SUMO Modification of STAT1 and Its Role in PIAS-mediated Inhibition of Gene Activation*

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The PIAS (protein inhibitors of activated STAT) family of proteins were first discovered as inhibitors of activated signal transducers and activators of transcription (STATs). More recently these proteins have been shown to function as E3 ligases that promote the SUMO modification of a number of transcription regulators. We have investigated the relationship between the effects of PIAS proteins on STAT1 transcriptional activity and the ability of the PIAS proteins to function as SUMO E3 ligases. We demonstrate that STAT1 is a substrate for SUMO modification and that PIASx-α, but not PIAS1, functions as an E3 ligase to promote STAT1 modification. In addition, we have mapped the major site for SUMO modification on STAT1 to lysine 703. This lysine residue is in close proximity to the regulatory tyrosine residue at position 701, whose phosphorylation mediates STAT1 activation in response to cytokine signaling. Mutation of lysine 703 to arginine abolishes SUMO modification of STAT1 both in vitro and in vivo. However, this mutation does not affect the activation of STAT1 or the ability of either PIAS1 or PIASx-α to function as an inhibitor of STAT1-mediated transcription activation. Our findings demonstrate that inhibition of STAT1 by PIAS proteins does not require SUMO modification of STAT1 itself. SUMO modification of STAT1 may nonetheless be functionally important given the close proximity between the SUMO modification site and tyrosine 701.

STAT1 proteins comprise a family of transcription factors that regulate gene expression in response to a variety of extracellular signals, mainly cytokines and growth factors (2, 3). The STAT proteins reside dormant in the cytoplasm of cells until they are activated in response to ligand binding to specific cell surface receptors. The pathway that leads to the activation of STAT1 involves a series of tyrosine phosphorylation events mediated by Janus kinase proteins. Phosphorylation of STAT1 by Janus kinase proteins at tyrosine 701 mediates STAT1 homodimerization and its subsequent translocation into the nucleus. In the nucleus, STAT1 regulates the transcription of numerous genes that are instrumental in controlling development, cell growth, and cell differentiation (for review, see Refs. 4 and 5). In addition, the Janus kinase-STAT1 pathway is critical for cellular responses to microbial and viral infection (6).

The general features of STAT1 activation are fairly well characterized; however, the mechanisms leading to STAT1 inactivation are less well defined. Recently, a family of proteins termed “protein inhibitors of activated STATs” (PIAS) was identified through their direct interactions with the STAT proteins (7, 8). Functional characterization of the PIAS proteins revealed that they inhibit the transcription activation of STAT-regulated genes. There are currently five unique PIAS proteins (PIAS1, PIAS3, PIASγ, PIASx-α, and PIASx-β), each with distinct activities toward the different STAT family members. PIAS1 and PIAS3, for example, specifically inhibit STAT1- and STAT3-mediated gene activation, respectively (7, 8). The specific mechanism by which the PIAS proteins inhibit STAT activity in vivo is unclear, although in vitro studies suggest that they may regulate interactions between STATs and DNA (7).

After their identification as regulators of STAT function, the PIAS proteins were shown to function as regulators of SUMO (small-ubiquitin like modifier) modification (9–15). Like ubiquitination, SUMO modification occurs through a series of enzymatic steps (16). The first step involves the ATP-dependent formation of a thioester bond between SUMO and the E1 heterodimer consisting of Aos1 and Uba2. The second step involves the transfer of SUMO to the E2-conjugating enzyme Ubc9. In the third and final step, SUMO is covalently linked to a lysine residue in the targeted substrate. Recognition of the substrate involves direct interactions between Ubc9 and the substrate (1, 17) but may also be facilitated by proteins functioning as E3-like recognition factors. PIAS proteins have been shown to enhance the SUMO modification of a growing list of proteins that includes p53 (12, 13), c-Jun (10, 13), Sp3 (11), androgen receptor (10, 18), glucocorticoid receptor (19), LEF1 (20), IRF1 (21), and C/EBP (CCAAT/enhancer-binding protein) (22), and they have therefore been proposed to function as SUMO E3-like factors.

