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MAPK - INDUCED SER727 PHOSPHORYLATION PROMOTES SUMOYLATION OF STAT1

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Abbreviations: CBP, CREB-binding protein; ERK, extracellular signal regulated kinase; Elk, Ets-like-protein; GAS, gamma-activated sequence; HSF, heat shock factor; IFN, interferon; IRF1, interferon regulatory factor 1; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinase; MKK, mitogen-activated protein kinase-kinase; PIAS, protein inhibitor of activated STAT; PKC δ, protein kinase C δ; SOCS, suppressor of cytokine signalling; STAT1, Signal transducer and activator of transcription 1; SUMO, small ubiquitin-related modifier.

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ABSTRACT

Signal transducer and activator of transcription 1 (STAT1) is a critical mediator of interferon-γ (IFN-γ)-induced gene responses, and its function is regulated through phoshorylation of Tyr701 and Ser727. Mitogen-activated protein kinase (MAPK) pathways mediate phosphorylation of Ser727 in response to microbial infections, stress stimuli and growth factors. Recently, STAT1 was found to become modified by PIAS mediated SUMO-1 conjugation at Lys703, but the regulation of this modification is largely unknown. Here, we have investigated the role of MAPK-induced Ser727 phosphorylation in regulation of STAT1 sumoylation. Activation of p38MAPK pathway by upstream activating kinase, mitogen-activated protein kinase kinase-6 (MKK6) or osmotic stress, enhanced sumoylation of STAT1, which was counteracted by the p38MAPK inhibitor SB202190 or by dominant negative p38MAPK. Activation of extracellular signal regulated kinase (ERK) 1/2 pathway by Raf-1 also enhanced Ser727 phosphorylation and sumoylation of STAT1, and this induction was counteracted by PD98059 inhibitor. Mutation of Ser727 to Ala abolished the p38MAPK-induced sumoylation. Furthermore, Ser727Asp and Ser727Glu mutations, that mimic the phosphorylation of Ser727, enhanced the basal sumoylation of STAT1, and interaction between PIAS1 and STAT1. Taken together, these results identify Ser727 phosphorylation as a regulator of STAT1 sumoylation, and highlight the central role of Ser727 in co-ordination of STAT1 functions in cellular responses.

INTRODUCTION

Interferon-γ (IFN-γ) has an important function in immune responses against microbes by regulating macrophage functions and Th1 differentiation. IFN-γ-induced gene responses are largely dependent on signal transducer and activator of transcription 1 (STAT1). Activation of STAT1 requires phosphorylation on Tyr701 by JAK kinases, and the tyrosine phosphorylated STAT1 form dimers that are translocated to the nucleus and bind to specific elements termed γ-activated sequence (GAS) on IFN-γ -responsive promoters[1, 2]. Regulation of STAT1 activation involves also phosphorylation of Ser727 in the transactivation domain (TAD) and this posttranslational modification modulates the interaction of STAT1 with coactivator proteins and selectively affects IFN-induced gene responses[3, 4]. STAT1 Ser727 phosphorylation can be induced by various kinases activated by IFN stimulation or through extracellular signals like mediators of inflammation, stress and

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growth signals. p38-mitogen-activated protein kinase (MAPK) mediates the microbial and stress responses[3], while CaM kinase II and protein kinase C δ (PKC-δ) have been shown to phosphorylate STAT1 in response to IFN-α and IFN-γ stimulations, respectively[5, 6]. During anti-microbial response the combinatorial input of these pathways secures optimal functional response [7, 8].

