Stat1 and SUMO modification

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Many proteins are known to undergo small ubiquitin-related modifier (SUMO) modification by an E1-, E2-, and E3-dependent ligation process. Recognition that protein inhibitor of activated signal transducers and activators of transcription (STATs) (PIAS) proteins are SUMO E3 ligases raised the possibility that STATs may also be regulated by SUMO modification. Consistent with this possibility, a SUMO-ylation consensus site (ΨKxE; Ψ indicates hydrophobic residue, and x indicates any residue) was identified in Stat1 (ie, ^{702}IKTE^{705}), but not in other STATs. Biochemical analysis confirmed that Stat1 K^{703} could be SUMO modified in vitro. Mutation of this critical lysine (ie, Stat1^{K703R}) yielded a protein that, when expressed in Stat1$^{-/-}$ mouse embryonic fibroblasts (MEFs), exhibited enhanced DNA binding and nuclear retention. This was associated with modest changes in transcriptional and antiviral activity. However, mutation of the second critical residue in the SUMO consensus site, E^{705} (ie, Stat1^{E705A}), yielded a protein with wild-type DNA binding, nuclear retention, and transcriptional and antiviral activity. Similar observations were made when these mutants were expressed in primary Stat1$^{-/-}$ macrophages. These observations suggest that although Stat1 can uniquely be SUMO-ylated in vitro, this modification is unlikely to play an important role in regulating Stat1 activity in vivo. (Blood. 2006;108:3237-3244)

Introduction

Characterization of the ability of type I interferons (IFNs) (eg, IFN-α) to rapidly activate genes led to the identification of ISGF-3, a transcription factor consisting of Stat1, Stat2, and an IFN regulatory factor-9 (IRF-9) DNA binding protein.[1] Subsequently, IFN-γ was shown to induce genes through Stat1 homodimers.[2] To date, 7 signal transducers and activators of transcription (STATs) have been identified in vertebrates, all of which are activated by phosphorylation on a single tyrosine (Y701 in Stat1; reviewed in Levy and Darnell[3] and Kisseleva et al[4]). Activation drives STAT dimerization by directing a stable and specific association between the phosphotyrosine of one STAT and the src homology 2 (SH2) domain of a partner STAT.[5] Residues located at positions +1, +3, +5, +6, and +7 carboxy terminal to this phosphotyrosine (ie, amino acids 702, 704, 706, and 707 for Stat1) determine the specificity of this interaction.[6] Dimerized STATs translocate to the nucleus, where they bind to members of the gamma-activated site (GAS) family of enhancers, culminating in the induction genes.[3,4]

The regulation of STAT signal decay has also been an area of active investigation. Four major classes of counterregulatory molecules have been identified, including phosphatases,[3,4,7] nuclear "transportases,"[8-10] covalent modifiers,[4,11,12] and specific STAT counterregulatory proteins (eg, suppressor of cytokine signaling [SOCS] and protein inhibitor of activated STATs [PIAS] proteins[13,14]). Studies on SOCS-1 have provided significant evidence for a critical role in down-regulating IFNγ-Stat1–dependent signals, but studies on PIAS proteins have yielded less direct mechanistic insight into Stat1 regulation.[14-16] More recent studies have determined that PIAS proteins are small ubiquitin-related modifier (SUMO) E3 ligases, raising the possibility that STAT activity is regulated through SUMO modification.[17-19]

SUMOs are approximately 100–amino acid peptides which, like ubiquitin, become covalently attached to cellular target proteins (reviewed in Kim et al,[17] Melchior et al,[18] and Müller et al[19]). However, in contrast to ubiquitin, SUMO modifications do not target proteins for degradation, but rather promote protein–protein interactions and direct subcellular localization, and/or serve to antagonize ubiquitin-dependent degradation. SUMO conjugation entails the formation of a reversible isopeptide bond between the C-terminus of the SUMO peptide (SUMO-1, SUMO-2, or SUMO-3) and the ϵ amino group of the lysine found in the consensus sequence ψKxE (ψ indicates hydrophobic residue, and x indicates any residue; Table 1). Analogous to ubiquitin, SUMO conjugation is mediated by an ATP-dependent E1-activating complex (ie, Aos1 + Uba2), an E2 ligation complex (ie, Ubc9) and an E3 conjugation complex. The relative specificity exhibited by Ubc9 for some SUMO substrates is likely to account for E3-independent SUMO conjugation observed in vitro.[20,21] Finally, isopeptidases from the SUSP/SENP family assure that SUMO modification is reversible.[18,22]

Sequence analysis revealed 2 potential SUMO modification sites, ^{109}LKEE^{112} and ^{702}IKTE^{705}, in Stat1, but not in other STATs (Table 1). In vitro SUMO conjugation studies determined that Stat1 is SUMO modified at lysine 703, but not lysine 110. A subsequent functional analysis of 2 SUMO-ylation–resistant Stat1 mutants,

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Table 1. Comparison of SUMO modification consensus sites

	Modification
Potential SUMO conjugation sites; ψKxE	
Stat1	107-SC**LKEE**RKI
Stat1	700-GY**IKTE**LIS
Stat2	693-GY**VPSV**FIP
Stat3	704-PY**LKTK**FIC
Stat4	688-KYLKHKLIV
Wild-type and mutant SUMO conjugation sites; ψKxE	
p53	320-HKKLMF**K**TEGPDSD
$p53^{K386M}$	320-HKKLMF**M**TEGPDSD
Stat1	697-KGTGYI**K**TELISVS
$Stat1^{K703R}$	697-KGTGYI**R**TELISVS
PO4-Stat1	697-KGTGYI**K**TELISVS
Stat3	701-SAAPYL**K**TKFICVT

$Stat1^{K703R}$ and $Stat1^{E705A}$, revealed 2 distinct phenotypes. $Stat1^{K703R}$ exhibited enhanced DNA binding, prolonged nuclear retention, and modest changes in the biologic response to IFN-γ, as recently reported.[23] In contrast, $Stat1^{E705A}$ exhibited wild-type DNA binding, nuclear retention, and biologic response to IFN-γ. These observations suggest that lysine 703, located at the critical interface between Stat1 homodimers,[6] plays a fortuitous and structurally important role in Stat1 DNA binding activity, and that Stat1 activity is not likely to be significantly regulated by SUMO conjugation in vivo.