The link between PIAS proteins and STAT has led us to hypothesize that STAT1 is a SUMO substrate and that its ability to activate transcription may be regulated by SUMO modification. In this paper we demonstrate that STAT1 is modified by SUMO both in vitro and in vivo and that PIASx-α acts as an E3 ligase for STAT1 modification. We mapped the site for SUMO modification of STAT1 to lysine 703, a residue that is in close proximity to the regulatory tyrosine 701. Phosphorylation of tyrosine 701 in response to cytokine signaling is essential for STAT1 dimerization and nuclear translocation, suggesting that SUMO modification of lysine 703 may play an
important regulatory role. In *vivo* transcription studies, however, indicate that SUMO modification at lysine 703 is not required for PIAS-mediated inhibition of STAT1 transcription activation. Our results demonstrate that the inhibition of STAT1 by PIAS proteins occurs through alternate mechanisms.

**MATERIALS AND METHODS**

**Plasmid Constructions**—Plasmids for expressing pCMV5-FLAG-murine PIAS1 and pCMV5-FLAG-PIASx-α were generous gifts from Dr. Ke Shuai (University of California, Los Angeles, CA). pCMV-STAT1-FLAG, pcDNA1/SUMO-3, pcDNA6/STAT1, pEGFP-C1/SUMO-1, pEGFP-C1/SUMO-2, pGEX-4T-1/STAT1, pGEX-4T-1/PIAS1, pGEX-4T-1/PIASx-α, and pGEX-4T-1/STAT1 were all produced using standard PCR and sub-cloning techniques. Site-directed mutagenesis of STAT1 constructs was performed using the QuickChange site-directed mutagenesis system (Stratagene, La Jolla, CA). All mutants were verified by DNA sequence analysis.

**Protein Expression and Purification**—Recombinant SUMO-1, Aos1/Uba2, and Ubc9 were expressed and purified as previously described (1). Recombinant PIAS1 and PIASx-α were produced in bacteria by transforming the appropriate pGEX-4T-1 vector and inducing expression with 0.1 mM isopropyl-β-D-thiogalactopyranoside at 20 °C for 6 h. Recombinant proteins were purified by affinity chromatography on glutathione-Sepharose beads (Amersham Biosciences). Recombinant PIAS1 and PIASx-α were subsequently cleaved from the beads by thrombin protease as outlined by the manufacturer (Amersham Biosciences). STAT1 was transcribed and translated in rabbit reticulocyte lysate in the presence of [35S]methionine as outlined by the manufacturer (Promega Corp., Madison, WI).

**In Vitro SUMO Modification Assays**—In *vitro* SUMO modification of STAT1 was performed using two individual assay conditions. Assays performed using high concentrations of Aos1/Uba2 and Ubc9 contained 0.5 μM Aos1/Uba2, 12 μM Ubc9, 10 μM mature SUMO, 2 mM ATP, 5 mM MgCl₂, 1% glycerol, 50 mM Tris, pH 7.5, and 2 μl of in vitro translated STAT1. Assays performed using limiting concentrations of Aos1/Uba2 and Ubc9 contained 0.3 μM Aos1/Uba2, 0.3 μM Ubc9, 7.7 μM mature SUMO, 2 mM ATP, 5 mM MgCl₂, 1% glycerol, 50 mM Tris, pH 7.5, and 2 μl of in vitro translated STAT1 or 200 ng of recombinant STAT1. Reactions were incubated at 37 °C for 15 min. Reactions were quenched with SDS-PAGE sample buffer and subsequently analyzed by SDS-PAGE and autoradiography or by immunoblot analysis with a STAT1 antibody (Sigma).

**Cell Culture, Transient Transfection, and Transcription Reporter Assays**—U3A cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Cells were plated onto 12-well tissue culture plates 12 h before transfection and transfected with the indicated plasmids (300 ng of STAT1, 300 ng of SUMO, 100 ng of PIAS) using Lipofectin as described by the manufacturer (Invitrogen). Total DNA concentrations in each transfection experiment were kept constant by adding empty vector DNA to 1.6 ng of PIAS (14). Transfections were performed using high concentrations of Aos1/Uba2 and Ubc9 contained 0.3 μM Aos1/Uba2 and 12 μM Ubc9. Transient transfection with a STAT1 luciferase control plasmid was transfected into all cells to normalize luciferase expression.

