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Regulation of STAT1 through Post-translational Modifications

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ACADEMIC DISSERTATION
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“Ihminen joka varjelee ystäviään ei anna
koskaan elämän myrskyjen lannistaa:
hänellä on voimaa voittaa vaikeudet
ja jatkaa eteenpäin”

Valon soturin käsikirja, Paulo Coelho

CONTENTS

1. ABSTRACT

Cytokines are produced at multiple sites in the organism and various cytokines act to regulate the development, growth and activation of hematopoietic cells. About 50 members of the hematopoietin family in mammals exert their biological effects through the activation of specific receptors to trigger the janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway that represents a rapid membrane to the nucleus signalling system. The JAK-STAT pathway uses a mechanism in which cytosolic latent transcription factors STATs are tyrosine phosphorylated by receptor affiliated JAKs, allowing STAT protein dimerization and localization into the nucleus, where STAT modulates the expression of target genes. Interferon-γ (IFN-γ) plays an important function in immune responses against microbes. IFN-γ-induced gene responses are largely dependent on STAT1. The function of STAT1 is regulated by regulatory proteins such as suppressors of cytokine signalling (SOCS), protein tyrosine-phosphatases (PTP) and protein inhibitors of signal transducer and activator of transcription (PIAS) and by various posttranslational modifications that act in a highly coordinated fashion to combine signals from secreted cytokines and also from microbial agents. SUMO (small ubiquitin-like modifier) modification has emerged as an important transcriptional regulation mechanism of proteins and an increasing number of transcription factors and co-regulators has been reported to be regulated by SUMO modification.

The mechanism by which the PIAS proteins inhibit STAT activity is unclear. Recent findings have demonstrated that all PIAS proteins and Siz proteins in yeast function as SUMO E3 ligases and promote SUMO conjugation to several substrates. These findings prompted us to investigate the possibility that PIASs may have E3 ligase function towards STAT1 and promote its sumoylation. STAT1 was shown to be sumoylated through the E3 ligase function of PIAS proteins. SUMO-1 modification occurs *in vivo* at a single, evolutionary conserved C-terminal amino acid residue Lys703. Mutation of Lys703 (STAT1-KR) resulted in increased IFN-γ-mediated transactivation thus suggesting a negative regulatory function of sumoylation in STAT1. More detailed analysis of IFN-γ target genes indicated that SUMO deficiency of STAT1 selectively modulated the induction of genes showing a similar pattern of gene regulation as observed in PIAS1$^{-/-}$ mice. Molecular modelling of STAT dimer indicated that the SUMO consensus site is well exposed to SUMO-conjugation and SUMO moiety could affect the promoter binding affinity of STAT1. Experimental analysis indicated that SUMO deficient STAT1 displayed higher DNA-binding activity towards guanylate binding protein (GBP) -1 promoter which is well in line with the molecular model. Our results also identified stress induced Ser727 phosphorylation as a regulator of PIAS1 binding and SUMO conjugation to STAT1. These results identify Ser727 phosphorylation promoted sumoylation and PIAS1 binding as a co-operative mechanism for fine-tuning post-translational regulation of STAT1.

1. TIIVISTELMÄ

Sytokiinit joita tuotetaan eliön useissa eri solutyypeissä säätelevät solujen erilaistumista, kasvua ja aktiivisuutta. Hematopoieettisten sytokiinien vaikutukset välittyvät soluissa pääsääntöisesti spesifisten solureseptorien aktivaation ja sitä seuraavan JAK ja STAT signalointiproteiinien aktivaation kautta. Yleinen mekanismi jolla JAK-STAT proteiinit välittävät signaalin tumaan on riippuvainen reseptoriin liittyneiden JAK proteiinien aktivaatiosta sekä niiden välittämästä STAT transkriptiotekijöiden tyrosiinifosforylaatiosta. Fosforyloituneiden STAT molekyylien muodostama dimeeri lokalisoituu solun tumaan, jossa se sitoutuu kohdegeenien säätelyalueeseen yhdessä muiden kofaktoreiden kanssa säätelemään kyseisten geenien ilmentymistä. Interferoni-γ sytokiinillä on tärkeä rooli immuunivasteissa ja sen säätelemät geeni-ilmentymät välittyvät suurilta osin STAT1 transkriptiotekijän kautta. STAT1 proteiinin toiminta on tarkan säätelyn alainen ja useat eri säätelyproteiinit kuten SOCS, PTP ja PIAS1 ja erityyppiset translaation jälkeiset muokkaukset säätelevät sen toimintaa vaikuttaen joko positiivisina tai negatiivisina säätelytekijöinä.

PIAS1 proteiini toimii STAT1 transkriptiotekijän negatiivisena säätelijänä, joka todennäköisimmin rajoittaa STAT1 proteiinin sitoutumista kohdegeenin säätelyalueeseen. Useista tutkimuksista huolimatta tarkka mekanismi jolla PIAS proteiinit säätelevät STAT proteiinien aktiivisuutta on jäänyt epäselväksi. Kaikki PIAS proteiinit ja Siz proteiinit, jotka ovat PIAS proteiinien vastineita hiivassa, toimivat E3 ligaatio entsyymeinä useiden proteiinien kovalenttisessa SUMO konjugaatiossa. Sumolaatio on proteiinin translaation jälkeinen muokkaus, joka on tärkeä mekanismi useiden muiden solunsisäisten toimintojen lisäksi proteiinien transkription aktiivisuuden säätelyssä. Tässä tutkimusprojektissa halusimme tutkia mahdollisen PIAS välitteisen SUMO konjugaation toimintaa STAT1 proteiinin aktiivisuuden säätelyssä. Tulokset osoittivat, että STAT1 proteiini sumoloidaan C-terminaaliseen ja konservoituneeseen lysiini 703 aminohappotähteeseen ja PIAS proteiinit toimivat E3 ligaaseina SUMO:n konjugaatioreaktiossa. Lysiini 703:n mutaatio sumoloitumattomaksi arginiiniksi aiheutti lisääntyneen IFN-γ välitteisen transkription aktiivisuuden osoittaen, että sumolaatio on STAT1 proteiinin toiminnan negatiivinen säätelijä. Tarkempi IFN-γ:n kohdegeenien tarkastelu osoitti, että sumoloitumaton STAT1 mutantti sääteli selektiivisesti geenien ilmentymistä osoittaen samankaltaisuutta havaintoihin PIAS1 -/- hiiressä. DNA:han sitoutuneen STAT1 dimeerin molekulaarinen mallitus ja sen tarkastelu osoittivat, että sumolaatio konsensussekvenssi sijaitsee proteiinin pinnalla, jossa lysiini 703:n sivuketju osoittaa DNA:n kaksoisjuostetta kohden. SUMO:n konjugaatio tähän kohtaan voi häiritä STAT1 dimeerin DNA sitoutumista ja siten vähentää transkription aktiivisuutta. Tämä rakennemalli tuki työn kokeellisia löydöksiä jotka osoittivat, että sumoloimattomalla STAT1 mutantilla oli korkeampi affiniteetti STAT1:n kohdegeenin GBP1:n promoottori elementtiin. Työssä tutkimme myös STAT1 sumolaation säätelymekanismia ja tuloksemme osoittivat, että STAT1:n MAPK riippuvainen seriini 727 fosforylaatio lisää sumolaatiota. Tulostemme perusteella seriini 727 fosforylaatio säätelee PIAS1:n

sitoutumista joko suoraan tai epäsuorasti SUMO konjugaatiokompleksin muiden proteiinien välityksellä, mikä vuorostaan näkyy lisääntyneenä sumolaationa. Nämä havainnot osoittavat, että sumolaatio on tärkeä STAT1 transkriptiotekijän hienosäätelymekanismi, joka toimii osittain kooperatiivisesti PIAS1 proteiinin kanssa negatiivisena regulaattorina.

2. LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original communications, referred to in the text by their Roman numerals I-IV:

I Ungureanu D[1], **Vanhatupa S[1]**, Kotaja N, Yang J, Aittomaki S, Janne OA, Palvimo JJ, Silvennoinen O: PIAS proteins promote SUMO-1 conjugation to STAT1.
Blood. 2003 Nov 1;102(9):3311-3

II Ungureanu D, **Vanhatupa S**, Gronholm J, Palvimo JJ, Silvennoinen O: SUMO-1 conjugation selectively modulates STAT1-mediated gene responses. Blood. 2005 Jul 1;106(1):224-6.

III **Vanhatupa S**, Ungureanu D, Paakkunainen M and Silvennoinen O: MAPK-induced Ser727 phosphorylation promotes sumoylation of STAT1. Biochem J. 2008 Jan 1; 409: 179-185

IV **Vanhatupa S[1]**, Ungureanu D[1], Väliaho J, Laitinen T, Valjakka J and Silvennoinen O:
Structural and Functional Analysis of SUMO-modification in STAT1
Submitted

[1] Authors contributed equally to this work.
The publication No II has also been used in the doctoral thesis of Daniela Ungureanu

3. ABBREVIATIONS

AR	Androgen receptor
ARIP3	Androgen receptor interacting protein-3
CBP	CREB binding protein
DBD	DNA-binding domain
DNA	Deoxyribonucleic acid
DMEM	Dulbecco´s modified Eagle´s medium
EMSA	Electrophoretic mobility shift assay
ERK1/2	Extracellular-signal-regulated kinase ½
FBS	Foetal bovine serum
FERM	Band four point one, ezrin, radixin and moesin
GAS	Gamma activated sequence
GBP1	Guanylate binding protein 1
GR	Glucocorticoid receptor
HDAC	Histone deacetylase
HSF	Heat shock factor
IFN	Interferon
IL	Interleukine
IKK	I kappa B kinase
IRF	Interferon regulatory factor
ISGF3	Interferon-stimulated gene factor
ISRE	Interferon-stimulated response element
JAK	Janus kinase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MKK	MAP kinase kinase
NES	Nuclear export signal
NF-κB	Nuclear factor κB
NLS	Nuclear localization signal
NPC	Nuclear pore complex
Pc2	Polycomb 2
PDSM	Phosphorylation dependent sumoylation motif
PIAS	Protein inhibitor of signal transducer and activator of transcription
PKC	Protein kinase C
PML	Promyelocytic leukemia
PTP	Protein tyrosine phosphatase
SCID	Severe combined immunodeficiency
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
SENP	Sentrin-specific protease
SH2	Src homology-2
SIM	SUMO interacting motif
SMAD	SMA- and MAD-related protein
SOCS	Suppressors of cytokine signaling

STAT	Signal transducer and activator of transcription
SUMO	Small ubiquitin-like modifier
TAD	Transactivation domain
TAP1	Transporters associated with antigen presentation-1
TNF	Tumor necrosis factor
Ubc9	Ubiquitin conjugating enzyme

4. INTRODUCTION

Cells depend on a continuous flow of signals from the environment for normal growth. Cytokines are an important set of signals for many cell types, especially those of the hematopoietic lineage. Cytokines are commonly described as small glycoproteins that function as messenger molecules that convey information from cell to cell. Cytokines have multiple and diverse biological functions in the regulation of activity, growth, differentiation and development of cells.

Interferons (IFN) are secreted proteins that are important in combating microbial infections. They can be subdivided into three functional classes, type I IFNs, type II IFNs and IL-10 family and constitute the largest and most divergent subfamily of cytokines. The studies focusing on the set of genes induced by IFN-γ led to the identification of a distinct response element, the gamma activated sequence (GAS). IFN-γ signalling is initiated when liganded receptor dimerize, bringing the associated kinases to apposition. The signal is transduced into the nucleus through the JAK (Janus kinase)/STAT (Signal transducer and activator of transcription) pathway. GAS elements are directly bound by STAT1 homodimers, which promotes the induction of GAS driven target genes.

Post-translational modifications of STATs provide an important regulation mechanism. STATs have been reported to undergo several covalent modifications such as tyrosine and serine phosphorylation, acetylation, methylation, ubiquitination, ISG15ylation and O-glycosylation. Additionally, activity of STAT1 can be negatively regulated in the nucleus by at least two distinct mechanisms dephosphorylation by protein tyrosine phosphatases and repression of STAT1-dependent transcription by protein inhibitor of signal transducer and activator of transcription (PIAS1) protein. The mechanism by which the PIAS proteins inhibit STAT activity is unclear.

Small ubiquitin-like modifier (SUMO) modification has emerged as an important transcriptional regulation mechanism of proteins. Recent findings have demonstrated that all PIAS proteins function as SUMO E3 ligases and promote SUMO conjugation to several substrates. The specific aim of this study was to analyse the putative sumoylation site and the mechanistic function of SUMO modification on STAT1 activity.

5. REVIEW OF THE LITERATURE

5.1 Overview of cytokines

Signals from the extracellular environment regulate the growth, differentiation, proliferation and apoptosis of cells in multicellular organisms. The development and survival of a multicellular organism is a highly regulated process with interplay between different cells. In mammals, these interactions are mediated either through direct cell-cell or cell-matrix contacts or through soluble mediators. The soluble mediator proteins transmit signals from the surrounding environment into the cell through activation of specific receptor molecules. These mediators can be divided into systemically acting endocrine hormones and locally acting growth factors and cytokines. The biological effects of growth factors and cytokines are mediated through activation of transmembrane receptors.

Cytokines are a large family of secreted glycoproteins that function as messenger molecules conveying information from one cell to another (Ihle, 1995). Most cytokines have multiple and diverse biological functions in the regulation of the activity, growth, differentiation and development of cells. Most cells in the organism both produce and respond to cytokines and many cytokines are produced by a variety of cell types. An important group of cytokines is hematopoietic cytokines, including over 50 members, mainly regulating hematopoietic cell differentiation and activity (Bazan, 1991, Schindler, 1999). One way to classify cytokines is based on the similarities in receptor structure, since structurally related receptors show similarity both in signalling mechanism and biological function (Ihle et al., 1995). Hematopoietic cytokine receptors are transmembrane glycoproteins, composed of an extracellular ligand binding domain, a hydrophobic transmembrane part and a cytoplasmic intracellular domain (Silvennoinen et al., 1997) Cytokine receptors can be divided into five subcategories according to composition of receptor subunits. These include single chain receptors (e. g. erythropoietin (Epo), thrombopoietin (Tpo), growth hormone (GH)), common β chain receptors (interleukine (IL)-3, IL-5, colony-stimulating factor for granulocyte-macrophages (GM-CSF)), gp130 family (IL-6, IL-11, IL-12), the common γ chain family (IL-2, IL-4, IL-7, IL-9) and interferon receptors (IFN-α-,β-,γ and IL-10 family) (Ihle et al., 1995). In addition of cytokines, hematopoiesis is regulated by polypeptide growth factors like M-CSF (macrophage-colony-stimulating factor) that bind to receptors with an intrinsic tyrosine kinase activity which are classified as receptor tyrosine kinases (RTK). Contrary to RTKs, hematopoietic cytokine receptors are lacking intrinsic kinase activity and they use receptor mediated kinase proteins to activate the signaling cascade inside the cell (Ihle et al., 1995).

5.1.1. Biological effects of cytokines

Cytokines are produced at multiple sites in the organism and their production is stimulated during infections and immune responses. Various cytokines act to regulate the development, growth and activation of all types of blood cells, and they act in a highly integrated manner. Though almost all cells in the organism are influenced by cytokines, the hematopoietic cells are their main targets. Hematopoiesis is a complex process strictly regulated by the surrounding microenvironment that includes cell-cell interactions, extracellular matrix-cell interactions and exposure to cytokines. The life span of most blood cells is short, and must therefore be constantly renewed in a process called constitutive hematopoiesis taking place in bone marrow. Cytokines are critical regulators of the proliferation and development and maintenance of blood cells. Hematopoietic cells of main blood cell lineages derive from pluripotent hematopoietic stem cells that have to proceed into differentiation cascade (Cheng et al., 1996, Krause et al., 2002, Rothenberg and Anderson, 2002).

A key function of the hematopoietic system is to provide the organism with the defensive mechanisms. Cytokines play a crucial role in the initiation and extension of immune responses in the organism. Cytokine production is triggered by recognition of the microbes and tumor cells by the immune system. Cytokines stimulate the proliferation of immune effector cells, such as macrophages, and activate them to destroy harmful invaders. In innate activation the macrophage recognizes microbial components that are absent in the host, e.g. lipopolysaccharide by its pattern recognition receptor. Ligation of these receptors enhances phagocytosis and induces cells to produce pro-inflammatory cytokines. In classical activation the macrophage is exposed to cytokine IFN-γ and a microbial trigger like lipopolysaccharide (LPS). As a result, the production of nitric oxide, reactive oxygen species and pro-inflammatory cytokines like interleukines-6 and -1, increases the ability of the cell to kill and degrade microbes. Stimulation with IL-4 and IL-13 results in macrophage activation involved in humoral immunity, allergic responses and tissue repair (Gordon, 2003, Mosser, 2003).

5.2 Interferons

Interferons (IFNs) have been shown to play an important role in both innate and adaptive immune resposes but also in the inhibition of cell growth and regulation of apoptosis. IFNs belong to the type II cytokine family and can be divided into three major classes, type I and type II IFNs (IFN-γ) and the IL-10 family (Kotenko and Pestka, 2000, O'Shea, Gadina and Schreiber, 2002, Sheppard et al., 2003). Type I IFNs represents a large family of widely expressed ligands that signal through a single receptor expressed in most tissues. This receptor consists of IFNAR1 and IFNAR2 chains. IFNAR1 knockout mice have confirmed the role of this chain in the mediation of biological responses to IFN-Is and highlighted the role of IFNs in the regulation of responses in viral infection and adaptive immunity (Hwang et al., 1995). Among type I IFNs, IFN- α is a family of closely related proteins encoded by a gene cluster and IFN-β is a single protein encoded by a more distantly related gene. IFN-α and IFN-β are found across many species, whereas expression of IFN-ω and other type I IFNs is mostly restricted to different species or cell types (Chesler and Reiss, 2002, Levy and Darnell, 2002). The signalling

process of the type I IFNs (IFN-α and IFN-β) leads to the activation of pairs of Janus kinases JAK1 and Tyk-2 and transcription factors STAT1 and STAT2, which form dimers (Bach, Aguet and Schreiber, 1997). Dimers further associate with a third protein, IRF-9 (interferon regulatory factor 9; p48), to form the interferon stimulated gene factor 3 (ISGF3) complex of the proteins. ISGF3 complex activates transcription through binding to interferon-stimulated response elements (ISRE) present on the promoters of the IFN-α/β responsive genes such as PKR (the dsRNA dependent protein kinase), PML (promyelocytic leukemia), guanylate binding protein (GBP), iNOS (inducible nitric oxide synthetase) and ISG (interferon stimulated gene)-15. ISGF3 has been shown to bind only ISRE sequence, which appears to be a characteristic feature of type I IFN-induced signalling (Bach, Aguet and Schreiber, 1997, Muller et al., 1993, Schindler et al., 1992a, Schindler et al., 1992b).

The founding member, IL-10, of the IL-10 family of cytokines (IL-19, IL-20, IL-22, IL-24, IL-26 and IFN-λs) was initially identified as an inhibitor of T helper (Th)1 cytokine production but subsequent studies demonstrated IL-10´s anti-inflammatory activity and its role in regulating immune tolerance (Kotenko and Pestka, 2000). The IL-10 initiated signal is transduced through the sequential activation of IL-10R1/IL-10R2 associated Tyk2/JAK1 and STAT1/STAT3 and the roles of IL-10R2, JAK1 and STAT3 have been confirmed by gene targeting studies. Studies of IL-10 family members in JAK-STAT signalling suggest that STAT3 is likely to play a critical role as a regulator of gamma activated sequence (GAS) -driven genes (Conti et al., 2003, Meraz et al., 1996, Takeda et al., 1998).

5.2.1 Interferon-γ

IFN-γ is known as a type II IFN and in contrast to type I IFNs there is only one gene located in the long arm of human chromosome 12, which codes for a single protein of 17 kDa (Bach, Aguet and Schreiber, 1997). IFN-γ is synthesized by natural killer cells (NK), CD4 Th1 cells, CD8 cytotoxic lymphocytes, dendritic cells and macrophages. IFN-γ functions as a noncovalent N-glycosylated homodimer that consists of two 17 kDa-polypeptide chains. IFN-γ mediates its action through a cognate cell surface receptor composed of an extracellular domain, cell membrane spanning domain and an intracellular domain. The IFN-γ receptor (IFNGR) is structurally related to type I IFN receptor and it consists of two heterologous subunits IFN-γ receptor 1(IFNGR-1) and IFNGR-2 chains in association with Jak1 and Jak2 kinases. The binding of the homodimeric IFN-γ to two IFN-γ receptor chains 1 (IFNGR-1) brings about a receptor complex consisting of two IFNGR-1 and two IFNGR-2 chains. Receptor dimerization leads to the activation of JAK1/2 and STAT1 signal transduction pathway and results in a regulation of IFN-γ responsive genes (Bach, Aguet and Schreiber, 1997, Kisseleva et al., 2002b, Muller et al., 1994). Analysis of IFN-γ induced cells by DNA microarray technique has revealed that IFN-γ regulates the expression of hundreds of genes in different types of cells. IFN-γ mediates the induction of iNOS, transcription factors IRF-1, IRF-9, IRF-8/ICSBP and CIITA (MHC class II transactivator), suppressor of cytokine

signaling (SOCS) 1, MHC class I pathway proteins like transporters associated with antigen presentation (TAP)1 and 2 and GBP1. However, some genes such as cell cycle genes (cyclin D and A) and matrix metalloproteinases are suppressed by IFN-γ (Decker et al., 2002, Shtrichman and Samuel, 2001).

IFN-γ plays important roles in both innate and adaptive immunity. Knockouts of the IFNGR-1, IFNGR-2 or the IFN-γ ligand genes produced mice that were impaired in their ability to effectively respond against microbial and viral pathogens. IFN-γ induces the activation of macrophages and the stimulation of specific cytotoxic immunity based on the recognition of cell-surface-bound antigen in association with MHC proteins. Differentiation of naïve T-cells into Th1-type cells and Ig isotype switching in B-cells is also promoted by IFN-γ (Dalton et al., 1993, Muller et al., 1994). IFN-γ has been shown to have a growth inhibitory role and pro-apoptotic activities promoting apoptotic cell death. The mechanism by which IFN-γ mediates cell death involves transcriptional activation of death-modulating genes such as caspases, death receptors, iNOS and Bcl-xL and cell-cycle arrest genes such as p21waf1. IFNs can also induce an indirect anti-tumor mechanism by activating immune effector cells such as cytotoxic T cells and natural killer (NK) cells (Bowman et al., 2000).

5.2.2 Interferon-γ signal transduction pathway

The IFN-γ signalling pathway (Figure 1.) is a paradigm for most aspects of JAK-STAT signalling. The transmembrane IFN-γ receptor, expressed on the surface of all cell types, consists of two different polypeptide chains, ligand-binding subunit IFNGR-1 and IFNGR-2 subunit. Homodimerized IFN-γ binds to two IFNGR-1 subunits, which also function as signal transducing units (Bach, Aguet and Schreiber, 1997, Kotenko and Pestka, 2000). Ligand binding promotes change in receptor conformation, leading to the activation of receptor associated JAK1 (IFNGR-1) and JAK2 (IFNGR-2), through a transphosphorylation event. Presumably, receptor bound JAKs phosphorylate conserved tyrosine residues in the intracellular domain of the IFNGRs. Phosphorylated Tyr440 residue then serves as docking sites for the recruitment of the Src homology 2 (SH2) domain of the cytoplasmic transcription factor STAT1 (Darnell, Kerr and Stark, 1994). At the receptor, STAT1 is phosphorylated by JAKs on a single tyrosine (Tyr701) residue and this is followed by release from the receptor. The phosphorylated Tyr701 of one monomer and Arg602 of the SH2 domain of the adjacent monomer are critical residues for the formation of the dimer interface in the STAT1 homodimer (Chen et al., 1998). The dimerized STAT1 translocates into the nucleus, where it binds to GAS promoter element to regulate IFN-γ responsive genes. Several GAS-like elements that possess the palindromic core sequence $TTCN_{2-4}GAA$ are known and in addition to STAT1 homodimers this consensus sequence also provides a binding site for other STATs activated by other cytokines with the exception of STAT2, which appears to be defective in DNA binding. In part this specificity of STAT binding is determined by spacing (N_{2-4}) between palindromic half sites. STATs are also able to bind cooperatively to tandem GAS elements divergent from consensus sequence contributing to DNA binding

specificity. After the decay of stimulus STAT1 is exported back to the cytoplasm in a process requiring STAT1 dephosphorylation, and some activated STAT1 may also undergo targeted degradation (Darnell, Kerr and Stark, 1994, Kotenko and Pestka, 2000).

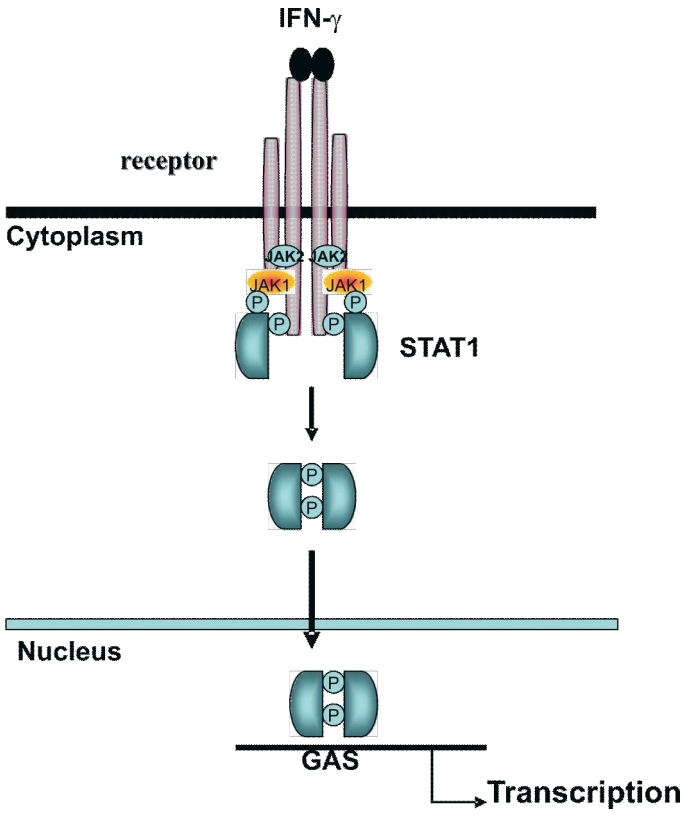

Figure1. Schematic representation of the IFN-γ signaling pathway. IFN-γ binds to the receptor chains which lead to the activation of JAK1/2 and Tyr701 phosphorylation of STAT1. Phosphorylated STAT1 forms dimer and translocates into the nucleus. STAT1 dimer binds to the promoter GAS element, where it regulates the transcription of IFN-γ target genes.