Materials and methods

Cell culture

HEK-293T, Vero, and L929 cells were from ATCC (Manassas, VA); and *Stat1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) and macrophages were harvested from *Stat1*$^{-/-}$ mice (generously provided by D. Levy, New York University, NY). Cells were cultured in Dulbecco modified Eagle medium, supplemented with 10% fetal bovine serum from GIBCO Laboratories (Grand Island, NY). Bone marrow (from mouse femurs)–derived macrophages were cultured in 20% L929 cell–conditioned media for 5 to 10 days.[24] After 16 hours in culture, adherent bone marrow cells were infected (3 times) with retrovirus freshly prepared from HEK-293T transfectants (below). $Stat1^{-/-}$ MEFs were infected twice with pMIG retroviruses encoding Stat1, $Stat1^{K703R}$, and $Stat1^{E705A}$ in the presence of polybrene (8 μg/mL; Sigma, St Louis, MO), as previously reported.[25] High-titer viral supernatants were prepared through transient transfection in HEK-293T cells by calcium phosphate precipitation.[9] Retroviral infection efficiency, as determined by fluorescence-activated cell-sorter (FACS) (green fluorescent protein–positive [GFP^+] cells; FACS-Calibur; BD Biosciences, San Jose, CA), varied between 90% to 95% in MEFs and 25% to 35% in bone marrow macrophages (BMMs).

For viral response assays, MEFs were infected with vesicular stomatitis virus (VSV, Indiana strain; gift from R. Pine, Public Health Research Institute, Newark, NJ) prepared in Vero cells. Viral yield was measured 24 hours after infection by titering on Vero cells overlayed with 1.5% methyl cellulose for 36 hours.[26] Expression of the reporter GFP gene and major histocompatibility complex (MHC) II (after staining with anti-mouse $I\text{-}A^b$; BD Pharmingen, San Diego, CA) was evaluated by flow cytometry (FACS-Calibur).[9,26] Nitric oxide (NO) production was evaluated at day 10 of culture and 72 hours after IFN-γ (50 U/mL) and/or LPS (2 μg/mL) stimulation, as previously described.[27]

Biochemical studies

Recombinant SUMO-1, E1, and E2 were expressed, purified, and assayed as previously described.[20] The IR1-M-IR2 domains of Nup358 (aa 2596-2836) and full-length PIAS1 (aa 1-651) were cloned and expressed as Smt3 fusion proteins, cleaved by Ulp1, and purified by anion exchange and gel filtration chromatography.[28] To assay for Stat1 SUMO-ylation, purified recombinant Stat1 (0.5 μM) was incubated with SUMO-1 (2 μM), E1 (Uba2/Aos1; 0.3 μM), E2 (Ubc9; 0.3 μM), and E3 (PIAS1 or Nup358 at 0.3 μM) in a buffer with 5 mM $MgCl_2$, 20 mM HEPES (pH 7.5), 1 mM DTT, 2 mM ATP, and 0.5 U pyrophosphatase (Sigma) for 1 hour at 37°C. To assay for peptide SUMO-ylation, p53 (residues 323-393), $p53^{K386M}$ (residues 320-393) Stat1/phospho-Stat1 (residues 697-711), or Stat3 (residues 701-714) peptides (at 500 μM) were incubated with E1 and either wild-type or mutant Ubc9 for 5 or 24 hours at 37°C, as previously described.[29] Samples were fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Blue or Sypro (4-12–hour staining, > 1 hour destaining; Bio-Rad, Hercules, CA). Sypro-stained gels were imaged by UV illumination and the band intensity was quantified (Bio-Rad UV system; Quantity One Software; Bio-Rad).

Overexpression assays entailed transfecting protamine complementary DNA (pcDNA; Invitrogen, Carlsbad, CA) expression plasmids encoding hemagglutinin (HA)–SUMO-1, His–SUMO-1, Ubc9, and/or PIAS-y (gifts from S. Goff, Columbia University, New York, NY) into HEK-293T cells by calcium phosphate precipitation or MEFs with LipofectAmine Plus regent (Invitrogen). For oligonucleotide "pull-down" assays 20 μg GAS oligonucleotide (Table S1, available on the *Blood* website; see the "Supplemental Table" link at the top of the online article) coupled to 75 μL biotinylated agarose beads (Sigma) was incubated with 400 μL whole-cell extracts (WCEs) for 2 hours at 4°C, after blocking the beads with 1% bovine serum albumin (BSA) for 1 hour at 4°C, as previously reported.[30] For immunoprecipitation, 400 μL WCEs was incubated with primary antibody for 2 to 16 hours followed by protein A agarose (Sigma), and then collected, as previously reported.[9,26] WCE or precipitates were fractionated by SDS-PAGE and then evaluated by immunoblotting with the appropriate antibody.[9] For nickel pulldown, 800 μL WCEs was incubated with 100 μL ProBond nickel beads, washed, and eluted as per the manufacturer's instructions (Invitrogen). For electrophoretic mobility shift assay (EMSA), extracts were incubated with a GAS probe and fractionated by native PAGE as previously described.[9,26] Antibodies were directed against Stat1,[1] phosphotyrosine Stat1 (Cell Signaling Technology, Beverly, MA), MHC II (BD Pharmingen), HA (Covance, Berkeley, CA), and β-actin (Sigma).

Plasmid constructs

pClEco and pMIG were provided by J. Luban (Columbia University).[25] Murine Stat1, $Stat1^{K703R}$, and $Stat1^{E705A}$ were cloned into the *Xho*I site in pMIG. $Stat1^{K703R}$ and $Stat1^{E705A}$ were prepared by site directed mutagenesis (Quickchange kit; Stratagene, La Jolla, CA) and confirmed by sequencing (Table S1 for primer sequences).

Immunofluorescence

Cells were cultured on sterile cover slips until 20% to 25% confluent, fixed in formaldehyde, and stained as previously reported[9] with Stat1-specific antibodies (1:250 fold dilution) and a Cy3-conjugated secondary antibody (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA). Slides were examined under a Nikon Eclipse TE300 microscope (Nikon, Melville, NY) after excitation at 550 nm (Cy3) and excitation at 495 nm (GFP).