**Nickel Affinity Chromatography**—Cells were lysed in 100 μl of buffer containing 6 M guanidine hydrochloride, 10 mM Tris, 100 mM NaH₂PO₄, 300 mM NaCl, and 10 mM imidazole at pH 8.0 24 h post-transfection. Cell lysates were mixed gently with 25 μl of nickel nitrilotriacetic acid-agarose (Qiagen, Valencia, CA) for 1 h at room temperature. Beads were washed 3 times with buffer containing 8 M urea, 10 mM Tris, 100 mM NaH₂PO₄, 300 mM NaCl, and 20 mM imidazole at pH 6.3. Bound proteins were eluted from the beads using 40 μl of SDS-PAGE sample buffer. Immunoblot analysis was performed using the STAT1α p91 (C-24) antibody (Santa Cruz Biotechnology).

**RESULTS**

**STAT1 Is a Substrate for SUMO Modification**—The recent findings that PIAS proteins function as SUMO E3 ligases for a variety of transcription factors led us to address the question of whether STAT1 may be a substrate for SUMO modification. A SUMO modification assay containing recombinant SUMO-1, Aos1/Uba2, and Ubc9 was used to determine whether STAT1 could serve as a substrate for SUMO modification *in vitro*. STAT1 was translated in rabbit reticulocyte lysate in the presence of [35S]methionine. As demonstrated by SDS-PAGE, the 91-kDa full-length STAT1 was produced as well as several smaller protein products possibly resulting from translation initiation at internal methionines or from proteolysis of the full-length protein (Fig. 1, lane 1). Translated STAT1 was added to an assay reaction containing 10 μM SUMO, 12 μM Ubc9, and 0.5 μM Uba2/Aos1, conditions under which most SUMO substrates can be modified in the absence of exogenously added E3 ligases. When analyzed by SDS-PAGE, a novel protein band could be detected migrating with a molecular mass of ~106 kDa (Fig. 1, lane 2). Conjugation of a single molecule of SUMO to protein substrates resulted in an ~15-kDa shift in molecular mass, suggesting that the 106-kDa protein corresponds to STAT1 conjugated to SUMO at a single lysine residue. The presence of the 106-kDa band was dependent on SUMO, because it was not detected in reactions containing only exogenously added E1 and E2 enzymes (Fig. 1, lane 2). Modification reactions containing glutathione S-transferase-SUMO produced an appropriately higher molecular mass band of ~135 kDa (Fig. 1, lane 4), presenting further evidence that the shifted bands correspond to SUMO-modified STAT1.

STAT1 was modified in the above assay in the presence of relatively high concentrations of E1 and E2 enzymes and in the absence of an exogenously added E3 enzyme. This is consistent with the findings for most SUMO substrates, where E3 ligases are required only when E1 and E2 enzyme concentrations are significantly lower. To assay for the ability of PIAS proteins to function as E3 ligases for STAT1, we therefore repeated the above assay using reduced concentrations of Aos1/Uba2 and Ubc9. Translated STAT1 was added to an assay containing 7.7 μM SUMO, 0.3 μM Ubc9, and 0.3 μM Uba2/Aos1. A faint protein band was detected by SDS-PAGE, indicating that STAT1 was not efficiently modified by SUMO under these reduced E1 and E2 enzyme conditions (Fig. 2A, lane 2). Assays using the same

