2 and SUMO-3 have very similar sequences and are therefore often termed SUMO-2/3. SUMO-1 seems to be the most conjugated isoform under normal conditions, whereas SUMO-2/3 appears to be conjugated to proteins under stress conditions. The cells contain a large pool of free SUMO2/3, but very little free SUMO-1. The recently identified SUMO-4 isoform is encoded by a sequence within the human TAB2 gene and is strongly expressed in kidney cells (Saitoh and Hinchey 2000, Eaton et al. 2003, Azuma et al. 2003, Bohren et al. 2004). SUMO-2/3 and SUMO-4 share a SUMO attachment consensus site ψKXE in their N-terminal extension and have been shown to form SUMO chains *in vivo* and *in vitro*, both characteristics being absent in the case of SUMO-1 (Bohren et al. 2004, Tatham et al. 2001).

The structure of human SUMO-1 has been determined by NMR and compared to the crystal structure of ubiquitin. The sequence identity between SUMO and ubiquitin is relatively low, 18%, but both proteins share a characteristic ββαββαβ structure fold and a C-terminal di-glycine motif. SUMO is distinguished by a long and flexible N-terminal extension (Bayer et al. 1998). All SUMO genes encode a precursor having a short C-terminal peptide, which is cleaved off by SUMO-cleaving protease to produce the mature Gly-Gly C-terminus found in most ubiquitin-related proteins.

5.8.1 SUMO conjugation pathway

SUMO is attached to most substrates at the Lys residue in a conserved ψKXE sequence (Melchior 2000). The Lys residue and the Glu residue are the most conserved in the consensus sequence. The ψKXE motif is sufficient to target an artificial substrate for sumoylation and is bound directly by the SUMO conjugation enzyme E2, indicating that the requirement for SUMO conjugation may be very simple (Rodriguez et al. 2001, Sampson et al. 2001). The ψKXE motif is very short and can be found in many proteins, most of which cannot be modified by SUMO, indicating that other interactions are likely to be critical in substrate selection (Johnson 2004). The enzymes required for reversible SUMO conjugation (often termed sumoylation) were first characterized in yeast *S.*

cerevisiae, and some of them such as SUMO-activating enzyme E1 and SUMO-conjugating enzyme E2 have sequence similarity with their counterparts in the ubiquitin system.

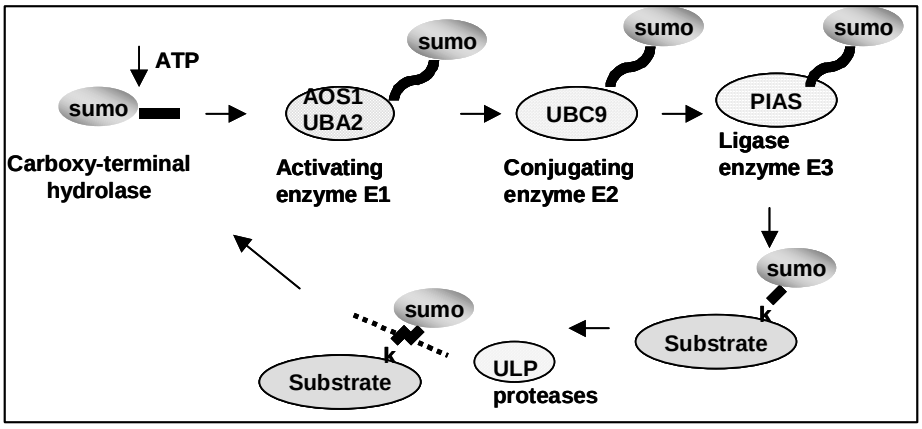

Figure 6. Schematic representation of SUMO conjugation pathway. SUMO conjugation utilizes E1 activating enzyme complex termed SAE1/SAE2 and an E2 conjugating enzyme Ubc9 as well as a group of E3 ligases, such as a PIAS proteins. E3 ligases facilitate the transfer of SUMO from E2 conjugation enzyme Ubc9 to the substrate by catalyzing the conjugation reaction.

SUMO activating enzyme (E1)

The SUMO activating enzyme required for conjugation of all SUMO variants to all substrates is a heterodimer of Aos1 (SAE1, Sua1) and Uba2 (SAE2), proteins with sequence similarities respectively to the N- and C-terminal parts of ubiquitin activating enzymes (Dohmen et al. 1995, Johnson et al. 1997, Okuma et al. 1999, Desterro et al. 1999, Azuma et al. 2001). The SUMO activating enzyme catalyzes a three-part reaction: first, formation of the SUMO C-terminal adenylate in an ATP-dependent manner, second, the thiol group of the active cysteine residue in E1 attacks the SUMO-adenylate forming a high-energy thioester bond between E1 and the C-terminus of SUMO, and third, the activated SUMO is transferred to a cysteine in E2.

SUMO conjugating enzyme (E2)

The second step in the SUMO conjugation pathway involves the transfer of SUMO from E1 to the active site cysteine of the SUMO conjugating enzyme (E2), forming the SUMO-E2 intermediate. So far, Ubc9 has been characterized as the only SUMO conjugating enzyme in yeast and invertebrates and most likely in vertebrates as well. Ubc9 shares a high sequence similarity with ubiquitin E2 enzymes and also has the same folded structure, although Ubc9 has a strong overall positive charge (Desterro et al. 1997, Johnson and Blobel 1997, Tong et al. 1997, Hayashi et al. 2002, Jones et al. 2002). Also, mapping of the binding sites of mammalian SUMO-1 and SUMO2/3 revealed that Ubc9 does not discriminate between them (Tatham et al. 2003).

SUMO E3 ligases

Three types of SUMO E3 ligases have been described, PIAS (protein inhibitor of activated STAT), RanBP2 and polycomb group protein PC2, all of which interact with Ubc9 and enhance sumoylation *in vivo* and *in vitro* (Jackson 2001, Hochstrasser 2001, Pichler et al. 2002, Kagey et al. 2003). These SUMO E3 ligases can bind to E2, bind to the substrate and promote the transfer of SUMO from E2 to the substrate. However, *in vitro* conjugation of SUMO can take part in the absence of E3 ligase. Similar to the RING domain-containing E3 ligases involved in ubiquitination, SUMO E3 ligases do not form covalent intermediates with SUMO, but act by bringing together Ubc9 and the substrate, and may also activate Ubc9 (Okuma et al. 1999, Desterro et al. 1999).

Recently, PIAS proteins were shown to function as E3-type ligases for SUMO (Takahashi et al. 2001, Johnson et al. 2001, Kotaja et al. 2002). PIAS1 was shown to enhance SUMO-1 conjugation

to p53 *in vitro* and *in vivo*, and was found to bind with SUMO-1 and Ubc9. However, a mutant PIAS1 lacking the RING finger-like region was unable to promote transfer of SUMO-1 to acceptor proteins *in vitro* and *in vivo* (Kahyo et al. 2001, Sachdev et al. 2001). PIAS proteins also contain a short motif of hydrophobic amino acids followed by acidic amino acids called SXS domain or SIM (SUMO interaction motif). SIM has been implicated in binding directly to SUMO and its deletion can affect their localization and transcriptional effects but not the ability of PIAS to promote SUMO conjugation (Minty et al. 2000, Sachdev et al. 2001, Kotaja et al. 2002). PIAS proteins may promote the attachment of different SUMO isoforms. For example, PIASy preferentially conjugates SUMO-2 rather than SUMO-1 to the transcription factors LEF1 and GATA-2, and can enhance overall SUMO-2 conjugation (Sachdev et al. 2002, Chun et al. 2003). In S. *cerevisiae*, Siz1 is required for sumoylation of the septin family of cytoskeletal proteins and replication factor PCNA, whereas Siz2 can sumoylate some other, so far unidentified, proteins (Hoege et al. 2002, Johnson and Gupta 2001, Takahashi et al. 2001). PIAS E3 ligases share important similarities with RING-type ubiquitin ligases. While RING-type ubiquitin ligases require the RING-like domain for their ligase activity, PIAS ligases depend for their integrity on the RING-like domain for SUMO conjugation (Takahashi et al. 2001a, 2001b, Kahyo 2001, Kotaja et al. 2002). Interestingly, PIAS proteins themselves are subject to SUMO modification and this process requires the integrity of the RING-like domain, suggesting a similarity with the RING-dependent autoubiquitination of RING-type ubiquitin ligases (Kotaja et al. 2002, Smith and Muller 2002). However, ubiquitin and SUMO E3 ligases differ in some important aspects, one of these being the substrate specificity. PIAS proteins are less important determinants for substrate specificity in the SUMO conjugation pathway. Furthermore, PIAS proteins do not seem to be essential for the sumoylation of substrates, but rather function as a factor that stimulate SUMO conjugation. In this context, it has been suggested that in the SUMO conjugation reaction, Ubc9 contributes to substrate specificity, whereas PIAS proteins stabilize the interaction between Ubc9 and the substrate, as demonstrated by the finding that PIAS proteins can make physical contact with Ubc9 and most substrates (Kotaja et al. 2002, Sapetchnig et al. 2002, Schmidt and Muller 2003). The identification of a SUMO-recognition motif in PIAS, adjacent to the SP-RING finger suggested that sumoylation of a target protein enhances its interaction with PIAS (Minty et al. 2000). In this context, PIAS proteins seem to function as E3 SUMO ligases and also as SUMO tethering factors, probably contributing to the formation of a large protein complex. All mammalian PIAS proteins concentrate in subnuclear structures resembling PML (Rodel et al. 2000, Sachdev et al. 2001, Kotaja et al. 2002). More specifically, in the case of PIASy it has been demonstrated that at least a subset of PIASy-containing foci colocalises with a subpopulation of PML nuclear bodies. Proper localization of PIAS proteins to nuclear foci depends on the integrity of the SP-RING domain, indicating that the ligase activity is implicated in the targeting process. A growing number of transcription factors have been shown to be modified by PIAS-mediated SUMO conjugation, including c-Jun, MDM2, Sp3, SATB2, C/EBPb, Tcf4, IRF-1, HSF1, p300, p73, c-Myb, as well as androgen receptor, progesterone receptor and glucocorticoid receptor (Dohmen 2004).