RNA expression analysis

Total RNA was prepared from MEFs with Trizol (Invitrogen) extraction. RNA (4 μg) was treated with RQ1 DNAse (Promega, Madison, WI) and then reverse-transcribed with SuperScript II (Invitrogen). The cDNA was quantitatively amplified in an ABI Prism 7700 polymerase chain reaction (PCR) system with a SYBR green master mix (Applied Biosystems, Foster City, CA) as previously described.[24] Gene expression was normalized to a β-actin control.

Luciferase reporter assay

MEFs were seeded in 24-well plates (7.5×10^5/well) and transfected with 400 ng of an IRF-driven luciferase reporter (ie, B2WT3[31]) and

40 ng pRL-tk renilla in LipofectAmine Plus (Invitrogen). Later (24 hours), transfectants were stimulated with IFN-γ (5 U/mL for 6 hours), resuspended in a passive lysis buffer, and evaluated in a luminometer (TD 20/20; Turner Systems, Sunnyvale, CA), as previously reported.[31] Experiments were done in triplicate and luciferase activity was normalized to renilla activity.

Results

Stat1 can be SUMO modified

Recent studies identifying PIAS proteins as SUMO E3 ligases suggested that STATs may also be SUMO modified.[17-19] Analysis of all mammalian STAT sequences identified 2 potential SUMO modification sites: Stat1 residues ^{109}LKEE^{112} and ^{702}IKTE^{705} (Table 1). Efforts to exploit SUMO-1–specific antibodies to detect endogenous SUMO-modified Stat1 were unsuccessful. To improve the chances of detecting this product, Ubc9 and HA–SUMO-1 were overexpressed in HEK-293T cells. Under these conditions SUMO-Stat1 conjugates were readily recovered in Stat1 immunoprecipitates (Figure 1A) as previously reported.[32,33] Coexpression of PIAS-y, however, impeded recovery of SUMO-Stat1 conjugates, most likely because of reduced levels of SUMO-1 and Ubc9 expressed in triple transfectants.

One potential SUMO modification site, lysine 703 (K703), lies adjacent to the tyrosine that is phosphorylated during Stat1 activation, (ie, Y701). It was therefore important to determine whether SUMO conjugation affected Stat1 phosphorylation and subsequent DNA binding activity. As anticipated, phosphorylated Stat1 was readily recovered, by either immunoprecipitation or oligonucleotide precipitation, from prepared extracts of IFN-γ–stimulated HEK-293T cells overexpressing Ubc9 and HA–SUMO-1 (Figure 1B, left panel). Although slower migrating SUMO-Stat1 conjugates were readily recovered in the Stat1 immunoprecipitates, they were not detected in the oligonucleotide precipitates, suggesting that SUMO-modified Stat1 will not bind DNA. Likewise, phosphorylated Stat1 was readily recovered in Stat1 immunoprecipitates prepared from IFN-γ–stimulated HEK-293T cells overexpressing Ubc9 and His–SUMO-1. Yet, even though Stat1 was readily collected by nickel–His-SUMO-1 pull-down and phospho-Stat1 was abundant, no phospho-Stat1 was recovered in the nickel pull-downs (Figure 1B, right panel). These observations suggest that Stat1 modification by tyrosine phosphorylation and SUMO-ylation are mutually exclusive.

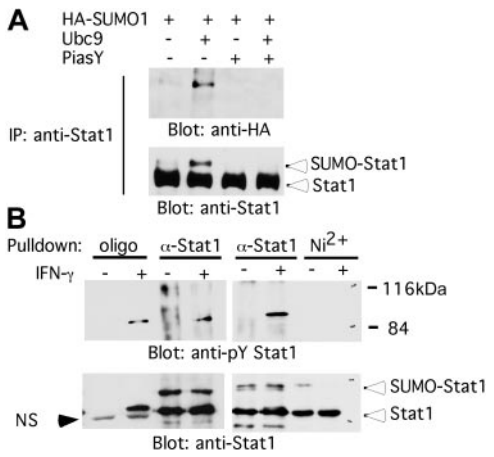

Figure 1. Stat1 SUMO-ylation in HEK-293T cells. (A) HEK-293T cells were transfected with HA–SUMO-1, Ubc9, and/or PIASy. Later (48 hours), they were stimulated with IFN-γ (3 ng/mL for 30 minutes). WCEs were immunoprecipitated with an anti-Stat1 antibody and sequentially immunoblotted with HA and Stat1 antibodies. (B) Left panel: 48 hours after transfection with HA–SUMO-1 and Ubc9, HEK-293T cells were stimulated with IFN-γ (3 ng/mL for 30 minutes). WCEs were either precipitated with a biotinylated GAS oligonucleotide (Oligo; Table S1) or Stat1-specific antibody (α-Stat1). Precipitates were fractionated by SDS-PAGE, and sequentially immunoblotted with Stat1-phosphotyrosine (top) and Stat1-specific antibodies (bottom). Right panel: WCEs were prepared 48 hours after transfection with His–SUMO-1 and Ubc9; HEK-293T cells were stimulated with IFN-γ (3 ng/mL for 30 minutes) and either precipitated with a Stat1-specific antibody (anti-Stat1) or ProBond nickel beads, and sequentially immunoblotted with Stat1-phosphotyrosine (top) and Stat1-specific antibodies (bottom). Nonspecific (NS) band is indicated. Data are representative of 3 independent studies.