![Fig. 1. STAT1 is a SUMO substrate.](https://example.com/fig1.png)
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STAT1 Is Modified at a Single Lysine Residue at Position 703—STAT1 has a modular structure with the amino terminus containing a coiled-coil domain, the central region containing the DNA binding domain, and the carboxyl terminus containing an SH2 domain followed by a transactivation domain (Fig. 4A). STAT1 contains two potential SUMO modification sites defined by the consensus sequence $\text{KXE}$, where $\text{K}$ is a hydrophobic residue, $\text{E}$ is glutamic acid, and $\text{X}$ is any amino acid, and $\text{E} = \text{glutamic acid} (17, 24). This consensus sequence defines the site modified in many (but not all) SUMO substrates. The consensus sequences in STAT1 reside around lysine 110 in the amino terminus and lysine 703, which is in the carboxyl-terminal region between the SH2 domain and the transactivation domain (Fig. 4A). The consensus sequence surrounding lysine 703 is of particular interest because of its close proximity to tyrosine 701, the tyrosine residue phosphorylated in response to cytokine stimulation. To determine whether either of these sites in STAT1 is modified by SUMO, we mutated lysine residues 110 and 703 to arginine. Mutant and wild-type STAT1 proteins were translated in rabbit reticulocyte lysate in the presence of $[^{35}\text{S}]$methionine, and their ability to be modified by SUMO was determined using in vitro assays followed by SDS-PAGE (Fig. 4B). Both wild-type STAT1 and the lysine 110 to arginine mutant STAT1 could be modified by SUMO, as determined by the appearance of a 106-kDa STAT1-SUMO conjugate (Fig. 4B, lanes 1–2 and 4–5). In contrast the 106-kDa band corresponding to SUMO-modified STAT1 was not detected with the lysine 703 to arginine mutant STAT1 (Fig. 4B, lanes 7–8). These SUMO modification assays were performed in the presence of high concentrations of E1 and E2 enzymes and no added E3, although similar results were also obtained with low concentrations of E1 and E2 enzymes. These results indicate that lysine 703 is the major site for SUMO modification of STAT1 in vitro. The results also indicate that PIASx-α enhances specifically the modification at lysine 703.

STAT1 Is Constitutively Modified by SUMO in Vivo—The above results indicate that STAT1 is modified by SUMO in vitro and indicate that PIASx-α functions directly as an E3 ligase to promote STAT1 modification. STAT1 Is Modified at a Single Lysine Residue at Position 703—STAT1 has a modular structure with the amino terminus containing a coiled-coil domain, the central region containing the DNA binding domain, and the carboxyl terminus containing an SH2 domain followed by a transactivation domain (Fig. 4A). STAT1 contains two potential SUMO modification sites defined by the consensus sequence $\text{KXE}$, where $\text{K}$ is a hydrophobic residue, $\text{E}$ is glutamic acid, and $\text{X}$ is any amino acid, and $\text{E} = \text{glutamic acid} (17, 24). This consensus sequence defines the site modified in many (but not all) SUMO substrates. The consensus sequences in STAT1 reside around lysine 110 in the amino terminus and lysine 703, which is in the carboxyl-terminal region between the SH2 domain and the transactivation domain (Fig. 4A). The consensus sequence surrounding lysine 703 is of particular interest because of its close proximity to tyrosine 701, the tyrosine residue phosphorylated in response to cytokine stimulation. To determine whether either of these sites in STAT1 is modified by SUMO, we mutated lysine residues 110 and 703 to arginine. Mutant and wild-type STAT1 proteins were translated in rabbit reticulocyte lysate in the presence of $[^{35}\text{S}]$methionine, and their ability to be modified by SUMO was determined using in vitro assays followed by SDS-PAGE (Fig. 4B). Both wild-type STAT1 and the lysine 110 to arginine mutant STAT1 could be modified by SUMO, as determined by the appearance of a 106-kDa STAT1-SUMO conjugate (Fig. 4B, lanes 1–2 and 4–5). In contrast the 106-kDa band corresponding to SUMO-modified STAT1 was not detected with the lysine 703 to arginine mutant STAT1 (Fig. 4B, lanes 7–8). These SUMO modification assays were performed in the presence of high concentrations of E1 and E2 enzymes and no added E3, although similar results were also obtained with low concentrations of E1 and E2 enzymes. These results indicate that lysine 703 is the major site for SUMO modification of STAT1 in vitro. The results also indicate that PIASx-α enhances specifically the modification at lysine 703.