The function of STAT1 is negatively regulated by several transacting proteins such as protein tyrosine phosphatases, suppressor of cytokine signalling 1 (SOCS1), STAT-interacting LIM protein 1 (SLIM1)[9] as well as by protein inhibitor of activated STAT (PIAS) proteins. The family of PIAS proteins consists of five members, PIAS1, PIAS3, PIASxα, PIASxβ, and PIASy, that have been implicated in the regulation of several nuclear signaling pathways. PIAS1 and PIAS3 were identified as interaction partners for STAT1 and STAT3, respectively, and they have been shown to inhibit the DNA-binding activity of activated STATs[10-13]. Analysis of PIAS1$^{-/-}$ mice showed that disruption of PIAS1 resulted in enhanced immune responses to viral or bacterial infections. PIAS1 was shown to selectively regulate a subset of IFN-γ and IFN-β responsive genes in macrophages by interfering with recruitment of STAT1 to the promoters that show low affinity to STAT1[14]. Interestingly, all PIAS proteins exert SUMO-E3 ligase activity towards various SUMO-targets. 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 [9]. The RING domain-containing E3 ligases facilitate the transfer of SUMO from E2 conjugation enzyme Ubc9 to the substrate by catalyzing the conjugation reaction [13, 15-17].

The functional consequences of sumoylation vary significantly from protein to protein, and the modification has been implicated in regulation of protein-protein interactions, protein stability, localization and transactivation capacity [10]. Recently, STAT1 was shown to become sumoylated by the 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[18, 19]. More detailed analysis of IFN-γ target genes indicated that sumoylation selectively modulated the induction of genes with low affinity promoter (GBP-1) to STAT1, while high affinity promoter (interferon

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regulatory factor 1; IRF-1) was not significantly affected, thus showing a similar pattern of gene regulation as observed in PIAS1$^{-/-}$ mice. [14, 20].

Appropriate biological responses to external challenges such as infections require the convergence and crosstalk of different signaling pathways. Phosphorylation is an important mechanism for regulation of other posttranslational modifications such as ubiquitination and acetylation[21, 22]. Recently, phosphorylation has been also implicated in regulation of SUMO-modification. Taking into account the diverse functional roles of sumoylation in cells, it is not surprising that the effect of phosphorylation on sumoylation varies between different proteins. Serine phosphorylation of c-Jun, p53, IκBα and PML have been reported to inhibit their sumoylation[23-25], and in the case of transcription factor Elk-1 (Ets-like-protein-1), Erk-MAPK induced phosphorylation results in desumoylation of Elk-1 and increased transcriptional activation[26]. Phosphorylation has also been reported to positively regulate SUMO-1 conjugation, and a well characterized example is heat shock factor 1 (HSF1), where stress induced phosphorylation of a serine residue enhances the sumoylation of an adjacent lysine residue [27].

In this study, we have investigated the regulation of SUMO-1 modification in STAT1. Our results indicate that MAPK-induced phosphorylation of Ser727 enhance PIAS1 binding and SUMO-1 conjugation to STAT1**.** The MAPK-pathway mediated regulation of STAT1 sumoylation suggests that the interplay between sumoylation and Ser727 phosphorylation is involved in fine-tuning the transcriptional responses during immune responses to bacterial infections, stress stimuli and growth signals.

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MATERIALS AND METHODS

Cell culture, transfections and plasmids

COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS; Gibco BRL, Life Technology, Gaithersburg, MD) and 100 units/ml of penicillin and 100 μg/ml of streptomycin. COS-7 cells were seeded onto 6-well plates and transfected using FuGene reagent according to manufacturer's instructions (Roche Molecular Biochemicals, IN) with STAT1-WT-HA or different mutants (0.7 μg), SUMO-1 (0.2 μg), MKK6b (E) (0.03-0.2μg) or 0.1μg of MKK6b (E), MKK1, Raf-1 (Fig. 3A and B). His-SUMO-1, STAT1-WT-HA (pEBB-HA) and STAT1-K703R-HA (pEBB-HA) plasmids were previously described [18, 28]. STAT1Y701F-, STAT1S727A-mutantions and Flag-PIAS1 have been previously described [28, 29]. Dominant negative p38MAPKα (p38AF) and constitutively active MKK6b (MKK6b (E)) were kindly provided by Dr.J.Han. Constitutively active MKK1, Raf-1 were provided by Dr. J.Westermarck