RanPB2 (Ran binding protein 2) is located at the cytoplasmic filaments of the nuclear pore complex and interacts with sumoylated RanGAP and the GTPase Ran. RanPB2 was one of the first SUMO substrates to be identified (Saitoh et al. 1998). It has been shown that RanPB2 represents an NPC-associated SUMO ligase that couples sumoylation with nuclear import (Pichler et al. 2002, Pichler and Melchior 2002). The RanBP2/RanGap-SUMO complex is relocalized to kinetochores during mitosis in a microtubule- and SUMO-dependent manner (Joseph et al. 2004, 2002).

The polycomb group (PcG) protein PC2 was recently reported to be a third type of SUMO E3 ligase and to be apparently structurally unrelated to the other SUMO E3 ligases and ubiquitin ligases (Kagey et al. 2003). PcG proteins were initially identified in *Drosophila*, where it mediates the

silencing of homeotic gene expression by modifying histones (Muller et al. 2002, Cao et al. 2002). PcG proteins form a large multimeric complex called PcG bodies located within cell nuclei. The human PC2 recruits CtBP and Ubc9 to PcG bodies and stimulates CtBP sumoylation *in vivo* and *in vitro*. Also, PC2 itself is modified by sumoylation (Kagey et al. 2003, Lin et al. 2003).

5.8.2 Desumoylation

SUMO-cleaving enzymes (also called isopeptidases) play an important role in the reversible sumoylation cycle by removing the SUMO from substrates and providing free SUMO to be used for another cycle of sumoylation. Newly synthesized SUMO must be first cleaved to remove the short C-terminal peptide (Matunis et al. 1996, Johnson et al. 1997, Li and Hochstrasser 1999,). All SUMO-cleaving enzymes contain a C-terminal Ulp domain, which has SUMO cleaving activity and is distantly related to a number of viral proteases, but does not share sequence similarity with other enzymes that cleave ubiquitin. The N-terminal domain is variable and has a regulatory role by targeting the enzyme at different parts of the cells (Mossessova and Lima 2000, Strunnikov et al. 2001, Hang and Dasso 2002, Zhang et al. 2002, Li and Hochstrasser 2003, Panse et al. 2003,). In the mammalian genome, seven genes have been identified to encode proteins with Ulp domains. These enzymes include SENP3 (SMT3IP1), SENP6 (SUSP1), SENP1 and SENP2 (Axam, SMT3IP2/Axam2, SuPr-1), all localizing in different parts of the cell and having a divergent N-terminal domain (Yeh et al. 2000, Nishida et al. 2000, Kim et al. 2000, Bailey and O'Hare 2002, Best et al. 2002, Mendoza et al. 2003). SENP1 has been colocalized with a herpes virus protein ICP0 during early infection and this colocalization may be involved in the ICP0-induced loss of SUMO-1-modified PML in infected cells (Bailey et al. 2002). Also, SENP1 contains a basic motif within the N-terminus of the protein that is critical for its efficient nuclear localization, and mutation of cysteine residue 603 to serine within the predicted catalytic core inactivates SENP1, resulting in a dominant negative effect (Bailey et al. 2004).

6. AIMS OF THE STUDY

Post-translational modifications play an essential role in all biological processes, from replication and gene expression to morphogenesis of organisms. The importance of post-translational modifications in regulation of cytokine receptor signaling has obtained increasing recognition.

The specific aims of this study were:

1) To characterize the regulatory mechanism of JAK2 activation and the role of ubiquitination in cytokine receptor signaling.
2) To characterize the role and functional consequences of STAT1 sumoylation.

7. MATERIALS AND METHODS

7.1 Cell lines

Human HeLa cells and monkey Cos-7 cells were cultured in Dulcecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100U/ml penicillin and 50μg/ml streptomycin (Gibco-BRL). Human fibrosarcoma U3A cells (kindly provided by Dr. I. Kerr) (Muller et al. 1993) were cultured in DMEM supplemented with 10% Cosmic calf serum (HyClone) and antibiotics. IL-3 dependent myeloid progenitor 32D cells and Ba/F3 cells were maintained in RPMI medium supplemented with 4% WEHI supernatant plus 10% FCS and antibiotics. Prior to cytokine stimulation, the cells were washed with PBS and starved in medium containing 0.5% serum. The stimulation was carried out in 0.5% serum containing medium.

7.2 Antibodies and cytokines

The following antibodies were used: anti-phosphotyrosine clone 4G10 (Upstate Biotechnology, Lake Placid, NY), polyclonal anti-JAK2 antibody, kind gift from Dr. Ihle, anti-influenza virus hemagglutinin (HA)-epitope-antibody (clone 16B12, Berkeley-Antibody, Richmond, CA), anti-ubiquitin mouse monoclonal antibody mAb-Ubi-1 (Sigma-Aldrich RBI, USA), anti-SOCS1 and anti-SOCS3 monoclonal antibodies, kind gift from Dr. Hilton, monoclonal anti-STAT1 C-terminus and N-terminus and polyclonal anti-phospho-STAT1 (Tyr701) (Transduction Laboratories, BD Bioscience), anti-SUMO-1 (mouse anti-GMP1) (Zymed, San Francisco, CA), anti-FLAG (Sigma-Aldrich RBI, USA), biotinylated anti-mouse and anti-rabbit (Dako A/S, Denmark) and streptavidin-biotinylated horseradish peroxidase (Amersham Pharmacia Biotech, Buckingamshire, UK). Human IFN-γ was from Immugenex and murine IL-3 was from R & D System.

7.3 DNA constructs

HA-tagged JAK2WT has been cloned in pCI-Neo (Silvennoinen et al. 1993) and used as a template to clone HA-tagged JAK2-YF and HA-tagged JAK2KN mutants. The JAK2 Y1007F mutant was created by direct PCR mutagenesis using of the following primers: 5'TGC CGC AGG ACA AAG AAT TCT ACA AAG TAA AGG AGC CA and 3'TGG CTC CTT TAC TTT GTA GAA TTC TTT GTC CTG CTG CGG CA. The JAK2KN-HA has K882E substitution created also by direct PCR mutagenesis as described (Silvennoinen et al. 1993).
The plasmid pMT-$(HA\text{-}ubiquitin)_8$ encoding HA-tagged ubiquitin and pMT- His6-c-ubiquitin were kind gifts from Dr. Bohmann (EMBL Heidelberg, Germany). Mouse SOCS1 and SOCS3 have been cloned in pEF-FLAG-I plasmid (Starr et al. 1997). SOCS1ΔSB (SOCS1 lacking the SOCS-box) was cloned by PCR amplification of SOCS1 N-terminal region and SH2 domain into pEF-FLAG-I vector (Starr et al. 1997).
STAT1 expression plasmids were constructed by cloning the STAT1 cDNAs into EF-BOS vector containing HA-tag (hemagglutinin) epitope tag (Dr. C. Schindler). The STAT1 K703-to-R mutation was created from STAT1-WT-HA using direct PCR mutagenesis with the following primers:
5'GGAACTGGATATATCAGGACTGAGTTGATTTCTGTGTCTG-3' and
5'-CAGACACAGAAATCAACTCAGTCCTGATATATCCAGTTCC-3'. STAT1-I702R-HA (Ile-to-Arg) and STAT1-E705A-HA (Glu-to-Ala) were constructed from STAT1-WT-HA using PCR mutagenesis with the following primers:
5'- GGAACTGGATATAGGAAGACTGAGTTGATTTCTGTGTCTGAA-3' and
5'- TTCAGACACAGAAATCAACTCAGTCTTCCTATATCCAGTTCC-3'
5'-GGAACTGGATATATCAAGACTGCGTTGATTTCTGTGTCTGAA-3' and
5'-TTCAGACACAGAAATCAACGCAGTCTTGATATATCCAGTTCC-3'