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The above results indicate that STAT1 is modified by SUMO in

![Fig. 2](image-url) SUMO modification of STAT1 is enhanced by PIASx-α. A, $[^{35}\text{S}]$methionine-labeled STAT1 was expressed in rabbit reticulocyte lysate (lane 1), and SUMO modification was assayed in the presence of the indicated components (assay mix containing 0.3 μM Aos1/Uba2, 0.3 μM Ubc9, 7.7 μM SUMO-1). PIAS1 and PIASx-α were added at a concentration of 0.3 μM. Molecular mass markers (kDa) and full-length AR/106 kDa larger that unmodified glutathione S-transferase-STAT1) (Fig. 3, lane 3). These results further demonstrate that

![Fig. 3](image-url) PIASx-α stimulates SUMO modification of recombinant STAT1. Recombinant STAT1 was expressed in bacteria, and SUMO modification was assayed using purified recombinant factors. STAT1 was incubated with buffer alone (lane 1), with 0.3 μM Aos1/Uba2, 0.3 μM Ubc9, 7.7 μM SUMO-1 (lane 2), or with 0.3 μM Aos1/Uba2, 0.3 μM Ubc9, 7.7 μM SUMO-1, and 0.3 μM PIASx-α (lane 3). Molecular mass markers (kDa), and recombinant STAT1 and SUMO-1-modified recombinant STAT1 are indicated.
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*a* schematic diagram of STAT1 protein structure (ND, amino-terminal domain; DNA, DNA binding domain; LD, linker domain; SH2, Src homology 2 domain; Y, tyrosine 701; S, serine 727. The two SUMO modification consensus sequences present in STAT1, surrounding lysine residues 110 and 703, are indicated. B, wild type (WT) and lysine to arginine mutant STAT1s were expressed in rabbit reticulocyte lysate in the presence of [35S]methionine (lanes 1, 4, and 7). SUMO modification of the expressed proteins was characterized by incubation with either an assay mix (+ +) containing 0.5 μM Aos1/Uba2, 12 μM Ubc9, and 10 μM SUMO-1 (lanes 2, 5, and 8) or (+) containing 0.3 μM Aos1/Uba2, 0.3 μM Ubc9, 7.7 μM SUMO-1, and 0.3 μM PIASx-α (lanes 3, 6, and 9).

**Fig. 4.** STAT1 is modified by SUMO at lysine residue 703. A, **STMN-1 is modified by SUMO in vitro and that this modification, which occurs on a lysine residue only two amino acids away from tyrosine 701, may be functionally significant. Therefore, we next performed experiments to assess whether STAT1 is modified by SUMO in vitro and also whether its modification may be influenced by PIAS overexpression and/or by cytokine stimulation. U3A cells, which do not express endogenous STAT1, were transfected with plasmids encoding for either Myc-His-tagged wild-type STAT1 or Myc-His-tagged lysine 703 to arginine mutant STAT1. 24 h after transfection, cells were lysed in guanidine hydrochloride, and STAT1 protein was purified by nickel affinity chromatography. Immunoblot analysis of the affinity-purified proteins with an anti-STAT1 antibody revealed unmodified STAT1 but no detectable higher molecular mass bands corresponding to possible STAT1-SUMO conjugates (Fig. 5, lanes 2 and 6). Overexpression of SUMO by transient transfection can enhance the modification of many SUMO substrates in vivo. Therefore, cells were co-transfected with an Myc-His STAT1 plasmid, GFP-SUMO plasmid, and a plasmid encoding for FLAG-PIASx-α. Cells could be lysed 24 h after transfection, and STAT1 was purified by nickel affinity chromatography. Immunoblot analysis revealed a significant enhancement of the SUMO modification of wild-type STAT1 in the presence of overexpressed PIASx-α (Fig. 5, lane 4 versus 5). No SUMO-modified STAT1 could be detected in cells transfected with the lysine 703 to arginine STAT1 mutant (Fig. 5, lane 9) or in cells overexpressing only STAT1 and PIASx-α but not SUMO (Fig. 5, lanes 3 and 7). These results indicate that PIASx-α stimulates the SUMO modification of STAT1 in vivo and further demonstrate that STAT1 is modified on lysine 703. The expression of transfected plasmids was confirmed by immunoblot or immunofluorescence analysis (Fig. 5B; data not shown).