Stat1 is SUMO modified at K703

To develop more direct evidence for Stat1 SUMO modification and to map the SUMO-ylation site, we turned to an effective in vitro conjugation assay that overcomes limitations imposed by SUMO deconjugating enzymes.[20,22,34] Purified recombinant Stat1 was incubated with purified active preparations of human recombinant SUMO-1, E1 (Uba2/Aos1), E2 (Ubc9), and E3 (Nup358 or PIAS1).[20,21,35] After 1 hour, the products were fractionated by SDS-PAGE and immunoblotted with a Stat1-specific antibody. As shown in Figure 2, the anticipated approximately 105-kDa SUMO-Stat1 conjugate was readily formed in an ATP-dependent, but not E3-dependent manner (Figure 2A). Analogous results were obtained with a purified p53 control (data not shown). The SUMO-Stat1 conjugates (approximately 3%-4% of the total Stat1) and the remaining nonreacted (ie, native) Stat1 were then excised, digested with trypsin, and evaluated by mass spectrometry. The specific loss of peptides spanning K703, but not K110 indicated that Stat1 had been SUMO modified at K703 (data not shown). To confirm that K703 was the SUMO-ylation site, a Stat1 peptide spanning residues 697-711 (ie, KGTGYIKTELISVS) was evaluated in an in vitro SUMO conjugation system where a defective E2 (ie, Ubc9^{C93S}) served as a negative control.[29] Several additional peptides were evaluated. This included the analogous peptide from Stat3 (ie, AAPYLKTKFICVT) and a Stat1 peptide in which K703 was mutated to arginine (ie, KGTGYIRTELISVS). To explore whether Stat1 Y701 phosphorylation precludes SUMO modification, as suggested by HEK-293T overexpression studies (Figure 1), a Stat1 phosphopeptide (ie, phospho-Y701) was also evaluated. Again, p53 peptides spanning a well-characterized SUMO-ylation site served as important controls.[20] After 5 and 24 hours of SUMO conjugation, only the wild-type Stat1 peptide was SUMO-ylated as effectively as the control wild-type p53 peptide (Figure 2B). More detailed kinetic studies provided further evidence that wild-type Stat1 was an effective SUMO substrate, whereas Stat3, phospho-Stat1, and the mutant peptides were poor substrates (Figure 2C). These data confirmed K703 as the SUMO modification site, but only when the adjacent Y701 was not phosphorylated. The failure to SUMO-ylate the Stat3 peptide was consistent with our inability to modify purified preparations of recombinant Stat3[9] (data not shown), reflecting a critical divergence in the corresponding potential Stat3 SUMO conjugation site (Table 1).

Stat1^{K703R} exhibits enhanced DNA binding activity

To explore the potential role of Stat1 SUMO modification in vivo, a SUMO-ylation–resistant Stat1^{K703R} mutant was generated. Retroviral vectors directed expression of Stat1^{K703R} and wild-type Stat1 at physiologic levels in *Stat1*$^{-/-}$ MEFs[36] in more

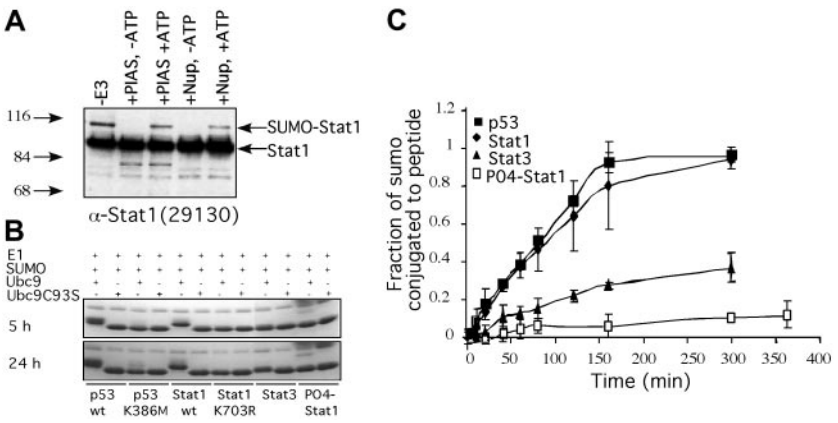

Figure 2. In vitro SUMO conjugation assay. (A) Purified recombinant Stat1 (0.5 μM) is SUMO-ylated in vitro after incubation with SUMO-1 (2 μM), E1 (Uba2/Aos1; 0.3 μM), E2 (Ubc9; 0.3 μM), and E3 (PIAS1 or Nup358 at 0.3 μM) for 1 hour at 37°C. Samples were fractionated by SDS-PAGE and immunoblotted with anti-Stat1. Mobility of molecular weight markers and Stat1 isoforms are indicated. (B) Wild-type and mutant p53, Stat1, PO_4-Stat1, and Stat3 peptides (500 μM) were SUMO-ylated, as in panel A, in the absence of E3, with either wild-type or mutant Ubc9 for 5 or 24 hours at 37°C. Samples were fractionated by SDS-PAGE and stained with Coomassie Blue. (C) Graphic representation of a more detailed kinetic SUMO-ylation assay of peptides from panel B (t = 0, 10, 40, 60, 90, 160, and 300 minutes). Maximal conjugation is 1.0. Products were detected by staining the gel with Sypro (Bio-Rad). The images were quantified on Quantity One Software (Bio-Rad). Error bars indicate standard deviation.

than 95% of infected cells (data not shown; Figure 3A-B). Upon brief (ie, 0.5 hours) stimulation with IFN-γ, both Stat1 and Stat1^{K703R} were rapidly activated (data not shown; Figure 3B). However, when these extracts were evaluated by EMSA, the DNA binding activity of Stat1^{K703R} was significantly more prolonged than that of wild-type Stat1 (data not shown; Figure 3C). To determine whether Stat1^{K703R} exhibited an enhanced affinity for GAS elements, DNA dissociation studies (ie, "off-rate") were performed. Stat1^{K703R} displayed a 4-fold slower off-rate than wild-type Stat1 (ie, $t_{1/2} > 120$ vs 30 minutes; Figure 4A-B). Likewise, Stat1^{K703R} exhibited an increased relative GAS binding activity (approximately 4-fold) compared with wild-type Stat1 (Figure 4C-D). As anticipated, no differences were observed in ISGF3-ISRE DNA binding activity in IFN-α–stimulated extracts, where IRF-9 mediates DNA binding