SUMO-1-Flag and SUMO-1-Flag-Flag cloned in pcDNA3 vector were kind gift from Dr. Yasuda (Tokyo University, Japan). pFLAG-PIAS1 and pFLAG-PIAS3 were gifts from Dr. K. Shuai (Chung et al. 1997, Liu et al. 1998). Flag-PIAS1mut (PIAS1Δ310–407) was constructed by amplifying the coding sequence for the PIAS1 C-terminus (amino acids 408 to 651) by PCR as described (Kotaja et al. 2002). Flag-ARIP3 and Flag-ARIP3mut (ARIP3Δ347-418) were created by PCR as described (Kotaja et al. 2002). pSG5-His-SUMO-1 was provided by A. Dejean (Muller et al. 2000).
The GAS-luc luciferase construct contains the GAS site from the IRF-1 gene promoter inserted upstream of the TK (thymidine kinase) promoter driving the firefly luciferase (luc) coding region (Pine et al. 1994).
GAL4-STAT1-TAD was constructed by PCR the TAD domain from STAT1-WT or STAT1-KR (amino acids 680-750) and cloned into *BamHI/EcoR1* sites in GAL4-DBD plasmid. FLAG-tagged SENP1 and SENP1 C603S mutant were previously described (Bailey et al. 2002, 2004) pCI-Neo expression vector was from Promega (Madison, WI) and pSG5 expression vector was from Stratagene (La Jolla, CA).

7.4 Transfection of cell lines

7.4.1.Calcium phosphate co-precipitation

U3A cells were transfected using calcium phosphate co-precipitation. For this, cells were plated 24 hours prior transfection at specific density. Plasmids DNA, 62µl of 2M $CaCl_2$ and water up to a final volume of 500µl were mixed up with the same volume of 2X HEPES solution (280mM NaCl, 1.5mM Na_2HPO_4, 55 mM N-2-hydroxyethylpiperazine-N'-2-ethanosulfonic acid, pH 7.0) and the final mixture was added to 60% semiconfluent cells. 24 hours after transfections, the cells received fresh medium of 1% FCS and either left untreated or treated with IFN-γ for another 6-8 hours before harvesting and luciferase assay.

7.4.2 Electroporation

Cos-7 cells were transfected by electroporation with BioRad Gene Pulser apparatus at 260V, 960µF, with 0.5cm cuvettes. Certain amount of plasmid DNA was mixed with herring sperm DNA added up to 40µg, and mixed with the cells in 250µl final volume (3-5 x 10^6 cells) prior to electroporation. Afterwards, the cells were plated in 10cm plates and analyzed for the expression of target proteins after 24-48 hours.

7.5 Immunoprecipitation and Western blot

For the detection of JAK2 ubiquitination and interaction with SOCS proteins, Cos-7 cells were transfected with 4 µg JAK2 plasmid, 6 µg SOCS plasmid, 2 µg HA-ubiquitin or His-tagged-ubiquitin plasmids. The cells were harvested in ice-cold PBS and lysed in NP-40 lysis buffer (50 mM Tris-HCl pH 7.4, 10% glycerol, 50 mM NaCl, 0.5% Na-deoxycholate, 1% NP-40, 20 mM NaF, and 0.2 mM Na3VO4) supplemented with protease inhibitors. After 30 minutes incubation on ice, lysates were cleared by centrifugation for 20 minutes at 4°C. Ubiquitin-aldehyde was added in lysis buffer at concentration of 20 µg/ml in order to inhibit isopeptidase activity. Alternatively the cells were lysed by boiling in PBS-2% SDS buffer, added directly onto cell-pellets, the DNA was sheared by strong pipeting and the samples were boiled for 10 minutes followed by centrifugation. The protein amount was determined using BioRad Dc protein assay Kit (Bio-Rad Laboratories)
Immunoprecipitations were carried out by incubating the lysates (1 mg total protein) with anti-JAK2 antibody, anti-HA antibody and anti-SOCS1 antibody for at least 3 hours at 4°C on shaker

rotation. For anti-ubiquitin detection, immunoprecipitations were performed in the presence of 25 μM MG132.

After SDS-PAGE electrophoresis the proteins were transferred to a nitrocellulose membrane (Protran, Schleicher & Schuell GmbH, Germany) and blocked with 5% non-fat dried milk in TBS 0.1% Tween20. The membranes were incubated with specific primary antibodies diluted in TBS 0.05% Tween20 followed by secondary biotinylated antibody and streptavidin-biotin horseradish peroxidase conjugate. Immunodetection was performed using enhanced chemiluminescence method (Amersham Pharmacia Biotech, Buckingamshire, UK). The stripping was performed by incubating the filters with stripping buffer (62.5 mM Tris-HCl, 100 mM 2-mercaptoethanol, 2% SDS) for 45 minutes at 56°C, followed by extensive washings and blocking with 5% non-fat dry milk in TBS.
For detection of STAT1 sumoylation in Cos-7 cells, the cells were transfected with 2 μg STAT1, 0.6 μg SUMO-1, 2.5 μg PIAS1, 1 μg PIAS1mut, 4 μg PIAS3, 4 μg ARIP3 and 0.5 μg ARIP3mut, in order to yield similar expression levels. The cells were lysed in Triton X lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 10% glycerol, 1% Triton X-100) supplemented with protease inhibitors and 5 mM *N*-ethylmaleimide (NEM). Immunoprecipitation and immunobloting were performed as described above.

7.6 Luciferase assay

Reporter gene assay was done in U3A cells were transfected with different STAT1 constructs, pCMV-βgal as an internal transfection efficiency control and GAS-luc reporter plasmid. After 24 hours, the cells were treated with IFN-γ for 6-8 hours and lysed in reporter lysis buffer (Promega), according to the manufacturer's instructions. Luciferase activity was measured using a luminometer with Promega luciferase assay reagent and normalized against β-galactosidase activity of the lysates, determined using ONPG (O-nitrophenyl-β-D- galactopyranoside) as a substrate and measuring the absorbance at 420nm.

7.7 Electrophoretic mobility shift assay (EMSA)

For gel-mobility shift assay, stable U3A clones either unstimulated or stimulated for different time points with IFN-γ were washed in ice-cold PBS and lysed in WCE lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1 mM EDTA, 50 mM NaF, 10% glycerol, 0.5% NP-40, 0.5 mM Na_3VO_4) supplemented with protease inhibitors. The GAS element from the murine IRF-1 gene (IRF-GAS, made by annealing the oligonucleotide 5' – CTA GAG CCT GAT TTC CCC GAA ATG ATG AG –3' and its complement) were end-labeled with T4-polynucleotide kinase using γ-^{32}P ATP and used as a probe. The binding assay was performed by incubating the cell extracts on ice with herring sperm DNA for 15 min followed by an additional 15 min incubation with GAS ^{32}P-labelled oligonucleotides. Reaction were resolved by 4.5% TBE-PAGE (2.2x TBE concentration) and visualized by autoradiography.

7.8 *In vitro* ubiquitination reaction

In vitro JAK2 ubiquitination was performed using immunoprecipitated JAK2 from transfected Co-7 cells bound to protein A-Sepharose beads. After extensive washes, the beads were resuspended in 30 μl buffer containing 40 mM Tris-HCL (pH 7.5), 5 mM MgCl2, 2 mM DTT, 2 mM ATP, 5 μg/ml ubiquitin, 12.5 μM MG132, and 20 μg/ml ubiquitin-aldehyde. To deplete endogenous ATP, hexokinase (1 μg/ml) and 2-deoxy-glucose (20 μM) were also included. The final reaction was

completed by adding 5 µl of crude rabbit reticulocyte lysates and incubation for 1.5 hour at 30°C. The beads were then extensively washed and resuspended in SDS-loading buffer.
For *in vitro* ubiquitination reaction using cell-lysate, crude cell-lysates from transfected Cos-7 cells were prepared by lysing the cell pellet in 250µl of lysis buffer (20 mM Hepes, pH 7.2, 10 mM KCl, 1.5 mM MgCl2, 1 mM DTT, 25 µM MG132 and protease and phosphatase inhibitors) followed by sonication for two cycles of 30 sec and centrifugation for 30 min. Ubiquitination reaction was performed in 50µl reaction buffer (20 mM Hepes, pH 7.2, 10 mM MgCl2, 1 mM DTT, 1 mM ATP, 1 µg/ml hexokinase, 20µM D-glucose, 25 µM MG132, 5µg/ml ubiquitin) using 100 µg of crude cell-lysates and immunprecipitated JAK2 as described above. The reaction was incubated for 90 min at 30°C followed by washing and resuspention in SDS-loading buffer.