5.3 JAKs and STATs

5.3.1 JAKs

JAKs are non-receptor protein kinases that are constitutively associated with the intracellular region of many cytokine receptors, where they remain in an inactive state in the absence of ligand. The JAK kinases are activated through members of the cytokine receptor superfamily consisting of receptors for interleukins (IL), interferons (IFN) and a

number of other cytokines like erythropoietin, prolactin and growth hormones. Ligand binding and conformational change in the cytokine receptor (homo or heterodimerization) is required for auto/ transphosphorylation of JAKs on critical tyrosine residues within the activation loop of the kinase domain and activation of JAKs. In heterodimeric receptor complexes JAKs are activated through interplay of different JAKs, whereas in homodimeric receptor two similar JAKs can activate one another (JAK2) (Ihle, 1995, Schindler, 1999). In mammals four JAKs (JAK1, JAK2, JAK3 and TYK2) have been identified. JAK2 has two transcripts and multiple alternatively spliced forms of JAK3 have been detected. However, the functional significance of the variants is not known. JAK1, JAK2 and TYK2 are ubiquitously expressed whereas, JAK3 has more restricted tissue expression in cells of hematopoietic origin, such as NK, T, B and myeloid cells (Darnell, Kerr and Stark, 1994, Ihle, 1995). JAK homologs have been identified in carp, zebrafish and in Drosophila, where the gene was termed Hop (*hopscotch)* (Binari and Perrimon, 1994, Yin and Kwang, 2000).

JAKs are large kinases with molecular weights of 120-140kDa. JAK kinases share a characteristic domain structure, which includes an amino terminal band-4.1, ezrin, radixin, moesin (FERM) domain, an SH2-like region, a pseudokinase domain and a carboxyterminal kinase domain. These structural domains have historically been termed JAK homology (JH) domains1-7, numbered from carboxyl to the amino terminus. The overall three-dimensional structure of JAKs has not been determined, but the crystal structure of JAK3 tyrosine kinase domain (JH1) was recently characterized (Boggon et al., 2005, Hubbard and Till, 2000). The JH1 domain of JAKs has an activation loop (A-loop) that contains a conserved double tyrosine motif which is phosphorylated in response to cytokine stimulation (JAK1, Y1038/1039; JAK2, Y1007/1008; JAK3, Y980/Y981, Tyk2, Y1054/Y1055) (Leonard and O'Shea, 1998). Adjacent to the JH1 domain is a catalytically inactive pseudokinase or kinase-like domain (JH2) which is highly conserved in all JAK kinases. Although the pseudokinase domain lacks catalytic activity, many patient-derived and artificial mutations of this domain have suggested it to have an essential regulatory function. The N-terminal part of JAKs contains an SH2-like domain (JH3-JH4) and a FERM homology domain (JH6-JH7). While the function of SH2-like domain is unknown at present, the FERM domain has been suggested to have a role in the association of JAKs with the cytokine receptor (Hilkens et al., 2001, Kampa and Burnside, 2000, Saharinen and Silvennoinen, 2002, Saharinen, Takaluoma and Silvennoinen, 2000).

5.3.1.1 Biological role of JAKs

Mouse knockout models have demonstrated the physiological role of JAKs in cytokine signalling. JAK1 plays an important role in the biological response to members of the IL-6, IL-2 and IFN/IL-10 receptor families. JAK1 deficient mice exhibit a perinatal lethal phenotype as a result of neurological defects. These mice also have impaired lymphoid development, disminished number of T and B cells. They also fail to respond to IFNs and to cytokines using receptors with common γc subunit and the gp130 subunit (Rodig et al., 1998). JAK2 deficiency results in embryonic lethality at day 12.5 as a result of a failure

in erythropoiesis. JAK2 deficient cells fail to respond to EPO, TPO, IL-3, GM-CSF and IFN-γ. Mice deficient in EPO or EPOR have similar phenotype to JAK2 deficient mice, suggesting an important role for JAK2 in EPO signalling (Neubauer et al., 1998). JAK3-deficient mice are viable, but they have severe defects in T, NK and B cell development and activity. The phenotype of JAK3 knockout resembles the phenotype of γc receptor subunit deficient mice and both deficiencies abrogate signalling by the family of cytokines using γc-receptors (Nosaka et al., 1995, Park et al., 1995). JAK3 deficiency was first identified in humans with autosomal recessive severe combined immunodeficiency (SCID) disease, where mutation in the FERM domain of JAK3 abrogates its binding to γc-receptor. The phenotype of JAK3 mutation caused SCID is similar to X-linked SCID resulting from mutations in γc-receptors, suggesting that JAK3 has an essential role in γc-dependent lymphoid development (Macchi et al., 1995, Russell et al., 1995, Thomis et al., 1995). Tyk2 deficient mice exhibit only subtle defects in IFN-α/β and IL-10 responses, but profound defects in responses to IL-12 and LPS, and they show impairment in innate and adaptive immune responses (Shimoda et al., 2000).

JAK signalling is under strict control and tightly regulated in various steps by several proteins such as protein tyrosine phosphatases (PTPs), SOCS and small proteins such as ubiquitin and ISG15. This indicates that downregulation of JAKs is important for normal cell function. A gain-of-function mutation in *Drosophila Hop* JH2-domain results in a leukemia-like phenotype in the affected flies (Harrison et al., 1995). Moreover, TEL-JAK2 fusion protein with constitutive kinase activity is associated with lymphoid and myeloid leukemias (Lacronique et al., 2000). Recently, JAK2 mutation V617F in JH2 pseudokinase domain has been associated with polycythemia vera and other myeloproliferative disorders. V617F mutation results in constitutive tyrosine phosphorylation of JAK2 kinase domain that promotes cytokine hypersensitivity (Baxter et al., 2005, James et al., 2005).

5.3.2 STATs

The STAT family of transcription factors was first identified through the purification of factors involved in interferon (IFN)-regulated gene expression. STATs are latent cytoplasmic proteins that are activated by overlapping but distinct sets of cytokines and growth hormones. Cytokine or growth hormone induced activation of receptor associated JAK proteins phosphorylate a SH2 docking site for the binding of STATs. JAKs phosphorylate the receptor associated STATs, which dissociates from the receptors and translocates to the nucleus to bind specific promoter elements on the target gene promoters. The identification of a STAT gene in *Drosophila* and *Dictyostelium discoideum*, *Xenopus laevis* and *Danio rerio* suggest that the family arose from a single primordial gene. In *Drosophila* a single *stat*92E (STAT) and *Hop* (JAK) plays an important role in many aspects of development such as sex determination, segmentation, blood cell development and immunity (Kisseleva et al., 2002a). Seven STAT proteins have been identified in mammalian cells and genes of STATs are localized in three clusters on three different chromosomes. Mouse STAT1 and STAT4 are located on chromosome 1, STAT2 and STAT6 on chromosome10 and STAT3, STAT5a and

STAT5b on chromosome 11. Presumably these genes in three clusters have been formed by tandem duplications and the STAT5 gene has undergone more recent duplication to STAT5a and STAT5b (Copeland et al., 1995).

The degree of sequence identity divides STATs into two groups. STAT1, STAT2, STAT3 and STAT4 are grouped together by their 41 to 54% sequence identity. STAT5a and STAT5b are 42 to 45% identical to STAT6 and form the second group. Identity between these two groups is much lower, 29 to 34%. STAT5a and STAT5b have been reported to have an identity of 94%, indicating that they are products of a recent duplication event. The various STAT family members are more related to the STATs of other species than to other family members within the same species. For example, human STAT3 is more related to zebrafish STAT3 than to any other human STATs. The evolutional history of the STAT gene family is still elusive, but presumably STAT3 and STAT5 appeared early in the evolution of multicellular organisms, where they were generated by the first gene duplication event. Further duplication events took place early in mammalian evolution and generated other STAT family members (Hou et al., 2002, Pascal et al., 2001). STAT1, 3, 4, 5A and 5B have shorter isoforms (STAT1β, 3β, 4β, 5Aβ and 5Bβ) generated by alternative splicing of the 3´end of the gene transcripts. These shorter isoforms lack a transcriptional activation domain but they have the ability to occupy promoters of target genes. The short isoforms of STAT3 and STAT5 have been shown to exhibit increased DNA binding affinity and to form more stable dimers. These truncated forms of STATs might compete with full length STATs for DNA binding sites and through this mechanism inhibit transcriptional activation of target genes. However, STAT1β can combine with STAT2 and IRF9 to form the transcription complex ISGF3, where it has a function as a transcriptional activator. Also, STAT3β and c-Jun can bind cooperatively to an IL-6 responsive promoter element to activate the gene expression (Schaefer, Sanders and Nathans, 1995, Schindler et al., 1992b).

5.3.2.1. Biological role of STATs

The JAK-STAT pathway plays an important role in the regulation of immune responses and cellular homeostasis in human health and diseases. The activity of JAK-STAT signalling cascade has been shown to be tightly regulated in various steps and unrestricted JAK-STAT signalling has been associated with dysregulated cell growth and immune disorders (Ihle, 2001). The generation of null mice has revealed that the STAT family members have non-redundant biological roles. STAT1 deficient mice are viable with no developmental defects, but they show elevated susceptibility to infection by viruses and microbial pathogens and also tumor formation (Durbin et al., 1996). Subtle mutations of STAT1 in humans lead to impaired resistance to mycobacterial infections. STAT1 deficient mice fail to induce transcription of target genes in response to both IFN-γ and IFN-α and also ISGF-3 target genes in response to IFN-α. STAT1 deficient mice also developed spontaneous and chemically induced tumors more readily than wild type animals (Chin et al., 1997, Meraz et al., 1996). The major function of STAT2 is in IFN-α/β signalling, where it forms a complex with STAT1 and IRF9 to bind ISRE. STAT2 deficient mice are fertile and viable, but they are susceptible to viral infections, having

impaired IFN-α/β responsiveness. STAT1 upregulation and induction of GAS-dependent genes in response to IFN-α is also impaired in STAT2-deficient cells except macrophages expressing normal levels of STAT1 (Park et al., 2000) (Bluyssen and Levy, 1997). In clear contrast to other STAT family members, STAT3 deficiency is embryonic lethal at day 7.5 which make the function more difficult to define (Takeda et al., 1998). Tissue specific targeting of STAT3 did not reveal major developmental defects but some abnormalities were apparent. STAT3 was found to be essential for IL-6-induced suppression of apoptosis of T cells, and IL-2-induced T cell proliferation. Furthermore, STAT3 deficient macrophages do not respond to IL-10 and have increased sensitivity to LPS, indicating on essential role for STAT3 in anti-inflammatory responses. STAT3 conditional knockouts have also revealed that STAT3 deficiency affects apoptosis of mammary epithelial cells and in brain it resulted in perinatal lethality due to neuronal defects. Selective deletion of STAT3 in skin resulted in impaired wound healing, associated with defected migration of epidermal cells (Chapman et al., 1999, Sano et al., 1999, Takeda et al., 1999; Takeda et al., 1998). STAT4 deficient mice are viable but Th1 cell development is severely impaired and STAT4 deficient NK and T cells were unresponsive to IL-12 (Kaplan et al., 1996a). The products of the closely related STAT5a and STAT5b genes are activated by a wide range of cytokines. Mice deficient in each of these genes had a very specific and limited phenotype. STAT5a deficient mice have a loss of prolactin-dependent mammary gland development (Liu et al., 1997). The deletion of STAT5b results in a phenotype with similarities to that observed in growth hormone receptor deficient mice having sexually dimorphic retardation (Udy et al., 1997) and this phenotype is further accentuated in STAT5a/b deficient mice. In addition to this, STAT5a/b double knockout mice exhibited a more severe phenotype. Many of these mice die within a few weeks after birth or they have reduced levels of insulin-like growth factor (IGF-1) and reduced growth in males and females. STAT5a/b deficiency also results in the loss of development of corpus luteum and female fertility and in these repects phenotype is identical to that observed in prolactin receptor deficient mice. Despite normal blood cell counts these double mutants also have multiple defects in the development of myeloid and lymphoid lineages (Liu et al., 1997, Teglund et al., 1998). STAT6 deficient mice lack most of the physiological functions associated with IL-4. These mice exhibit a defect in T-cell development toward Th2 cells and lymphoid cells lose the responsiveness to IL-4 and IL-13 but have normal response to other cytokines. The most striking phenotype is the inability of B cells to undergo class switching and produce IgE constant region of the heavy chain locus (Kaplan et al., 1996b).

Tyrosine kinases are among the most frequently activated oncoproteins in cancer cells. Consequently, persistent activation of STAT signalling has been shown in a number of human cancers such as leukemias, lymphomas, breast cancer, prostate cancer, head and neck tumors, brain tumors etc. The earliest indication that STATs contribute to oncogenesis was the finding that STAT3 is constitutively activated in SRC-transformed cells and interruption of STAT3 signalling blocks the transformation of mouse fibroblasts by the Src oncoprotein (Bowman et al., 2000, Bromberg et al., 1999). In addition to STAT3, constitutive activation of STAT1 and STAT5 has been shown in human cancers. STAT5 signaling has been shown to promote oncogenesis in chronic myelogenous leukemia (CML) and myeloproliferative diseases induced by oncogenic fusion proteins

BCR-Abl and TEL-Jak. Recently, JAK2 somatic mutation V617F has been associated with polycythemia vera and other myeloproliferative disorders. V617F mutation results in constitutive tyrosine phosphorylation of JAK2 that promotes cytokine hypersensitivity and constitutive activation of STAT5 (Garcon et al., 2006). STAT activation and cell transformation may also be induced by viruses and viral proteins such as HTLV-1 virus, v-Abl, v-Ros and Epstein-Barr virus (Campbell et al., 1997, Danial, Pernis and Rothman, 1995, Garcia et al., 1997). Regardless of the mechanism of constitutive activation of STATs in tumor cells, the consequence is upregulation of genes such as Bcl-xl, Mcl-1, Bcl-2, cyclin D1, c-Myc, $p21^{WAF1/CIP1}$ and VEGF. Activation of these genes contributes to malignant progression by inhibiting apoptosis, enhancing cell proliferation, inducing angiogenesis and disabling the function of immune mechanisms (Bowman et al., 2001, Bromberg et al., 1999, Niu et al., 2002).

5.4 STAT1

5.4.1. Structure of STAT1

STATs are 750-850 amino acid long proteins with functionally and structurally conserved domains: N-terminal domain, coiled-coil domain, DNA binding domain (DBD), linker domain, SH2-domain and carboxy (C) terminal transactivation domain (TAD). STAT1 is the founder member of the STAT family and a paradigm for most aspects of the structure of STATs. STAT1 has a critical role in interferon (IFN) signalling when IFN-γ activates STAT1 through JAK1/2-mediated tyrosine phosphorylation on Tyr701. Activated STAT1 homodimerizes and translocates to the nucleus to initiate the transcription of IFN-γ-regulated genes (Darnell, 1997).

Analysis of the crystal structure of the DNA complex of phosphorylated STAT1 (aa 132-713) and STAT3 (aa 127-722) has revealed that STAT forms a contiguous C-shaped clamp around DNA stabilized by specific interactions between SH2 domain and tyrosine phosphorylated C-terminal tail segment (residues 700-708) of monomers. In the dimer interface the C-terminal tail segments form an antiparallel β sheet structure and dimer structure is stabilized by hydrophobic interactions between SH2 domains. Crucial interaction is formed between the SH2-domain and phosphotyrosine of adjacent monomer so that the phosphate group is recognized by strictly conserved Arg 602 residue rising from the interior of SH2 domain. The adjacent helical linker domain (aa 488-575) connects SH2 domain (aa 576-683) to DNA binding domain (aa 317-488) that contains several β sheets folded similarly to the DNA binding domain of nuclear factor κb (NF-κB) and p53. Structural analysis has revealed that there are relatively few direct contacts between STAT1 and DNA bases. However, DNA binding of STAT1 involves four loop structures per monomer, which contact the DNA backbone and DNA-bases. Three of these loops protrude from the β-barrel domain, while the fourth loop links the β-barrel and connector (linker) domains. This loop has Asn460 residue (Asn466 in STAT3), presumably the most important determinant of the base specificity that recognizes bases from both DNA strands (Becker, Groner and Muller, 1998, Chen et al., 1998).

Structural analysis of 130 N-terminal residues of STAT4 has revealed that it is a stable, highly conserved folding unit consisting of eight helixes. The N-terminal domain forms a dimer in the solution and this ability enables N terminus to mediate cooperative binding of STATs to target sites and the formation of higher-ordered STAT complexes (Vinkemeier et al., 1998). Recently, the crystal structure for an unphosphorylated STAT1 (aa 1-683) with a phosphopeptide derived from IFN-γ receptor revealed that unphosphorylated STAT1 is dimerized through interactions between two N-domains or two core fragments (aa 132-683) which allows parallel and anti-parallel orientations of monomers (Mao et al., 2005, Zhong et al., 2005). Rearrangement of phosphorylated dimer to anti-parallel orientation has also been proposed to be required for efficient presentation of STAT1 phospho-tyrosine to phosphatases in the nucleus. The N-terminal domain has also been implicated in interactions with co-activators such as CREB binding protein CBP/p300 and Protein Inhibitors of Activated STAT (PIAS) (Liao, Fu and Shuai, 2000, Zhang et al., 1996). The N-terminal domain is connected through a flexible linker to the adjacent 4-α-helical bundle of coiled-coil domain (aa 136-317; STAT1). These α-helixes form a large hydrophilic surface capable of mediating interactions with other helical proteins such as N-myc interacting protein (NMI) and interferon regulatory factor 9 (IRF9) (Kisseleva et al., 2002a, Zhang et al., 2000). The coiled-coil domain is also involved in the nuclear transport of STAT and STAT1 has a nuclear export sequence (NES) which comprise residues 302-314 (Schindler, 2002)

The structure of TAD has remained elusive, but it is known as the least conserved domain among STAT family members ranging from 38 (STAT1) to 180 (STAT6) amino acids in length and β forms of STATs lack the TAD domain. This sequence diversity has been proposed to contribute to the specific activation of genes by different STATs (Schindler, 2002, Shen and Darnell, 2001). The transactivation domains of STATs have been found to interact with a number of proteins involved in gene transcription and some of these associations require serine phosphorylation of TAD. For example, the interaction of mini-chromosome maintenance (MCM) protein 5 and CBP/p300 protein is regulated by phosphorylation of STAT1 Ser 727 (Ramsauer et al., 2007, Zhang et al., 1996, Zhang et al., 1998). Also serine phosphorylation of STAT3 and STAT4 has shown to increase they transcription potential (Decker and Kovarik, 2000b)

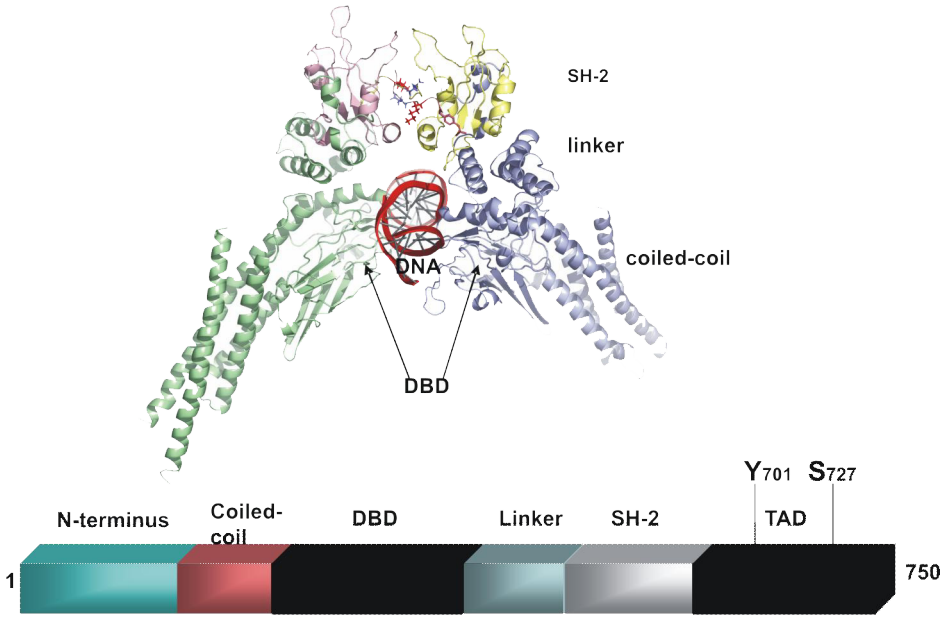

Figure 2. Schematic representation of STAT1 domain structure and 3D-structure of the STAT1 homodimer bound to DNA (PDB code:1bf5, Chen et al. 1998; figure generated with program PyMol). DBD represents the DNA binding domain of the STAT1 and is followed by linker and SH-2 domain. The C-terminal transactivation domain (TAD) including Ser727 residue and N-terminal domain are not shown in the 3D structure. The coiled-coil domain is located adjacent to DBD and together with TAD is involved in binding of proteins.

5.5 Regulation of STAT1 signalling

A characteristic feature of STAT signalling is its highly restricted and rapid turn on and off cycles that are regulated by various mechanisms. The majority of STATs expressed in unstimulated cells reside in the cytoplasm and activated STATs are rapidly imported into the nucleus. After the signal has decayed the STATs are re-exported back to the cytoplasm. The activity of STATs has been reported to be regulated by dephosphorylation, nuclear trafficking, feedback inhibition by SOCS and interactions by negative regulators including PIAS and SLIM (STAT interacting LIM protein). Post-translational modifications of STATs constitute another important regulation mechanism. STATs have been reported to undergo several covalent modifications, such as tyrosine and serine phosphorylation, acetylation, methylation, ubiquitination, ISG15ylation and O-glycosylation (Decker and Kovarik, 2000a, Levy and Darnell, 2002, Schindler, Levy and Decker, 2007).

5.5.1 Post-translational modifications of STAT1

5.5.1.1 Tyrosine phosphorylation

The function of STAT1 is regulated by various posttranslational modifications that act in a highly coordinated fashion to combine signals from secreted cytokines as well as from microbial agents. Phosphorylation of the Tyr701 residue in STAT1 and other canonical tyrosine residues around position 700 in other STATs is a most important step in the JAK-STAT paradigm of signal transduction. IFN-γ stimulated Tyr701 phosphorylation activates STAT1 and induces its binding to palindromic GAS element (Darnell, Kerr and Stark, 1994). The crystal structure of STAT1 homodimer revealed the molecular consequence of Tyr phosphorylation of STAT1. In process of the dimerization, the phosphorylated tail segment of one monomer spans to reach Arg602 residue in the SH2 domain of the other monomer that recognizes phosphate group bonded to tyrosine. This is a basis for IFN-γ induced STAT1 activation (Chen et al., 1998). Evidence from the targeted disruption of JAK genes in mice suggests that JAKs are the most important STAT tyrosine kinases (Rodig et al., 1998). However, STAT1, STAT3 and STAT5 have been shown to be tyrosine phosphorylated in JAK deficient cells by EGF and PDGF receptors having intrinsic tyrosine kinase activity (Leaman et al., 1996, Vignais et al., 1996). Tyrosine phosphorylated STATs are also frequently found in transformed cells expressing oncogenic tyrosine kinases such as v-Src, v-Abl, BCR-Abl, but it is unclear to what extent STATs are direct substrates of these kinases (Bowman et al., 2000, Danial and Rothman, 2000, Reddy et al., 2000).

5.5.1.2 Serine phosphorylation

All Stats except STAT2 are phosphorylated on at least one serine residue in their TAD domain (Decker and Kovarik, 2000a). Conserved phosphorylation sites included a PMS*P motif in STAT1, STAT3 (Ser727) and STAT4 (Ser721), PS*P motif in STAT5a (Ser725) and STAT5b (Ser730), and a SS*PD motif in STAT6 (Ser756) (Wang et al., 2000). Additionally, STAT1 and STAT5 possess other Ser phosphorylation sites in their TADs Ser708 and Ser779 respectively (Beuvink et al., 2000, ten Hoeve et al., 2002). STAT1 Ser727 phosphorylation can be induced by various kinases activated by IFN stimulation or through extracellular signals like mediators of inflammation, stress and growth signals. Kinases that induce Ser727 phosphorylation of STAT have been identified using inhibitors or dominant negative alleles for specific pathways, and STAT serine phosphorylation is regulated at least by p38-mitogen-activated protein kinase (MAPK), protein kinase C (PKC) δ, calcium dependent calmodulin kinase (CaM) II and I kappa B kinase (IKK) ε. α-isoform of MAPK has been shown to phosphorylate Ser727 in numerous cellular responses to inflammatory stimuli such as LPS and TNF or as a consequence of cellular stress (UV irradiation or hyperosmolarity) (Deb et al., 2003, Kovarik et al., 1998, Kovarik et al., 1999, Nair et al., 2002). Nevertheless, IFN-induced Ser phosphorylation of STAT1 was not affected by the absence of p38MAPK, suggesting that p38MAPK is not a STAT serine kinase requiring a tyrosine phosphorylated

substrate and is not part of IFN signalling pathway. CaM kinase II and PKC-δ have been shown to function as serine kinases that phosphorylate Ser727 residue of STAT1 in response to IFN-α and IFN-γ stimulations (Deb et al., 2003, Nair et al., 2002). These proteins have also been proposed to function as serine kinases for other Stats. p38MAPK has been suggested to phosphorylate Ser721 of STAT4 in response to IL-12 stimulation. The ERK (extracellular-signal regulated kinase) family of MAPKs has been shown to phosphorylate STAT3 at Ser727 in response to growth factors. Also phosphorylation of STAT1 at Ser727 occurs through ERKs, but evidently STAT3 was a superior substrate when compared to STAT1. Interestingly, Ser727 phosphorylation negatively modulates STAT3 tyrosine phosphorylation required for dimer formation and DNA binding activity of STAT3 (Chung et al., 1997b).