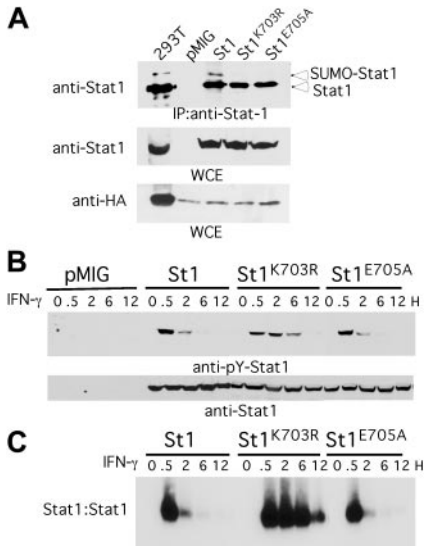

Figure 3. IFN-γ–dependent Stat1, Stat1^{K703R}, and Stat1^{E705A} activation. (A) Stat1$^{-/-}$ MEFs, infected with an empty vector (pMIG) or retroviral vectors directing expression of either Stat1 (St1), Stat1^{k703R} (St1^{K703R}), or Stat1^{E705A} (St1^{E705A}), were subsequently transfected with HA–SUMO-1 and Ubc9 cDNA constructs. WCEs were prepared, immunoprecipitated with anti-Stat1, fractionated on 7% SDS-PAGE, and immunoblotted for Stat1 (top panel); or extracts were directly fractionated on 12% SDS-PAGE and sequentially immunoblotted with anti-Stat1 and anti-HA (bottom panels). WCEs from 293T cells transfected with HA–SUMO-1 and Ubc9 from Figure 1 served as positive controls. (B) *Stat1*$^{-/-}$ MEFs from panel A were stimulated with IFN-γ (50 U/mL for 0.5-12 hours). WCEs were prepared, fractionated by SDS-PAGE, and sequentially immunoblotted with antibodies specific for phosphotyrosine-Stat1 (top panel) and total Stat1 (bottom panel). (C) Extracts from panel B were evaluated by EMSA with a GAS probe. Data are representative of 3 independent studies in MEFs.

(data not shown). In sum, these studies demonstrate that mutation of lysine 703 to arginine significantly enhanced Stat1 GAS binding activity in response to IFN-γ stimulation, recently also reported in human U3A cells.[23]

Analysis of additional Stat1 SUMO-ylation–defective mutant

To develop additional evidence that Stat1 was SUMO modified at K703 in vivo, a second critical amino acid in the Stat1 SUMO consensus modification site (ie, E705), which is also not involved in dimerization, was mutated to alanine.[6,17,18] Again, retroviral vectors directed a physiologic expression of Stat1^{K703R}, Stat1^{E705A}, and wild-type Stat1 in more than 95% of Stat1$^{-/-}$ MEFs (Figure 3A, top panel). To confirm that both mutants were defective in SUMO modification, HA–SUMO-1 and Ubc9 were overexpressed in these MEFs. Despite modest transfection efficiency in these MEFs (Figure 3A, bottom panel), the studies clearly demonstrate the formation of the approximately 105-kDa SUMO-Stat1 conjugate in cells expressing wild-type Stat1, but not in those cells expressing the SUMO-ylation–defective mutants Stat1^{K703R} and Stat1^{E705A} (Figure 3A, top panel).

Next, the IFN-γ–dependent activation of Stat1^{E705A} was evaluated. Stat1^{E705A} exhibited a pattern of activation (ie, tyrosine phosphorylation) and DNA binding that was identical to that of wild-type Stat1, with continuous (ie, up to 12 hours) IFN-γ stimulation (Figure 3B-C). Likewise, Stat1^{K703R} exhibited an essentially normal pattern of tyrosine phosphorylation, notable for a slight delay at early time points and more robust activity at later time points (Figure 3B).

However, these modest changes in IFN-γ–dependent Stat1^{K703R} tyrosine phosphorylation correlated with a striking increase in DNA binding activity (Figure 3C). Thus, SUMO-ylation–defective Stat1^{E705A} exhibits an activation profile that is distinct from Stat1^{K703R}, but similar to wild-type Stat1.

The rapid and transient translocation of Stat1 to the nucleus is another characteristic feature of Stat1 signaling activity. Since several studies have implicated SUMO modification in the regulation of nuclear trafficking,[34,37,38] a set of Stat1 immunolocalization studies were carried out. Analogous to the pattern observed with wild-type Stat1, both Stat1^{K703R} and Stat1^{E705A} exhibited a predominately cytoplasmic distribution in unstimulated cells and robust nuclear accumulation with IFN-γ stimulation (ie, 0.5 hours; Figure 5). After stimulation (6 hours), both Stat1 and Stat1^{E705A} were fully re-exported back to the cytoplasm, rendering those cells ready for another round of stimulation. In contrast, Stat1^{K703R} continued to exhibit a strong nuclear retention, consistent with its enhanced DNA binding activity.[10] These studies demonstrate that the 2

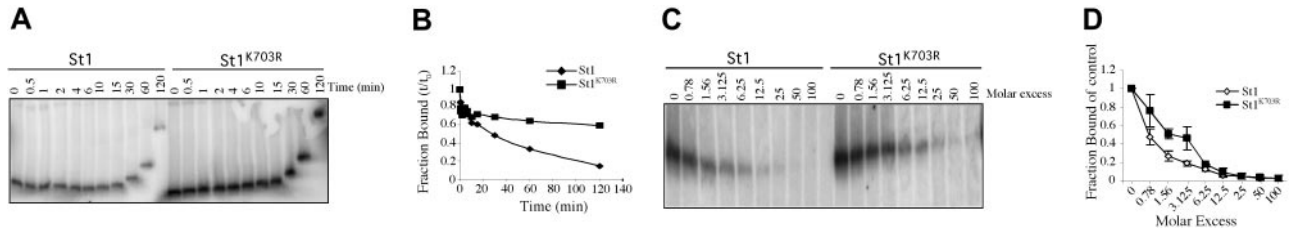

Figure 4. Kinetics of IFN-γ–dependent Stat1 and Stat1^{K703R} DNA binding. (A) *Stat1*$^{-/-}$ MEFs, infected with retroviral vectors directing expression of either Stat1 (St1) or Stat1^{K703R} (St1^{K703R}), were stimulated with IFN-γ (66 U/mL for 30 minutes). Dissociation kinetics of Stat1 DNA binding activity were determined by incubating the IFN-γ–stimulated extracts with a labeled GAS probe (0.1 pmol for 20 minutes at 22°C) and then chasing with a 100-fold excess of cold probe. Aliquots were removed at indicated times and loaded onto a running gel. (B) Quantification of DNA binding from panel A was determined by ImageQuant (BD Molecular Dynamics, Santa Clara, CA) and plotted as fraction bound versus time. (C) Relative affinity was determined by simultaneously incubating IFN-γ–stimulated WCEs (from panel A) with a labeled GAS probe (0.1 pmol) and increasing molar excess of cold probe, as indicated (20 minutes at 22°C). Samples were then run on EMSA gel. (D) Quantification of DNA binding results from panel C was determined by imagequant and plotted as fraction bound versus molar excess of cold probe. Data are representative of 3 independent studies. Error bars indicate standard deviation.