Our *in vitro* findings indicated that PIASx-α could act as an E3 ligase and stimulate the SUMO modification of STAT1. We therefore examined whether overexpression of PIASx-α could enhance SUMO modification of STAT1 *in vivo*. U3A cells were co-transfected with an Myc-His STAT1 plasmid, GFP-SUMO plasmid, and a plasmid encoding for FLAG-PIASx-α. Cells were lysed 24 h after transfection, and STAT1 was purified by nickel affinity chromatography. Immunoblot analysis revealed a significantly enhanced modification of wild-type STAT1 in the presence of overexpressed PIASx-α (Fig. 5, lane 4 versus 5). No SUMO-modified STAT1 could be detected in cells transfected with the lysine 703 to arginine STAT1 mutant (Fig. 5, lane 9) or in cells overexpressing only STAT1 and PIASx-α but not SUMO (Fig. 5, lanes 3 and 7). These results indicate that PIASx-α stimulates the SUMO modification of STAT1 *in vivo* and further demonstrate that STAT1 is modified on lysine 703. The expression of transfected plasmids was confirmed by immunoblot or immunofluorescence analysis (Fig. 5B; data not shown).

Previous studies indicate that PIAS proteins may act specifically on activated STAT dimers (25), suggesting that STAT1 SUMO modification may be up-regulated in response to STAT1 activation. To determine whether STAT1 activation and dimerization had any influence on SUMO modification, we treated cells with γ-interferon (IFN-γ) for 30 min after transfection with plasmids encoding for STAT1 or STAT1 and Myc-tagged SUMO-3 (again we have observed that STAT1 is equally modified by SUMO-1, SUMO-2, or SUMO-3 *in vitro* and *in vivo* experimental conditions; data not shown). GFP-tagged SUMO was used because it causes a unique shift in molecular mass of ~45 kDa. Cells were again lysed 24 h after transfection, and STAT1 was purified by nickel affinity chromatography. Under these conditions, a faint protein band with the predicted molecular mass of a STAT1-GFP-SUMO conjugate was detected by immunoblot analysis, but only in cells transfected with wild-type STAT1 (Fig. 5, lane 4) and not in cells transfected with the lysine 703 to arginine STAT1 mutant (Fig. 5, lane 8). These results indicate that a small fraction of STAT1 is modified by SUMO at lysine 703 *in vivo*. **Fig. 5.** STAT1 is modified by SUMO *in vivo*. A, U3A cells lacking endogenous STAT1 were transiently co-transfected with expression vectors coding for wild-type (WT) STAT1 (lanes 2–5) or with a lysine 703 to arginine mutant STAT1 (lanes 6–9) and the indicated SUMO and PIAS expression vectors. Lane 1 represents a control in which cells were transfected with only empty vector.
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Our results demonstrate that STAT1 can be modified by SUMO at lysine 703. We were unable to demonstrate directly using SUMO antibodies that the higher molecular mass forms of STAT1 are in fact SUMO conjugates. However, collectively, the data provide compelling evidence that STAT1 is modified by SUMO at lysine 703 in vivo. First, detection of the higher molecular mass forms of STAT1 was enhanced in cells overexpressing SUMO. Second, detection of the higher molecular mass forms of STAT1 was further enhanced by co-expression of PIASx-α, which we found promotes SUMO modification of STAT1 in vitro. Third, the higher molecular mass forms of STAT1 that were detected corresponded to the predicted molecular masses of STAT1-SUMO or STAT1-GFP-SUMO, depending on the form of SUMO overexpressed in the cells. Finally, the higher molecular mass forms of STAT1 were not detected in cells expressing the lysine 703 to arginine mutant STAT1, which we showed is not modified by SUMO in vitro.