7.9 Pulse-chase experiments

For pulse-chase experiments, transfected Cos-7 cells were preincubated with proteasome inhibitors MG132 (12.5 µM) or β-lactacystin (5 µM) for one hour and maintained throughout the pulse and chase experiments. Cells were then transferred to methionine-free and cysteine-free DMEM (Gibco-BRL) for 30 min, pulsed with 0.1 mCi of [^{35}S] methionine and [^{35}S] cysteine (ProMix, Amersham) for 15 min and chased with DMEM. JAK2 proteins were immunoprecipitated using anti-HA antibody and detected by autoradiographic exposure.

7.10 Quantitative RT-PCR

Total RNAs from stable U3A clones either treated or untreated with IFN-γ were extracted using TRIZOL reagent (Gibco-BRL) and 3 µg of total RNAs were used for first-strand cDNA synthesis with the First strands cDNA synthesis kit (MBI, Fermentas). The PCR amplifications were performed using the LightCycler instrument (Roche Applied Science) with the QuantiTect SYBR Green Kit (Qiagen). For normalization, the amount of mouse *G3pdh* mRNA was measured in each cDNA sample. Standard curves were generated using *G3pdh* cDNAs.

7.11. Immunofluorescence detection

Stably transfected U3A clones plated at 60% confluence were serum starved and stimulated with 100 ng/ml huIFN-γ. Cells were fixed in p-formaldehyde (4%) and methanol (100%) and stained with anti-phospho-STAT1 antibody (1:300 dilution) and anti-STAT1 (N-term) (1:1000 dilution) over night followed by TexasRed and Alexa 488 staining and microscopy fluorescence detection.

8. RESULTS AND DISCUSSION

8.1 The molecular mechanism of JAK2 ubiquitination (I)

8.1.1 JAK2 is ubiquitinated *in vivo* and its ubiquitination is regulated by cytokines

JAK2 plays an essential role in EPO, IL-3 and IFN-γ signaling (Schindler and Darnell 1995, Ihle et al. 1995, Parganas et al. 1998). Studies focused on the mechanisms of regulation of cytokine signaling have suggested that proteasome-mediated degradation could be involved in the modulation of cytokine actions. Treatment of Ba/F3 cells with the proteasome inhibitor N-acetyl-L-leucinyl-L-leucinyl-norleucinal (LLnL) led to stable tyrosine phosphorylation of IL-3 receptor beta common chain (βc) and STAT5 following stimulation as a result of prolonged activation of JAK2 and JAK1 (Callus and Mathey-Prevot 1998). We had previously observed that enhanced tyrosine phosphorylation of JAK2 resulted in a significant decrease in JAK2 protein levels (Saharinen et al. 2000). Therefore it was of interest to analyze if JAK2 activation is directly regulated through the ubiquitin-proteasome pathway and what mechanism is involved in the process of JAK2 ubiquitination.

The role of proteasome-mediated degradation in the regulation of JAK2 activation was studied in IL-3 dependent myeloid progenitors 32D cells and Ba/F3 cells. Two different proteasome inhibitors were used, MG132, a reversible proteasome inhibitor and β-lactacystin (*clasto*-lactacystin β-lactone), which targets the 20S proteasome by an irreversible modification and also blocks the activity of isopeptidases. 32D cells were lysed using non-denaturing buffer containing both proteasome inhibitors and ubiquitin aldehyde, an isopeptidase inhibitor. JAK2 was immunoprecipitated and subjected to anti-ubiquitin and anti-Jak2 immunoblotting. The results showed that proteasome inhibitors stabilized IL-3-induced tyrosine phosphorylation of endogenous JAK2 in 32D cells and Ba/F3 cells as previously reported (Callus and Mathey-Prevot 1998). The same effect was observed in Cos-7 cells after IFN-γ stimulation in the presence of MG132 pretreatment. Attachment of polyubiquitin chains to a certain substrate results in increased molecular weight and can be detected in Western blot as a ladder or smear of high molecular weight bands.

A protein corresponding to the molecular weight of JAK2 was detected by anti-ubiquitin antibody in both unstimulated and IL-3-stimulated 32D cells. Stripping and reblotting with anti-Jak2 antibody indicated that the protein was JAK2. Based on the molecular weight this band is likely to represent a monoubiquitinated form of JAK2. IL-3 stimulation induced a smear above the Jak2 band, which is characteristic of polyubiquitination. This smear could also be detected using anti-phosphotyrosine antibody in Western blot. The presence of ubiquitin aldehyde in the lysis buffer was critical for the detection of the polyubiquitinated form of JAK2. Pretreatment of 32D cells with MG132 did not affect the level of monoubiquitinated JAK2, but increased the accumulation of polyubiquitinated JAK2. Tyrosine phosphorylation stimulated both monoubiquitination and polyubiquitination of JAK2, and proteasome inhibitor treatment stabilized the polyubiquitinated form of JAK2. In our experiments JAK2 protein levels were not significantly downregulated in 32D cells after IL-3 stimulation. One possible explanation could be that only a small fraction of the cellular JAK2 becomes activated and tyrosine phosphorylated after cytokine stimulation, and the degradation of this fraction is likely to be difficult to detect on the total protein level.

The effect of proteasome inhibitors on cytokine receptor expression or activation was a possible mechanism for increased JAK2 activity. Therefore ubiquitination of JAK2 was investigated in Cos-

7 cells, where JAKs can be activated by overexpression in a receptor-independent mechanism. Cos-7 cells were transiently transfected with plasmids encoding HA-epitope tagged wild type JAK2 (JAK2WT-HA) and ubiquitin. JAK2 protein was immunoprecipitated using anti-Jak2 antibody and subjected to immunoblotting with anti-ubiquitin and anti-HA antibodies. We observed that overexpression of JAK2WT enhanced the ubiquitination of JAK2. Ubiquitin co-expression did not have any significant effect on the level of JAK2WT ubiquitination, suggesting that the availability of ubiquitin is not a rate limiting step in the ubiquitination reaction. Proteasome inhibitor treatment resulted in accumulation of multiubiquitinated forms of JAK2 detected as the characteristic high molecular weight smear.

8.1.2 Polyubiquitination and degradation of JAK2 is regulated through tyrosine phosphorylation

In order to directly examine the role of tyrosine phosphorylation in the process of ubiquitination and degradation of JAK2, we used JAK2 kinase inactive mutant (JAK2KN), in which the conserved Lys882 residue was substituted with Glu (K882E). For these experiments the expression level of JAK2 was titrated to a level which allowed the regulation of JAK2 tyrosine phosphorylation by tyrosine phosphatase inhibitor pervanadate. Transiently transfected Cos-7 cells with HA-tagged JAK2WT and JAK2KN were treated with different concentrations of pervanadate to induce tyrosine phosphorylation of JAK2. JAK2 was immunoprecipitated using anti-HA antibody and analyzed by Western blotting. We compared the kinetics of phosphorylation between JAK2WT and JAK2KN after pervanade treatment in the presence or absence of proteasome inhibitor pretreatment. Overexpression resulted in tyrosine phosphorylation and activation of JAK2WT and pervanadate treatment induced in a dose-dependent manner ubiquitination of JAK2WT, which correlated with enhanced tyrosine phosphorylation of JAK2WT. Furthermore, at high concentration or with longer treatment, pervanadate caused phosphorylation and polyubiquitination of JAK2WT and disappearance of the monoubiquitinated form of JAK2. Proteasome inhibitor treatment enhanced both polyubiquitination and tyrosine phosphorylation of JAK2WT and stabilized the phosphorylated JAK2WT protein. JAK2KN was found to be monoubiquitinated at low levels in Cos-7 cells. JAK2KN is not significantly tyrosine phosphorylated when overexpressed in Cos-7 cells, but high concentration of pervanadate treatment resulted in low levels of tyrosine phosphorylation of JAK2KN, probably due to transphosphorylation. A modest increase in mono- and polyubiquitination of JAK2KN was detected after pervanadate treatment, and β-lactacystin pretreatment enhanced ubiquitination as well as tyrosine phosphorylation of JAK2KN. Taken together, these results demonstrated the correlation between tyrosine phosphorylation of JAK2 and its ubiquitination and degradation through the proteasome pathway.

Our results demonstrated that tyrosine phosphorylation is a signal for JAK2 ubiquitination and degradation through the proteasome pathway. Ubiquitination and phosphorylation have several features in common: first, both are reversible forms of covalent modification; second, both occur rapidly, often within minutes or seconds of stimulation; third, both are highly specific and controlled by a specific set of enzymes; fourth, both ubiquitination and phosphorylation can be detected by specialized domains that bind to ubiquitin or phospho-amino acids respectively (Sun and Chen 2004). It is well established that tyrosine phosphorylation is an important signal that targets proteins for ubiquitination and subsequent degradation. Examples of this regulatory mechanism are cell surface receptors, Src kinases, IκB-α, p53, β-catenin, c-Jun and RNA-polymerases (Orford et al. 1997, Fuchs et al. 1997, Ratner et al. 1998, Laney and Hochstrasser 1999, Magnani et al. 2000, Marmor and Yarden 2004, Giannini and Bijlmakers 2004, Dai and Lu 2004, Saville et al. 2004). In our case, tyrosine phosphorylation stimulated JAK2 polyubiquitination, and this was stabilized by proteasome inhibitor treatment, indicating that the polyubiquitinated JAK2 is rapidly degraded through the proteasome pathway.