STAT1 functions as a critical regulator of the innate immunity and immune responses during microbial infections. Microbial infections induce Ser727 phosphorylation through the p38MAPK pathway positioning Ser727 as a converging point in immune surveillance. STAT1 combines signals derived from IFN-γ and LPS receptors during macrophage activation, and phoshorylation of Ser727 appears to mediate important fine-tuning of these responses (Kovarik et al., 1998, Kovarik et al., 1999). Mice expressing mutated STAT1S727A show reduced antibacterial immunity due to diminished or delayed expression of some IFN-γ/STAT1 target genes such as GBP-1, TAP-1 and IRF-1 without affecting SOCS1 and MCH I. When challenged with *Listeria monocytogenes* bacterium these mice display increased mortality and diminished ability to clear the bacteria from liver and spleen compared to wild-type mice. However, Ser727 phosphorylation is not critical for host defence against a milder pathogenic infection. When STAT1S727A mice were infected with lower doses of bacteria these mice exhibited similar survival rates to wild type control mice. These findings suggests that Ser727 phosphorylation has a specific role restricted to certain functions of STAT1 (Varinou et al., 2003). Alternatively serine phosphorylated Stats can exhibit biological activity in the absence of tyrosine phosphorylation. Transcriptional activity has been reported for STAT1 and STAT3 mutants defective in tyrosine phosphorylation, where Ser727 phosphorylated STAT1 and 3 tyrosine mutants recruited other transcription factors like NFκB to regulate transcription (Yang et al., 2005). Additionally, Ser727 phosphorylation, but not Tyr701 phosphorylation is required for some STAT1-dependent apoptotic responses (Decker and Kovarik, 2000a). Nevertheless, phosphorylation of Ser727 in the transactivation domain (TAD) has been shown to modulate the interaction of STAT1 with coactivator proteins and to selectively affect IFN-induced gene responses. Recent reports clearly demonstrate that CBP is recruited to GBP promoter chromatin in a STAT1 Ser727 phosphorylation dependent manner and the CBP function correlated with the hyperacetylation of promoter histones (Schindler, Levy and Decker, 2007, Varinou et al., 2003). Leu724 has been proposed to be essential for gene activation mediated by STAT1. Leu724 modulates Ser727 phosphorylation, and Leu724 as well as phosphorylated Ser727 are essential for the recruitment of the transcriptional coactivator CBP/p300 to STAT1 at GBP-1 and IRF-1 promoters (Zhang et al., 1996). Ser727 phosphorylation of STAT1 has been shown to regulate the binding of other transcriptional regulators like MCM5 (Zhang et al., 1998). Recent studies have implicated IFN-inducible Ser708 phosphorylation of STAT1 by IKKε kinase in antiviral

transcriptional responses. Mice lacking IKKε are hypersusceptible to viral infections because type I IFN dependent ISGF3 complex has impaired ISRE promoter binding and target gene activation. IFNβ activated IKKε has been proposed to directly phosphorylate Ser708, Ser744 and Ser747 of STAT1, and Ser708 phosphorylated STAT1 has been proposed to favour the formation of STAT1-STAT2 heterodimers necessary for the assembly of ISGF3 complex (Tenoever et al., 2007).

5.5.1.3 Ubiquitination

Protein degradation is a complex process involving multiple different enzymes generating polyubiquitinated proteins, which are recognized and degraded by the 26S proteasomes in an adenosine triphosphate dependent-manner. The ubiquitin-proteasome pathway involves ubiquitin activation step by E1 enzyme, transfer of ubiquitin residue to conjugation enzyme E2 and transfer of ubiquitin by E3 ligase enzyme to the ε-amino group of lysine residue of the substrate. Polyubiquitinated proteins are then degraded by the 26S proteasomes. Many proteins of signal transduction pathways have been shown to be substrates for enzymatic ubiquitin-proteasome degradation (Ciechanover, 1998). Proteasome inhibitors increase the duration of cytokine effects by modulating signalling events at multiple steps. For example, turnover of certain cytokine receptors is controlled by proteasomes and at the receptor complex activated JAKs have been shown to become polyubiquitinated and degraded SOCS box-dependently, mediated by the E3 ligase complex (Krebs and Hilton, 2001, Ungureanu et al., 2002). Ubiquitin-proteasome pathway has also been implicated in the regulation of activated STAT proteins (Kim and Maniatis, 1996). IFN-γ activated STAT1 proteins were shown to be stabilized by a proteasome inhibitor and ubiquitinated *in vivo* (Kim and Maniatis, 1996). Recently a novel nuclear protein, STAT-interacting LIM (SLIM), was shown to function as an ubiquitin E3 ligase acting on STAT1 and STAT4 and cause their proteosome-mediated degradation, while SLIM-deficiency resulted in increased STAT expression and enhanced IFN-γ production by Th1 cells. SLIM was also shown to enhance dephosphorylation of STATs, suggesting that SLIM may be involved in the recruitment of tyrosine phosphatases (Tanaka, Soriano and Grusby, 2005, Ungureanu and Silvennoinen, 2005). Paramyxoviruses can also target STATs for ubiquitin-mediated proteasomal degradation. Their host evasion mechanism is attributed to the V proteins having a function as ubiquitin E3 ligase with high specificity for STAT1, STAT2, or STAT3 (Li et al., 2006)**.**

5.5.1.4 Arginine methylation

The N-terminal Arg residue at position 31 is conserved in all STATs, and this residue in STAT1, STAT3 and STAT6 have been proposed to be methylated. It was suggested that arginine 31 methylation of STAT1 by protein arginine methyl-transferase (PRMT1) enhances its DNA binding by diminishing the association with PIAS1 (Mowen et al., 2001). However, this finding is still controversial. Another report provided evidence contradicting these results. MALDI spectra analysis of Strep-tagged STAT1 did not

support the existence of Arg31 methylated STAT1 because mass peaks which would match with Arg31 methylated sequence were not present, suggesting that Arg31 is not significantly methylated (Meissner et al., 2004).

5.5.1.5 Acetylation, O-glycosylation and ISGylation

STAT1, STAT3 and STAT6 have been reported to be targets for lysine acetylation. Acetylation of STAT1 and STAT3 is involved in nuclear factor- κB (NFκB) signalling causing a pro-apoptotic effect in the case of STAT1 and an anti-apoptotic effect in the case of STAT3 (Kramer et al., 2006, Nadiminty et al., 2006). O-glycosylation site is conserved in STAT1, STAT3, STAT5 and STAT6 and O-glycosylation of STAT5 has been associated with the recruitment of the coactivator protein CBP (Gewinner et al., 2004). The ISG (highly interferon-inducible gene) 15 protein is conjugated to a variety of cellular proteins such as STAT1. The consequence of STAT1 ISGylation has been proposed to increase the activity of STAT1 in response to type I IFN, but the clear indication of the function has remained unclear (Malakhova et al., 2003).

5.5.2 Protein phosphatases

Several tyrosine phosphatases have been identified to negatively regulate JAK/STAT pathway. Dephosphorylation of Stats occurs in the nucleus and is an important signal for the export back to the cytoplasm. During a full response to cytokine stimulation, an individual STAT1 molecule undergoes several cycles of phosphorylation, nuclear import, dephosphorylation and export from the nucleus (Haspel and Darnell, 1999). For STAT1 and STAT3, there is evidence implicating TC45 (T-cell protein-tyrosine phosphatase) as a nuclear phosphatase. Cells lacking this enzyme have prolonged tyrosine phosphorylation of STAT1. TC45 has also been implicated in regulating cytoplasmic dephosphorylation of JAK1 and JAK3. SHP-2 (SH2-containing phosphatase 2), TCPTP (T cell protein-tyrosine phosphatase) and PTP 1B (protein-tyrosine phosphatase 1B) have also been proposed as cytoplasmic regulators of JAK and STAT phosphorylation (David et al., 1995, Irie-Sasaki et al., 2001, Meyer, Gavenis and Vinkemeier, 2002, You, Yu and Feng, 1999, ten Hoeve et al., 2002). Interestingly, SHP-2 has been demonstrated to dephosphorylate STAT1 at both tyrosine and serine residues (Wu et al., 2002). However, how the specificity of these phosphatases is determined is not so far known.

5.5.3 Nucleocytoplasmic transport

The movement of macromolecules in a cell is regulated by a nuclear membrane functioning as a barrier separating the nucleus from the cytoplasm. The movement of molecules larger than 50kDa is regulated through passageways called nuclear pore complexes (NPC). NPCs allow passive diffusion of small molecules but require a nuclear localization signal (NLS) for the transport of large molecules into the nucleus (Gorlich and Kutay, 1999). DNA replication and transcription are separated from other cellular

processes by compartmentalization of DNA binding factors and regulators by the nuclear membrane. STAT1 resides mainly in latent state in the cytoplasm. Upon stimulation by IFN-γ and dimerization caused by tyrosine phosphorylation, STAT1 accumulates in the nucleus. Within 15-30 min after stimulation STAT1 is almost completely translocated in the nucleus. Mutations preventing Tyr701 phosphorylation or dimerization prevents this translocation (Improta et al., 1994). STAT1 and other STATs do not have the classic NLS motif in their amino acid sequences but presumably tyrosine phosphorylation and dimerization alter the conformation so that it is recognized by an importinα family member, importinα5. Association of STAT1 dimers with importinα5 bound to importinβ1 results in the transport of STAT1 through NPCs into the nucleus (Melen et al., 2003). STAT1 has been proposed to have an unconventional NLS. Mutations within a small region of the DNA binding domain have been found to disrupt nuclear import in response to cytokine stimulation. Mutations in Leu407 or a double mutation in Lys410 and Lys413 produced a STAT1 form that even though Tyr701 phosphorylated remained in the cytoplasm (Fagerlund et al., 2002, Melen et al., 2003). Although unphosphorylated STAT1 is predominantly in the cytoplasm, there is also a low level in the nucleus. The nuclear localization mechanism of unphosphorylated STAT1 is distinct from that of the tyrosine phosphorylated form (Kumar et al., 1997, Meyer, Gavenis and Vinkemeier, 2002). The nuclear redistribution of phosphorylated STAT1 is transient and subsequently STAT1 is actively exported back to the cytoplasm. STAT1 has been suggested to have two different leucine-rich nuclear export signals (NES), one in the coiled-coil domain (aa 302-314) and the other in the DNA binding domain (aa 400-409). It has been suggested that when STAT1 is bound to DNA NES sequence is masked, which prevents association with export receptor CRM1 (chromosome region maintenance 1) and the nuclear export of STAT1. Conversely, when STAT1 is dephosphorylated in the nucleus by PTP, STAT1 dissociates from the DNA and STAT1 becomes accessible to CRM1-mediated export (Begitt et al., 2000, McBride et al., 2002, McBride, McDonald and Reich, 2000).

5.5.4 Suppressors of cytokine signalling, SOCS

The SOCS family includes at least eight members (SOCS-1-7 and cytokine induced SH2 protein CIS). All eight SOCS-family members have a central SH2 domain, an amino-terminal domain of variable length and a 40-amino-acid motif at the carboxy terminus known as the SOCS box. SOCS proteins are induced by a variety of cytokines, and they inhibit cytokine signalling via a number of different mechanisms (Greenhalgh and Hilton, 2001). SOCS1 directly interacts with JAKs and inhibits their catalytic activity (Endo et al., 1997). CIS is thought to bind receptors and to block the recruitment and activation of STATs (Matsumoto et al., 1997). SOCS proteins also interact with the ubiquitination machinery through the SOCS box and may direct proteins, such as JAKs and receptors, for proteasomal degradation. The SOCS box acts as an adaptor region that couples SOCS to elongins B and C, which in turn recruit the complex to the proteasome (Kamura et al., 1998). The SOCS proteins act in the negative feedback loop to extinguish signal transduction (Greenhalgh and Hilton, 2001). SOCS expression is inducible in response to a range of cytokines, IFN-γ among them. STAT proteins mediate SOCS gene expression, as several SOCS genes have a STAT binding site in their promoter. For example, STAT1

binds to the promoter region of SOCS1 gene. As an indication of negative feedback mechanism SOCS1 has been demonstrated to be an essential regulator of IFN-γ signalling. Gene targeting studies of SOCS1 have underscored the important role that SOCS1 plays in antagonizing responses to IFN-γ and STAT1. SOCS1 deficient mice die within three weeks of birth owing to fatty degeneration of the liver and hematopoietic infiltration of multiple organs, such as the pancreas, heart and liver. Severe lymphopaenia and reduced number of lymphocytes were evident. These mice exhibited excessive responses similar to those induced by elevated levels of IFN-γ and phenotype was temporarily attenuated by the lack of IFN-γ or STAT1 (Alexander et al., 1999).

5.5.5 Protein inhibitors of activated STAT, PIAS

The Protein inhibitor of activated STAT (PIAS) proteins regulates the activity of many transcription factors. STATs, NF-κB, SMA- and MAD-related proteins (SMADs) and the tumor-suppressor protein p53 are key families of transcription factors widely used downstream of cytokine-signalling to regulate gene-responses. PIAS proteins regulate these transcription factors, either positively or negatively, through various mechanisms: inhibition of DNA binding activity, recruiting transcriptional corepressors or coactivators, and promoting protein sumoylation (Hay, 2005, Johnson, 2004, Sharrocks, 2006). Four mammalian genes encoding PIAS proteins, PIAS1, PIAS3, PIASx and PIASy have been described. PIAS3 has a splice variant called PIAS3β or KChaP (potassium-channel-associated protein) and PIASx also produces two isoforms derived from alternative splicing, designated PIASxα (androgen receptor interaction protein 3, ARIP3) and PIASxβ (Msx-interacting-zinc finger protein 1, Miz1) (Chung et al., 1997a, Johnson, 2004, Liu et al., 1998). The PIAS family of proteins shares significant sequence homology and highly conserved domains (Figure 3.). The most conserved domain of this family is RING- finger-like zinc-binding domain (RLD) in the central part of PIAS. The PIAS RDL substantially differs from RING domains present in many E3 ubiqiuitin ligases and is required for the SUMO-E3-ligase activity of PIAS proteins (Hochstrasser, 2001, Jackson, 2001a). In the N-terminus PIAS proteins contain putative domain named SAP (SAF-A/B Acinus and PIAS), which is conserved among species and shared by other chromatin binding proteins, such as SAFA and SAFB. In SAFA and SAFB SAP can recognize and bind (A+T)-rich DNA sequences present in scaffold-attachment regions (SARs) frequently found close to gene enhancers (Kipp et al., 2000, Sachdev et al., 2001, Tan et al., 2002). PIAS1 and PIASy can bind (A+T)-rich DNA sequences *in vitro*. Within the SAP domain, an LXXLL signature motif is present in all PIAS proteins. It has been shown to mediate interactions between nuclear receptors and their co-regulators and it is required for PIASy mediated transcriptional repression of STAT1 and androgen receptor (Heery et al., 1997, Moilanen et al., 1999, Torchia et al., 1997). The carboxy terminal region of PIAS proteins is the most diverse region containing a highly acidic domain (AD) and a serine and threonine rich (S/T) region. In addition, a putative SUMO1 interaction motif (SIM) is present in the AD motifs in all PIASs except PIASy (Minty et al., 2000). The functional role of these motifs is not clear, as the removal of the SIM of PIASxα had no effect on its SUMO-E3-ligase activity and they remain functional in promoting the conjugation of SUMO to proteins (Kotaja et al., 2002, Sachdev et al.,

2001). Many regions in PIASs are involved in protein-protein interactions and C-terminal region (aa 392-541) containing AD and SIM-motifs has been reported to bind N-terminal region of STAT1 containing amino acids 1- 191. Binding of PIAS1 to STAT1 has been proposed be negatively regulated by methylation of Arg 31 in the N-terminus of STAT1 but these results have remained controversial (Liao, Fu and Shuai, 2000, Mowen et al., 2001).

Co-immunoprecipitation assays have been used to examine endogenous PIAS-STAT interactions. PIAS family members have been shown to be involved in the regulation of STAT signalling. For example, both PIAS1 and PIASy interact with activated STAT1 upon cytokine treatment and PIAS3 and PIASx interacts with activated STAT3 and STAT4 respectively. The PIAS-STAT interaction is clearly cytokine dependent and PIASs do not bind STATs in unstimulated cells, which can be explained by the finding that PIAS1 binds to tyrosine phosphorylated dimeric STAT1 (Chung et al., 1997a, Liu et al., 1998, Liu et al., 2001). It has been proposed that PIAS1 and PIAS3 function by blocking the DNA-binding activity of STAT1 and STAT3 respectively, but the detailed molecular basis of this mechanism has not been determined (Chung et al., 1997a, Liu et al., 1998). Gene targeting studies have been performed to understand the physiological role of PIAS proteins in cytokine signalling. Analysis of $PIAS1^{-/-}$ mice showed that disruption of PIAS1 resulted in enhanced immune responses to viral or bacterial infections. DNA-microarray analyses from wild type and PIAS1 deficient macrophages revealed that PIAS1 selectively regulates only a subset of IFN-γ and IFN-β responsive genes by interfering with the recruitment of STAT1 to the promoters showing low affinity with STAT1 such as GBP1, but does not affect promoters with stronger affinity binding sites (IRF1) (Liu et al., 2004). PIASy deficient mice are phenotypically normal, but they have slight defects in IFN-γ induced transcriptional responses . The role of PIAS as a negative regulator of STAT signalling has been supported by studies in *Drosophila*. *Drosophila* PIAS, dPIAS (Zimp), has been shown to negatively regulate the JAK-STAT pathway and the removal of PIAS from *Drosophila* resulted in defects in eye development and increased tumor formation. Hyperactive mammalian JAK-STAT pathway has also been implicated in blood-cell tumor formation (Betz et al., 2001, Mohr and Boswell, 1999).

PIAS proteins have been shown to regulate other transcription factors through a variety of molecular mechanisms. PIASs has been proposed to regulate transcription by promoting the sumoylation of transcription factors. All PIAS proteins exert SUMO-E3 ligase activity towards various SUMO targets. The SUMO family of proteins (SUMO-1,-2,-3,-4) are small ubiquitin-related proteins covalently conjugated to the ε-amino group of lysine residues in target proteins. The RING domain-containing E3 ligases facilitate the transfer of SUMO from E2 conjugation enzyme Ubc9 to the substrate by catalyzing the conjugation reaction (Hay, 2005, Johnson, 2004). Members of the PIAS family interact with SMADs to either negatively or positively regulate their transcriptional activity. PIASy has been proposed to inhibit SMAD-mediated transcription by recruiting histone deacetylase (HDAC) 1 which regulates transcription by modifying chromatin. PIASy has also been found to promote sumoylation of SMAD3 and to inhibit transcription (Long et al., 2003). On the other hand, PIAS promoted sumoylation of

SMAD3 increased its stability and transcriptional activity . PIAS3 has also been shown to increase the transcriptional activity of SMAD3 by recruiting p300 or CBP (Lee et al., 2003, Long et al., 2004). The function of PIAS1 in the regulation of activity of tumor suppressor p53 is also controversial. PIAS1 has been shown to increase p53-mediated transcription and p53-dependent cell cycle arrest in the G1 phase. However, another group reported that PIAS1 repressed the transcriptional activity of p53 (Megidish, Xu and Xu, 2002, Schmidt and Muller, 2002). PIAS1 and PIAS3 have been proposed to regulate NF-κB signalling through interaction with REL family transcription factor p65 by modulating its DNA binding activity. Disruption of PIAS1 also resulted in the upregulation of a subgroup of NF-κB-dependent genes (Jang et al., 2004, Liu et al., 2005).

Figure 3. Domain structure of the mammalian PIAS protein. Within the SAP domain there is a conserved LXXLL signature motif. RING: RING finger-like zinc binding domain. AD/SIM:acidic domain. Within the AD, a SUMO1 Interaction Motif (SIM). S/T: serine-threonine rich region.

5.6 Sumoylation of proteins

As discussed in the chapter 5.5.1, posttranslational protein modifications provide a reversible tool to rapidly regulate protein functions in response to changes in a cell state or its environment. Without altering the protein synthesis, cells can control their activity, localization, stability and interactions with other proteins through posttranslational modifications. Posttranslational modification of transcription factors is also a mechanism used to achieve dynamic regulation of gene expression. Ubiquitin and a structurally and mechanistically related protein, small ubiquitin-like modifier (SUMO), can be covalently linked to specific proteins by similar enzymatic conjugation reaction. Ubiquitylation of proteins is generally associated with proteasomal protein degradation. By contrast, SUMO modification appears to play important roles in diverse processes such as cell division, DNA replication and repair, localization and formation of subnuclear structures, and regulation of activity of transcription factors (Hay, 2005, Johnson, 2004). Lysine residues subject to SUMO modification have been found within a SUMO modification consensus motif ψKxE and some substrates have been reported to have phosphorylation dependent sumoylation motif ψKxExxSP (Hay, 2005, Hietakangas et al., 2006, Stankovic-Valentin et al., 2007). Although it is clear that modification of most substrates takes place within the SUMO consensus motif, certain substrates are modified even when the surrounding sequence does not conform to consensus (Melchior, 2000).

5.6.1 SUMO-conjugation pathway

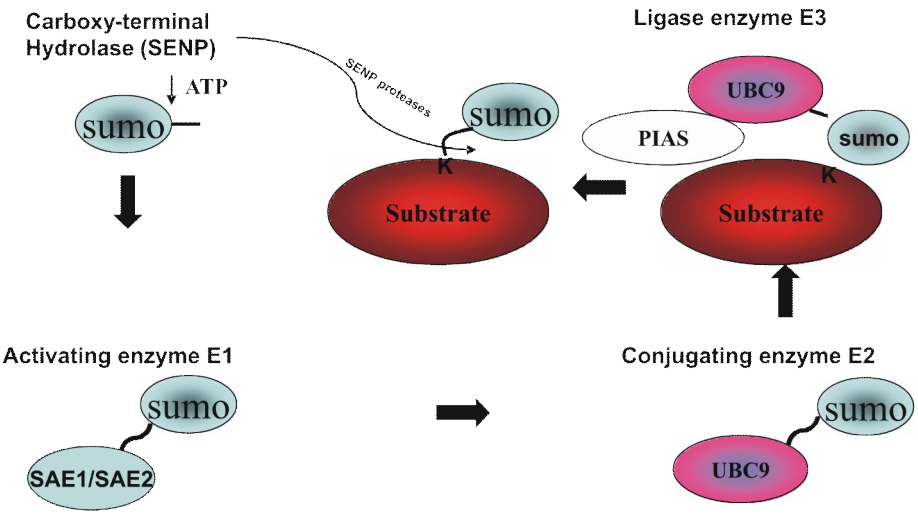

Figure 4. Schematic representation of the SUMO conjugation pathway. SUMO is activated by the E1 activating enzyme, a heterodimer consisting of SAE1 and SAE2 (in mammals). SUMO is subsequently transferred to the E2 enzyme, Ubc9, and conjugated to substrates in a reaction catalyzed by a variety of E3 enzymes including PIAS. SUMO is removed from substrates in reactions catalyzed by SENPs.

SUMO is a ~100 amino acids long protein distantly related to ubiquitin (18 % identity). Regardless of low sequence identity, the overall three-dimensional structures are very similar (Bayer et al., 1998). SUMO was first identified in mammals, where it was covalently linked to GTPase activating protein RanGAP1 (Matunis, Coutavas and Blobel, 1996). SUMO is covalently attached to lysine residues in proteins in a process analogous to, but distinct from, ubiquitination. In yeast, insects and nematodes, only one SUMO gene is expressed, whereas in plants up to eight versions of SUMO are expressed. In vertebrates four paralogs SUMO-1 (Smt3c), SUMO-2 (Smt3a), SUMO-3 (Smt3b) and SUMO-4 are expressed. Mammalian SUMO-2 and SUMO-3 share 95% sequence identity with each other and are 50% identical to SUMO-1. The majority of SUMO-1 proteins exist in conjugated form, whereas, SUMO-2 and SUMO-3 exist primarily as free proteins that are subjected to rapid conjugation after cellular stress. This also suggests that mammalian SUMO isoforms may perform distinct functions in the cell (Azuma, Arnaoutov and Dasso, 2003, Bohren et al., 2004, Eaton and Sealy, 2003, Saitoh and Hinchey, 2000). A consensus SUMO-acceptor sites consists of sequence ψKXE, where ψ

is a large hydrophobic amino acid and K is the SUMO conjugation site (Melchior, 2000). SUMO-2 and SUMO-3 as well as SUMO-4 presumably form poly-SUMO chains by conjugation with Lys 11, which is part of the SUMO consensus motif in SUMO, thus the role of poly-SUMO conjugation has remained unclear (Bohren et al., 2004, Saitoh and Hinchey, 2000, Tatham et al., 2001).

SUMO is synthesized as a precursor bearing a C-terminal extension that is cleaved by ubiquitin-like modifier proteases (Ulps) to produce an exposed di-glycine motif. The activity of these same proteases releases SUMO from conjugates allowing it to re-enter the conjugation cycle (Li and Hochstrasser, 2003). The SUMO activating enzyme (E1) is a heterodimer containing SAE1 and SAE2 subunits known as Aos1 and Uba2 in yeast (Okuma et al., 1999). Most organisms contain a single SUMO-activating enzyme, which is required for the conjugation of all SUMO variants with all substrates. Interestingly, both components of E1 heterodimer are related to ubiquitin E1 enzyme, which is a monomer. SAE1 resembles the N-terminus, while SAE2 corresponds to the C-terminus of ubiquitin E1 enzyme. The SUMO modification reaction is initiated by a reaction in which SAE1/SAE2 catalyzes the formation of covalent linkage between C-terminal carboxyl group of SUMO and AMP (Figure 4.). Breakage of this bond is followed by the formation of an intermediate thioester bond between the C-terminal carboxyl group of SUMO and the sulphydryl group of a cysteine residue in Uba2 (SAE2) (Desterro et al., 1999, Johnson et al., 1997, Johnson, 2004).

In the second step of the conjugation pathway, SUMO is transferred from the Uba2 subunit of activating enzyme E1 to a single SUMO conjugation enzyme (E2) known as ubiquitin conjugating enzyme 9 (Ubc9), through the formation of a SUMO-E2 thioester intermediate, where SUMO binds to cysteine residue 93 in Ubc9 (Figure 4.). Ubc9 is the only SUMO conjugating enzyme in yeast, invertebrates and presumably also in vertebrates. A structural comparison of Ubc9 to ubiquitin specific E2 enzymes (such as Ubc4 and Ubc7) revealed that the surface of Ubc9 that is involved in SUMO binding is positively charged, whereas the corresponding regions in ubiquitin E2 have negative or neutral potentials, and this difference most likely determines the specificity in binding to these modifiers. This positively charged patch surrounding the active site cystein 93 of Ubc9 binds directly to the ψKXE consensus sequence in the substrate (Johnson and Blobel, 1997, Okuma et al., 1999, Tatham et al., 2001).