SUMO Modification of STAT1 at Lysine 703 Is Not Required for PIASx-α or PIAS1-Mediated Inhibition of STAT1—Previous studies using in vivo reporter assays indicate that PIAS proteins can inhibit STAT-mediated gene activation (7, 25, 26). Our results demonstrate that STAT1 can be modified by SUMO at lysine 703 in vivo and that PIASx-α can act as an E3 ligase to stimulate modification of STAT1 at this residue. To determine whether SUMO modification of STAT1 at lysine 703 is functionally related to PIAS-mediated STAT1 inactivation, luciferase assays were performed using a reporter construct containing a (3x)Ly-6E promoter fused to the firefly luciferase gene. (3x)Ly-6E is a construct consisting of three copies of a promoter element found in the murine Ly-6/ER gene, which is transcriptionally induced by treatment of cells with IFN-α, β, or γ (27).

We first performed experiments to determine whether the lysine 703 to arginine mutant STAT1 could activate gene expression in a manner comparable with wild-type STAT1. U3A cells were transiently transfected with FLAG-tagged wild-type STAT1 or a lysine 703 to arginine mutant STAT1 and the (3x)Ly-6E reporter construct. The transfected cells were treated with IFN-γ for 6 h, and cell lysates were then assayed for luciferase activity. Cells co-transfected with wild-type STAT1 and (3x)Ly-6E yielded a ∼4.6-fold increase in luciferase expression in response to IFN-γ compared with a ∼5-fold increase when the lysine 703 to arginine mutant STAT1 was co-transfected with the reporter construct (Fig. 7A). These results indicate that a lysine to arginine substitution at residue 703 in STAT1 does not inhibit STAT1 ability to activate transcription in response to IFN-γ stimulation. The slight, but reproducible increase in transcription activation observed with the lysine 703 to arginine mutant STAT1 relative to the wild-type STAT1 suggests that SUMO modification of lysine 703 may play an inhibitory role.

We next assayed for the ability of PIAS1 and PIASx-α to inhibit gene activation induced by wild-type STAT1. It has previously been shown that PIAS1 is able to inhibit STAT1-mediated gene activation (7, 25, 26); however, the affects of
PIASs on STAT1 gene activation have not been definitively characterized. When FLAG-tagged PIAS1 was co-transfected in increasing amounts with wild-type STAT1, PIAS1 reduced IFN-γ-stimulated transcriptional activation in a dose-responsive manner (Fig. 7B), similar to that previously observed by others (7, 25, 26). When FLAG-tagged PIASx-α was co-transfected with wild-type STAT1, a reduction in gene activation was also observed (Fig. 7B). Both PIAS1 and PIASx-α were expressed at comparable levels in these experiments, as determined by immunoblot analysis (data not shown). At these expression levels PIASx-α acted as a more potent inhibitor of STAT1 gene activation, reducing induced expression of the reporter gene by 2.1-fold at the lower protein concentration compared with 1.1-fold inhibition by PIAS1.

Finally, we examined the ability of PIAS1 and PIASx-α to inhibit gene activation mediated by the lysine 703 to arginine mutant STAT1 that cannot be modified by SUMO. Cells were co-transfected with the lysine 703 to arginine STAT1 mutant, the (3x)LY-6E reporter construct, and PIAS1 or PIASx-α. Cell lysates were again prepared 6 h after IFN-γ treatment for luciferase assays. These assays revealed that the lysine 703 to arginine mutant STAT1 is inhibited by both PIAS1 and PIASx-α (Fig. 7C). Most significantly, the levels of inhibition were comparable with the levels of inhibition observed with wild-type STAT1. These results demonstrate that the PIAS proteins are able to inhibit interferon-induced gene activation by STAT1 in a manner that is independent of STAT1 SUMO modification.

**DISCUSSION**

Because the discovery that PIAS proteins act as E3 ligases for SUMO modification, there has been speculation that STAT1 may be a SUMO substrate. We have demonstrated that STAT1 can be modified by SUMO at lysine residue 703 in vitro and also provide compelling evidence that STAT1 is modified at this same lysine residue in vivo. We have also found that PIASx-α (but not PIAS1) can function as an E3 ligase to enhance the SUMO modification of STAT1. Overexpression of PIASx-α, like PIAS1, was able to inhibit STAT1-mediated gene activation in cultured cells. Significantly, however, both PIAS1- and PIASx-α-mediated inhibition of STAT1 gene activation was found to be independent of SUMO modification of STAT1 itself.