One interesting finding during the studies of JAK2 ubiquitination was the monoubiquitination of JAK2, as detected to variable degrees in different cells using different antibodies in Western blotting, and also using different lysis conditions. Tyrosine phosphorylation was not required for monoubiquitination of JAK2, since both JAK2KN and endogenous JAK2 from unstimulated cells were found to be monoubiquitinated. However, induction of tyrosine phosphorylation stimulated both monoubiquitination and polyubiquitination of JAK2. At this moment, the target Lys residue in JAK2 that is modified by ubiquitin conjugation is at present unknown. Also, it is not known if JAK2 is ubiquitinated at a single Lys or several Lys residues. Studies analyzing the downregulation of the EGFR by c-Cbl have revealed that monoubiquitination plays a role in the regulation of RTK endocytosis and lysosomal degradation. Cbl was shown to mediate monoubiquitination of EGFR *in vitro*, demonstrating that monoubiquitination is sufficient to induce endocytosis and lysosomal degradation of RTK. Cbl-mediated ubiquitination of RTK seems to be more critical for the endosomal sorting than for the initial internalization step (Stang et al. 2000, Longva et al. 2002, Haglund et al. 2003, Mosesson et al. 2003). Analysis of endocytosis of the G-protein coupled receptor CXCR4 supports the idea that ubiquitination is required for late endosomal sorting but not for internalization, since mutation of the ubiquitin-acceptor Lys residue did not result in internalization defect, but rather in a major inhibition of degradation (Marchese and Benovic 2001). Furthermore, identification of ubiquitin-interacting motifs (UIMs) in many endocytic adaptors suggested that monoubiquitination could be a recognition signal for endocytic components (Hofman and Falquet 2001). Most UIM-containing proteins undergo monoubiquitination, however the mechanism of monoubiquitination but not polyubiquitinaltion is not yet clear. It will be of great interest to define if JAK2 contains any of the UIM motifs and also to identify the role of those motifs in the process of activation-mediated ubiquitination of JAK2. Since JAK2 was found to be constitutively monoubiquitinated, it is possible that the monoubiquitination reaction involves different E3 ubiquitin ligases than the SOCS1-mediated polyubiquitination reaction. Our results suggested that monoubiquitination could increase the efficiency of polyubiquitination reaction. In this context, JAK2 monoubiquitination may "sensitize" the activated kinase and accelerate the subsequent phosphorylation-mediated polyubiquitination, ensuring that activated JAK2 is degraded in a timely fashion. Characterization of the acceptor Lys residue within the EGFR has shown that an EGFR mutant containing Lys only within the kinase domain underwent Cbl-mediated ubiquitination and degradation, suggesting that one or several Lys within this region can serve as ubiquitination sites (Mosesson et al. 2003). Similarly, site-specific mutation of Lys residues within JH1 or JH2 domain of JAK2 would help to identify the target Lys residue (s) important for JAK2 ubiquitination.

Recently, reversal of ubiquitin conjugation or deubiquitination emerged as another important regulatory mechanism for ubiquitin-dependent protein modification. Like protein phosphorylation, protein ubiquitination is dynamic, involving enzymes that add ubiquitin (ubiquitin conjugating enzymes) and enzymes that remove ubiquitin (deubiquitinating enzymes) (D'Andrea and Pellman 1998, Liu 2004). In our experiments, IL-3 induced activation and ubiquitination of JAK2 could be detected more easily after using β-lactacystin proteasome inhibitor in order to inhibit the isopeptidase activity. This suggests that JAK2 ubiquitination is immediately reversed by isopeptidases. However, further studies are needed in order to identify the specific isopeptidases that reverse the ubiquitination of JAK2 and also their functional mechanisms.

Ubiquitination and ubiquitin-mediated degradation emerged as important mechanisms in regulation of receptor activation. Several cytokine receptors have been shown to be ubiquitinated upon ligand stimulation and their turnover and intracellular sorting is regulated through proteasome degradation (Verdier et al. 1998, Yeh et al. 2000). A classical example is the EGFR activation and endocytosis,

a process that can be modulated through association with c-Cbl, which can function as E3 ubiquitin ligase promoting ubiquitination and degradation of EGFR. A likely mechanism for the tyrosine phosphorylation-dependent regulation of JAK2 ubiquitination is the specific recruitment of ubiquitination enzymes to the protein. Proteasome inhibitors stabilized the tyrosine phosphorylated form of JAK2, which suggests that the physiological function of the ubiquitin-proteasome pathway is to remove the active tyrosine kinase and prevent aberrant cytokine signaling.

8.1.3 SOCS1 regulates the degradation of JAK2 through the proteasome pathway

Previous studies have shown that SOCS1 is a negative regulator of JAK2 activation. SOCS1 binds to the phosphorylated activation-loop tyrosine in JAK2 inhibiting its catalytical activity (Yasukawa et al. 1999). Our studies indicated that tyrosine phosphorylation regulates the degradation of JAK2 via the ubiquitin-proteasome pathway, and therefore it was logical to investigate if SOCS proteins were involved in ubiquitin-mediated degradation of JAK2. In transient transfection system in Cos-7 cells, co-expression of SOCS1 with JAK2WT resulted in reduced JAK2WT ubiquitination, phosphorylation and protein levels. MG132 pretreatment partially stabilized JAK2WT protein levels and also the ubiquitination and phosphorylation of JAK2WT in the presence of co-transfected SOCS1. Interestingly, SOCS1 protein levels were not affected by pervanadate or proteasome inhibitor treatment, suggesting that SOCS1 itself is stable and its interaction with phosphorylated JAK2WT does not affect its protein level.

We used JAK2 mutants (JAK2KN and JAK2Y1007F) that do not become activated, and therefore do not interact or interact very weakly with SOCS1. JAK2Y1007F has the target Tyr1007 substituted with Phe (Y1007F), therefore is not phoshorylated and activated. We analyzed the association between different JAK2 mutants and SOCS1 in transient transfection system in Cos-7 cells. JAK2 was immunoprecipitated and analyzed by Western blot with antibodies for SOCS1 in order to detect the co-immunoprecipitation between JAK2 and SOCS1. The results confirmed that pervanadate treatment enhanced the association between SOCS1 and JAK2WT, and MG132 pretreatment stabilized JAK2WT-SOCS1 complex. Also, SOCS1 does not associate with JAK2KN or JAK2YF, suggesting that the interaction between SOCS1 and JAK2 requires phosphorylation of JAK2 on Tyr1007, as previously demonstrated (Yasukawa et al. 1999). These results were validated by performing co-immunoprecipitation using anti-SOCS1 antibody and Western blot with anti-HA antibody.

The half-life of different JAK2 mutants was studied by overexpressing the HA-tagged proteins in Cos-7 cells followed by metabolic labeling with [^{35}S] methionine and [^{35}S] cysteine. JAK2 proteins were immunoprecipitated using anti-HA antibody and detected by autoradiographic exposure. We found that JAK2KN has the shortest half-life, 2 hours, compared with JAK2WT and JAK2YF, which show a half-life of around 4 hours, as previously reported (Siewert et al. 1999). However, the degradation of JAK2WT was accelerated in the presence of SOCS1 co-transfection, but JAK2KN and JAK2YF were not affected. The results clearly indicated that SOCS1 co-expression decreases JAK2WT protein levels, but do not affect the protein levels of JAK2KN and JAK2YF.

8.1.4 The SOCS-box domain is required for ubiquitin-mediated degradation of JAK2

The SOCS-box of SOCS1 interacts with Elongins B and C and may target the bound molecules for proteasomal degradation (Zhang et al. 1999). Therefore we wanted to analyze the role of SOCS-box of SOCS1 in JAK2 ubiquitination and degradation. For this purpose, Cos-7 cells were transiently transfected with JAK2WT-HA and SOCS1 or a mutant SOCS1 lacking the C-terminal conserved

SOCS-box (SOCS1ΔSB). The cells were pretreated with proteasome inhibitor and tyrosine phosphorylation of JAK2 was induced by pervanadate treatment. JAK2 was immunoprecipitated and analyzed by Western blot with anti-ubiquitin and anti-HA antibodies. SOCS1 co-expression induced a decrease in JAK2WT ubiquitination and protein levels, especially upon pervanadate treatment. Co-expression of SOCS1ΔSB had less effect on JAK2WT degradation. However, tyrosine phosphorylation of JAK2WT was downregulated by co-expression of both SOCS1 and SOCS1ΔSB respectively. Pretreatment of cells with MG132 enhanced the ubiquitination of JAK2WT, whether JAK2WT was expressed alone or together with SOCS1 or SOCS1ΔSB. On the other hand, co-expression of SOCS1 or SOCS1ΔSB had no effect on ubiquitination or protein levels of JAK2KN and JAK2YF, indicating that SOCS1 induces ubiquitination of JAK2WT, and this requires the phosphorylation of JAK2 and the presence of the SOCS-box of SOCS1.