Antibodies and reagents

Anti–SUMO-1 antibody was purchased from Zymed and anti-HA (anti-influenza virus hemagglutinin, clone 16B12) antibody was purchased from Berkeley-Antibody, Richmond, CA. Anti-Flag M2 monoclonal antibody was purchased from Sigma. 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) was from Cell Signaling Technology. Rabbit polyclonal anti-p38MAPK (N-20) and rabbit polyclonal anti-IgG were from Santa Cruz Biotechnology. 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 was purchased from R&D systems. p38MAPK inhibitor SB202190, was purchased from Calbiochem and PD98059 (Calbiochem) was kindly provided by Dr. J. Westermarck

Western blotting

Transiently transfected COS-7 cells were starved and left unstimulated or stimulated with IFN-γ (100ng/ml) for 30 min. To perform osmotic shock cells were treated with 0.3 M sorbitol and then washed in ice cold PBS and lysed in Triton X lysis buffer (50

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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 protein amount 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% Tween20. The filters were incubated with the specific antibodies diluted in TBS 0.05% Tween20. Immunodetection was performed using enhanced chemiluminescence system ECL (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Mutagenesis

STAT1Ser727Asp and STAT1Ser727Glu were created from STAT1-WT-HA using Site-Directed Mutagenesis kit (Stratagene) and following primers were used in the mutagenesis PCR:´5ACAACCTGCTCCCCATGGATCCTGAGGAGTTTGAC3´, ´5GTCAAACTCCTCAGGATCCATGGGGAGCAGGTTGT3´(Ser727Asp-mutation) and ´5ACAACCTGCTCCCCATGGAACCTGAGGAGTTTGAC3´,´5GTCAAACTCCTCAGGT TCCATGGGGAGCAGGTTGT3´ (Ser727Glu-mutation).

Co-immunoprecipitation

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 (Sigma) and rabbit polyclonal anti-IgG (Santa Cruz Biotechnology) 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 (clone 16B 12; BabCO).

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RESULTS

p38MAPK activation enhances SUMO-1 conjugation to STAT1

MAP kinases and serine phoshorylation have been implicated in both positive and negative regulation of sumoylation [23, 24, 26, 27]. The role of p38MAPK pathway in phosphorylation of Ser727 in STAT1 prompted us to examine whether p38MAPK is involved in regulation of SUMO-1 modification of STAT1. To investigate the effect of p38MAPK activation on STAT1 sumoylation, COS-7 cells were transiently transfected with increasing amounts of constitutively active mutant of MKK6 (MKK6b E) plasmid together with HA-tagged STAT1-WT and SUMO-1 plasmids. After 36h the cells were left unstimulated or stimulated with IFN-γ for 30 min. Total cell lysates were resolved by SDS-PAGE gel and immunoblotted with anti-HA, anti-P-S727-STAT1, anti-P-p38MAPK, anti-p38MAPK and anti–SUMO-1 antibodies. Expression of MKK6b (E) resulted in activation of p38MAPK and induction of Ser727 phosphorylation of STAT1 (Fig. 1). In addition, expression of MKK6b (E) also enhanced SUMO-1 conjugation to STAT1 (Fig. 1). The levels of sumoylation as well as Ser727 phosphorylation of STAT1 were further increased after IFN-γ stimulation thus supporting the concept of combinatorial input of different signalling pathways in regulation of STAT1. Importantly, immunoblotting with anti-P-S727-STAT1 antibody detected STAT1-SUMO-1 moiety, indicating that the sumoylated form of STAT1 is also Ser727 phosphorylated (Fig. 1; anti-P-S727-STAT1 blot).