The specific mechanism by which PIAS proteins inhibit STAT1-activated transcription in vitro has not previously been defined. In vitro experiments suggest that direct interactions between PIAS1 and STAT1 may interfere with the STAT1 ability to bind DNA (7). The recent discoveries that PIAS proteins function as SUMO E3 ligases, however, has raised the possibility that PIAS-mediated inhibition of STAT1 transcription may involve direct SUMO modification of STAT1. Although our findings indicate that STAT1 can be modified by SUMO, several lines of evidence indicate that this modification does not play a direct role in the PIAS-mediated inhibition of transcription measured in our assays. First, our results indicate that both PIASx-α and PIAS1 are able to inhibit STAT1-mediated gene activation in vitro but that only PIASx-α acts as a SUMO E3 ligase that enhances the SUMO modification of STAT1. Most significantly, PIAS1 and PIASx-α were able to inhibit both wild-type STAT1 and a mutant STAT1 not able to be modified by SUMO.

Together these findings indicate that the PIAS proteins can inhibit STAT1 through a mechanism that is not dependent on direct SUMO modification of STAT1. Inhibition may involve interactions between STAT1 and the PIAS proteins that prevent DNA binding and/or interaction with other regulatory factors, as previously suggested (7). Alternatively, it is also possible that the observed inhibition of STAT1 may involve PIAS-mediated SUMO modification of transcription regulators other than STAT1. Notably, overexpression of both PIAS1 and PIASx-α in vivo enhances the SUMO modification of many proteins (data not shown) (10). Although the precise identity of these SUMO conjugates is not known, they are likely to include numerous transcriptional regulators known to undergo SUMO modification. It is conceivable that the enhanced modification of some of these factors could indirectly regulate STAT1 activity. Consistent with this model, LEF1 (20), p53 (13), C/EBPα (22), and the progesterone receptor (15) are all inhibited by PIAS protein overexpression, independent of their ability to be directly modified by SUMO.

Although our findings do not reveal a relationship between SUMO modification of STAT1 and PIAS-mediated inhibition of its transcriptional activity, our findings do nonetheless establish that STAT1 is a SUMO substrate. To address the effects of SUMO modification on STAT1, more specific approaches to selectively enhance the levels of STAT1 SUMO modification in vivo are required. However, the position of modification at lysine 703 provides insights that allow us to speculate on the role for SUMO modification of STAT1. Analysis of the crystal structure of STAT1 reveals that residues encompassing the SUMO modification site are located at the dimer interface (28). Significantly, however, amino acid residues likely to be directly involved in interactions with Ubc9 and subsequent SUMO modification (isoleucine 702, lysine 703, and glutamic acid 705) are exposed on the surface of the protein dimer (Fig. 8; Ref. 28).

The positions of these residues suggest that the phosphorylated STAT1 dimer could be a substrate for SUMO modification, a suggestion that is supported by preliminary in vitro studies (data not shown). The enzymes involved in SUMO conjugation (including the PIAS proteins) are concentrated in the nucleus, making it reasonable to hypothesize that SUMO modification of STAT1 is a nuclear event. SUMO modification of STAT1 dimers in the nucleus could have a stabilizing effect by protecting the dimer from phosphatases, or it could play a role in recruiting co-factors that regulate STAT1-mediated transcription activation. Alternatively, SUMO modification at lysine 703 could...
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In conclusion, we have shown that STAT1 is a SUMO substrate and that PIASx-α is able to act as an E3 ligase and enhance its SUMO modification. Most importantly, we have demonstrated that inhibition of STAT1 by overexpression of PIAS proteins does not require direct SUMO modification of STAT1 itself. We propose a model in which PIAS overexpression leads to the SUMO modification of other transcription regulators that indirectly affect STAT1-mediated gene activation. Identification of these other regulatory SUMO substrates will be an important task for the future. In addition, it will also be of great interest to determine how SUMO modification of STAT1 at lysine residue 703 regulates its functions.

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