Three types of SUMO ligases have been described; PIAS/Siz, RanBP2 a large vertebrate nuclear pore protein and Pc2 polycomp group protein. They all interact with Ubc9 and enhance sumoylation both *in vivo* and *in vitro*. These proteins bind the E2 conjugation enzyme and its substrate and promote the transfer of SUMO from the E2 to the substrate. These E3 ligases do not form covalent intermediates with SUMO, but appear to act by bringing Ubc9 and the substrate together (Hay, 2005, Johnson, 2004). The PIAS class of SUMO ligases was discovered in *S. cerevisiae* where Siz1 and Siz2 E3-ligase proteins were shown to mediate the majority of the sumoylation. However, Siz1 and Siz2 deletion mutants did not have significant growth defects, and it has been proposed that Ubc9 may be sufficient to execute the essential function of SUMO conjugation in the absence of these E3 enzymes. Additionally unrecognized SUMO ligases exists in *S. cerevisiae*

(Hochstrasser, 2001, Johnson and Gupta, 2001, Pichler et al., 2004, Takahashi et al., 2001, Tatham et al., 2005). Siz1 and Siz2 share an area of sequence homology with PIAS proteins. This homology region includes a region that resembles the RING domains of ubiquitin ligases and is called SP-RING (Siz/PIAS RING). This domain binds to Ubc9 and is required for the E3 activity and characterization of SP-RING domain led to identification of PIAS proteins as SUMO ligases (Hochstrasser, 2001, Jackson, 2001b). Mammalian cells have been reported to contain four PIAS proteins and they splice variants which are also negative regulators of JAK-STAT signalling pathway (described in Section 5.5.5). A second type of SUMO E3 ligases is the nucleoporin RanBP2 (Ran binding protein 2, Nup358). Although RanBP2 does not interact directly with the substrate, it has been proposed that it possibly functions through importin and Ubc9. RanBP2 is not sequentially similar to PIAS SUMO ligases. Contrary to PIASs, which are mainly nuclear proteins, RanBP2 proteins are located at the cytoplasmic part of the NPCs where they accelerates the modification of proteins such as RanGAP1, PML, Sp100 and HDAC4 by SUMO-1. *In vitro* results indicate that RanBP2 and PIAS proteins have mostly a distinct set of substrates, suggesting that they may have different specificities (Hay, 2005, Johnson, 2004, Pichler et al., 2002). The polycomp chromatin-modifying complex mediates transcriptional repression and E3 ligase activity has been reported in the Pc2 component of this complex. Like RanBP2, Pc2 is not a RING-containing E3 ligase but however the transcriptional corepressor CtBP associates with PcG bodies via Pc2, and Pc2 stimulates sumoylation of CtBP *in vivo* and *in vitro*. Overexpressed Pc2 in cells causes accumulation of SUMO and Ubc9 into PcG bodies, suggesting that PcG bodies may be major sites for this sumoylation event (Kagey, Melhuish and Wotton, 2003, Lin et al., 2003b).

5.6.2 Regulation of sumoylation

The pattern of SUMO conjugates is dynamic and changes upon the cell state. SUMO isopeptidases have two functions in the regulation process: They remove SUMO from proteins to make modification reversible and they process newly synthesized SUMO by cleaving the short C-terminal peptide (Hay, 2005, Seeler et al., 2001, Yeh, Gong and Kamitani, 2000). *S. cerevisiae* has two desumoylating enzymes, Ulp1 localized in NPCs and required for cleaving both SUMO precursors and SUMO conjugates, and nuclear Ulp2 which appears to desumoylate a distinct set of conjugates but not precursors (Li and Hochstrasser, 1999, Takahashi et al., 2001). Seven mammalian genes encode proteins with C-terminal Ulp domains with SUMO cleaving activity. They all have diverse N-terminal domains and distinct distribution in cells, suggesting that they may desumoylate different proteins. These desumoylating enzymes in mammals include SENP1, SENP3 (SMT3IP1) localized in the nucleus, SENP6 (SUSP1), primarily in the cytoplasm, and SENP2 (Axam, SMT3IP2/Axam2, SuPr-1) which have three different isoforms derived from alternative splicing. SENP2/Axam binds to the nucleoplasmic side of NPC, Axam2 /SMT3IP2 localizes to the cytoplasm and SuPr1 localizes to promyelocytic leukemia (PML) bodies. Diverse subcellular localization of these proteins will target them to discrete sets of substrates and allow selection of modified proteins for deconjugation

(Bailey and O'Hare, 2002, Best et al., 2002, Di Bacco and Gill, 2006, Kadoya et al., 2002, Kim et al., 2000).

Sumoylation can be regulated either positively or negatively by other post-translational modifications such as phosphorylation and ubiquitylation or through binding of macromolecules to substrates. Phosphorylation of c-Jun, PML, and IκB correlates with reduced SUMO attachment (Johnson, 2004). However, phosphorylation has the opposite effect on sumoylation of the heat shock transcription factor 1 (HSF1), where phosphorylation of Ser303 stimulates sumoylation of an adjacent Lys298. The sumoylation site in HSF1 has been shown to form phosphorylation dependent sumoylation motif (PDSM, ψKxExxSP) and sumoylation of this motif efficiently represses the transactivation capacity of HSF1. This motif is also found in numerous other SUMO substrates such as HSF-4b, c-Myb**,** and MEF (myocyte-specific enhancer factor) 2 family of proteins, and can be used to predict novel SUMO substrates (Hietakangas et al., 2006). Lysine serves as an attachment site for other modifications than sumoylation, such as ubiquitylation and acetylation. These modifications may regulate each other by competing for the same lysines. For example, SUMO conjugation to IκBα can prevent binding of ubiquitin and protein degradation because SUMO and Ubi have the same target lysine (Desterro, Rodriguez and Hay, 1998) and transcription factor Sp3 contains a lysine that can be either acetylated or sumoylated (Sapetschnig et al., 2002). Sumoylation of some proteins is regulated by interactions with other macromolecules. Sumoylation of the base excision repair protein thymine DNA glycosylase (TDG) *in vitro* is stimulated both by DNA and a downstream enzyme in the repair pathway. However, Sp3 is resistant to sumoylation when bound to DNA (Hardeland et al., 2002, Sapetschnig et al., 2002).

5.6.3 Transcription regulation by SUMO

The transcriptional effects of SUMO in mammalian systems are a result of SUMO modification of factors involved in gene expression such as transcriptional activators, repressors, coactivators, corepressors as well as other proteins involved in signal transduction pathways and components of PML (promyelocytic leukemia) nuclear bodies. SUMO modification of transcription factors can lead to transcriptional activation, but is more often associated with transcriptional repression. For example, sumoylation of transcription factors Elk-1, Sp-3, c-myb and androgen receptor (AR) decrease transcription from responsive promoters (Johnson, 2004). The glucocorticoid receptor (GR) and several other transcription factors have synergy control motifs, peptide motifs that reduce GR-dependent transcription from promoters containing multiple GR binding elements. These motifs contain SUMO conjugation sites and their mutation increases transcriptional synergy, thus indicating SUMO as a negative regulator of the synergy control motifs (Tian et al., 2002). The mechanism by which SUMO regulates the activity of transcription factors is unclear, but some results suggest that SUMO is a mediator for protein interactions in repressive transcriptional complexes. Candidates for such factors include HDACs, the repressor protein Daxx and PIAS proteins. For example, HDAC6 has been reported to bind to the repressor domain of p300 only when

sumoylated, resulting in deacetylation of core histones and inhibition of transcription. HDAC1 and HDAC4 are also sumoylated, which enhances their transcriptional repression activities. Some PIAS proteins interact with HDACs and also directly with SUMO and sumoylated proteins (Johnson, 2004, Kerscher, 2007). The consequences of SUMO modification of SMAD proteins appear to be promoter specific. On some promoters SUMO modification of SMAD4 activates transcription, whereas on other promoters transcription is repressed (Hay, 2005, Lin et al., 2003a). HSF1 and 2 are SUMO targets and SUMO modification of HSF2 loop structure contributes to negative regulation of its DNA binding, indicating that sumoylation of HSF has a negative regulatory role in gene expression (Anckar et al., 2006). An alternative mechanism to explain the repressive effect of SUMO on transcription is that sumoylated proteins are recruited into subnuclear domains such as PML bodies. A number of SUMO target proteins localize to PML nuclear bodies such as Daxx, Sp100, p53, CBP and homeodomain interacting protein kinase2 (HIPK2). PML protein itself is modified by SUMO on three lysine residues and SUMO conjugation is crucial for formation of mature nuclear bodies (NB) and the recruitment of other proteins. This suggests that PML bodies are storage depots for nuclear factors or sites for the modification and assembly of transcription factors (Duprez et al., 1999, Kamitani et al., 1998, Seeler et al., 2001, Verger, Perdomo and Crossley, 2003).

6. AIMS OF THE STUDY

Post-translational protein modifications regulate multiple cellular functions of proteins in response to changes in a cell state. SUMO modification has emerged as an important transcriptional regulation mechanism of proteins and the increasing number of transcription factors and co-regulators has been reported to be regulated by SUMO modification. The general aim of this study is to investigate the role of sumoylation in the regulation of transcriptional activity of STAT1.

The specific aims of the study were:

1) To analyse the putative sumoylation site and the functional effect of SUMO-modification on STAT1 activity

2) To study the mechanism of SUMO modification and crosstalk between sumoylation and phosphorylation in the regulation of transcriptional activity of STAT1

7. MATERIALS AND METHODS

7.1 Plasmid constructs

STAT1-mutant expression plasmids were constructed from STAT1 in pEF-BOS vector (Dr. C.Schindler) containing HA-tag using Site-Directed Mutagenesis kit (Stratagene). The following primers were used in the mutagenesis PCR,

STAT1-K703R (Lys703Arg) mutation:
5´-GGAACTGGATATATCAGGACTGAGTTGATTTCTGTGTCTG-3´and
5´-CAGACACAGAAATCAACTCAGTCCTGATATATCCAGTTCC-3´
STAT1-I702R (Ile702Leu) mutation:
5´-GGAACTGGATATAGGAGGACTGAGTTGATTTCTGTGTCTGAA-3´and
5´-TTCAGACACAGAAATCAACTCAGTCTTCCTATATCCAGTTCC-3´
STAT1-E705A (Glu705Ala) mutation:
5´-GGAACTGGATATATCAAGACTGCGTTGATTTCTGTGTCTGAA-3´and
5´-TTCAGACACAGAAATCAACGCAGTCTTGATATATCCAGTTCC-3´
STAT1-S727D (Ser727Asp) mutation:
5´-ACAACCTGCTCCCCATGGATCCTGAGGAGTTTGAC-3´ and
5´-GTCAAACTCCTCAGGATCCATGGGGAGCAGGTTGT-3´
STAT1-S727E (Ser727Glu) mutation:
5´-ACAACCTGCTCCCCATGGAACCTGAGGAGTTTGAC-3´ and
5´-GTCAAACTCCTCAGGTTCCATGGGGAGCAGGTTGT-3´
STAT1-Y701F-mutation and STAT1-S727A-mutation have been previously described (Aittomaki et al., 2000, Wen, Zhong and Darnell, 1995)**.**

SUMO-1-Flag and SUMO-1-Flag-Flag cloned in pcDNA3 vector were a kind gift from Dr. Yasuda. pFLAG-PIAS1 and pFLAG-PIAS3 were gifts from Dr. K. Shuai**.** Flag-PIAS1mut (PIAS1Δ310-407), 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-luciferase reporter construct contains a GAS site TTTCCCCGAAA from the IRF-1 promoter inserted upstream of the thymidine kinase (TK) promoter driving the firefly luciferase (luc) coding region (Pine, Canova and Schindler, 1994). Flag-SENP1 and SENP1 C603S mutant were described (Bailey 2002 and O´Hare, 2004). Dominant negative p38MAPKα (p38AF) and constitutively active MAP-kinase kinase MKK6b (MKK6b (E)) were kindly provided by Dr. J. Han. Constitutively active MKK1 and Raf-1 were provided by Dr. N. Ahn and Dr. U. Rapp.

7.2 Antibodies, cytokines and inhibitors

Anti–SUMO-1 (mouse anti-GMP1) antibody was purchased from Zymed (San Francisco, CA) and anti-HA (anti-influenza virus hemaglutinin, clone 16B12) antibody was purchased from Berkeley-Antibody (Richmond, CA). Anti-Flag M2 monoclonal antibody was purchased from Sigma (St-Louis, MO). Rabbit polyclonal anti-P-S727-STAT1

antibody was from Calbiochem, (San Diego, CA), rabbit polyclonal anti-P-p38MAPK (Thr180/Tyr182) antibody and mouse monoclonal anti-P-ERK1/2 (Thr202/Tyr204) were from Cell Signaling Technology (Danvers, MA). Rabbit polyclonal anti-p38MAPK (N-20) and rabbit polyclonal anti-IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-STAT1 antibody, N-terminus was form Transduction Laboratories, BD Bioscience (Lexington, KY) and polyclonal anti-P-Y701-STAT1 was from New England Biolabs (Beverly, MA, USA). Biotinylated anti-mouse antibody for Western blot was from Dako A/S (Glustrup, Denmark) and streptavidin-biotin horseradish peroxidase-conjugate was from Amersham Pharmacia Biotech (Buckinghamshire, UK). Human and mouse interferon-γ were purchased from R&D systems (Minneapolis, MN) or Immunogenex (Los Angeles, CA). p38MAPK inhibitor SB202190, was purchased from Calbiochem and PD98059 (Calbiochem) was kindly provided by Dr. J. Westermarck.

7.3 Cell lines and culture

Human HeLa and monkey COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS; Gibco BRL, Life Technology, Gaithersburg, MD), 100 units/ml of penicillin and 100 μg/ml of streptomycin. Human fibrosarcoma U3A cells (kindly provided by Dr. I. Kerr) and U3A-STAT1 stable cell lines were cultured in DMEM containing 10% Cosmic calf serum (HyClone Labs Inc., Logan, Utah) and antibiotics.

7.4 Transfection of cell lines

7.4.1 Calcium phosphate coprecipitation

Transfection of Hela-, U3A cells and U3A- STAT1-stable clones was performed using the calcium phosphate precipitation method. Cells were plated 60% confluent 24 hours before transfection. Plasmid DNA, 62 μl of 2 M $CaCl_2$ and water up to a total of 500 μl were mixed with 500 μl of 2X HEPES solution and the mixture was added to semiconfluent cells. 24 hours after transfection, the cells were either left untreated or treated with IFN-γ for 6-8 hours before luciferase assay.

7.4.2 FuGene 6

1.5×10^5 or 5.0×10^5 COS-7 cells were plated onto 6-well or 10 cm plates and 24 hours later transfected with FuGene 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) according to manufacturer`s instructions. 36 hours after transfection cells were either left untreated or treated with IFN-γ or together with sorbitol.

7.4.3 Electroporation

COS-7 cells transfected by electroporation with Bio-Rad GenePulser apparatus, 260V, 960 μF, with 0.5 cm cuvettes. Plasmid DNA was mixed with herring sperm DNA added up to 40 μg and mixed with the cells in 250μl final volume (4×10^6 COS-7 cells) prior to electroporation. Cells were plated onto 10 cm plates and after 48 hours cells were either left untreated or treated with IFN-γ.

7.5 Reporter gene assay

HeLa cells, U3A cells and U3A-STAT1 stable cells were plated onto 24-well dishes and transfected with 0.25 μg pCMV-βgal as an internal transfection efficiency control and 0.25 μg GAS-luc. Stable U3A clones were transfected with an increasing amount of CBP (0.25, 0.5, 1.0μg). After 24 hours the cells were stimulated with 10 ng/ml human IFN-γ (R & D systems) for an additional 6 hours and lysed in Promega's (Madison, Wisconsin) reporter lysis buffer according to the manufacturer's instructions. Luciferase activity was measured using Luminoscan Ascent (ThermoElectron Corporation, Finland) and normalized against the β-galactosidase activity of the lysates.

7.6 Electrophoretic mobility shift assay (EMSA)

U3A clones were either left unstimulated or stimulated at different times with IFN-γ and then washed in ice-cold PBS. Cells were 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´-CTAGAGCCTGATTTCCCCGAATGATGA-3´ and its complement) was end labelled with [γ-^{32}P] ATP using T4-polynucleotide kinase. The binding assay was performed by incubating the cell extracts on ice with herring sperm DNA followed by incubation with GAS [γ-^{32}P] labelled oligonucleotides. The reaction was resolved by 4.5 % TBE-PAGE and visualized using autoradiography.

7.7 Immunoprecipitation and Western blot analysis

Immunoprecipitation
For the detection of STAT1 sumoylation COS-7 cells were transfected with 2 μg STAT1, 0.6 μg of SUMO-1, 2.5 μg PIAS1, 1 μg PIAS mut, 4 μg ARIP3, 0.5 ARIP3 mut in order to obtain similar expression levels. To detect the effect of SENP1 Cos-7 cells were transfected with 2 μg STAT1-WT, 1 μg SUMO-1, 2 μg SENP1 and 2 μg SENP1 C603S mutant. 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. The lysates were incubated with anti-STAT1 antibody (monoclonal anti-STAT1 N-terminus) or anti-HA antibody. The

immunocomplex was washed and subjected to Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were detected with immunoblotting. STAT1 and sumoylated STAT1 protein levels were determined with anti-STAT1 and anti-HA (clone 16B12) antibodies. SENP1 protein levels were determined by immunoblotting of total cell lysates with primary anti-FLAG antibody. STAT1 protein levels from luciferase samples were analysed by immunoblotting using anti-HA antibody.

Co-immunoprecipitation

To study PIAS1 binding to STAT1 the transfected COS-7 cells were left unstimulated or stimulated with IFN-γ (50ng/ml) and sorbitol (0.3M) for 30 min. Cells were resuspended in NP-40 lysis buffer (50mM Tris-HCl, pH 7.5, 150mM NaCl, 1mM EDTA, 0.5% NP-40, 10% glycerol and 50mM NaF) supplemented with PMSF, aprotinin and NEM (N-ethylmaleimide). Flag-tagged PIAS1 was immunoprecipitated from lysates with anti-Flag M2 monoclonal antibody and rabbit polyclonal anti-IgG was used as a control. Immunoprecipitated proteins were separated by SDS-PAGE. PIAS1 was detected by immunoblotting with anti-FlagM2 antibody and co-precipitated STAT1 was detected by anti-HA antibody.

Western blotting

To study the effect of Ser727 phosphorylation on sumoylation of STAT1, transiently transfected Cos-7 cells were starved and left unstimulated or stimulated with IFN-γ (100ng/ml) for 30 min. To cause on osmotic shock, the cells were treated with 0.3 M sorbitol and then washed in ice cold PBS and lysed in Triton X lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 1% Triton X-100 and 10% glycerol) supplemented with protease inhibitors and 10 mM NEM (N-ethylmaleimide) (Calbiochem, San Diego, CA). The lysates were cleared by centrifugation for 20 min at 4°C. The amount of protein was determined using Bio Rad Dc protein assay Kit (Bio-Rad Laboratories).

Immunodetection: after SDS-PAGE gel electrophoresis, the proteins were transferred onto a nitrocellulose membrane (Protran, Schleicher & Schuell GmbH, Germany) and blocked with 5% non-fat dried milk in TBS 0.1% Tween 20. The filters were incubated with the specific antibodies diluted in TBS 0.05% Tween 20. Immunodetection was performed using enhanced chemiluminescence system ECL (Amersham Pharmacia Biotech, Buckinghamshire, UK).

7.8 GAS-oligoprecipitation

COS-7 cells were transfected with 2 μg STAT1-WT or STAT1- KR- or STAT1-EA-mutants, 0.7 μg SUMO-1 and left unstimulated or stimulated with 50 ng/ml of hIFN γ (Immugenex, Los Angeles, CA) for 30 minutes and together with osmotic shock (0.3M sorbitol) for 15 minutes. The cells were lysed in Triton X lysis buffer (0.2% Triton X-100, 10 mM HEPES pH 8, 2 mM EDTA, 1 mM EGTA, 0.3 M KCl, 1 mM DTT, 10% glycerol, 1 mM NaF) supplemented with protease inhibitors. The lysates were diluted threefold with lysis buffer lacking KCl.

For the binding assay, a biotinylated oligonucleotide containing GAS from the human GBP1 (guanylate binding protein gene, 5´-GGATCCTACTTTCAGTTTCATATTACTCTAAAT-3´) was annealed and 3 nmols of

biotinylated oligonucleotide duplex were incubated with Neutravidin agarose to form GAS-agarose affinity beads. Diluted cell extracts were precleared with Neutravidin beads and then incubated with GAS-agarose affinity beads. Bound proteins were analysed by SDS-PAGE and immunoblotting. STAT1 was detected with anti-HA and anti- Tyr701-phospho-STAT1 (New England Biolabs, Beverly, MA) antibodies. STAT1 and sumoylated STAT1 levels were detected from lysates with anti-HA antibody.

7.9 Chromatin immunoprecipitation

Stable U3A-STAT1-WT-HA and U3A-STAT1-KR-HA clones were starved and left unstimulated or stimulated with IFN-γ for 1h. Chromatin was cross-linked with 1% formaldehyde and the reaction was stopped with 100 mM glycine. Cells were lysed and chromatin was sonicated to a length of 200-500 bp using Vibra Cell 500 watt sonicator (Sonics and Materials, Inc., Newton, CT). Samples were precleared with protein A beads and immunoprecipitated using anti-acetyl-histone H4 antibody (Upstate Biotech, Lake Placid, NY) or as a control, anti-IgG antibody (Santa Cruz Biotechnology). Immunocomplexes were collected onto protein A beads and DNA was phenol/chloroform-extracted. Ethanol-precipitated DNA was analysed for human GBP-1 promoter by quantitative real time PCR with the following primers: 5´-AGCTTCTGGTTGAGAAATCTTT-3´ and 5´-CCCTGGACTAATATTTCACTG-3´. Quantitative PCR was done using SYBR green I kit from Qiagen (Valencia,CA) according to manufacturer's instructions. The values from ChIP assays were normalized to the total input DNA

7.10 Quantitative RT-PCR

Stable U3A clones were either treated or untreated with IFN-γ and total RNAs were extracted using TRIZOL reagent (Gibco-BRL). The first-strand cDNA synthesis was performed from 3 μg of total RNA using First Strands cDNA Synthesis Kit (MBI, Fermentas, Ontario, Canada). The PCR amplifications were performed using the LightCycler instrument (Roche Applied Science) with the QuantiTect SYBR Green Kit (Qiagen). For normalization, the amount of GAPDH3 mRNA was measured in each cDNA sample. Standard curves were generated using G3*pdh* cDNAs.

7.11 Immunofluorescence detection

Stable U3A clones, plated 60% confluent, were serum starved and either treated or untreated with IFN-γ. Cells were fixed and permeabilized in 4% p-formaldehyde 0.2 % Triton-X 100 in PEM buffer (100mM PIPES, 5mM EGTA, 2 mM $MgCl_2$, pH 6.6) and methanol (100%) and stained with anti-P-STAT1 antibody and anti-STAT1 (N-term) -antibody for 1 h followed by TexasRed anti-rabbit and Alexa 488 anti-mouse stainings for additional 1 h. Subcellular localization of STAT1 was visualized under an Olympus IX70 confocal microscope equipped with a 100X/1.4 oil immersion objective (Olympus,

Melville, NY). Green emission was detected through 488mm/10mm and 525 mm/50mm filters. Red emission was detected through 568 mm/10 mm and 608 mm/ 45 mm filters (Perkin-Elmer Life Sciences, Waltham, Massachusetts)

7.12 Molecular modelling

A 3D-structure of STAT1-dimer with DNA was built using high-resolution crystal structure of tyrosine phosphorylated STAT-1-DNA complex (PDB code:1BF5) (Chen et al., 1998). The molecular geometry of the loop 684-699 in the SH2 domain was calculated using the Sybyl program with Amber 7 FF99 force field parameters. The initial structure for the loop region was constructed using the crossover loop structure from the SUMO-1-TDG (PDB code:1wyw) (Baba et al., 2005) as a model. First, during the energy and geometry minimization for the loop all hydrogen atoms and non-constraints were included in the protocol. Second, during the molecular dynamic refinement the constraints were applied for the outer part of the loop in the SH2 domain. After the loop modelling we used the deposited coordinates of SUMO1 (PDB code:1wyw) (Baba et al., 2005) in our model. The SUMO1 was set nearby the constructed loop so that its C-terminal residue (Gly97) was in the vicinity of the Lys703 of the STAT1. The disordered loop was also modelled with InsightII (2005, Accelrys Inc. InsightII - molecular modelling program. http://www.accelrys.comomain). The entire structure was then subjected to energy minimization using the molecular mechanics force field CVFF (consistent valence force field) and the steepest descent algorithm implemented under InsightII Discover program. During the minimization, the DNA and the atoms of the STAT1 residues 136-686, 700-710 were fixed.

8. RESULTS

8.1 Lys 703 residue of STAT1 is well exposed to SUMO-conjugation

Phosphorylation of STAT1 by Janus kinase proteins at Tyr701 mediates STAT1 homodimerization and its subsequent translocation into the nucleus. The JAK-STAT pathway is critical for cellular responses to microbial and viral infections where STAT1 regulates numerous of IFN induced genes (Darnell, Kerr and Stark, 1994). The activity of STAT1 is under strict control and PIAS1 and PIASy proteins have been shown to inhibit the transcriptional activity of STAT1 through direct interaction. The mechanism by which the PIAS proteins inhibit STAT activity is unclear, although PIASs have been suggested to regulate interaction between STAT and DNA (Liu et al., 1998, Liu et al., 2001). Recent findings in several laboratories have demonstrated that all PIAS proteins and Siz proteins in yeast function as SUMO E3-ligases and promote SUMO conjugation to several substrates (Hay, 2005). These findings prompted us to investigate the possibility that PIASs may have E3-ligase function towards STAT1 and promote its sumoylation.

8.1.1 The SUMO consensus site in the C-terminus of STAT1 is well exposed to sumoylation (I, II, IV)

Sequence alignment revealed that STAT1 contains two potential SUMO modification sites defined by consensus sequence ψKXE. These consensus sequences reside around Lys110 in the N-terminus and Lys703 in the C-terminus of STAT1. The consensus sequence surrounding Lys703 was of particular interest because of the close proximity to Tyr701 residue (I, Fig. 1B). To analyse the conformation of this consensus site we used information from the published structure of tyrosine phosphorylated STAT1 dimer in a complex with DNA (Chen et al., 1998). The SH2-domain and phosphotyrosine 701 of an adjacent monomer forms an interface between STAT1 monomers, where the phosphate group is recognized by the strictly conserved Arg602 residue of adjacent SH2 domain. Analysis of the SUMO conjugation site in this structure demonstrated that the side chains of Lys703 of both monomers formed a projection in the same direction as DNA, and formed a suitable site for covalent isopeptide bond between STAT1 and SUMO. Investigation of the structure and position of the side chain of Glu705 revealed that due to its hydrophilicity it was also pointing towards the protein surface, where its negative charge provides a site for interaction with Ubc9 conjugation enzyme (IV, Fig. 1).