Inhibition of p38MAPK activity abrogates the enhancement of STAT1 sumoylation

To confirm that p38MAPK is mediating Ser727 phosphorylation and the enhanced sumoylation of STAT1, we made use of dominant negative form of p38MAPK, (p38MAPK-DN) and p38MAPK specific inhibitor SB202190. COS-7 cells were transiently transfected with HA-tagged STAT1-WT, SUMO-1 and MKK6b (E) plasmids as indicated and activation of p38MAPK pathway, sumoylation of STAT1 as well as Ser727 phosphorylation were analyzed by immunoblotting with anti-HA, anti-P-S727-STAT1, anti-P-p38MAPK and anti-p38MAPK antibodies, respectively. As shown in Fig. 2A and B, expression of MKK6b (E) resulted in activation of p38MAPK (anti-P-p38MAPK blot; Fig. 2A lane 2 and B lanes 2-4) and enhanced Ser727 phosphorylation (anti-P-S727-STAT1 blot; Fig. 2A and B lane 2) and sumoylation of STAT1 (anti-SUMO-1 blot; Fig 2A and B lane 2). Furthermore, expression of increasing amounts of p38MAPK-DN inhibited activation of

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p38MAPK and inhibited both Ser727 phosphorylation and SUMO-1 conjugation to STAT1 (Fig. 2A. lanes 3-4). Similar results were obtained using p38MAPK inhibitor SB202190. As shown in Fig. 2B, enhanced Ser727 phosphorylation and SUMO-1 conjugation to STAT1 were reduced by the treatment with SB202190.

To investigate whether stress-induced activation of p38MAPK affects SUMO-1 conjugation to STAT1, COS-7 cells were transiently transfected with HA-tagged STAT1-WT and SUMO-1 plasmids, and subjected to osmotic shock treatment using 0.3 M sorbitol (Fig. 2C) for the indicated time points. Total cell lysates were resolved by SDS-PAGE gel electrophoresis and immunoblotted with anti-HA, anti-P-S727-STAT1, anti-P-p38MAPK and anti-p38MAPK antibodies. Sorbitol treatment enhanced Ser727 phosphorylation of STAT1 (anti-P-S727-STAT1 blot; Fig. 2C, lanes 2, 3) and activation of p38MAPK (anti-P-p38MAPK blot; Fig. 2C, lanes 2,3) which correlated with the increase in SUMO-1 conjugation to STAT1. Furthermore, SB202190 treatment inhibited the enhanced sumoylation of STAT1 after sorbitol treatment (anti-SUMO-1 blot; Fig 2C, lane 4). Collectively, these results demonstrate that activation of p38MAPK pathway enhances sumoylation of STAT1 in stress responses.

ERK1/2 activation induces Ser727 phosphorylation and sumoylation of STAT1

The reported phosphorylation of STAT1 Ser727 by ERK1/2[30, 31]prompted us to investigate whether sumoylation of STAT1 could also be modulated by ERK1/2. To investigate the effect of different MAPK pathways on Ser727 phosphorylation and sumoylation of STAT1, COS-7 cells were transiently transfected with constitutively active upstream kinases MKK6b (E), Raf-1 or MKK1 together with HA-tagged STAT1-WT and SUMO-1 plasmids. Sumoylation of STAT1, Ser727 phosphorylation, p38MAPK activation were analyzed by immunoblotting with anti-HA, anti-P-Ser727-STAT1, anti-P-p38MAPK and anti-p38MAPK antibodies, respectively. As shown in Fig. 3A expression of MKK6 and Raf-1, but not MKK1, resulted in enhanced Ser727 phosphorylation (anti-P-S727-STAT1 blot; lanes 2, 4) and sumoylation of STAT1 (anti-SUMO-1 blot; lanes 2, 4). As expected, phosphorylation of p38MAPK was induced only in response to MKK6 activation (anti-P-p38MAPK blot; lane 2). Ectopic expression of Raf-1 enhanced ERK1/2 phosphorylation, Ser727 phosphorylation and sumoylation of STAT1 (Fig 3A, lane 4). Next, we wanted to investigate the effect of MEK inhibitor PD98059 on Raf-1-induced phosphorylation and sumoylation of STAT1. For this purpose COS-7 cells were transiently transfected with HA-

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tagged STAT1-WT, SUMO-1 and Raf-1 or MKK6b (E) plasmids as indicated. Treatment of cells with PD98059 or SB202190 inhibitors (Fig. 3B lanes 3, 6) reduced phosphorylation of Ser727 and ERK1/2 as well as sumoylation of STAT1. Furthermore, SB202190 or PD98059 treatments also inhibited the sorbitol-induced enhancement of sumoylation and Ser727 phosphorylation of STAT1 (Fig. 3C, lanes 5-6). These findings demonstrate that Ser727 phosphorylation and sumoylation of STAT1 are also modulated by activation of ERK1/2 pathway.