Phosphorylation has been shown to trigger proteasome-mediated protein degradation, but whether SOCS proteins are subjected to phosphorylation-mediated degradation is not yet fully understood. In our experiments, we did not detect phosphorylation or protein degradation of SOCS1. However, SOCS3 became phosphorylated after pervanadate treatment. Previous studies have indicated that tyrosine phosphorylation decreases the half-life of SOCS3 by disrupting the interaction between SOCS3 and Elongin C (Haan et al. 2003). Sequence analysis revealed a high degree of conservation for the tyrosine-phosphorylated residues within the SOCS-box, suggesting that the SOCS-box may play a role in regulating the stability of SOCS proteins. So far, except for the SOCS3, it is not known if other SOCS proteins can be degraded as a result of phosphorylation. It has been proposed that the interaction between Elongin C and SOCS proteins promotes their E3 ligase activity, thereby targeting their degradation by the proteasome. In this context, mutations or post-translational modifications of SOCS1 disrupting the interaction with Elongin C stabilize the protein (Kamura et al. 1998, Chen et al. 2002). However, others have suggested that Elongin C may stabilize SOCS protein expression, and disruption of this interaction leads to proteasome-mediated degradation of SOCS (Zhang et al. 1999). *In vitro* overexpression studies have also demonstrated that the deletion of the SOCS-box decreases the half-life of SOCS1, suggesting that the interaction between the SOCS-box and Elongins B and C stabilizes the SOCS protein (Narazaki et al. 1998, Kamura et al. 1998).

SOCS1 can mediate proteasomal degradation of substrates other than JAKs, and regulates the half-life of Vav as well as IRS-1/2 (De Sepulveda et al. 2000, Rui et al. 2002). Expression of SOCS1 in cells transformed with oncogenic fusion-protein TEL-JAK2 suppresses factor-dependent growth and tumorigenecity through the SOCS-box dependent ubiquitination and degradation of TEL-JAK2 (Kamizono et al. 2001, Frantsve et al. 2001). A mutant form of SOCS1 (F59D-JAB) augments the cytokine-induced JAK-STAT pathway by accelerating the degradation of wild-type SOCS1 through the SOCS-box (Hanada et al. 2001). Interestingly, *in vitro* overexpression studies have shown that deletion of the SOCS-box from SOCS1 and SOCS3 had little impact on the inhibition of cytokine signal transduction (Nicholson et al. 1999, Narazaki et al. 1998). Generation of a mouse model by deleting the DNA encoding the SOCS box from the *SOCS1* gene has shown that animals homozygous for this allele still respond to IFNs (Zhang et al. 2001). This suggests that the interaction between JAKs and SOCSs (via the N-terminal and SH2 domains) contributes to signal termination. However, targeting JAK for degradation through the interaction of the SOCS-box with other components of the E3 ubiquitin ligase complex plays a physiologically important role in signal regulation (Zhang et al. 2001, Kile et al. 2002). In Cos-7 cells detection of endogenous SOCS1 by Western blot was not detected in our experiments, suggesting that some other molecules than SOCS1 could function as E3 ubiquitin ligase for JAK2 ubiquitination. In line with this hypothesis, ubiquitination of JAK2 was also detected in $SOCS1^{-/-}$ T cells isolated from $SOCS1^{-/-}$ mice and stimulated with IFN-γ.

It has already been demonstrated that SOCS are not highly expressed in unstimulated cells, but their genes are rapidly induced following cytokine stimulation and their protein products block the signaling cascade by inactivating the JAK-STAT pathway (Johnston 2004). An interesting property of the SOCS proteins is that they specifically affect the kinetics of JAK-STAT signaling termination, but have no effect on the initial activation process or signaling strength. For example, in the absence of SOCS proteins, cytokine-induced STAT1 activation is prolonged, which in turn might lead to altered cytokine responses. Also, earlier studies have indicated that both SOCS1 and SOCS3 have shown a high specificity *in vivo* in the regulation of cytokine signaling. For example, in *SOCS1*$^{-/-}$ macrophages the activation of STAT1 by IL-6 is prolonged, whereas activation of STAT-1 by IFN-γ is normal (Croker et al. 2003, Lang et al. 2003, Yasukawa et al. 2003). However, in *SOCS3*$^{-/-}$ macrophages the situation is exactly the opposite, suggesting that SOCS proteins have specificity for cytokines, but not for JAKs or STATs.

SOCS1 mediated ubiquitination and degradation of JAK2 shows some analogy to the c-Cbl-mediated desensitization of EGFR (Levkowitz et al. 1999). The phosphorylated Y1045 on EGFR serves as a docking site for RING finger protein c-Cbl, which regulates the ubiquitination of the receptor. Phosphorylation of c-Cbl on Tyr371 flanking its RING finger domain is critical for the ubiquitination and subsequent degradation of the activated EGFR. The JAK2-SOCS1 interaction does not involve direct tyrosine phosphorylation of SOCS1, but whether the function of SOCS1 is regulated by other post-translational modifications or protein interactions remains to be investigated.

8.2 The molecular mechanism of STAT1 sumoylation (II and III)

8.2.1 Sumoylation of STAT1 in intact cells

PIAS1 and PIAS3 were identified as interaction partners with activated STAT1 and STAT3 respectively, and they we found to inhibit transcriptional activation by interfering with the DNA-binding of STATs (Chung et al. 1997, Liu et al. 1998). However, it has become obvious that the cellular function of PIAS proteins goes far beyond inhibitors of STATs, as demonstrated by the growing number of cellular proteins that were identified as binding partners for PIASs (Smith and Muller, 2003). At the same time when PIASs were characterized as inhibitors of activated STATs, our collaborators identified PIAS proteins as modulators of steroid receptor-mediated transcription (Kotaja et al. 2002). During the time of these studies, several laboratories demonstrated that all mammalian PIAS proteins and the yeast Siz protein exert SUMO-ligase activity towards various SUMO-targets proteins (Melchior 2000). Therefore, the SUMO E3 ligase function of PIAS proteins prompted us to examine whether STAT1 is subjected to sumoylation.

As a member of the ubiquitin-related proteins, SUMO-1 binds covalently to targeted proteins at Lys residue (Melchior 2000). The attachment of SUMO-1 to its substrates often involves a minimal consensus sequence, ψK*x*E, where ψ stands for an aliphatic amino acid residue Leu, Val or Ile and *x* stands for any amino acid residue. The level of free SUMO-1 in cells is limited and co-expression of SUMO-1 increases the sumoylation level of target proteins (Melchior 2000). Sumoylation of STAT1 was detected in Western blot as a ~112 kDa band in Cos-7 cells co-transfected with STAT1 and SUMO-1, and this band corresponds to the covalent attachment of one SUMO-1 moiety to STAT1. Using sequence alignment, the consensus SUMO binding motif ψK*x*E was identified between residues 109-112 and 702-705 in STAT1 and the Lys residues at positions 110 and 703 were proposed as a potential conjugation sites for SUMO-1. To validate this hypothesis, the target Lys residue was mutated to Arg and the mutant STAT proteins were used for an *in vitro*

sumoylation reaction. Indeed, we observed that STAT1 703 Lys-to-Arg (STAT1-KR) mutant did not show any SUMO-1 conjugation, indicating that sumoylation of STAT1 occurs at Lys703. In similar studies, STAT1 110 Lys-to-Arg mutant was still sumoylated in an *in vitro* sumoylation reaction, suggesting that the only Lys modified by SUMO in STAT1 is at position 703 (Rogers et al. 2003). The specificity of SUMO conjugation to its consensus *IKTE* motif in STAT1 was validated by mutating the other residues apart from Lys703. The mutant proteins STAT1-I702R (702 Ile-to-Arg) and STAT1-E705A (705 Glu-to-Ala) were used as substrates for SUMO-1 conjugation. In Western blot, STAT1-I702R and STAT1-E705A were not conjugated by SUMO-1, indicating that the Ile702 and the Glu705 residues are essential to create the *IKTE* motif for a specific SUMO-1 conjugation reaction.