To determine whether STAT1 is modified by SUMO, we mutated Lys703 residue to Arg and STAT1-WT (wild type) and STAT1-K703R mutant were transfected to COS-7 cells together with small amounts of SUMO-1. Ectopic expression of SUMO-1 was used because the detection of endogenous SUMO-1 conjugation has been proven to be difficult due to low amounts of unconjugated SUMO-1 in cells and additionally reversal of conjugation by SENPs. Covalent attachment of SUMO-1 to STAT1 was detected in the Western blot as a slower migrating, ~115 kDa band. By contrast, the band

corresponding to SUMO-modified STAT1 was not detected with the STAT1-K703R mutant (I, Fig 1A). Moreover, sumoylation experiments in cell-free conditions were performed. *In vitro* sumoylation of STAT1-WT was readily observed, whereas STAT1-K703R mutant was not sumoylated (I, Fig. 1C). To verify that the effect observed was due to conjugation sequence ^{702}IKTE^{705}, Ile in position 702 was mutated to Arg and Glu in position 705 to Ala. The *in vivo* SUMO-1 conjugation of these mutants was investigated together with STAT1-WT, and, as expected, STAT1-I702R and STAT1-E705A mutants failed to conjugate SUMO-1 (II, Fig 1A). In another similar study, where lysine 110 residue from the putative N-terminal sumoylation site was mutated to arginine, was still sumoylated *in vitro* (Rogers et al., 2003). Together these findings indicate that Lys703 is the major site for sumoylation. Furthermore, ectopic expression of SUMO-1 induced sumoylation of endogenous STAT1 in HeLa cells, suggesting that sumoylation modifies and regulates STAT1 *in vivo* (I, Fig 1D).

8.1.2 PIAS proteins function as E3 ligases in SUMO conjugation of STAT1 (I)

PIAS proteins are known to interact with STATs and to have SUMO E3 ligase activity towards several proteins. This prompted us to study whether PIAS proteins function as E3 ligases towards STAT1. COS-7 cells were transfected with STAT1-WT and SUMO-1 together with Flag-tagged PIAS proteins such as PIAS1, PIAS3, ARIP3 and RING-finger domain deleted mutant proteins, PIAS1mut and ARIP3mut. PIAS1, PIAS3 and ARIP3 proteins enhanced the sumoylation of STAT1 but RING-finger mutants (PIAS1mut and ARIP3mut) could not promote sumoylation. These results suggest that PIAS proteins function as SUMO E3 ligases and promote SUMO conjugation to STAT1 (I, Fig. 2A).

8.2 STAT1 activity is regulated by sumoylation (I, II)

The PIAS proteins promote sumoylation of transcription factors such as p53, AR and c-Jun and through this mechanism it modulates their transcriptional activity. We wanted to investigate whether sumoylation regulates the transcriptional activity of STAT1 and whether STAT1 sumoylation is regulated through endogenous IFN-γ receptor. For this purpose COS-7 cells were transiently transfected with STAT1 and a small amount of SUMO-1 and left untreated or treated with hIFN-γ for the times indicated. IFN-γ treatment readily induced sumoylation of STAT1 within 30 minutes and the peak was observed in 2 hours, indicating that IFN-γ induced activation of STAT1 serves as a signal for SUMO-conjugation (I, Fig 2B). The effect of sumoylation on STAT1 mediated transcriptional activity was studied in U3A cells, transiently or stably expressing STAT1-WT or the STAT1-K703R mutant. When GAS-Luc-reporter construct was introduced to the stable cells, the clones expressing the STAT1-K703R-mutant showed enhanced transcriptional response to IFN-γ compared to STAT-WT clones (I, Fig 2D). In transient transfection to U3A cells STAT-K703R mutant and another sumoylation deficient mutant STAT1-E705A were also more potent transactivators than STAT1-WT, indicating that sumoylation has a repressive effect on STAT1 activity (II, Fig 1B).

8.2.1 STAT1-mediated gene responses are selectively modulated by sumoylation (II)

To examine whether STAT1 sumoylation has a general or specific impact on the regulation of IFN-γ induced genes, expression levels of interferon regulatory factor (IRF)-1, GBP1 and transporters associated with antigen presentation (TAP)-1 were analysed. For this purpose RNA from IFN-γ treated U3A stable clones was extracted and expression levels was analysed by using Quantitative-real time-PCR. STAT1-K703R mutant clones showed enhanced induction of GBP1 and TAP1 genes compared to STAT1-WT clones. Interestingly, K703R mutation did not have effect on IRF-1 gene activity, indicating that sumoylation of STAT1 selectively regulates IFN-γ responses (II, Fig. 1D).

8.2.2 SUMO-1 conjugation modulates IFN- γ induced subcellular distribution of STAT1 (II)

In unstimulated cells STAT1 is mainly a cytoplasmic protein which undergoes rapid import to the nucleus and export from the nucleus to the cytoplasm upon IFN-γ stimulus. Several studies have proposed that some SUMO substrates have different localization in the cell after SUMO conjugation. To analyse whether STAT1-WT and STAT1-K703R have distinct subcellular localization in cells, U3A stable clones were stimulated with IFN- γ for different times. After fixation the cells were stained with anti-P-Tyr701-STAT1 and anti-STAT1 antibodies and P-Tyr701-STAT1 and STAT1 were detected by immunofluorescence microscopy. After stimulation for 1 hour, STAT1-WT and STAT1-K703R-mutant were mainly located in the nucleus. A clear difference in cellular distribution was observed after 2 hours, when STAT1-WT relocated to the cytoplasm, whereas cytoplasmic relocalization of STAT1-K703R-mutant was delayed, when it remained in the nucleus for up to 4 hours (II, Fig 2).

8.2.3 Sumoylation inhibits DNA binding activity of STAT1 (II, IV)

To investigate the mechanism of sumoylation mediated inhibition of STAT1 we conducted DNA binding experiments. EMSA assay was performed to study DNA binding of STAT1-WT and STAT1-K703R-mutant. In U3A stable clones, the STAT1-K703R-mutant showed enhanced and prolonged DNA-binding activity after IFN-γ stimulation (II). We also conducted an oligoprecipitation experiment in COS-7 cells transiently transfected with STAT1-WT, K703R or E705A-mutants together with SUMO-1 plasmid. Cells were either left untreated or treated with IFN-γ together with osmotic shock to achieve strongly enhanced sumoylation. STAT1-WT, K703R or E705A-mutants were oligoprecipitated with biotinylated GBP-1-oligo and both STAT1-K703R and E705A-mutants showed enhanced DNA binding compared to STAT1-WT. Additionally, no sumoylation was detected in the DNA bound fraction of STAT1, suggesting that SUMO-1 moiety in STAT1 influences its DNA-binding properties (IV, Fig. 3 A, B).

To validate the DNA binding experiments further we analysed whether sumoylated STAT1 is a substrate for SUMO protease SENP1. STAT1, SUMO-1 and wild-type SENP1 or SENP1 C603S mutant were transfected into COS-7 cells. Co-transfection of SENP1, but not the enzymatically inactive SENP1 C603S completely removed conjugated SUMO-1 from STAT1. These findings indicate that sumoylation of STAT1 is a reversible process and SENP1 can act as a SUMO-specific isopeptidase for STAT1. The identification of STAT1 as a substrate for SENP1 prompted us to investigate whether SENP1-mediated desumoylation affects the transcriptional activity of STAT1. For this purpose, STAT1 deficient U3A cells were transiently transfected with STAT1-WT and different amounts of SENP1 and SENP1 C603S mutant together with GAS-luc reporter plasmid. The wild-type SENP1 augmented STAT1-mediated transcription, whereas the catalytically inactive SENP1 C603S mutant slightly repressed transcription. Similar results were obtained in HeLa cells. Collectively these results indicate that desumoylation of STAT1 enhances its transcriptional activity and these results are well in line with the molecular model constructed (8.2.4) and DNA binding experiments (IV, Fig 5).

8.2.4 Molecular model of SUMO conjugated STAT1 (IV)

The molecular model of STAT1 dimer together with SUMO-1 was constructed to validate the DNA binding experiments. The structure of STAT1 dimer (PDB code: 1bf5) has a linker region (aa 684-699; loop structure) that is invisible on the electron density maps. The immediate vicinity of the sumoylation site to loop residues caused us to investigate and remodel this loop. To gain insight on this, we constructed a model of sumoylated STAT1 dimer using previously published coordinates of conjugated SUMO-1. The loop, amino acids 684-699, was reconstructed using two programs Sybyl with Amber7 FF99 force field and InsightII. SUMO-1 was positioned at conjugation distance, and the loop structure constructed was presented adjacent to the β-sheet structure of SUMO-1**.** This model proposes that interface between SUMO and the loop structure of STAT1 may direct the SUMO towards DNA and which, in turn, may directly or indirectly affect DNA binding of sumoylated STAT1 (IV, Fig. 2).

8.3 Sumoylation of STAT1 is regulated by phosphorylation of Ser727 residue (III)

Phosphorylation is an important mechanism for the regulation of posttranslational modifications. Recently, phosphorylation of several SUMO substrates has also been implicated in the regulation of their sumoylation. These findings prompted us to investigate if STAT1 sumoylation is also regulated through Ser727 phosphorylation. Our results revealed that STAT1 sumoylation was clearly enhanced due to Ser727 phosphorylation when COS-7 cells were transiently transfected with STAT1, SUMO-1 together with upstream kinases of p38MAPK or ERK1/2 pathways (III, Fig 3B). Sorbitol induced osmotic shock also enhanced both the phosphorylation and the sumoylation of STAT1. MAPK pathway specific induction of Ser727 phosphorylation and sumoylation was counteracted when cells were treated with p38MAPK inhibitor SB202190 and

ERK1/2 upstream (MEK) inhibitor PD98059. Sorbitol induced phosphorylation and sumoylation were counteracted by both of these inhibitors, suggesting that p38MAPK and ERK pathways can equally induce these modifications (III, Fig. 3C). Mutation of Ser727 to Ala abolished the p38MAPK-induced sumoylation of STAT1, indicating that the effect of p38MAPK activation on SUMO-1 modification is mediated through Ser727 phosphorylation of STAT1 (III, Fig. 4 A, B).

To validate the role of Ser727 phosphorylation in the regulation of sumoylation of STAT1, two mutations, Ser727Asp and Ser727Glu, which mimic phosphorylation of the Ser residue, were constructed. COS-7 cells were transiently transfected with STAT1-WT, STAT1-S727E, STAT1-S727D or STAT1-S727A together with SUMO-1 plasmid, and subjected to osmotic shock or left untreated. In contrast to STAT1-WT, Ser727Asp and Ser727Glu mutants showed clearly enhanced sumoylation in untreated samples, which were not markedly affected by osmotic shock. Next we investigated the underlying mechanism of Ser727 phosphorylation regulated sumoylation of STAT1. For this purpose, COS-7 cells were transiently transfected with STAT1-WT, STAT1-S727D or STAT1-S727A together with SUMO-1 and PIAS1. Cells were either left untreated, or treated with IFN-γ together with sorbitol. The interaction of PIAS1, STAT1-WT and different STAT1 mutants was studied by co-immunoprecipitation and after stimulation STAT1-WT was shown to interact with PIAS1. In all the conditions to which samples were subjected, STAT1-S727A mutant failed to significantly coimmunoprecipitate with PIAS1. The phosphomimetic S727D mutant coimmunoprecipitated with PIAS1 already in unstimulated cells and stimulation further increased this protein interaction (III, Fig 5). Taken together, these results indicate that phosphorylation of Ser727, directly or indirectly, enhances the interaction between STAT1 and PIAS1. This finding provides a mechanistic explanation for the Ser727 phosphorylation promoted STAT1 sumoylation.

9. DISCUSSION

9.1 Multifunctional roles of sumoylation

Proteins with essential roles in diverse cellular processes are reversibly modified by SUMO. Many key proteins in nuclear transport, subnuclear targeting, DNA metabolism, chromatin structure, genome stability and transcriptional regulation are subjected to sumoylation. Perturbation of the SUMO conjugation system has dramatic consequences for the cells, including defects in the nuclear organisation and chromosome segregation (Hay, 2005). The E2 enzyme Ubc9 is an essential component of the SUMO conjugation cascade and it is essential for viability in most eukaryotes. Recent studies on Ubc9 deficient mice have revealed that lack of Ubc9 leads to dramatic dysfunction of the SUMO conjugation system. Ubc9 deficient mice embryos die shortly after the blastocyst stage and in culture, mutant blastocysts are viable for up to 2 days before the cells undergo apoptosis. Mutant cells exhibit chromosome defects and disassembled PML nuclear bodies, misshapen nuclei and abnormal localization of RanGAP1 (Nacerddine et al., 2005). Interestingly, homozygous PIAS mutants of *Drosophila* exhibited defects resembling the Ubc9 deficiency (Hari, Cook and Karpen, 2001). The mutation of one homolog of mouse deconjugation enzyme SENP1/SuPr-2 was reported to cause placental abnormalities incompatible with embryonic development (Yamaguchi et al., 2005). These findings indicate that the SUMO pathway is crucial for nuclear architecture, accurate chromosome segregation of cells and for embryonic viability.

The tumor suppressor PML protein was originally identified in patients with acute promyelocytic leukemia (APL). Sumoylation of PML proteins is essential for the assembly of subnuclear structures called PML-nuclear bodies (NBs). PML-NBs have been implicated in a variety of cellular processes, including transcriptional regulation, DNA repair, genome stability, apoptosis, tumor suppression and have been proposed to serve as nuclear depots where different factors are targeted at or released from. A large proportion of PML and other proteins in PML-NBs are targets for covalent conjugation by SUMO (Johnson, 2004). Furthermore, several of these proteins, including PML, TDG, Ubc9, Daxx and SP100 interact non-covalently with SUMO. The recently identified SUMO interaction motif (SIM, V/I-V/I-X-V/I/L) enables PML to interact non-covalently with sumoylated proteins to form a PML-protein network. The combination of covalent conjugation of SUMO and non-covalent binding through SIM motif enables interaction of PML-NB proteins to establish the NB scaffold (Johnson, 2004, Kerscher, 2007).

9.2 Mechanism and function of STAT1 sumoylation

The biological effects and functions of sumoylation are diverse, but in the case of many transcription factors, SUMO modification negatively regulates transcriptional activity. Consistent with this idea, removal of SUMO by SENPs positively regulates the activity of several transcription factors (Hay, 2007, Johnson, 2004). Our findings, together with reports from other groups, indicate that STAT1 is modified by SUMO at a single lysine

residue 703. Another putative SUMO modification site in the N-terminus of STAT1 was also studied by Roger et al. Mutant STAT1, where lysine 110 was mutated to arginine was sumoylated as wild type STAT1 *in vitro*. Together with our findings this indicates that Lys 703 is the only sumoylation site in STAT1. Both groups reported that PIAS proteins function as SUMO E3-ligases and promote SUMO conjugation to STAT1 (Rogers, Horvath and Matunis, 2003, I). In STAT1-mediated transcription, sumoylation appears to mediate a negative, promoter-dependent regulatory function. Analysis of IFN-γ target genes suggested that sumoylation selectively modulates the induction of genes with weak affinity promoter (GBP-1) to STAT1, while strong affinity promoter (interferon regulatory factor 1; IRF-1) is not significantly affected (I, II).

The structural aspects of sumoylation of STAT1 were analysed using a molecular model of DNA bound STAT1 dimer (Chen et al., 1998). Analysis of this model together with experimental results indicated that despite the close proximity of Lys703 to Tyr701 in linear sequence, phosphorylation of Tyr701 does not spatially prevent SUMO conjugation to STAT1. Analysis of the SUMO consensus site in DNA bound STAT1 dimer revealed that a side chain of Lys703 projects towards DNA (IV). Lys703 is located adjacent to the dimerization interface and this prompted us to investigate if mutation of Lys to Arg could affect the dimerization of STAT1. The STAT homodimer forms a nutcracker-like structure that binds to DNA. The monomers are held together by an interaction formed between SH2-domains (Chen et al., 1998). Both Lys703 and Glu705 residues have highly hydrophilic side chains facing away from the hydrophobic core of the SH2 interface, and these side chains are unlikely to be involved in the formation of the β-sheet structure between two C-tail segments. However, the positively charged Lys703 offers an optimal site for the approach of SUMO and mutation to arginine will interrupt the formation of the covalent bond. The side chain of Lys703 has an interaction face with Glu632 residue of SH2 domain of the adjacent monomer. Presumably, the interaction with Glu632 prevents rotation of this flexible side chain and keeps orientation favorable for the SUMO conjugation. The molecular model of sumoylated STAT1 dimer suggested that SUMO might functionally affect the DNA binding activity of STAT1. Consistent with the molecular model, experimental analysis of DNA binding ability of STAT1, STAT1-K703R mutant and STAT1-E705A mutant indicated that the sumoylation defective mutants showed higher DNA binding activity. These findings suggest that sumoylation of STAT1 decreases the affinity of STAT1 towards the response element (IV).

Another group recently investigated sumoylation of STAT1 by overexpression of sumoylation defective STAT1-K703R and STAT1- E705A mutants in Stat1-/- MEFs and macrophages. The K703R mutant showed enhanced DNA binding activity and biological responses, while the STAT1-E705A mutant exhibited DNA binding, nuclear retention and biological response to IFN-γ similar to STAT1-WT (Song et al., 2006). Examination of the structure of STAT1 dimer revealed that the mutation of Glu705 to Ala does not affect the β-sheet structure in STAT1 dimer, but Glu residue is highly reactive and may be involved in interactions with other proteins. Mutation of the highly hydrophilic and charged Glu to hydrophobic and relatively inert Ala may affect the ability of STAT1 dimer to form contacts with regulatory proteins and affect the function of STAT1 in a cell

type dependent manner (IV). Nevertheless, our experiments indicated that the STAT1-E705A mutant has a similar function in U3A and COS-7 cells, showing enhanced transcriptional activity and DNA binding similar to STAT1-K703R-mutant. Importantly, overexpression of SENP1 in STAT1-K703R mutant clones did not affect the transcriptional activity of STAT1, whereas SENP1 increased the activity of STAT1-WT. Collectively these results indicate that desumoylation of STAT1 enhances its transcriptional activity. These results are well in line with the molecular model constructed and DNA binding experiments indicating that desumoylation directly regulates STAT1 activation and sumoylation has a functional importance in the regulation of STAT1 responses (IV).

9.3 PIAS1 and SUMO, negative regulators of STAT1 signaling

PIAS proteins have been shown to regulate the activity of several proteins involved in controlling gene transcription. PIAS proteins also include SP-RING domain, which is related to the zinc-binding RING fingers present in many E3 ubiquitin ligases and is required for the SUMO-E3 ligase activity of PIAS proteins (Johnson, 2004). PIAS proteins exert SUMO-E3 ligase activity towards various transcription factors, thus raising the question of the role of SUMO conjugation in transcriptional regulatory function of PIAS. The binding of PIAS1 to STAT1 dimer has been shown to decrease the DNA binding activity of STAT1 (Liao, Fu and Shuai, 2000, Liu et al., 1998). Both Ser90 phosphorylation and SUMO ligase activity are required for the inhibition of promoter binding of p65 and STAT1 and the repression of gene regulation (Liu et al., 2007). PIAS1 has been shown to selectively regulate a subset of IFN-γ and IFN-β responsive genes in macrophages, and the absence of PIAS1 affected promoters showing low affinity to STAT1 such as GBP1 without affecting promoters with stronger affinity binding sites (interferon regulatory factor 1, IRF1). Thus, these results demonstrate that PIAS1 selectively regulates IFN-induced gene responses, but the mechanism of this modulation has not been firmly established (Liu et al., 2004). Analysis of the physiological effect of the SUMO deficiency of STAT1 on IFN-γ induced genes, such as IRF1, GBP1, TAP1 revealed similarities with results from PIAS1$^{-/-}$mice (Liu et al., 2004). STAT1 sumoylation deficient cells showed enhanced GBP1 and TAP1 expression levels, whereas IRF1 levels were unchanged, suggesting that PIAS1 and SUMO may share a common mechanism in STAT1 inhibition.

Our immunofluorescence experiments indicated that SUMO-1 is mainly a nuclear protein that colocalizes with STAT1-WT in the nucleus. This finding proposes that STAT1 is imported into the nucleus, where it associates with SUMO-conjugation machinery (unpublished data). Findings, demonstrating that PIAS1 interacts with tyrosine-phosphorylated STAT1 dimers, but not (tyrosine phosphorylated or unphosphorylated) STAT1 monomers (Liao, Fu and Shuai, 2000, Liu et al., 1998) are in line with our molecular model, where SUMO was proposed to be conjugated to tyrosine phosphorylated STAT1 dimer. These findings provide evidence for the importance of PIAS1 modulated sumoylation as a regulatory mechanism in physiological processes and

add an additional layer to the control of the transcriptional activity of STAT1 in the nucleus.

9.4 Convergence of signaling pathways in regulation of STAT1

STATs are activated by a variety of cytokines and inflammatory stimuli, which in turn induces the expression of immune responsive genes. The function of STAT1 is regulated by various posttranslational modifications that act in a highly coordinated fashion to combine signals from secreted cytokines as well as from microbial agents. IFN-γ stimulation induces Tyr701 phosphorylation and DNA-binding of STAT1, but the transcriptional response is also regulated by phoshorylation of Ser727 in the transactivation domain. Phosphorylation of Tyr701 and Ser727 are independent events, but they act in concert as tyrosine phosphorylation and presumably the dimer formation of STAT1 enhance serine phosphorylation (Kovarik et al., 1998, Kovarik et al., 1999, Varinou et al., 2003). Interferons have been reported to cause Ser727 phosphorylation in addition to tyrosine phosphorylation, but stress stimuli like UV, LPS, TNFα, and osmotic stress cause Ser727 phosphorylation of STAT1 independently of Tyr701 phosphorylation (Kovarik et al., 1998, Kovarik et al., 1999). In this IFN-independent pathway, the STAT1 phosphorylating serine kinase is most likely p38MAPK. These two groups of signals synergize and cause transcriptional response through STAT1.

In macrophage activation bacterial signals (lipopolysaccharide) synergize with IFN-γ, and this is required for the maximal induction of STAT1-mediated gene activation (Kovarik et al., 1998, Kovarik et al., 1999, Varinou et al., 2003). Microbial infection induces Ser727 phosphorylation through the p38MAPK pathway positioning Ser727 as a converging point in immune surveillance (Kovarik et al., 1998). This implication of Ser727 phosphorylation of STAT1 in p38MAPK activation and recent reports showing serine phosphorylation to regulate the sumoylation of several transcription factors (Desterro, Rodriguez and Hay, 1998, Hietakangas et al., 2003, Muller et al., 2001, Muller, Matunis and Dejean, 1998, Yang et al., 2003), prompted us to investigate if p38MAPK pathway regulates the sumoylation of STAT1. Our results clearly demonstrated that SUMO-1 modification of STAT1 is regulated through MAPKs, p38MAPK and ERK1/2, in a Ser727 phosphorylation dependent manner (III). STAT1 combines signals derived from IFN-γ and LPS receptors during macrophage activation. Our finding that LPS induced activation of p38MAPK enhanced Ser727 phosphorylation and sumoylation of endogenous STAT1 in RAW 264.7 macrophage cell line suggests that sumoylation may be of functional importance in immunological responses (unpublished data).

The activity of STAT1 is tightly regulated through negative regulatory proteins and the induction of negative feedback loops. For example, SOCS proteins are induced by inflammatory stimuli, which in return inhibit STAT signalling in the cytoplasm (Shuai and Liu, 2003). PIAS proteins are nuclear sensors that balance important immune responses by repressing the activity of transcription factors due to extracellular inflammatory stimuli. Pro-inflammatory and stress stimuli, such as TNFα, LPS and UV,

activate the serine kinase IKKα, which directly phosphorylates PIAS1 on Ser90 residue. The SUMO ligase activity of PIAS1 is required for IKKα-mediated PIAS1 phosphorylation and for PIAS1-mediated gene repression. Signals that trigger Ser90 phosphorylation of PIAS1 also induce p38MAPK mediated Ser727 phosphorylation of STAT1 (Kovarik et al., 1998, Liu et al., 2007). Interestingly, phosphorylation of Ser727 enhances the interaction between STAT1 and PIAS1, providing a likely explanation for the role of Ser727 phosphorylation as a promoting signal for sumoylation. Ser727 phosphorylation is at the convergence of STAT1 signalling and immune responses, and may function as a switch between activation and repression signals to retain the strict regulation of STAT1 activity. It is plausible that phosphorylated Ser727 is recognized by a sumoylation complex which induces PIAS binding and sumoylation of STAT1. This would posit PIAS1 as an important mediator of the crosstalk between IKKα and p38MAPK signalling pathways to control STAT1 dependent inflammatory responses.

The mechanism by which SUMO regulates the activity of transcription factors is unclear, but some results suggest that SUMO is a mediator of protein interactions in repressive transcriptional complexes. Candidate factors in these complexes are HDACs, the repressor protein Daxx and PIAS proteins. Daxx and PIASs have been reported to have functional SIM motifs. The functional relevance of the SIM motif of Daxx is in the tethering to and repression of promoter bound glucocorticoid receptor complexes. Daxx also interacts via its SIM motif with AR, Smad4 and CBP, and mediates their sumoylation dependent repression (Hay, 2005, Johnson, 2004, Lin et al., 2006). These findings suggest that other transcriptional complexes where the transcription factors or cofactors are sumoylated may also form repression complexes through their SIM motifs. Interestingly, structural analysis of phosphorylated STAT1 dimer revealed that STAT1 has a putative SIM motif. This motif is located in the DNA binding domain of STAT1, disclosing it an attractive candidate as a regulator motif for STAT1 activity. However, this motif is partially masked by a β strand, and conformational changes would be required for this motif to be exposed for binding of sumoylated protein. It is plausible that sumoylated STAT1 recruits cofactor complexes containing PIAS, and the fact that STAT1 and PIAS1 can be both covalently sumoylated and additionally that they have putative SIM motifs, gives an interesting perspective for future studies to define the mechanism behind this important interaction in the regulation of STAT1 activity.

9.5. CONCLUSIONS

Increasing evidence has demonstrated that the JAK-STAT signalling pathway is under strict control and negatively regulated at various stages. The activity of Stats has been reported to be regulated by dephosphorylation, interactions with negative regulators including SOCS, PIAS and SLIM (STAT-interacting LIM protein) and by post-translational modifications of Stats. Stats have been reported to undergo several covalent modifications, such as tyrosine and serine phosphorylation, acetylation, methylation, ubiquitination, ISG15ylation and O-glycosylation. The aim of this study was to characterize the mechanisms involved in the negative regulation of STAT1 specifically at the point of sumoylation, phosphorylation and PIAS inhibition.