Phosphorylation of Ser727 in STAT1 is required for p38MAPK induced SUMO-1 conjugation

Next we wished to determine whether the observed effect of MAPK-mediated enhancement of SUMO-1 conjugation to STAT1 was directly regulated by phosphorylation of Ser727. For this purpose we made use of different STAT1 mutants: sumoylation deficient mutant STAT1-K703R-HA, tyrosine phosphorylation mutant STAT1-Y701F-HA and serine phosphorylation mutant STAT1-S727A-HA, and analyzed the level of SUMO-1 conjugation and phosphorylation of Ser727 by Western blotting. The results in Fig. 4A show that STAT1-WT, as well as the STAT1-S727A and STAT1-Y701F mutants were all sumoylated at low basal level. Expression of MKK6b (E) induced sumoylation of STAT1-Y701F-HA at similar levels (lanes 7-9) as STAT1-WT-HA (lanes1-3), indicating that tyrosine phosphorylation *per se* was not required for sumoylation. However, expression of MKK6b (E) failed to enhance sumoylation of the S727A mutant (lanes 10-12). The critical role of Ser727 in MAPK-induced sumoylation was confirmed in experiments using sorbitol treatment. Conjugation of SUMO-1 to STAT1-WT-HA was increased after sorbitol treatment and this effect was counteracted by treatment with SB202190 inhibitor (Fig. 4B, lanes 1-4). In contrast, sorbitol treatment failed to enhance sumoylation of STAT1-S727A-HA (Fig. 4B, lanes 5-8). Taken together, these results show that the effect of p38MAPK activation on SUMO-1 modification is mediated through Ser727 phosphorylation of STAT1.

Ser727 phosphorylation promotes PIAS1 binding to STAT1

To validate the role of Ser727 phosphorylation in regulation of sumoylation of STAT1, we constructed two mutations Ser727Asp and Ser727Glu that mimic phosphorylation of the Ser residue. COS-7 cells were transiently transfected with HA-tagged STAT1-WT, STAT1-S727E, STAT1-S727D or STAT1-S727A together with SUMO-1 plasmid, and subjected to treatment with osmotic shock using 0.3 M sorbitol or left untreated

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as indicated. Sumoylation of STAT1, Ser727 phosphorylation, p38MAPK and p-p38MAPK levels were analyzed by immunoblotting. As expected, osmotic shock enhanced sumoylation of STAT1-WT (Fig. 5A, lanes1-2) but did not affect sumoylation of the Ser727Ala mutant (lanes 7-8). Ser727Asp and Ser727Glu mutants showed clearly enhanced sumoylation in untreated samples which was not markedly affected by osmotic shock (lanes 3-6). These findings provide strong evidence for a direct function of Ser727 phosphorylation as a regulator of STAT1 sumoylation.

Next we wanted to investigate the underlying mechanism of Ser727 phosphorylation in regulation of sumoylation, and a plausible mechanism could involve modulation of interaction between STAT1 and the SUMO-E3-ligase PIAS1. For this purpose, COS-7 cells were transiently transfected with HA-tagged STAT1-WT, STAT1-S727D or STAT1-S727A together with SUMO-1 and flag-tagged PIAS1. Cells were either left untreated, or treated with IFN-γ (50ng/ml) and sorbitol (0.3M) for 30 minutes to ensure maximal phosphorylation of STAT1. PIAS1 was immunoprecipitated from the lysates with anti-flag-antibody. Sumoylation of STAT1, Ser727 phosphorylation, and PIAS1 levels were analyzed by immunoblotting with anti-HA, anti-P-Ser727-STAT1 and anti-Flag antibodies. The results in Fig. 5B show that in untreated samples PIAS1 and STAT1 did not coimmunoprecipiate, but after stimulation STAT1 and PIAS1 are detected in the same complex (lanes 1, 2). The phosphomimetic S727D mutant, on the other hand, coimmunoprecipitated with PIAS1 already in unstimulated cells and stimulation further increased the protein complex formation between PIAS1 and STAT1-S727D (lanes 3, 4). In contrast, S727A mutant of STAT1 failed to significantly coimmunoprecipitate with PIAS1 even after stimulation (lanes 5, 6). Taken together, these results indicate that phosphorylation of Ser727 enhances the interaction between STAT1 and PIAS1, which provides a likely explanation for the role of Ser727 phosphorylation in enhancing the sumoylation of STAT1.