The consensus SUMO-1 target sequence was located in the C-terminal domain of STAT1, which includes the transactivation domain. Since the function of STAT1 is critically regulated by phosphorylation on Tyr701 and Ser727 residues (Shuai et al. 1993, Wen et al. 1995), we investigated if SUMO-1 conjugation at Lys703 is modulated by Tyr701 or Ser727 phosphorylation. We used STAT1-Y701F and STAT1-S727A mutants, which cannot be phosphorylated at Tyr701 and Ser727 respectively. In transient co-transfection in Cos-7 cells, both STAT1-Y701F and STAT1-S727A became modified by SUMO-1 conjugation. Also, the transcriptionally inactive isoform STAT1β, which lacks the 38 amino acids carboxyl terminal of TAD (Qureshi et al. 1995), was also sumoylated. These results indicate that Tyr701 and Ser727 or the C-terminal of the transactivation domain are not *per se* required for the covalent attachment of SUMO-1.

8.2.2. Sumoylation of STAT1 in response to interferon stimulation

Moreover, we investigated if sumoylation of STAT1 was regulated via its physiological activating ligand IFN-γ. The effect of IFN-γ was tested in Cos-7 cells transfected with STAT1-WT and low amounts SUMO-1 expression plasmid to ensure appropriate protein amounts for detection of the modification. The results showed that IFN-γ treatment enhanced the sumoylation of STAT1 with a peak at 2 hours, indicating that SUMO-1 conjugation to STAT1 is regulated through phosphorylation. The effect of loss of SUMO-1 conjugation on STAT1-mediated transcription activation was analyzed using reporter gene assay in STAT1-deficient U3A cells transiently transfected with different STAT1 plasmids. Both STAT1-K703R and STAT1-E705A mutants showed slightly increased transcriptional activity when compared to STAT1-WT, suggesting that sumoylation has a negative regulatory effect on STAT1-mediated transcription activation. STAT1-I702R did not become phosphorylated at Tyr701, probably due to the disruption of the phosphorylation motif or stearic hindrance by Arg702, and failed to activate transcription.

Sumoylation of STAT1 was easily demonstrated in the overexpression system, but sumoylation of endogenous STAT1 was difficult to detect. Detection of endogenous SUMO-1 conjugates has proven to be difficult due to low amounts of free, unconjugated SUMO-1 in cells (Matunis et al. 1996, Melchior 2000). Additionally, the modification is easily reversible by a group of highly active hydrolases that cleave the isopeptide bond between the C-terminus of SUMO-1 and the conjugating Lys residue (Matunis et al. 1996, Muller et al. 1998). We analyzed the sumoylation of endogenous STAT1 in HeLa cells either untreated or treated with pervanadate. In untreated cells, STAT1-SUMO-1 moiety could not be detected. However, when STAT1 was tyrosine phosphorylated by pervanadate treatment, the formation of a slow migrating band corresponding to the molecular mass of STAT1-SUMO-1 moiety was detected by Western blot, suggesting that STAT1 phosphorylation enhances SUMO-1 conjugation.

Our experimental settings using transient transfections in U3A cells were not very successful in the detection of the functional implication of STAT1 sumoylation at cellular level. We did not observe any difference in DNA binding in EMSA using total cell lysates from transiently transfected U3A cells with STAT1-WT and STAT1-KR plasmids (data not shown). Therefore, a series of stable clones expressing STAT1-WT-HA (U3A-WT) or STAT1-KR-HA (U3A-KR) was generated in U3A cells and the activation of STAT1 in response to IFN-γ stimulation was analyzed by reporter gene assay. U3A-KR clones showed enhanced transcriptional response after IFN-γ stimulation compared to U3A-WT clones and the magnitude was significantly higher than in U3A cells transiently transfected with STAT1-WT or STAT1-KR.

8.2.3 SUMO-1 conjugation regulates STAT1 DNA binding and nuclear localization

The close proximity of Lys703 to the phosphorylation site Tyr701 raised the possibility that the bulky SUMO-1 modification might potentially interfere with dimerization of STAT1. The K703R mutation did not affect the magnitude and kinetics of STAT1 tyrosine phosphorylation in transient transfection in U3A cells (data not shown). However, using STAT1 from stable U3A clones we observed that STAT1-KR shows more DNA-binding activity than STAT1-WT after IFN-γ stimulation in EMSA. Also, phosphorylation of Tyr701 was enhanced in STAT1-KR as detected by Western blot using specific anti-phospho-Tyr701 STAT1 antibody.

The subcellular localization of STAT1-WT and STAT1-KR during IFN-γ stimulation in U3A clones was analyzed using immunofluorescence microscopy. After one hour and two hours stimulation, phospho-STAT1-KR remained in the nucleus, whereas phospho-STAT1-WT began to relocate to the cytoplasm. After 4 hours, phospho-STAT1-KR was detected in the cytoplasm as well, but maintained a robust nuclear localization compared with phospho-STAT1-WT. Therefore, loss of SUMO-1 conjugation may have an impact on STAT1 nuclear-cytoplasmic redistribution. The cytoplasmic relocalization of STAT1-KR was delayed compared with STAT1-WT and this correlated with differences in the DNA binding activity and the induction of IFN-γ-dependent genes.

8.2.4 SUMO-1 conjugation to STAT1 selectively modulates STAT1-mediated gene responses

The impact of STAT1 sumoylation on IFN-γ-mediated gene induction was assessed in U3A stable clones by quantitative PCR and analysing previously characterized IFN-γ-induced genes interferon regulatory factor 1 (IRF1), guanylate-binding protein 1 (GBP1) and transporters associated with antigen presentation (TAP1 and TAP2) (Min et al. 1998, Brucet et al. 2004, Briken et al. 1995). Quantitative PCR using RNA from stable U3A clones stimulated with IFN-γ showed consistently enhanced induction for both GBP1 and TAP1 in U3A-KR clones compared with U3A-WT clones, while the effect on IRF1 was insignificant. These results indicate that STAT1-KR has a selective effect on IFN-γ-gene responses.

8.2.5 PIAS proteins function as SUMO E3 ligases for STAT1

Since PIAS proteins were shown to function as E3-type SUMO ligases (Hochstrasser et al. 2001, Jackson et al. 2001, Kotaja et al. 2002), we wished to investigate if PIAS proteins function as SUMO E3-type ligases for STAT1 *in vivo*. Co-transfection of PIAS1, PIAS3 and ARIP3 enhanced the sumoylation of STAT1, but the RING-like domain-deleted PIAS1 (PIAS1mut) and ARIP3 (ARIP3mut) could not promote the conjugation of SUMO-1 to STAT1 (data not shown). Together, these results indicate that PIASs function as SUMO-E3 ligases for STAT1 and the RING-finger like domain is required for the sumoylation reaction.

PIAS1 has been assigned two functions, those of an inhibitor of STAT1 activation and E3-type SUMO ligase. This raised an interesting question whether sumoylation would mediate the inhibitory function of PIAS1 on STAT1-mediated gene activation. We found that both PIAS1 and RING-like domain-deleted PIAS1 inhibited STAT1-mediated gene activation. However, the RING-like domain mutant showed a dimished inhibitory effect (data not shown). This finding would support the notion that PIAS1 utilizes a sumoylation-dependent as well as an SUMO-independent mechanism to regulate STAT1 function. Recently published data from PIAS1$^{-/-}$ mice showed that PIAS1 has a high specificity as a negative regulator of IFN-responsive genes containing weak STAT1 binding sites (GBP1) without affecting genes with high affinity STAT1 binding sites (IRF1) (Liu et al. 2004). Our results with sumoylation defective STAT1 show a similar pattern of selective gene responses, thus supporting the physiological regulatory role for sumoylation in fine-tuning of STAT1- and IFN-γ mediated gene resposnes. Studies on protein sumoylation in cells isolated from PIAS1$^{-/-}$ mice showed that lack of PIAS1 had no effect on total protein sumoylation, probably due to the redundant function of other PIAS proteins. Detailed gene activation and microarray studies showed a high specificity of PIAS1 in the regulation of IFN-γ or IFN-β-mediated gene activation. Functional studies have suggested that PIAS1 is important in interferon-mediated innate immunity to pathogenic infection.

8.2.6. Desumoylation of STAT1

A characteristic feature of sumoylation is the rapid turnover and dynamic nature of modification. SUMO-cleaving enzymes (also called isopeptidases) play an important role in SUMO cycle by removing the SUMO from substrates, thus making the modification reversible and providing a source of free SUMO to be used for another cycle of sumoylation (Matunis et al. 1996, Jonhston et al. 1997). In mammalian cells, SENP proteins were identified as SUMO-specific isopeptidases (Bailey and O'Hare 2002, Best et al. 2002, Mendoza et al. 2003). To determine whether SENP1 can deconjugate SUMO-1 from STAT1 in intact cells, we co-transfected Cos-7 cells with STAT1, SUMO-1 and wild-type SENP1 or catalytically inactive SENP1 C603S mutant (Bailey et al. 2004). Total cell lysates were immunoprecipitated with anti-STAT1 antibody and subjected to Western blot with anti-STAT1 and anti-SUMO-1 antibody. The results showed that SENP1, but not SENP1 C603S mutant, deconjugates SUMO-1 from STAT1, indicating that sumoylation of STAT1 is a reversible process and SENP1 can act as a SUMO-specific isopeptidase to desumoylate STAT1.