The mechanism by which the PIAS proteins inhibit STAT activity is unclear, although PIASs have been suggested to regulate interaction between STAT and DNA. Recent findings in different laboratories have demonstrated that all PIAS proteins and Siz proteins in yeast function as SUMO E3-ligases and promote SUMO conjugation to several substrates. These findings prompted us to investigate the possibility that PIASs might have E3-ligase function towards STAT1. Our studies identified STAT1 as a substrate for SUMO-1 conjugation and this posttranslational modification was shown to have a negative regulatory function in STAT1 mediated gene responses. The molecular model of sumoylated STAT1 and the DNA binding experiments suggested that the SUMO reduces binding of SUMO moiety of STAT1 to DNA**.** Interestingly, the PIAS family of E3 ligases was shown to promote STAT1 sumoylation.

Phosphorylation is an important modification in the regulation of other posttranslational modifications. Recently, phosphorylation of several SUMO substrates has also been implicated in the regulation of their sumoylation. These findings prompted us to investigate if STAT1 sumoylation is also regulated through Ser727 phosphorylation. Our results indicate that phosphorylation of Ser727 enhances STAT1 sumoylation and interaction between STAT1 and PIAS1.

These findings provide strong evidence for the importance of PIAS1 mediated sumoylation as a regulatory mechanism in physiological processes. PIAS promoted sumoylation of STAT1 adds an additional layer to control the transcriptional activity of STAT1 in the nucleus.

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PIAS proteins promote SUMO-1 conjugation to STAT1

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Brief report

PIAS proteins promote SUMO-1 conjugation to STAT1

Daniela Ungureanu, Sari Vanhatupa, Noora Kotaja, Jie Yang, Saara Aittomäki, Olli A. Jänne, Jorma J. Palvimo, and Olli Silvennoinen

Signal transducer and activator of transcription 1 (STAT1) is a critical mediator of interferon-γ (IFN-γ)–induced transcription that is regulated through posttranslational modifications and through transacting proteins such as protein inhibitor of activated STAT1 (PIAS1). PIAS proteins have been shown to function as E3-type small ubiquitin-like modifier (SUMO) ligases, and sumoylation has been identified as a modulatory mechanism for several transcription factors. Here we show that STAT1 is subject to SUMO-1 modification, and sumoylation occurs in vivo and in vitro at a single, evolutionary conserved amino acid residue Lys703. Members of the PIAS family of proteins were found to strongly stimulate sumoylation of STAT1. Furthermore, activation of STAT1 by IFN-γ or pervanadate induced SUMO-1 conjugation. Mutation of Lys703 in STAT1 resulted in increased IFN-γ–mediated transactivation, suggesting a negative regulatory function for sumoylation. These results indicate that STAT1 is covalently modified by SUMO-1 in cytokine signaling and that PIAS proteins promote SUMO-1 conjugation to STAT1. (Blood. 2003;102:3311-3313)

Introduction

Regulation of signal transducer and activator of transcription 1 (STAT1) involves posttranslational modifications such as phosphorylation of Tyr701 and Ser727 as well as arginine methylation.[1,2] STAT1 signaling is negatively regulated by protein tyrosine phosphatases (PTPs), suppressors of cytokine signaling (SOCS), and protein inhibitor of activated STAT1 (PIAS) proteins.[1-3] The family of PIAS proteins consists of 5 members that have been implicated in the regulation of several nuclear proteins. PIAS1 and PIAS3 were identified as interaction partners for STAT1 and STAT3, respectively, and they were found to inhibit the DNA-binding activity of activated STATs.[3-5] The other members, PIASxα/ARIP3 (androgen receptor–interacting protein 3), PIASxβ/Miz1, and PIASy, function as transcriptional coregulators for steroid hormone receptors.[6-8] Recently, several PIAS proteins have been shown to function as E3-type small ubiquitin-like modifier (SUMO) ligases.[9-12] SUMO-1, -2, and -3 are small modifier proteins that are covalently conjugated to specific lysine residues of target proteins.[13] A number of nuclear proteins, such as transcription factors AR, p53, and c-Jun, become modified by SUMO-1 conjugation, and this reversible posttranslational modification has been implicated in regulation of protein-protein interactions, protein stability, localization, or activity.[9,13,14] However, it is currently unknown whether STAT factors are modified through sumoylation.

Study design

Reagents

Antibodies used include anti–SUMO-1 (mouse anti–GMP-1) (Zymed, San Francisco, CA); anti-HA (clone 16B12) (Berkeley-Antibody, Richmond, CA); anti-Flag (anti-Flag M2) (Sigma Aldrich, St Louis, MO); anti-STAT1 (N terminus) (Transduction Laboratories, BD Biosciences); anti-STAT3 (Santa Cruz Biotechnology, Santa Cruz, CA) biotinylated antimouse (Dako, Glustrup, Denmark) and streptavidin-biotin horseradish peroxidase conjugate (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). Human interferon-γ (huIFN-γ) was purchased from Immugenex, Los Angeles, CA.

Plasmid constructs

SUMO-1–Flag and SUMO-1–Flag–Flag were a kind gift from Dr H. Yasuda.[15] The SUMO-1, Flag-PIAS1, Flag-PIAS1mut (PIAS1Δ310-407), Flag-PIAS3, Flag-ARIP3 Flag-ARIP3mut (ARIP3Δ347-418) plasmids[4,9] as well as STAT1-WT-HA and GAS-luc reporter construct have been previously described.[16] The STAT1 Lys703Arg mutation was created from STAT1-WT-HA using direct polymerase chain reaction (PCR) mutagenesis with the following primers: 5′GGAACTGGATATATCAGGACTGAGTTGATTTCTGTGTCTG-3′ and 5′-CAGACACAGAAATCAACTCAGTCCTGATATATCCAGTTCC-3′.

Transfections, luciferase assay, and immunodetection

COS-7 cells were electroporated using a Bio-Rad (Hercules, CA) gene pulser at 260 V and 960 microfarads (μF) and lysed in Triton X lysis buffer supplemented with 5 mM *N*-ethylmaleimide (NEM) (Sigma Aldrich). HeLa cells were transfected using a calcium phosphate method. For luciferase assay, U3A cells were transfected using a calcium phosphate method as described.[16] Immunodetection was performed as described.[16]

In vitro sumoylation reaction

GST–$SUMO_{GG}$-1, GST–Ubc-9, GST-SAE2, and GST-SAE1 were produced in *Escherichia coli* BL21-CodonPlus bacteria (Stratagene, La Jolla, CA) and purified with glutatione Sepharose 4B (Amersham Pharmacia Biotech).

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Purified proteins were eluted in buffer containing 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.8), 100 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, and 20 mM glutathione. The conjugation reaction was carried out as described.[9]

Results and discussion

We have previously analyzed PIAS proteins in regulation of steroid receptor and cytokine receptor–mediated transcription.[6,17] The SUMO E3 ligase function of PIAS proteins prompted us to examine whether STAT1 is subject to sumoylation. The level of free SUMO-1 in cells is low and apparently rate limiting, because ectopic expression of SUMO-1 increases the level of sumoylation of target proteins. To this end, COS-7 cells were transfected with HA-tagged STAT1 (STAT1-WT-HA) alone or together with SUMO-1, Flag-tagged SUMO-1 (Flag–SUMO-1), or tandem Flag–Flag–SUMO-1 (Figure 1A, lanes 1-4). STAT1 was immunoprecipitated with anti-HA antibody and analyzed by immunoblotting.

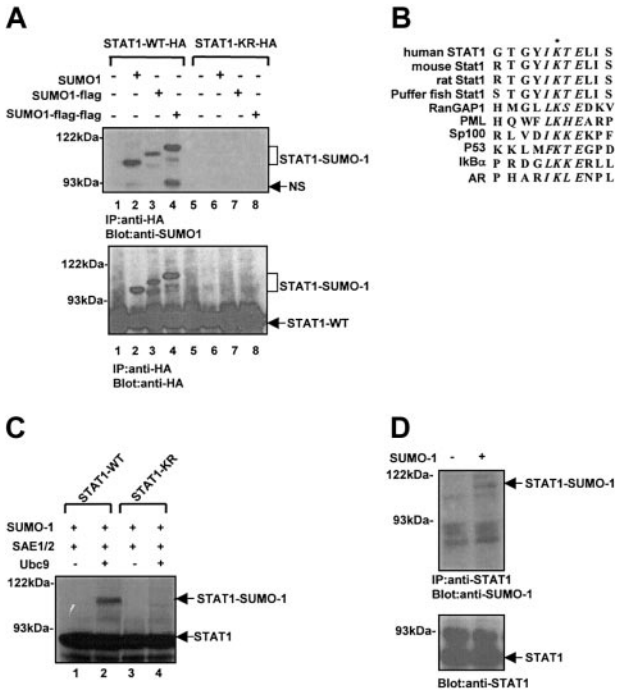

Figure 1. SUMO-1 conjugation to STAT1 in vivo and in vitro. (A) Sumoylation of STAT1-WT and STAT1-KR (Lys703Arg mutant) in COS-7 cells. COS-7 cells were transfected with 2 μg STAT1-WT-HA (lanes 1-4) or STAT1-KR-HA (lanes 5-8) together with different SUMO-1 constructs (2 μg) as indicated. After 36 hours the cells were lysed in Triton X lysis buffer (50 mM Tris [tris(hydroxymethyl)aminomethane]–HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA [ethylenediaminetetraacetic acid]; 50 mM NaF; 5 mM NEM; 1% Triton X-100; and 10% glycerol and protease inhibitors), and equal amounts of total cell lysates were immunoprecipitated with anti-HA antibodies and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with anti–SUMO-1 and anti-HA antibodies. (B) Sequence comparison of consensus SUMO-1 attachment sites in RanGAP1, PML, Sp100, p53, IκBα, AR, and STAT1. The consensus residues are marked in italics. The asterisk indicates the lysine that serves as a potential SUMO-1 attachment site. STAT1 sequence comparison starts at amino acid 698. (C) SUMO-1 conjugation of STAT1 in vitro. STAT1-WT (lanes 1-2) and STAT1-KR (lanes 3-4) were in vitro translated in the presence of [^{35}S]methionine using TNT-coupled transcription/translation system (Promega, Madison, WI). The in vitro–translated products were then used for an in vitro SUMO-1 conjugation reaction. The samples were resolved by SDS-PAGE gel and visualized by fluorography. (D) Sumoylation of endogenous STAT1. HeLa cells were untransfected or transfected with SUMO-1 (2 μg) plasmid using a calcium phosphate method. After 36 hours the cells were lysed as described in panel A. Sumoylation of endogenous STAT1 was analyzed after immunoprecipitation using anti-STAT1 antibody and Western blotting with anti–SUMO-1.

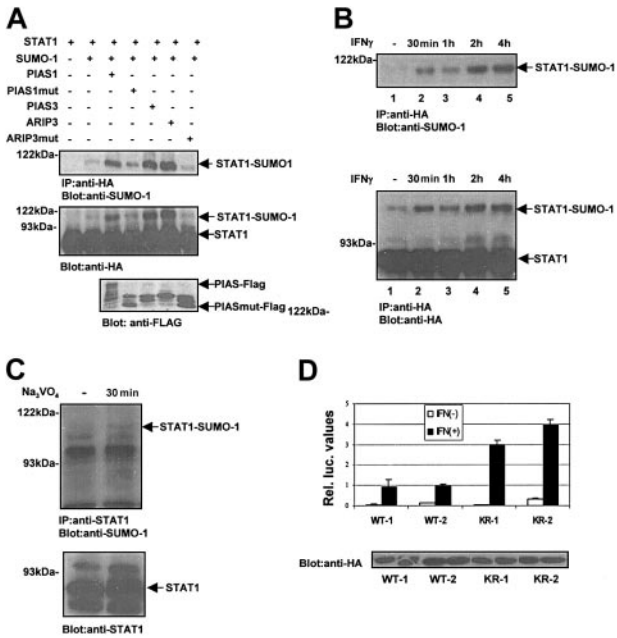

Figure 2. PIAS proteins and activation of STAT1 promote SUMO-1 conjugation. (A) PIAS proteins promote SUMO-1 conjugation to STAT1-WT in vivo. COS-7 cells were transiently transfected with plasmids encoding STAT1-WT-HA (2 μg) and SUMO-1 (0.6 μg) together with Flag-PIAS1 (2.5 μg), Flag-PIAS1mut (1 μg), PIAS3 (4 μg), ARIP3 (4 μg), and ARIP3mut (0.5 μg) as indicated. The plasmid amounts were adjusted to yield similar expression levels. After 36 hours the cells were lysed in Triton X lysis buffer, and STAT1 protein was immunoprecipitated using anti-HA antibody and analyzed by immunoblotting as indicated. PIAS and PIASmut protein levels were analyzed by immunoblotting of 15 μg total cell lysates with anti-Flag antibody. (B) IFN-γ stimulation enhances SUMO-1 conjugation to STAT1. COS-7 cells were transfected with plasmids encoding STAT1-WT-HA (2 μg) and SUMO-1 (1 μg). After 36 hours the cells were serum starved and stimulated with 100 ng/mL human IFN-γ for different time points as indicated. STAT1 was immunoprecipitated and analyzed by immunoblotting. (C) Pervanadate-induced phosphorylation of STAT1 enhances SUMO-1 conjugation. HeLa cells were starved overnight and left unstimulated or stimulated with 50 μM pervanadate for 30 minutes. Total cell lysates were prepared as described in Figure 1A, and STAT1 was immunoprecipitated with anti-STAT1 antibody, and immunoblotting was performed with anti–SUMO-1 or anti-STAT1 antibody. (D) The effect of Lys703Arg mutation on STAT1-mediated gene activation in response to IFN-γ stimulation. U3A clones stably expressing STAT1-WT-HA (U3A-WT1 and U3A-WT2) or STAT1-KR-HA (U3A-KR1 and U3A-HA-KR2) were transiently transfected with SUMO-1 (1 μg), GAS-luc reporter plasmid (0.5 μg), and pCMV-βGal. After 24 hours the cells were starved in 0.5% serum and left unstimulated or stimulated with 10 ng/mL human IFN-γ for 6 hours followed by luciferase measurement. The mean relative luciferase units ± SD from 3 independent experiments are shown. The lower panel shows the STAT1 levels in different clones.

Both anti–SUMO-1 and anti-HA antibodies reacted with a major band of about 110 kDa in cells transfected with STAT1-WT-HA and SUMO-1 (Figure 1A, lanes 1-2). The molecular mass of the recognized protein agrees with the covalent attachment of one SUMO-1 moiety to STAT1. In cells transfected with STAT1-WT-HA and Flag–SUMO-1 or Flag–Flag–SUMO-1, the mobility of the anti–SUMO-1 and anti-HA detected bands was shifted according to the presence of Flag sequences in the SUMO-1 expression constructs (Figure 1A, lanes 3-4). This conclusion was verified by immunoprecipitating the same lysates with anti-Flag antibody, where STAT1 was detected only in cells transfected with Flag-tagged SUMO-1 constructs (data not shown).

SUMO-1 is conjugated to targeted proteins at the ϵ-amino group of lysine residues.[13] The attachment of SUMO-1 to its substrates involves a minimal consensus sequence, ΨKxE (Figure 1B). The consensus SUMO-1 target sequence was identified around Lys110 and Lys703 in STAT1. The C-terminal sumoylation sequence is well conserved across species in STAT1, but it is not present in the transactivation domains of other STATs. To investigate whether Lys703 is a target for sumoylation, we constructed a STAT1

Lys703Arg mutation (STAT1-KR) and performed similar experiments as described above. The STAT1-KR mutant did not show any SUMO-1 conjugation (Figure 1A, lanes 5-8). Furthermore, we performed the sumoylation experiments under cell-free conditions (Figure 1C). In vitro sumoylation of STAT1–wild type (STAT1-WT) was readily observed, whereas STAT1-KR was not sumoylated. Together, these results indicate that STAT1 is subject to conjugation of a single SUMO-1 molecule at Lys703. Furthermore, ectopic expression of SUMO-1 induced sumoylation of endogenous STAT1 in HeLa cells (Figure 1D). While this paper was under review, sumoylation of STAT1 on Lys703 was reported also by Rogers et al.[18]

To investigate the regulation of STAT1 sumoylation and the role of PIAS proteins, the experimental conditions were modified by reducing the amount of exogenous SUMO-1. COS-7 cells were transfected with STAT1-WT-HA with or without different Flag-tagged PIAS proteins, PIAS1, PIAS3, ARIP3, and really interesting new gene (RING) fingerlike domain-deficient PIAS mutant (PIAS1mut, ARIP3mut) (Figure 2A). Cotransfection of different PIAS proteins strongly enhanced STAT1 sumoylation in a RING finger-dependent manner (Figure 2A). These results suggest functional redundancy of PIAS proteins as SUMO E3 ligases toward STAT1. Previously PIAS1 has been shown to promote sumoylation of several target proteins such as p53, AR, c-Jun.[9,14,15] Thus, various PIAS proteins can interact with several proteins and enhance sumoylation, and the specificity of these interactions and SUMO modification may also involve cell type–specific components.

Next we investigated whether sumoylation of STAT1 is regulated through endogenous IFN-γ receptor. As shown in Figure 2B, STAT1 was not detectably sumoylated in unstimulated COS-7 cells, but IFN-γ treatment readily induced sumoylation of STAT1 within 30 minutes with a peak at 2 hours. Furthermore, sumoylation of endogenous STAT1 was detected in HeLa cells after pervanadate treatment (Figure 2C), suggesting that activation of STAT1 serves as a signal for SUMO-1 conjugation.

The effect of sumoylation on STAT1-mediated transcriptional activity was assessed in U3A cell clones that stably express STAT1-WT or STAT1-KR mutant. The clones expressing the STAT1-KR showed enhanced transcriptional response to IFN-γ stimulation when compared with STAT1-WT clones (Figure 2D). Also in transient transfection system STAT1-KR was a more potent transactivator than STAT1-WT, but the difference was smaller (data not shown). These results suggest that sumoylation has a negative regulatory function for STAT1 and bears clear resemblance to the effects of sumoylation in AR, p53, and IRF-1–mediated gene activation.[6,14,15,19]

In summary, we show that STAT1 is subject to sumoylation in IFN-γ signaling through a single Lys703 residue. Interestingly, several proteins involved in modulation of IFN responses, such as interferon regulatory factor 1 (IRF-1)[19] as well as IFN-inducible proteins PML and SP100,[13] are subject to SUMO-1 conjugation. However, posttranslational modifications of STAT1 have been shown to mediate highly specific functions. Phosphorylation of Ser727 in STAT1 regulates only selected gene responses,[20] and ubiquitination of STAT1 is detected only in certain cell types.[21] The functions of PIAS proteins in cytokine and steroid receptor–mediated transcriptional regulation have been shown to involve both promoter- as well as cell type–specific components.[6] Likewise, SUMO-1 modification also may modulate highly specific and even cell type–restricted functions of STAT1.

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SUMO-1 conjugation selectively modulates STAT1-mediated gene responses

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Brief report

SUMO-1 conjugation selectively modulates STAT1-mediated gene responses

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Signal transducers and activators of transcription 1 (STAT1) is a critical mediator of interferon (IFN)–induced gene responses. Recently, STAT1 was found to become modified by small ubiquitin-like modifier 1 (SUMO-1) conjugation at Lys703 through the SUMO E3 ligase function of protein inhibitors of activated STAT (PIAS) proteins. However, the physiologic function of sumoylation in STAT1 is still unclear. Here, we show that mutations in the SUMO attachment site in STAT1 result in increased transcriptional activity in a fashion that is selective among IFN-γ target genes. The sumoylation-defective STAT1 mutant displayed increased induction of guanylate-binding protein 1 *(GBP1)* and transporters associated with antigen presentation 1 *(TAP1)* transcription but not interferon regulatory factor 1 *(IRF1)* transcription. Moreover, the sumoylation-defective mutant STAT1-KR showed a prolonged DNA-binding activity and nuclear localization in response to IFN-γ stimulation. These results suggest that sumoylation has a defined negative regulatory effect on selective STAT1-mediated transcription responses. (Blood. 2005;106:224-226)

Introduction

Signal transducers and activators of transcription 1 (STAT1) is critical for interferon-gamma (IFN-γ)–mediated immune responses, and its activation requires phosphorylation on tyrosine 701 by the receptor-associated Janus kinases (JAKs).[1] The function of STAT1 is also modulated by other posttranslational modifications, such as phosphorylation of Ser727 and, more recently, sumoylation at Lys703.[2-4]

Small ubiquitin-like modifier (SUMO)–1, –2, –3, and –4 are protein moieties covalently conjugated to specific lysine residues on substrate proteins through an enzymatic pathway.[5] Recently, the regulatory enzymes in these reactions have been characterized, and protein inhibitors of activated STAT (PIAS) proteins, initially identified as regulators of STAT and androgen receptor activation, were shown to function as E3-type SUMO ligases.[6,7] Sumoylation mediates divergent effects on transcription factors and can cause transcriptional repression or enhanced activation.[8] Previously, we and others showed that PIAS proteins promote the sumoylation of STAT1 at Lys703, but the effect of the Lys703 mutation on reporter gene responses varied, leaving the functional role of this modification elusive.[3,4] This study aimed to analyze the functional role of sumoylation in STAT1, and our results indicate that sumoylation mediates a negative, promoter-dependent regulatory function in STAT1-mediated transcription.

Study design

Reagents

Antibodies used were anti–SUMO-1 (mouse anti–GAP-modifying protein 1 [anti–GMP-1]; Zymed, San Francisco, CA); anti-HA (clone 16B12; Berkeley-Antibody, Richmond, CA); anti-Flag (anti-Flag M2; Sigma-Aldrich, St Louis, MO); anti-STAT1 (N-terminus; Transduction Lab, Becton Dickinson, Palo Alto, CA); anti–phospho-STAT1 (New England Biolabs, Beverly, MA); biotinylated antimouse (Dako A/S, Glostrup, Denmark); streptavidin-biotin horseradish peroxidase conjugate (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). Human IFN-γ (huIFN-γ) was purchased from Immugenex (Los Angeles, CA).

Plasmids

The SUMO-1, STAT1–wild-type–HA (STAT1-WT-HA), and STAT1-KR–HA plasmids have been previously described.[3]

STAT1-I702R-HA (Ile→Arg) and STAT1-E705A-HA (Glu→Ala) were constructed from STAT1-WT-HA using polymerase chain reaction (PCR) mutagenesis with the following primers: 5′-GGAACTGGATATAGGAAGACTGAGTTGATTTCTGTGTCTGAA-3′ and 5′-TTCAGACACAGAAATCAACTCAGTCTTCCTATATCCAGTTCC-3′; 5′-GGAACTGGATATATCAAGACTGCGTTGATTTCTGTGTCTGAA-3′ and 5′-TTCAGACACAGAAATCAACGCAGTCTTGATATATCCAGTTCC-3′.

Transfections and immunodetection

COS-7 cells were transfected using Fugene6 reagent and lysed in Triton-X lysis buffer supplemented with 5 mM NEM (N-ethylmaleimide; Sigma-Aldrich). HeLa cells were transfected using the calcium phosphate method. Immunodetection was performed as described.[3]

Quantitative RT-PCR and EMSA

Total RNA was extracted using TRIZOL (Gibco-BRL, Carlsbad, CA). Reverse transcription (RT) was performed using a First strands cDNA synthesis kit (MBI Fermentas, Burlington, ON, Canada). The primers for

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glyceraldehyde phosphate dehydrogenase (GAPDH), interferon regulatory factor 1 (IRF1), guanylate-binding protein 1 (GBP1), and transporters associated with antigen presentation 1 (TAP1) real-time PCR were previously described.[2] Detection of IFN-γ–activated sequence (GAS)–binding proteins by electrophoretic mobility shift assay (EMSA) has been previously described.[9]

Immunofluorescence detection

Stably transfected U3A clones 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) overnight followed by Texas Red and Alexa 488 staining and microscopy fluorescence detection.

Results and discussion

STAT1 is modified by SUMO-1 conjugation at Lys703 in vitro and in vivo in fibroblasts[3,4] and in hematopoietic myeloid cells (data not shown). Mutation of Lys703 to arginine (K→R) was found to enhance at variable degree the STAT1-dependent reporter gene activity in STAT1-deficient U3A cells, suggesting a negative regulatory function for sumoylation. To verify that the observed effect on STAT1 activity was due to and specific for SUMO modification, we mutated 2 amino acid residues within the SUMO-1 conjugation consensus *IKTE* (residues 702-705) motif of STAT1.[5] The in vivo SUMO-1 conjugation to the mutants was analyzed by transfecting HA-tagged STAT1-E705A and STAT1-I702R together with SUMO-1 into COS-7 cells followed by immunoblotting with anti–SUMO-1 and anti-HA antibodies (Figure 1A). As expected, STAT1-E705A and STAT1-I702R both failed to conjugate SUMO-1, indicating that the Ile702 and the Glu705 are essential to create the sumoylation motif in STAT1. The transcriptional activities of STAT1-WT, STAT1-K703R, and STAT1-E705A were analyzed using STAT1-dependent GAS-reporter gene assays in U3A cells. Both the K703R and the E705A mutants showed a similar increased transcriptional activity when compared with STAT1-WT (Figure 1B), implying that sumoylation has a repressive role on STAT1 transcriptional activity.

We wished to analyze the underlying mechanism for the effect of sumoylation on transcription. The close vicinity of Lys703 to the phosphorylation site Tyr701 raised the possibility that the bulky SUMO-1 moiety could interfere with the phosphorylation/dephosphorylation events on Tyr701. The K703 is not required for Y701 phosphorylation, since the K703R mutation did not significantly affect the magnitude and kinetics of STAT1 tyrosine phosphorylation in U3A clones (Figure 1C top panel). However, the sensitivity of Western blotting may not be sufficient to detect subtle changes. In stable U3A clones, the STAT1-KR mutation showed an enhanced and prolonged DNA-binding activity after IFN-γ stimulation when compared with STAT1-WT (Figure 1C middle panel).

Next, we wanted to analyze the physiologic effect of sumoylation on some well-characterized IFN-γ–induced genes, such as *IRF1*, *GBP1*, *TAP1*, and *TAP2*.[10-12] Quantitative RT-PCR using RNA from IFN-γ–treated U3A clones demonstrated an enhanced transcription of *GBP1* and *TAP1* in the STAT1-KR clones when compared with the wild-type clones (Figure 1D). Of interest, no significant differences between STAT1-WT and STAT1-KR were observed in the transcription of *IRF1* gene. These results suggest that sumoylation of STAT1 has a selective effect on IFN-γ–meditated gene responses.