SUMO-ylation–defective Stat1 mutants exhibit remarkably divergent phenotypes.

Evaluation of the biologic response mediated by Stat1^{E705A} and Stat1^{K703R} in MEFs

Next, several studies were undertaken to explore the potential role of SUMO-ylation in regulating biologic responses directed by Stat1. In the first set of studies, the ability of *Stat1*$^{-/-}$ MEFs expressing wild-type Stat1, Stat1^{K703R}, or Stat1^{E705A} to direct the IFN-γ–dependent induction of several target genes was explored (Figure 6). Wild-type Stat1 and Stat1^{E705A} promoted similar normal patterns of IRF1, TAP1, GBP1, and Mig expression.[24,39] However, cells expressing Stat1^{K703R} exhibited a delayed expression pattern, with significant reduction of target gene expression at 2 and 6 hours after stimulation, followed by normalization of expression by 12 hours (Figure 6A). Changes in the expression of a GAS-driven reporter gene, which tends to measure a more averaged transcriptional response, did not reveal any statistically significant differences between wild-type, Stat1^{E705A}, or Stat1^{K703R} (Figure 6B; Figure 6C illustrates that all Stat1s were expressed at equivalent levels). There was, however, a consistent trend toward diminished reporter expression in the Stat1^{K703R} cells. Thus, Stat1^{E705A} is functionally indistinguishable from wild-type Stat1, but the transcriptional kinetics in Stat1^{K703R} cells are notable for a reduced initial expression of target genes in the setting of a mutant with enhanced DNA binding activity.

IFNs stimulate a potent and physiologically important antiviral response, which represents an integrated response of numerous target genes.[4,26] To determine whether SUMO-ylation–defective Stat1 mutants direct an abnormal antiviral response to IFN-γ, *Stat1*$^{-/-}$ MEFs expressing either wild-type Stat1, Stat1^{K703R}, or Stat1^{E705A} were infected with VSV. In the first study, cells were pretreated with increasing doses of IFN-γ (0.5-50 U/mL) prior to infection with a fixed multiplicity of infection (MOI = 0.5; Figure 7A, top panel). As anticipated, Stat1$^{-/-}$ cells were not protected by IFN-γ pretreatment,[26,36] but Stat1^{E705A} and wild-type Stat1 directed the same robust antiviral response to IFN-γ (at 5 and 50 U/mL). Stat1^{K703R} demonstrated modestly enhanced antiviral activity, but only at low IFN-γ doses. Similarly, Stat1^{K703R} directed a modestly enhanced antiviral response to IFN-γ (5 U/mL) when the VSV MOI was varied from 0.001 to 1.0 (Figure 7A, bottom panel). Thus, SUMO-ylation–defective Stat1^{E705A} directs a wild-type antiviral response to IFN-γ. In contrast, Stat1^{K703R}, with its enhanced DNA binding ability, directs a modestly enhanced response.

Evaluation of the biologic response mediated by Stat1^{E705A} and Stat1^{K703R} in macrophages

Macrophages are an important physiologic target of IFN-γ.[4,27] To evaluate the biologic activity of the SUMO-ylation–defective Stat1 mutants, primary Stat1$^{-/-}$ BMMs were infected with retroviruses encoding wild-type Stat1, Stat1^{K703R}, or Stat1^{E705A}. Modest differences in the level of expression were noted that correlated with an infection efficiency, which ranged between 25% and 35% (not shown). First, these macrophages were assessed for a Stat1-dependent ability to induce NO production upon stimulation with IFN-γ plus LPS (Figure 7B, top panel).[26,27,40] Modest differences between the capacity of wild-type and mutant Stat1 to direct NO production appeared correlated with differences in the level of Stat1 expression. Next, the Stat1-dependent ability of IFN-γ to induce MHC II expression in Stat1 "transduced" (ie, GFP^{+}) macrophages was evaluated by FACS (Figure 7B, bottom panel).[26] Again, no significant differences were observed between

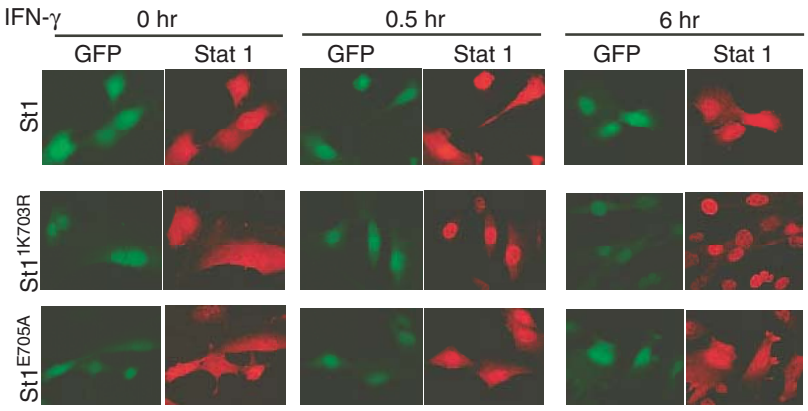

Figure 5. IFN-γ–dependent nuclear localization of Stat1, Stat1^{K703R}, and Stat1^{E705A}. Stat1$^{-/-}$ MEFs expressing physiologic levels of either Stat1 (St1), Stat1^{K703R} (St1^{K703R}), or Stat1^{E705A} (St1^{E705A}), as in Figure 3, were stimulated with IFN-γ (50 U/mL for 0, 0.5, and 6 hours). Cells were fixed and either imaged for the expression of a GFP reporter gene or after immunostaining with anti-Stat1. Cells were examined under a 40 × Nikon epifluorescence objective. Pictures were taken using a Nikon Plan Fluor ELWD 40×/0.60 objective lens and a SPOT RT color camera with SPOT advanced software (Diagnostic Instruments, Sterling Heights, MI). Data are representative of more than 3 independent studies.