Nuclear localization of SUMO-specific protease SENP1 prompted us to investigate if SENP1-mediated desumoylation of STAT1 affects the transcriptional activity of STAT1. For this purpose, U3A cells were transiently transfected with STAT1-WT and different concentrations of SENP1 and SENP1 C603S mutant together with GAS-luc reporter plasmid. Luciferase assay showed that SENP1 augmented STAT1-mediated transcription, whereas catalytically inactive SENP1 C603S mutant slightly repressed transcription, suggesting that desumoylation of STAT1 increases its transcriptional potential. Next, we determined whether SENP1 modulates, through desumoylation, the transcriptional activity of STAT1-TAD. For this purpose, we cloned the TAD domain (amino acids 680-750) from STAT1-WT and sumoylation defective STAT1-K703R into the GAL4-DBD plasmid, yielding GAL4-STAT1-TAD-WT and GAL4-STAT1-TAD-KR respectively. U3A cells were co-transfected with GAL4-STAT1-TAD-WT or GAL4-STAT1-TAD-KR and different concentrations of SENP1 or SENP-1 C603S mutant, together with $(GAL4)_3$-TK-luciferase plasmid. The results indicated that co-transfection of SENP1 increased the transcriptional activity of GAL4-STAT1-TAD-WT in a dose-dependent manner to almost 80%, whereas SENP-1 C603S mutant showed no effect. In contrast, the GAL4-STAT1-TAD-KR was less affected by co-expressed

SENP1. These results further confirm that removal of SUMO-1 from STAT1-TAD increases its transcriptional activity.

We have shown that sumoylation plays a negative role in the regulation of STAT1 activity, since sumoylation defective STAT1 mutant shows increased transcription activity compared with wild-type STAT1. Consistent with this idea, removal of conjugated SUMO-1 from STAT1 increases STAT1-mediated transcription, as demonstrated by our results with STAT1 and GAL4-STAT1-TAD-WT in reporter gene assays. However, the transcription activation of GAL4-STAT1-TAD-KR was also increased by SENP1 co-transfection, albeit at a reduced level, suggesting that SENP1-mediated SUMO deconjugation may affect the desumoylation of other components of the transcription machinery.

Post-translational modifications of STAT1 are highly specific processes, for example STAT1 Ser727 phosphorylation increases the IFN-γ-meditate response to bacteria or LPS, and enhances the histone acetylation and the expression of IFN-γ-induced genes (Kovarik et al. 2001). Thereby, SUMO-1 modification may mediate highly specific and even cell type determined functions in STAT1. An increasing number of cellular proteins were reported to undergo sumoylation, however the functional significance of this modification is not yet completely understood. Many SUMO substrates are mammalian proteins involved in signal transduction and transcriptional regulation, and sumoylation alters their activity, subcellular localization and/or interaction with other proteins (Dohnem 2004). Sumoylation plays an important role in the regulation of transcription factors by multiple mechanisms. The potency to activate transcription of some activators appears to be enhanced, whereas many others are inhibited by sumoylation. Inhibition has often been associated with redistribution of the transcriptional activators to nuclear bodies. Also, inhibition of transcriptional repressors has been linked to their sumoylation-dependent translocation to these nuclear reservoirs. In other cases, sumoylation may affect the interaction with co-repressors or co-activators. For example, SUMO-1 conjugation to Elk-1 recruits histone acetylase HDAC2 to promoters resulting in transcriptional repression (Yang et al. 2004). SUMO modification of histone H4 promotes recruitment of histone deacetylase and heterochromatin protein 1 and thereby mediates silencing (Shiio et al. 2003). However, sumoylation of HDAC1 and HDAC4 was shown to enhance their ability to repress transcription (David et al. 2002). Importantly, SUMO seems to play a crucial role in the compartmentalization of proteins within the nucleus. SUMO-modified proteins are frequently found in distinct subnuclear foci, the so-called PML nuclear bodies (NB also known as ND10 or PODs) indicating that SUMO directs proteins to NBs or that NBs are a site for sumoylation. The presence of transcription factors and transcription co-regulators suggests a role for NBs in modulation of transcriptional processes. Yet, NBs do not seem to be an active site of transcription, but rather represent storage or assembly sites for transcription factors and transcriptional co-regulators.

Both immunoblotting and immunostaining experiments indicate that only a relatively small fraction of STAT1 become SUMO modified, and the effects on gene responses are not likely to be general but rather be involved in fine-tuning the specificity of gene responses. One plausible explanation for the differences in gene responses is that sumoylation of STAT1 would cause its localization to defined nuclear compartments and its interaction with regulatory proteins. For example, sumoylation of glucocorticoid receptor affects the cooperative assembly of transcription factors and their coregulators in a promoter-dependent manner. We have observed that STAT1, but not STAT1-KR, colocalizes with SUMO-1 in punctuated structures in the nucleus (data not shown). HDACs have also been reported to assemble in nuclear bodies, and suggested to form protein complexes with sumoylated proteins. The sumoylated form of p300 interacts with HDAC6, and it is also possible that sumoylated STAT1 may recruit co-factor complexes containing HDAC activity

whereas the unsumoylated STAT1 has a preference to bind CBP and enhance transcription. Therefore sumoylation of STAT1 could be a transient process required to control its highly regulated transcriptional state in the nucleus. Our results showing the reversible nature of STAT1 sumoylation support the notion that transient sumoylation adds an additional layer to control the transcriptional activity of STAT1 in the nucleus.

8.3. Regulation of STAT protein turnover by SLIM (IV)

More recently, identification of SLIM as a regulator of STAT signaling and protein turnover provided more insight into the mechanism of negative regulation of STAT activation (Tanaka et al. 2005). SLIM contains a PDZ domain and an LIM domain, and interacts in the nucleus with tyrosine phosphorylated STAT molecules and specifically inhibits STAT4- and STAT1-mediated gene expression. Also, SLIM was found to promote both the ubiquitination and degradation of STAT1 and STAT4, a function attributed to its LIM domain, suggesting that SLIM could be the first E3 ubiquitin ligase with specificity towards STATs.

8.4 CONCLUSIONS

In order to ensure that the strength and duration of cytokine signaling is appropriately controlled, cells utilize multiple regulatory mechanisms to control the activation and inactivation of JAK tyrosine kinases and their downstream effectors, STAT transcription factors. Evidence accumulated over the past several years has demonstrated that the JAK-STAT signaling pathway can be negatively regulated at various steps, and implicated various post-translational modificiations such as ubiquitination and ubiquitin-like modifications, in the modulation of JAK-STAT pathway activation. The main focus of this study was to characterize the mechanism involved in the negative regulation of JAKs and STATs specifically aimed at the ubiquitination and sumoylation.

The present study identified that JAK2 activation is negatively regulated during cytokine signaling through the ubiquitin-proteasome pathway (I). JAK2 is the first member of JAK family to be identified as a target for ubiquitination. This modification required tyrosine phosphorylation of JAK2. Interestingly, JAK2 was found to be monoubiquitinated, as detected in unstimulated cells. Activation of JAK2 induces a negative regulatory pathway through the SOCS family of proteins. SOCS1 can inhibit JAK2 activation by two mechanisms, through direct interaction with the activated tyrosine residue in JAK2 and, as demonstrated by our studies, by targeting the activated JAK2 for degradation through the ubiquitin-proteasome pathway.

STAT activation can be negatively regulated by interaction with PIAS family members and by dephosphorylation by protein tyrosine phosphatases (PTPs). Our studies identified that STAT1 is a substrate for SUMO-1 conjugation and this post-translational modification has a negative regulatory role in STAT1-mediated gene responses (II and III). Members of the PIAS family E3-type ligases were shown to enhance SUMO-1 conjugation to STAT1. SUMO-modified STAT1 differentially affects IFN-γ regulated genes with low affinity promoters for STAT1 without affecting strong affinity promoter genes. Desumoylation of STAT1 by SUMO-specific protease SENP1 correlated with enhanced transcription activation, confirming the negative regulatory role for STAT1 sumoylation.

Although our previous understanding has placed tyrosine kinases and phosphatases at the center of JAK-STAT signaling, it is now becoming obvious that this regulation involves a plethora of interacting pathways, enzymatic activities and post-translational modifications. Ubiquitination, sumoylation and ISGylation have all been linked to JAK-STAT signaling. The identification of the SOCS family of proteins as a part of E3 ubiquitin ligase complex and PIAS proteins as E3-like SUMO ligases demonstrates the first molecular determinants of the specificity of these modifications that are responsible for the fine tuning and appropriate biological responses. Also, the identification SLIMs as specific E3 ubiquitin ligases for ceratin STATs is opening up a new and interesting avenue in the regulation of post-translational modification in cytokine signaling (IV).

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