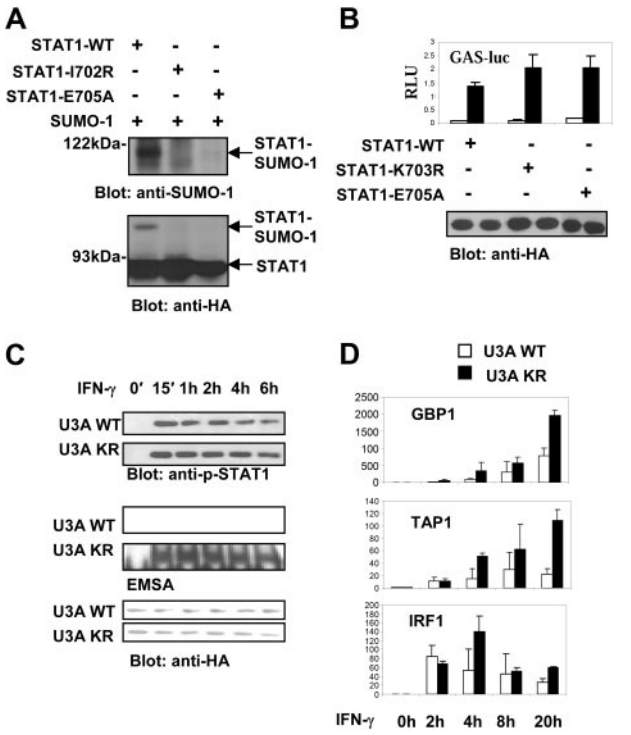

Figure 1. Mutation of the SUMO attachment site modulates STAT1-mediated transcription. (A) Point mutations in the SUMO consensus site affect SUMO-1 conjugation to STAT1. COS-7 cells were transfected with 0.7 μg of STAT1-E705A-HA, STAT1-I702R-HA, and STAT1-WT-HA together with SUMO-1 plasmid (0.2 μg) as indicated. After 36 hours the cells were lysed in Triton-X lysis buffer (50 mM tris(hydroxymethyl)aminomethane–HCl [Tris-HCl], pH 7.4; 150 mM NaCl; 1 mM EDTA [ethylenediaminetetraacetic acid]; 50 mM NaF; 5 mM NEM; 1% Triton-X 100; and 10% glycerol and protease inhibitors) and equal amounts of lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with anti–SUMO-1 and anti-HA antibodies. (B) K703R and E705A mutations increase the transcription activity of STAT1. STAT1-WT-HA, STAT1-K703R-HA, and STAT1-E705A-HA were transfected in U3A cells together with GAS–luciferase (luc) reporter plasmid (0.5 μg) and plasmid cytomegalovirus–βgalactosidase (pCMV-βGAL; 0.25 μg). After 24 hours the cells were starved and stimulated with 10 ng/mL huIFN-γ for 6 hours. The ± SD were calculated as a mean of 2 independent experiments. The bottom panel shows the STAT1 protein levels analyzed by Western blotting using anti-HA antibodies. RLU indicates relative luciferase units. (C) K703R mutation enhances DNA binding of STAT1. U3A STAT1-WT and STAT1-KR stable clones were treated with IFN-γ (100 ng/mL) as indicated and STAT1 DNA binding was analyzed by EMSA with ^{32}P-labeled GAS-IRF1 probe. The bottom panel indicates the STAT1 protein levels by anti-HA immunoblotting. (D) Analysis of GBP1, TAP1, and IRF1 gene activation in U3A STAT1-WT and STAT1-KR stable clones. Total RNA isolated from U3A stable clones stimulated with IFN-γ (10 ng/mL) for different times as indicated was subjected to RT-PCR using specific primers for GBP1, TAP1, and IRF1. The target gene expression levels were normalized against the expression values of GAPDH3. The results were calculated as the mean ± SD of 2 independent experiments for each type of clone.

Nuclear translocation is a marker for STAT1 activation. Latent STAT1 resides mainly in the cytoplasm of unstimulated cells and undergoes a rapid and transient nuclear accumulation after IFN-γ stimulation.[13] Nucleoplasmic STAT1 is actively exported to the cytoplasm in order to initiate another cycle of activation.[14,15] We analyzed the subcellular localization of STAT1-WT and STAT1-KR during IFN-γ stimulation in U3A stable clones using anti-STAT1 and anti–phospho-STAT1 antibodies by immunofluorescence microscopy (Figure 2). After one hour of IFN-γ stimulation, STAT1 is mainly translocated to the nucleus in both types of clones. However, after 2 hours of stimulation, STAT1-WT begins to redistribute to the cytoplasm whereas the STAT1-KR remains predominantly in the nucleus up to 4 hours after the stimulation. These results suggest that SUMO-1 conjugation affects nuclear

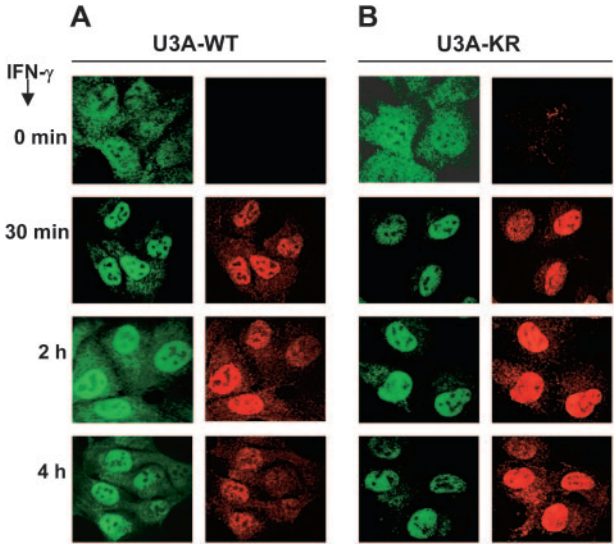

Figure 2. Differences in subcellular localization between STAT1-WT and STAT1-KR after IFN-γ stimulation. U3A STAT1-WT (A) and STAT1-KR (B) stable clones were serum starved overnight and stimulated with IFN-γ (100 ng/mL) for the indicated time points. STAT1 subcellular localization was visualized by immunofluorescence staining using specific anti-STAT1 antibody (left column of each panel) and anti–phospho-STAT1 (Y701) antibody (right column). Images were visualized under an Olympus IX70 confocal microscope equipped with a 100×/1.4 oil immersion objective lens (Olympus, Melville, NY) and an ULTRAPix ICX 085 camera (Perkin-Elmer, Wellesley, MA). Green emission was detected through 488 mm/10 mm and 525 mm/50 mm filters; red emission, through 568 mm/10 mm and 608 mm/45 mm filters (all filters from Perkin-Elmer Life Sciences, Cambridge, United Kingdom). UltraView 4.0 software (Perkin-Elmer) was used for image acquisition, and PhotoEditor software for Microsoft XP Office (Microsoft, Redmond, WA) was used for image analysis.

cytoplasmic redistribution of STAT1, which correlated with the observed differences in the DNA-binding activity. Nuclear export of STAT1 is dependent on functional nuclear export signal (NES) and requires dephosphorylation of STAT1 and dissociation from DNA.[14] Even though we did not detect differences in dephosphorylation of STAT1-WT in comparison to STAT1-KR using an in vitro dephosphorylation assay (data not shown), it remains possible that sumoylation affects the dephosphorylation in vivo.

Posttranslational modifications of STAT1 are highly specific processes as exemplified by the selective effect of STAT1-Ser727 phosphorylation on the expression of IFN-γ–induced genes.[2] The physiologic role of sumoylation of STAT1 is also interestingly related to the inhibitory function of PIAS1 on STAT1-mediated gene activation. Recent results from PIAS1$^{-/-}$ mice indicate that PIAS1 can function as a negative regulator of IFN-responsive genes and differentially affect the binding of STAT1 to various promoters of STAT1-dependent genes. PIAS1 had a profound impact on genes that contain weak STAT1 binding sites *(GBP1)* without affecting promoters with stronger affinity binding sites *(IRF1)*.[16] The precise molecular mechanism underlying the PIAS-mediated inhibition of DNA-binding activity is currently not known, but the inhibition is observed in EMSA where no complex formation between PIAS1 and STAT1 is detectable. This finding suggests that PIAS1 may induce modification of STAT1 that would diminish the DNA-binding activity. Our results with the sumoylation-defective STAT1 mutants are in line with the results from PIAS1$^{-/-}$ mice and support the concept that the SUMO E3-ligase function is a physiologic function of PIAS1, although they do not exclude the possibility that PIAS1 could also affect the STAT1 function through other mechanisms.

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Structural and Functional Analysis of SUMO-Modification in STAT1

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Running title: SUMO-1 modification of STAT1

Abbreviations: CBP, CREB-binding protein; GAS, gamma-activated sequence; IFN, interferon; IRF, interferon regulatory factor 1; PIAS, protein inhibitor of activated; STAT1, Signal transducer and activator of transcription 1; SUMO, small ubiquitin-related modifier.

Abstract

A distinct sumoylation consensus site (ψKxE; ^{702}IKTE^{705}) is localized in the C-terminus of STAT1, and Lys703 is a target for PIAS mediated SUMO-modification. PIAS1 negatively regulates STAT1 responses in a sumoylation-dependent manner, but the underlying mechanisms are still unknown. We have performed a structural and functional analysis of sumoylation in STAT1. Molecular modeling indicated, that SUMO consensus site is well exposed to SUMO-conjugation, and suggested that SUMO moiety is directed towards DNA which could affect the promoter binding affinity of STAT1. Consistently, experimental analysis indicated that sumoylation defective mutants of STAT1 display higher DNA-binding activity and enhanced histone acetylation of the GBP-1 promoter. Furthermore, deconjugation of SUMO by SENP1 enhanced the transcriptional activity of the wild-type STAT1 but not sumoylation defective mutant, thus confirming a negative regulatory effect of sumoylation on STAT1 activity. These results suggest that sumoylation participates in regulation of STAT1 responses by modulating DNA-binding properties of STAT1.

Keywords: Signal transduction, transcription factors, sumoylation

Introduction

Signal transducer and activator of transcription (STAT)1 is the founder member of the STAT family of transcription factors and plays a critical role in interferon (IFNs) regulated cellular gene responses[1]. IFN-γ activates STAT1 through Janus kinase (JAK)-mediated phosphorylation of Tyr 701. Activated STAT1 homodimerize and translocate to the nucleus to bind interferon-gamma activated site (GAS) element and to initiate the transcription of IFN-γ-regulated genes [1]. The structure of the DNA bound STAT1 dimer shows a contiguous C-shaped clamp around DNA, that is stabilized by specific interactions between SH2 domain and tyrosine phosphorylated C-terminal tail segment (residues 700-708) of monomers[2].

SUMO (small ubiquitin-related modifier) proteins belong to the family of ubiquitin-related protein modifiers, collectively termed Ubls, that are covalently attached to substrate proteins by a cascade of enzymatic reactions [3]. The conjugation is regulated by a number of SUMO specific enzymes such as E1 enzyme Aos1/Uba2 and the E2 conjugase Ubc9. The protein inhibitor of activated STAT (PIAS) family of proteins PIAS1, PIASxα, PIASxβ, PIASy have been shown to function as E3-type ligases to promote SUMO-1 conjugation[4-6]**.** PIAS1 functions as a negative regulator of STAT1 mediated transcription. This function is dependent on the E3-ligase function of PIAS1, but the molecular mechanisms underlying this regulation are presently unknown [7;8]. Recently, STAT1 was shown to become sumoylated by E3 ligase function of PIAS proteins and sumoylation was suggested to have a negative regulatory effect on the function of STAT1 [9-12]. However, clear indication of the functional mechanism of this modification has remained unclear. This study was aimed to analyze the structural and functional mechanism of sumoylation in STAT1. Molecular modeling indicated that SUMO consensus site is well exposed in STAT1 dimer, and accessible for interactions with regulatory proteins. The molecular model, together with experimental results also suggested, that SUMO-1 could form a non-covalent interface with the disordered loop (aa 684-699) in STAT1, that orients SUMO-1 towards DNA and affect the DNA-binding properties of STAT1.

Materials and methods

Plasmids.

STAT1-WT-HA (pEBB-HA), STAT1-K703R-HA (pEBB-HA) plasmids and STAT1-E705A-HA (pEBB-HA) were previously described [11]. pSG5-His-SUMO-1 was provided by A. Dejean[13]. The GAS-luc luciferase construct contains the IFN-γ regulated GAS element [14]**.** FLAG-tagged SENP1, SENP1 C603S mutant and CBP-HA were previously described [15;16].

Cell culture.

Human HeLa cells and monkey Cos-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 50 mg/ml streptomycin. Human fibrosarcoma U3A cells (kindly provided by Dr. I. Kerr) were cultured in DMEM supplemented with 10% Cosmic calf serum (HyClone, Logan, UT) and antibiotics. Stable U3A-STAT1-WT-HA and U3A-STAT1-KR-HA clones were previously described [10;11].

Reporter gene assays.

HeLa cells, U3A cells and U3A-STAT1 stable cells were plated onto 24-well dishes and (U3A cells were transfected with different STAT1 constructs), 0.25 μg pCMV-βgal as an internal transfection efficiency control and 0.25 μg GAS-luc. Stable U3A clones were transfected with increasing amount of CBP (0.25, 0.5, 1.0μg). After 24 hours the cells were stimulated with 10 ng/ml human IFN-γ (Immugenex, Los Angeles, CA) for additional 6 hours and lysed in Promega's reporter lysis buffer according to the manufacturer's instructions. Luciferase activity was measured using Luminoscan Ascent (ThermoElectron Corporation, Finland) and normalized against β-galactosidase activity of the lysates.

Immunoprecipitation and Immunoblotting.

Cos-7 cells were transfected with 2 μg STAT1-WT, 1 μg SUMO-1, 2 μg SENP1 and 2 μg SENP1 C603S mutant. 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. The lysates were incubated with anti-STAT1 antibody (monoclonal anti-STAT1 N-terminus, Transduction Laboratories, BD Bioscience) and the immunocomplex was washed and subjected to SDS-PAGE electrophoresis and proteins were detected with immunoblotting. STAT1 and sumoylated STAT1 protein levels were determined with anti-STAT1 (Transduction Laboratories, BD Bioscience) and anti-HA (clone 16B12) (Berkeley-Antibody, Richmond, CA) antibodies. SENP1 protein levels were determined by immunoblotting of total cell lysates with primary anti-FLAG antibody (Sigma-Aldrich, St Louis, MO). STAT1 protein levels from luciferase samples were analysed by immunoblotting using anti-HA antibody.

Oligoprecipitation.

Cos-7 cells were transfected with 2 μg STAT1-WT or STAT1- KR- or STAT1-EA-mutants, 0.7 μg SUMO-1 and left unstimulated or stimulated with 50 ng/ml of hIFN γ(Immugenex, Los Angeles, CA) for 30 minutes and together with osmotic shock(0.3M sorbitol) for 15 minutes. The cells were lysed in Triton X lysis buffer(0.2% Triton X-100; 10 mM HEPES pH 8; 2 mM EDTA; 1 mM EGTA; 0.3 M KCl; 1 mM DTT; 10% glycerol; 1 mM NaF) supplemented with protease inhibitors. The lysates were diluted threefold with lysis buffer lacking KCl.

For the binding assay, a biotinylated oligonucleotide containing the GAS from the human GBP1 (guanylate binding protein gene, 5´-GGATCCTACTTTCAGTTTCATATTACTCTAAAT-3´) was annealed and 3 nmols of biotinylated oligonucleotide duplex were incubated with Neutravidin agarose to form GAS-agarose affinity beads. Diluted cell extracts were precleared with Neutravidin beads and then incubated with GAS-agarose affinity beads. Bound proteins were analyzed by SDS-PAGE and immunoblotting. STAT1 was detected with anti-HA and anti- Tyr701-phospho-STAT1 (NEB) antibodies. STAT1 and sumoylated STAT1 levels were detected from lysates with anti-HA antibody.

Molecular modeling.

A 3D-structure of STAT1-dimer with DNA has been built using high-resolution crystal structure of tyrosine phosphorylated STAT-1-DNA complex (PDB code:1BF5)[2]. The molecular geometry of the loop 684-699 in the SH2 domain was calculated using the program Sybyl with Amber 7 FF99 force field parameters. The initial structure for the loop region was constructed using the crossover loop structure from the SUMO-1-TDG (PDB code:1wyw)[17] as a model. First, during the energy and geometry minimization for the loop all hydrogen atoms and non-constraints were included in the protocol. Second, during the molecular dynamic refinement the constraints were on for outer part of the loop in the SH2 domain. After the loop modeling we used the deposited coordinates of SUMO1 (PDB code:1wyw)[17] in our model. The SUMO1 were set nearby the constructed loop so that its C-terminal residue (Gly97) is in the vicinity of the Lys703 of the STAT1 and as well the SUMO can form an antiparallel β- sheet structure with the loop684-699. The disordered loop was also modeled with InsightII (2005, Accelrys Inc. InsightII - molecular modeling program. http://www.accelrys.comomain; 1wyw.pdb). The entire structure was then subjected to energy minimization using the molecular mechanics force field CVFF (consistent valence force field) and the steepest descent algorithm implemented under Insight II Discover program. During the minimization, the DNA and the atoms of the STAT1 residues 136-686, 700-710 were fixed.

Chromatin immunoprecipitation.

Stable U3A-STAT1-WT-HA and U3A-STAT1-KR-HA clones were starved and left unstimulated or stimulated with IFN-γ for 1h. Chromatin immunoprecipitation (ChIP) was performed as previously described [16] using anti-acetyl-histone H4 antibody (Upstate Biotech) or as a control, anti-IgG antibody (Santa Cruz Biotechnology). DNA was analyzed for human GBP-1 promoter by quantitative-RT-PCR with the following primers: 5´-AGCTTCTGGTTGAGAAATCTTT-3´ and 5´-CCCTGGACTAATATTTCACTG-3´. Qu-PCR was done using SYBR green I kit from Qiagen according to manufacturer's instructions. The values from ChIP assays were normalized to the total input DNA

Results

Analysis of SUMO consensus site in STAT1 dimer

To obtain insight into the mechanism of sumoylation and its function in STAT1, we used information from the published structure of tyrosine phosphorylated STAT1 dimer bound to DNA. The interaction between STAT1 monomers is formed by SH2-domain and phosphotyrosine 701 of an adjacent monomer so that the phosphate group is recognized by the strictly conserved Arg602 residue that rises up from the interior of the SH2 domain [2]. Analysis of the SUMO conjugation site (Figure 1A) in the structure demonstrated that the side chains of Lys703 of both monomers formed a projection on the same orientation with DNA, and formed a suitable site for covalent isopeptide bond between STAT1 and SUMO (Figure 1A, B). Formation of the β-sheet structure between two C-tail segments are mediated mainly through Glu705, Ile707 and Val709, and the dimer structure is stabilized by interactions in the hydrophobic core between SH2 domains[2]. Presumably, mutation of Lys703 to Arg does not interrupt interactions involved in dimerization interface, or DNA binding properties of STAT1 (Figure 1S).

Molecular model of the SUMO conjugated STAT1 dimer

The crystal structure of thymine DNA glycosylase (TDG) conjugated to SUMO-1 has revealed that TDG forms two dissimilar molecular interfaces with SUMO-1. The covalent contact to SUMO occurs at the Lys330 residue, but another interface is a β-sheet structure formed by β-strands of TDG and SUMO-1[17]. The structure of STAT1 dimer (PDB code: 1bf5) has a linker region (aa 684-699; loop structure) that is invisible in the electron density maps [2]. The immediate vicinity of sumoylation site to residues in both ´ends´ of the loop structure pointed us to investigate and remodel this loop. To get insight on this, we constructed a model of sumoylated STAT1 dimer using previously published coordinates of conjugated SUMO-1[17]. The loop aa 684-699 was reconstructed using two programs Sybyl with Amber7 FF99 force field (Figure 2) and InsightII, and the analysis resulted in two highly similar loop models**.** SUMO-1 was positioned on conjugation distance, and the constructed loop structure was presented adjacent to β-sheet structure of SUMO-1**.** This model proposes that interface between SUMO and the loop

structure of STAT1 can direct the SUMO moiety towards DNA which in turn can affect DNA binding of sumoylated STAT1.

Sumoylation defective STAT1-K703R and -E705A mutants show enhanced DNA binding activity

Glu705 is not directly involved in conjugation of SUMO but participates, together with Lys703, as a recognition site for the SUMO conjugation enzyme Ubc9 [3]. Investigation of the structure and position of the side chain of Glu705 revealed that due to its hydrophilicity it is also pointing towards protein surface (Figure 1B, Figure 1supplement)**.** Our previous results indicate that mutation of Lys703 and Glu705 residues abolish sumoylation of STAT1 and the mutants show enhanced transactivation[10;11]. To test the structural model of STAT1-SUMO-1 complex, and to address the question whether sumoylation affects the DNA-binding of STAT1, oligoprecipitation experiments were performed. Cells were transiently transfected with STAT1-WT, -KR- or -EA-mutants together with SUMO-1 plasmid and cells were either left untreated or treated with IFN-γ and osmotic shock to induce sumoylation (manuscript). STAT1-WT, -KR- or -EA-mutants were oligoprecipitated with biotinylated GBP-1-oligo (Figure 3A, B) and detected with anti-phospho-Tyr701 STAT1 antibody. Both STAT1-KR- and -EA-mutants showed enhanced DNA binding compared to STAT1 WT (Figure 3A). Additional oligoprecipitation experiments were carried out with larger protein amounts that would allow detection of sumoylated STAT1. Equal amounts of GBP1-oligo precipitated STAT1 and cellular STAT1 were analyzed with anti-HA antibody for the presence of SUMO-1 modification. As shown in Figure 3B, sumoylation was not detected in the DNA bound fraction of STAT1 while equal amount of cellular STAT1 was readily SUMO conjugated. These results suggest that SUMO-1 moiety in STAT1 influence DNA-binding properties.

Since STAT1-K703R mutant selectively enhances the IFN-γ induced transcriptional responses, we wanted to investigate the functional effect of enhanced DNA binding at the promoter level. To this end, we performed chromatin immunoprecipitation (ChIP) assays on the STAT1 target gene GBP-1. U3A cells stably transfected with STAT1-WT-HA or STAT1-K703R-HA, were left either unstimulated or

stimulated with IFN-γ. Immunoprecipitation of cross-linked and scattered chromatin was performed with anti-acetylated histone H4 antibodies or anti-rabbit IgG antibody as a control. The enhanced DNA binding of STAT1-KR resulted in increased acetylation of histone H4 when compared to STAT1-WT (Figure 4A). STAT1 interacts with the coactivator/ histone acetyl transferase CBP which selectively enhances IFN-γ induced transcriptional responses. To investigate the effect of CBP to transactivation of STAT1-KR and STAT1-WT we performed GAS-luc reporter assay. For this purpose, stable U3A–STAT1-WT and –STAT1-KR cells were transiently transfected with different amounts of CBP together with GAS-luc reporter plasmid (Figure 4B). Clear indication of enhanced DNA binding of STAT1-KR was observed when IFN-γ induced activation of STAT1-WT and STAT1-KR were compared. Interestingly, CBP enhanced transactivation of STAT1-KR whereas effect on STAT1-WT was modest. These findings indicate that activation of STAT1 is repressed by SUMO through impaired DNA binding of STAT1 that leads to reduced transcriptional activation. Enhanced DNA binding of sumoylation mutant (STAT1-KR) might lead to establishment of coregulator complexes that have histone acetylase function and thereby modulate the induction of IFN-γ target genes.

Desumoylation abolishes SUMO-dependent transcriptional inhibition of STAT1

To validate the constructed model and DNA binding experiments further we analyzed whether sumoylated STAT1 is a substrate for SUMO protease SENP1 *in vivo*. STAT1, SUMO-1 and wild-type SENP1 or SENP1 C603S mutant were transfected into COS-7 cells and total cell lysates were immunoprecipitated with anti-STAT1 antibody and subjected to SDS-PAGE electrophoresis. The filters were immunoblotted with anti-STAT1 and anti-SUMO-1 antibody (Figure 5A). Co-transfection of SENP1, but not the enzymatically inactive SENP1 C603S completely removed conjugated SUMO-1 from STAT1. These findings indicate that sumoylation of STAT1 is a reversible process and SENP1 can act as a SUMO-specific isopeptidase for STAT1. The identification of STAT1 as a substrate for SENP1 prompted us to investigate whether SENP1-mediated desumoylation affects the transcriptional activity of STAT1. For this purpose, STAT1 deficient U3A cells were transiently transfected with HA-tagged wild-type STAT1 and

different amounts of SENP1 and SENP1 C603S mutant together with GAS-luc reporter plasmid. As shown in Figure 5B, wild-type SENP1 augmented STAT1-mediated transcription, whereas the catalytically inactive SENP1 C603S mutant slightly repressed transcription. Similar results were obtained in HeLa cells co-transfected with GAS-luc reporter plasmid and different concentrations of wild-type SENP1 and SENP1 C603S mutant (Figure 5B), thus suggesting that SENP1 increases the activity of endogenous STAT1. Collectively these results indicate that desumoylation of STAT1 enhances its transcriptional activity and these results are well in line with constructed molecular model (Figure 2) and DNA binding experiments (Figure 3).

Discussion

Sumoylation is a common posttranslational modification of transcription factors, but in several proteins the physiological functions and molecular mechanisms of this modification remain elusive. This notion holds true also for STAT1, although several lines of evidence support the concept that sumoylation serves as a negative regulator of STAT1 [8-11]. In this study we aimed to obtain insight into the structural parameters involved in sumoylation of STAT1.

The STAT homodimer forms a nutcracker-like structure that binds to DNA. The monomers are held together by interface between SH2-domains [2]. The Lys703 is located adjacent to dimerization interface and this prompted us to investigate if mutation of Lys to Arg could affect dimerization of STAT1. Analysis of the SUMO conjugation consensus site in STAT1 dimer revealed that side chain of Lys703 formed a projection towards DNA. Both Lys703 and Glu705 residues have highly hydrophilic side chains which are converted away from hydrophobic core of SH2 interface and the β-sheet structure between two C-tail segments of STAT1 dimer is likely not affected by Lys703Arg mutation. However, mutation to arginine will interrupt formation of covalent bond with SUMO. The structural analysis revealed that side chain of Lys703 has an interaction face with Glu632 residue of SH2 domain of the adjacent monomer. Most probably this interface prevents rotation of this flexible side chain and keeps orientation favorable for the SUMO conjugation. The finding of controlled position of Lys703 also supports the importance of Lys703 as an SUMO acceptor site (Figure 2S).