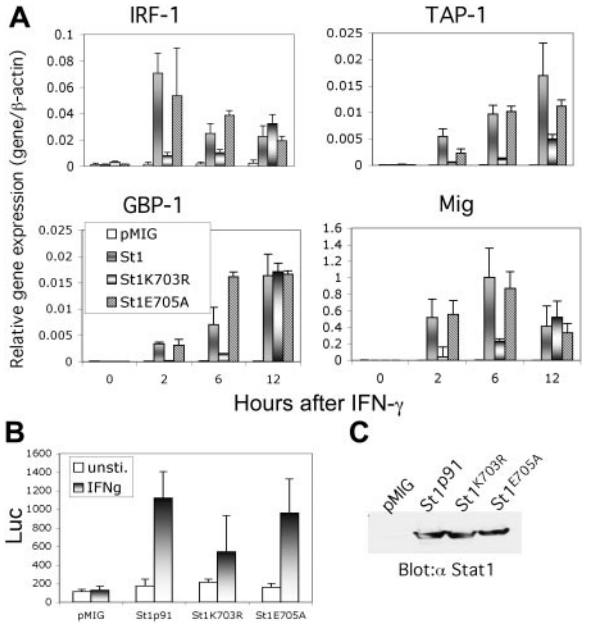

Figure 6. IFN-γ–dependent expression of target genes in Stat1, Stat1^{K703R}, and Stat1^{E705A} MEFs. (A) Total RNA was prepared from *Stat1*$^{-/-}$ MEFs ectopically expressing empty vector (pMIG), Stat1 (St1), Stat1^{K703R} (St1^{K703R}), or Stat1^{E705A} (St1^{E705A}), as in Figure 4, after stimulation with IFN-γ (50 U/mL for 0-12 hours), as indicated. The expression of target genes (*IRF1*, *TAP1*, *GBP1*, and *Mig*) was determined by quantitative PCR (Q-PCR) from cDNA templates. The relative expression of each gene was normalized to β-actin expression. (B) *Stat1*$^{-/-}$ MEFs, ectopically expressing empty vector (pMIG), Stat1 (St1), Stat1^{K703R} (St1^{K703R}), or Stat1^{E705A} (St1^{E705A}), were transiently transfected with a GAS-driven luciferase reporter (B2SH-WT3-Luc) in triplicate and stimulated with IFN-γ (50 U/mL for 6 hours). Samples were harvested and evaluated for luciferase and renilla activity (in arbitrary light units). Data are representative of 2 independent experiments. Error bars indicate standard deviation. (C) Immunoblot demonstrates that Stat1 expression (wild-type and mutants) was similar in all 3 Stat1-expressing lines.

wild-type Stat1 and the mutants, suggesting that Stat1^{K703R} and Stat1^{E705A} direct wild-type patterns of IFN-γ–dependent responses in primary macrophages.

Discussion

PIAS proteins were initially identified for their capacity to regulate STATs under conditions of overexpression.[14] However, recent studies identifying PIAS proteins as SUMO E3 ligase(s) raised the possibility that STATs may be regulated through SUMO modification.[19,34,37,38,41,42] When STAT sequences were scanned for the SUMO consensus modification site, only 2 potential sties were identified in Stat1, but not in other STATs (Table 1). Biochemical studies identified lysine 703, adjacent to the activation tyrosine (ie, Y701), as the SUMO conjugation site. However, the inability to recover tyrosine-phosphorylated SUMO-Stat1 conjugates in the HEK-293T cells raised concern over whether a single Stat1 molecule could be simultaneously modified at both Y701 and K703 (Figure 1). Consistent with this, a tyrosine-phosphorylated Stat1 peptide was a poor substrate for in vitro SUMO modification (Figure 2).

To evaluate the potential physiologic significance of SUMO Stat1 conjugation, SUMO-ylation–defective Stat1 mutants were generated. Fortuitously, the 2 most critical residues in the SUMO consensus modification site, K703 and E705, are exposed, suggesting they could be mutated without perturbing Stat1 activity.[6,32] Consistent with this, expression of the first mutant, Stat1^{K703R}, in *Stat1*$^{-/-}$ MEFs revealed a wild-type pattern of rapid IFN-γ–dependent activation. However, the activation of Stat1^{K703R} was associated with significantly enhanced DNA binding and a prolonged pattern of nuclear retention. This observation raised the