In addition to covalent conjugation to substrate Lys residues, SUMO binds to target protein also non-covalently [17-19]. We reconstructed the structure of the disordered loop of STAT1, and made a molecular model of sumoylated STAT1 dimer using x-ray structure of TDG-SUMO-1[17] as a template. This model suggested that the position of SUMO under the loop structure most likely affects DNA binding capability of STAT1. Oligoprecipitation experiments with GBP1-oligo supported this finding, and proposed that sumoylation may affect the affinity of STAT1 towards promoter elements**.** To test this hypothesis we studied a low affinity promoter GBP1 with ChIP experiments by using stably transfected U3A-STAT1-WT and U3A-STAT1-KR clones. As a consequence of enhanced DNA binding sumoylation mutant (STAT1-KR) recruited increased histone H4 acetylation activity to the GBP1 promoter (Figure 3C). Analysis of the molecular model of sumoylated STAT1, as well as the experimental results (Figure 3S), indicated that despite of the close proximity of Lys703 to Tyr701 in linear sequence, these two residues are pointing to different directions and phosphorylation of Tyr701 does not spatially prevent the SUMO conjugation of STAT1. In contrast, it seems likely that in most cases sumoylation occurs in tyrosine phosphorylated STAT1 dimers. In our experiments SUMO-1 was found to be nuclear protein and to colocalize with STAT1-WT (unpublished data), suggesting that STAT1 has to be imported into nucleus to associate with SUMO-conjugation machinery. In addition, PIAS1 has been shown to interact with tyrosine-phosphorylated STAT1 dimers [7], and dimer formation is likely to favor SUMO conjugation *in vivo*.

Recent results have clarified the physiological role of PIAS mediated sumoylation. PIAS1 was shown to associate with protein-tyrosine phosphatase 1B (PTP1B) and to catalyze its sumoylation, which resulted in a down regulation of the phosphatase activity and inhibited dephosphorylation of the insulin receptor. The PIAS1 mediated negative regulation of PTP1B was reversed by SENP1 [20]. In our experiments removal of conjugated SUMO-1 by SENP1 increased STAT1-mediated transcription, thus confirming the negative regulatory role of sumoylation for STAT1. Importantly, in U3A clones expression of SENP1 did not increase the transcriptional activity of sumoylation deficient STAT1-KR, indicating that desumoylation directly regulates STAT1 activation (data not shown). PIAS1$^{-/-}$ mice show enhanced immune responses to

viral and bacterial infections. The absence of PIAS1 selectively affected promoters that show low affinity to STAT1 such as GBP1 without affecting promoters with stronger affinity binding sites (interferon regulatory factor 1, IRF1) [21]. These results demonstrate that PIAS1 selectively regulates IFN-induced gene responses, but the mechanism of this modulation has not been firmly established. Sumoylation defective STAT1 mutants show comparable IFN-γ induced IRF1, GBP1, and TAP1 gene responses with PIAS1$^{-/-}$mice [9-11;21]. Binding of PIAS1 to STAT1 dimer has been shown to decrease the DNA binding activity of STAT1 [7;22] and PIAS proteins exert SUMO-E3 ligase activity towards various SUMO-targets thus raising the question of the role of SUMO conjugation in PIAS1 function. Recently, PIAS1 was found to become Ser90 phosphorylated by IKKα in response to various inflammatory stimuli, including IFN-γ and TNF-α. Phosphorylation of PIAS1 repressed activation of NF-κB and STAT1 responsive genes by affecting their DNA binding activity. Importantly, the SUMO ligase activity of PIAS1 was required for the transcriptional repression [8]. Taken together, these findings strongly suggest that sumoylation function as a negative regulator of STAT1 responses that appears to selectively modulate the formation of promoter binding protein complexes to coordinate inflammatory responses.

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Figure legends

Figure 1. Ribbon diagram of the STAT1 homodimer-DNA complex (PDB code:1bf5).
(A) SUMO-consensus element and amino acid (aa) sequence of SUMO-conjugation site in STAT1. The symbol ψ indicates large hydrophobic amino acid. (B) Coiled-coil domain, DNA-binding domain and linker region of one monomer are colored green and the SH2-domain is colored in salmon red. SH2 domain of adjacent monomer is colored light yellow and other domains are blue. DNA strand is labelled and colored in red. Residues, pTyr701 (salmon red), Lys703 (red) and Glu705 (blue) are shown in a stick representation**.** All ribbon diagrams presented in figure 1 are colored uniformly and produced using PyMOL (http://www.pymol.org).

Figure 2. Model of SUMO conjugated STAT1 dimer
The model was constructed by superimposing the structure of SUMO-1 from the complex of SUMO-1-TDG (PDB code:1wyw) on structure of DNA bound STAT1 dimer (PDB code:1bf5). The molecular geometry of the loop 684-699 in the SH2 domain was calculated using the program Sybyl with Amber 7 FF99 force field parameters (Supplementary data). Green and blue colours were used to distinguish monomers in a ribbon diagram of DNA bound STAT1 dimer. The loop structure and DNA are labeled and colored with red. The structure of SUMO-1 was incorporated between the loop and DNA in conjugation distance on both sides of dimer. SUMO-1 is represented in ribbon and surface models in yellow. Graphics were produced using PyMOL (http://www.pymol.org)

Figure 3. SUMO mutants show enhanced DNA binding.
(A) Binding of STAT1 WT, K703R- and E705A-mutants to DNA. Cos-7 cells were transiently transfected with expression vectors containing STAT1 WT, K703R-mutant, E705A-mutant and SUMO-1. Cells were stimulated as indicated and lysed. Total cell lysates were oligoprecipitated using biotinylated GBP-1-GAS-oligo and immunoblotted

using anti-HA antibody and anti-p-Tyr-STAT1 antibody. Sumoylation and expression levels of STAT1 were verified by immunoblotting (WB) total cell lysates with anti-HA. (B) Sumoylated STAT1 does not bind DNA. Cos-7 cells were transfected, stimulated, lysed and oligoprecipitated as described above. Oligoprecipitation samples were run on the gel together with lysates. Samples were subjected to immunoblotting with and anti-HA antibody.

Figure 4. SUMO mutant shows enhanced histone H4 acetylation and transcriptional activation of GBP-1 promoter

(A) Sumoylation defective STAT1-KR shows enhanced acetylation of histone H4 at the IFN-γ dependent GBP promoter. Stably transfected U3A-WT and U3A-KR clones were starved overnight and stimulated as indicated. Immunoprecipitation of cross-linked chromatin was performed with anti-acetyl H4 and anti rabbit IgG as a control antibody. DNA was extracted and analysed by Qu-PCR using primers specific for IFN-γ dependent GBP-1 promoter sequence. The values from immunoprecipitation were normalized to the total input DNA. The mean Qu-PCR values ± SD from three independent experiments are shown. (B) CBP cotransfection enhances the transcriptional activity of STAT1-KR. U3A-STAT1-WT and STAT1-KR stable cell lines were transiently transfected with increasing amounts of CBP (0.25, 0.5, 1.0 μg) together with GAS-luc reporter plasmid and pCMV-βGal for normalizing transfection efficiency. After 24 hours, the cells were serum starved and stimulated followed by luciferase measurement. The mean relative luciferase units ± SD from three independent experiments are shown.

Figure 5. Sumoylated STAT1 is target for SENP1 desumoylation

(A) SENP1 desumoylates STAT1 in intact cells. Cos-7 cells were transiently transfected with expression vectors containing STAT1, SUMO-1 and FLAG-tagged SENP1 or SENP1 C603S mutant as indicated. After 36 hours the cells were lysed and total cell lysates were immunoprecipitated with anti-STAT1 antibody and subjected to immunoblotting with anti-SUMO-1 and anti-STAT1 antibody. SENP1 protein levels were verified by immunoblotting with anti-FLAG antibody. (B) SENP1 co-transfection augments the transcriptional activity of STAT1. U3A cells were transiently transfected

with STAT1-WT and different concentrations of SENP1 or SENP1 C603S mutant together with GAS-luc reporter plasmid and pCMV-βGal for normalizing transfection efficiency. After 24 hours, the cells were serum starved and stimulated followed by luciferase measurement. HeLa cells were transiently transfected with different concentrations of SENP1 or SENP1 C603S mutant (1 μg and 2 μg) together with GAS-luc reporter plasmid and pCMV-βGal. The cells were treated as described above and luciferase assay was carried out as above. The mean relative luciferase units ± SD from two independent experiments are shown. SENP1 and STAT1 protein levels were verified by immunoblotting with anti-FLAG and anti-HA antibodies.

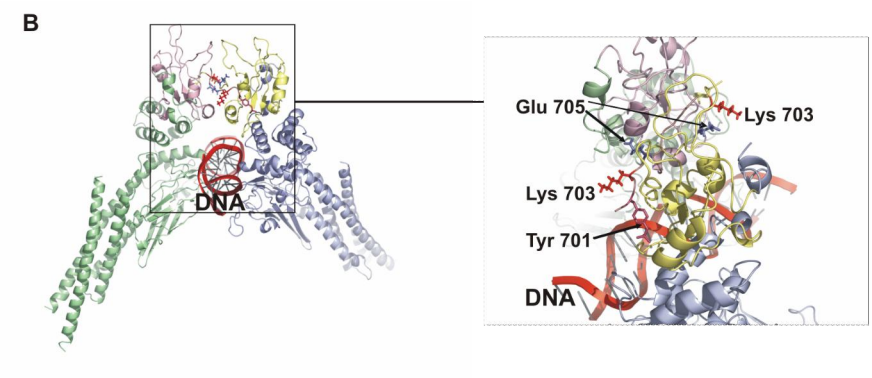
Fig 1.

A

SUMO-consensus ΨKxE

STAT1 aa sequence 701 YIKTELIS 708

B



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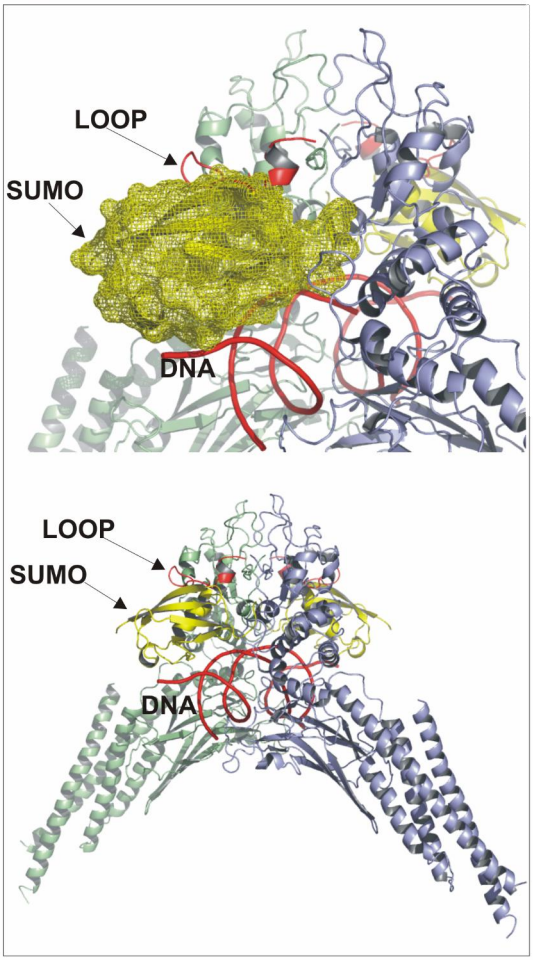
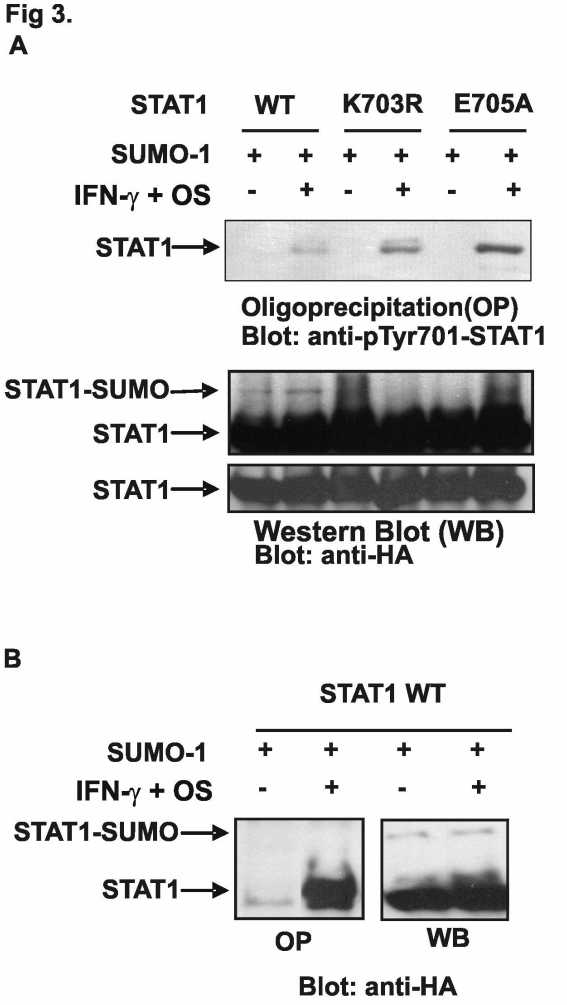


Fig 3.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K703R
E705A
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IFN-γ + OS
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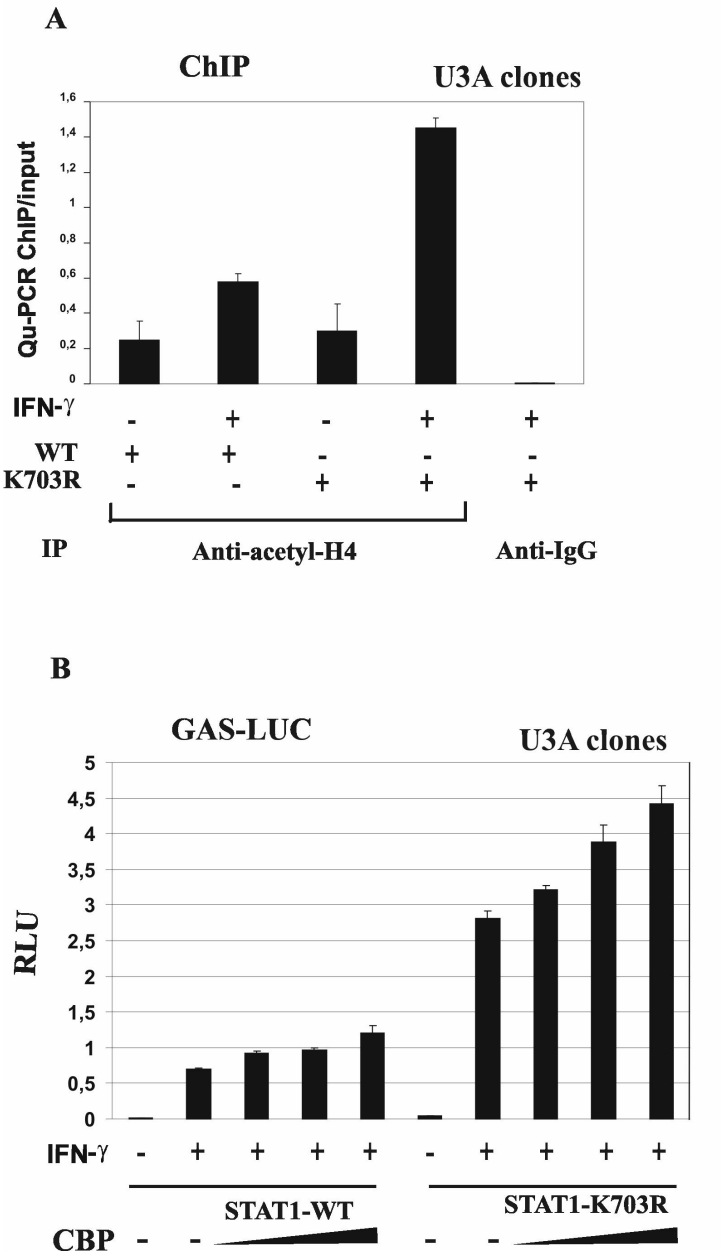
STAT1-SUMO
STAT1
STAT1
Western Blot (WB)
Blot: anti-HA
B
STAT1 WT
SUMO-1
+ + + +
IFN-γ + OS
- + - +
STAT1-SUMO
STAT1
OP
WB
Blot: anti-HA

Fig 4.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WT + + - - -
K703R - - + + +
IP
Anti-acetyl-H4
Anti-IgG
B
GAS-LUC
U3A clones
RLU
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3,5
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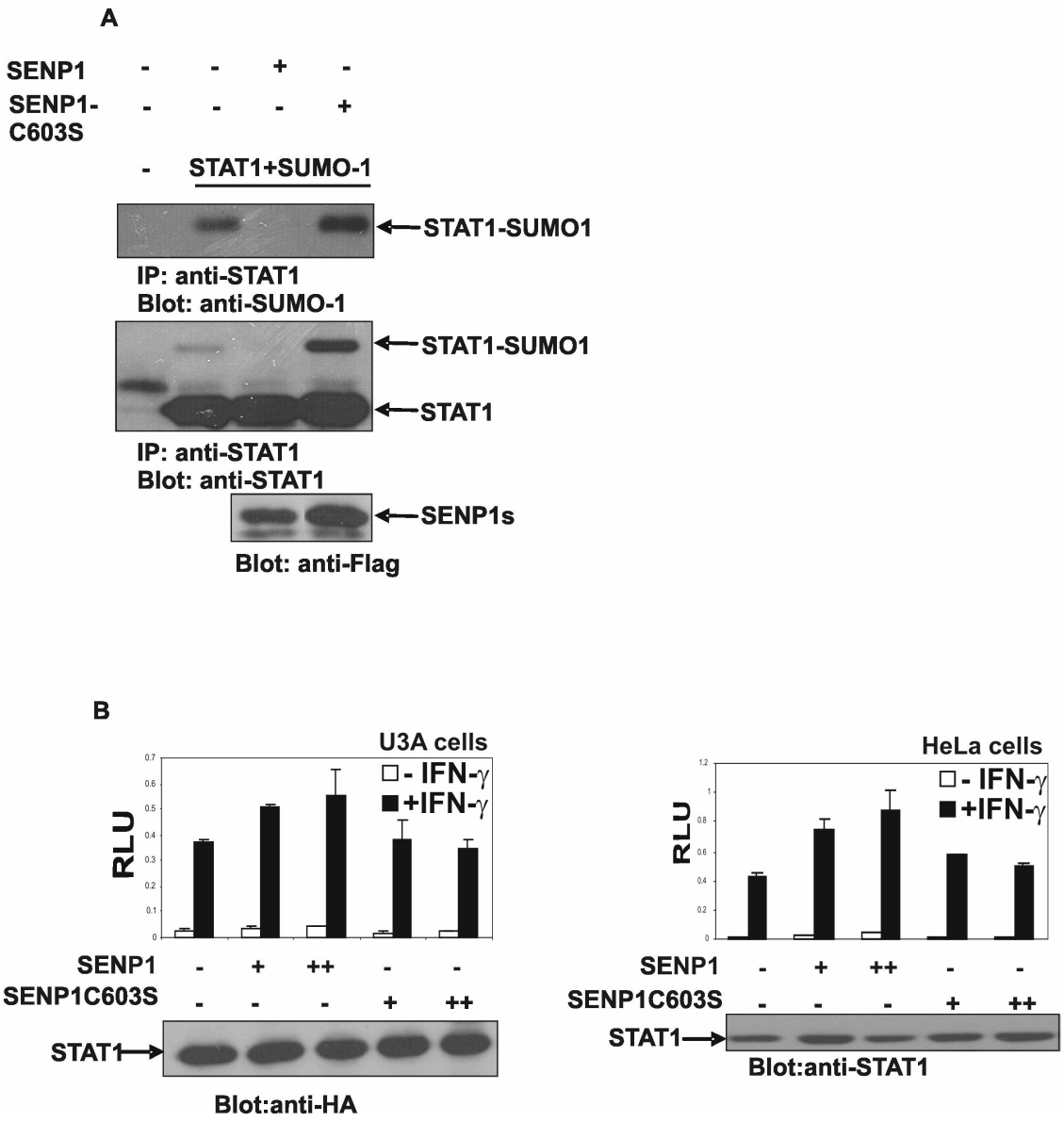
STAT1-WT
STAT1-K703R
CBP - - - -

Fig 5.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SENP1-C603S - - - +
- STAT1+SUMO-1
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IP: anti-STAT1
Blot: anti-SUMO-1
STAT1-SUMO1
STAT1
IP: anti-STAT1
Blot: anti-STAT1
SENP1s
Blot: anti-Flag
B
U3A cells
- IFN-γ
+IFN-γ
RLU
SENP1 - + ++ - -
SENP1C603S - - - + ++
STAT1
Blot:anti-HA
HeLa cells
- IFN-γ
+IFN-γ
RLU
SENP1 - + ++ - -
SENP1C603S - - - + ++
STAT1
Blot:anti-STAT1

Supplementary data

STRUCTURAL and FUNCTIONAL ANALYSIS of SUMO-MODIFICATION in STAT1

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Figures

Figure 1S

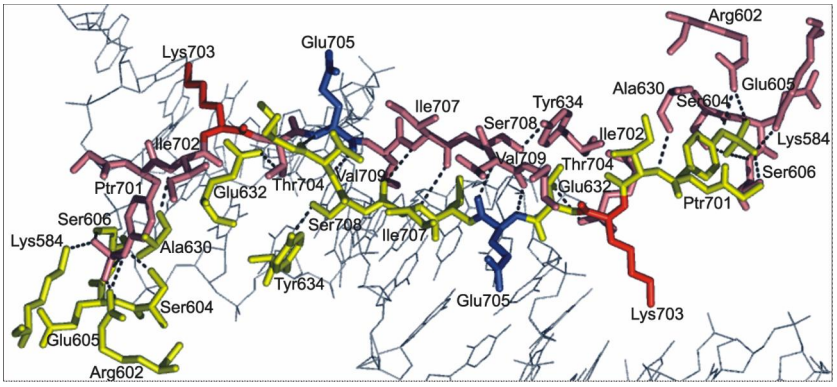

Figure 1S. Stick presentation of antiparallel β-sheet structure of the STAT1 dimer interface.

C-terminal tails of adjacent monomers in STAT1 dimer interface and other residues forming interactions with C-tail residues are coloured with light yellow (monomer 1) and salmon red (monomer 2).Glu705 is coloured with blue and Lys703 with red. β-sheet interactions formed between C-terminal tails of monomers and pTyr701 interactions with adjacent SH2 domain are indicated with dashed lines. DNA has been represented as a stick model under the monomer interface and coloured with light blue.

Figure 2S

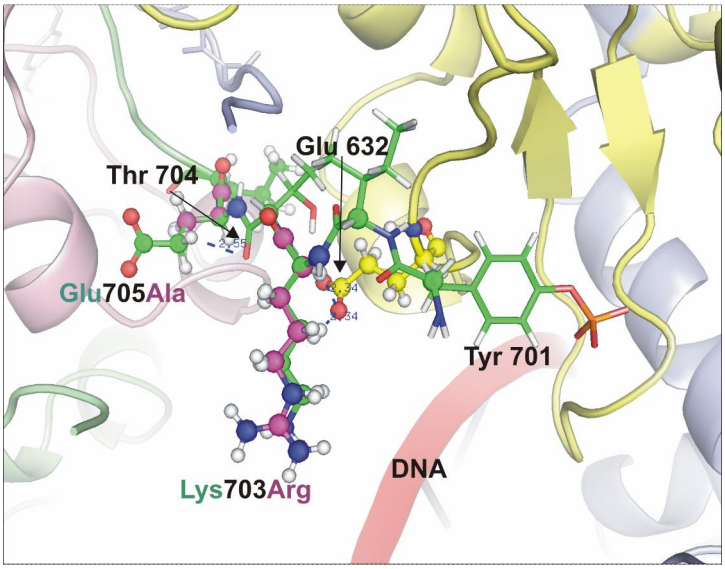

Figure 2S. Interfaces formed by Lys703 and Glu 705.

Details of the residues 701-705 in the C-terminal tail segment and SUMO consensus site of one monomer are shown in stick (backbone) and stick and ball (side chains) representation (green colour). Adjacent SH2 domain is showed in ribbon, except the interaction forming side chain of Glu632 is shown in stick and ball representation (yellow colour). Side chains of mutated Ala705 and Arg703 (purple colour) are shown together with side chains of wild type Glu705 and Lys703. Labels have the same colour as the residues they indicate. Thr704 and Glu632 residues are also indicated with arrows. Hydrogen atoms (H) are coloured in light grey, oxygen atoms (O) in red and nitrogen atoms (N) in blue. Interfaces are indicated with dashed lines between atoms.

Figure 3S

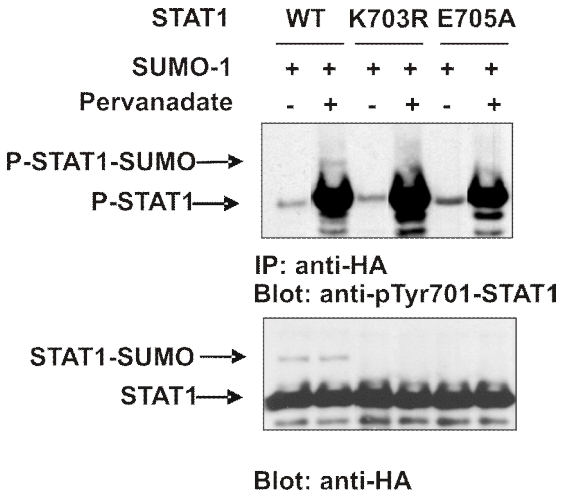

Figure 3S. Sumoylation of STAT1 does not interfere with its phosphorylation

Cos-7 cells were transiently transfected with expression vectors containing STAT1 WT, K703R-mutant, E705A-mutant (2μg) and SUMO-1 (1μg). After 36 hours, the cells were starved overnight and left untreated or treated with pervanadate as indicated. Cells were lysed in Triton X lysis buffer and total cell lysates were immunoprecipitated using anti-HA antibody and immunoblotted using anti-p-Tyr-STAT1 antibody (upper panel). Sumoylation and expression levels of STAT1 were verified by immunoblotting total cell lysates with anti-HA antibody (lower panel).