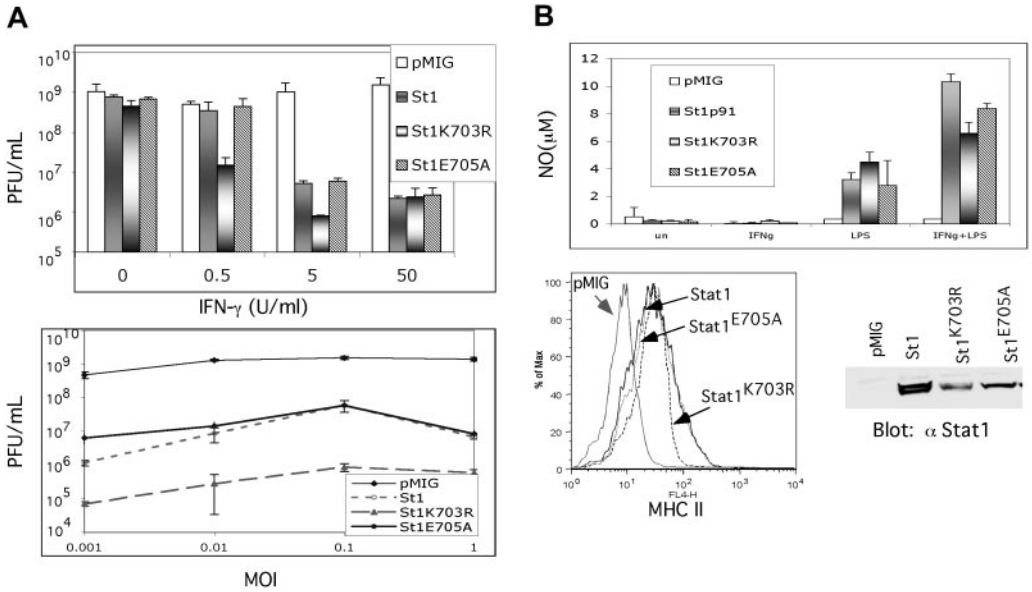

Figure 7. Biologic response of Stat1, Stat1^{K703R}, and Stat1^{E705A} in MEFs and macrophages. (A) IFN-γ–dependent antiviral response of Stat1, Stat1^{K703R}, and Stat1^{E705A} MEFs. Top panel: *Stat1*$^{-/-}$ MEFs ectopically expressing empty vector (pMIG), Stat1 (St1), Stat1^{K703R} (St1^{K703R}), or Stat1^{E705A} (St1^{E705A}), as in Figure 4, were infected with VSV (MOI = 0.5) after IFN-γ (0, 0.5, 5, and 50 U/mL for 16 hours) pretreatment. Viral yield in supernatants of infected cells was determined, in triplicate, by plaque assay on Vero cells. Data are presented as total recovered PFUs (plaque-forming units). Bottom panel: viral yield from pMIG, St1, St1^{K703R}, and St1^{E705A} MEFs infected in triplicate with VSV at varying MOIs (0.001, 0.01, 0.1, and 1) after IFN-γ (5 U/mL for 16 hours) pretreatment. Viral titer was determined as for the top panel and is representative of 3 independent studies. (B) IFN-γ–dependent Stat1, Stat1^{K703R}, and Stat1^{E705A} activity in macrophages. Top panel: *Stat1*$^{-/-}$ macrophages, infected with empty vector (pMIG), Stat1 (St1), Stat1^{K703R} (St1^{K703R}), or Stat1^{E705A} (St1^{E705A}), were evaluated for their capacity to produce NO 72 hours after stimulation with IFN-γ (50 U/mL) and/or LPS (2 μg/mL). Bottom left panel: surface MHC-II expression in GFP^{+} BMMs was determined by FACS 48 hours after stimulation with IFN-γ (50 U/mL). Bottom right panel: immunoblot demonstrates that Stat1 expression was similar in each set of transfectants. Data are representative of 3 independent studies. Error bars indicate standard deviation.

possibility that Stat1 SUMO modification might normally serve to promote Stat1 signal decay (eg, by driving DNA dissociation and thereby promoting dephosphorylation). Yet, biochemical studies failed to demonstrate that tyrosine-phosphorylated Stat1, the predicted SUMO-ylation target, could be SUMO modified. A second finding that was difficult to reconcile with this model was that the whole population of SUMO-ylation–defective Stat1^{K703R} exhibited dramatically enhanced DNA binding activity, even though only minute quantities of Stat1-SUMO conjugates were detected in wild-type cells, even under conditions of overexpression (Figure 1).[23,32,33] The most significant data to undermine the notion that SUMO conjugation regulates Stat1 activity came from the characterization of the second SUMO-ylation–defective mutant, Stat1^{E705A}. This mutant was essentially indistinguishable from wild-type Stat1. It exhibited a wild-type pattern of activation, signal decay, DNA binding, and nuclear retention. More importantly, in vivo studies demonstrated that Stat1^{E705A} and wild-type Stat1 were equivalent in their ability to direct the expression of IFN-γ target genes and IFN-γ–dependent antiviral responses. Similar results were obtained when Stat1^{E705A} was expressed in primary *Stat1*$^{-/-}$ macrophages. In sum, these observations provide strong evidence that SUMO modification does not play an important role in the regulation of Stat1 activity, either in fibroblasts or macrophages.

Upon completing our initial characterization of the Stat1 SUMO-ylation site, 2 other groups reported K703 as a Stat1 SUMO modification site.[32,33] Both found that Stat1^{K703R} exhibited relatively modest changes in Stat1^{K703R}-dependent transcriptional activity in primate cells. Subsequently, 1 of these groups confirmed our observation of enhanced IFN-γ–dependent DNA binding activity and nuclear retention with Stat1^{K703R}.[23] Analogous to our own observations, this correlated with a modest increase in the Stat1^{K703R}-dependent transcription of a reporter, as well as prolonged transcription of 3 endogenous genes (eg, *GBP1*, *IRF1*, and *TAP1*). A second SUMO-ylation–defective mutant, Stat1^{E705A}, was also evaluated and found to drive a modestly enhanced IFN-γ–dependent expression of a GAS-driven reporter gene (versus our finding of a wild-type response). However, companion DNA binding and nuclear retention studies on Stat1^{E705A} were not provided. We speculate that the modest differences between these published studies and ours reside in the cell type used. Our study used Stat1-deficient primary cells or their derivatives, and not tumor cells that had undergone extensive mutagenesis, as is the case with U3A cells.[43] Moreover, our study used a pool of stably "transduced" cells, rather than a limited number clonally selected cell lines. (Of note, in our hands the pattern of gene expression varied considerably among U3A clones; data not shown.) Finally, our studies demonstrated that IFN-γ–dependent biologic responses, including antiviral activity, NO production, and target gene expression, were not significantly perturbed in SUMO-ylation–defective Stat1 mutants.

Although the unusual properties of Stat1^{K703R} could easily be exploited to argue that Stat1 activity is regulated by SUMO conjugation of K703, we believe that our data provide compelling evidence to the contrary. Notably, even under idealized reaction conditions, with purified Stat1, only a modest fraction of Stat1 was SUMO modified (Figure 2). This contrasted the fully penetrant-enhanced DNA binding activity of Stat1^{K703R}, suggesting that this mutation causes a structural perturbation to Stat1 dimer that stabilizes DNA binding. Consistent with this, K703 residue lies in a critical location of the Stat1-Stat1 dimerization interface. More significantly, a second SUMO-ylation–defective Stat1 mutant fails to exhibit this phenotype, providing compelling evidence that the enhanced DNA binding is unrelated to a potential loss in the ability to be SUMO modified. Surprisingly however, the enhanced DNA binding activity of Stat1^{K703R} correlated with relatively meek changes in biologic response, including a modestly enhanced antiviral activity at the lowest doses of IFN-γ and a lack of differences in NO production. The enhanced antiviral activity suggests that increased DNA binding activity may compensate for low levels of activation (ie, when active Stat1-Stat1 homodimers are rate limiting). Yet, at more standard doses of IFN-γ, this advantage is lost. The lack of correlation between the enhanced DNA binding activity of Stat1^{K703R} and target gene expression was surprising. This may suggest that Stat1 plays a more important role in initiating transcription (ie, an "on switch") than in regulating the duration of a transcriptional response.[44] Moreover, the K703R mutation may impede recruitment of transcriptional cofactors, yielding an initially delayed response in target gene expression. Additional point mutations of K703 in Stat1, and closely related Stat3, will be important in exploring these possibilities.

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