DISCUSSION

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,

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but they act in concert as tyrosine phosphorylation and presumably dimer formation of STAT1 enhance the serine phosphorylation. [3, 4, 7]. Recently, sumoylation has emerged as another posttranslational modification that targets STAT1, but understanding of the function and regulation of SUMO-modification of STAT1 has remained incomplete [18, 19, 32, 33]. This study was aimed to investigate the regulation of SUMO-1 modification of STAT1, and our results demonstrate that MAPK-induced phoshorylation of Ser727 modulates SUMO-1 conjugation and PIAS1 binding to STAT1.

Sumoylation is emerging as a relatively common posttranslational modification for several proteins implicated mainly in transcriptional regulation. A rational approach to obtain insight into the mechanism of sumoylation is to analyze the regulatory mechanisms involved in this modification. Here we show that activation of p38MAPK-pathway, either through expression of activating upstream kinase (MKK6) or though external stimulations results in enhanced sumoylation of STAT1. The target for this regulation was Ser727 in the transactivation domain of STAT1. Also expression of ERK1/2 upstream activator resulted in increased Ser727 phosphorylation and sumoylation of STAT1 thus suggesting that activation of ERK1/2 might modulate sumoylation of STAT1 in growth factor responses.

In several proteins sumoylation is found to be regulated by phosphorylation. This regulation is well exemplified by HSF1, where phosphorylation of Ser303 stimulates sumoylation of an adjacent Lys298 [34] presumably by inducing a conformation change that favours the conjugation reaction. The sumoylation site in HSF1 was shown to form phosphorylation dependent sumoylation motif (ψKxExxSP) which is also found in multiple other SUMO substrates [27]. Also STAT1 sumoylation site (^{702}IKTELISV^{709}) resembles the phosphorylation dependent sumoylation motif. Recently Ser708 in STAT1 was found to become phosphorylated by IKKε kinase that was implicated in IFN-inducible antiviral transcriptional response [35]. However, mutation of Ser708 to Ala did not affect the sumoylation of STAT1 (unpublished data) indicating that Ser727 is the only phosphorylated residues in STAT1 involved in regulation of sumoylation.

Phosphorylation of Ser727 is generally considered to have a stimulatory effect on STAT1 mediated gene responses. However, phosphorylation of Ser727 in STAT1 has been shown to differentially affect basal and induced expression levels of target genes[3], but the underlying mechanism for the selective regulatory role of Ser727 in IFN-γ induced gene

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responses is currently not known. Thus, although Ser727 phosphorylation promotes sumoylation of STAT1, these modifications have distinct functional roles during immune response. Ser727 phosphorylation precedes the relatively slowly emerging sumoylation of STAT1 suggesting that Ser727 phosphorylation might induce negative feedback loop through SUMO-conjugation. Recently, the functional connection between sumoylation and PIAS1 received further strengthening, when IKKα induced phosphorylation of PIAS1 was shown to repress activation of STAT1 regulated genes in a sumoylation dependent manner [36]. Our present results indicate that phosphorylation of Ser727 modulates the interaction between PIAS1 and STAT1, either directly or indirectly, and thus provide an explanation for the Ser727 phosphorylation regulated sumoylation of STAT1. The Ser727 phoshorylation promoted recruitment of PIAS1 and sumoylation of STAT1 is likely to be involved in securing the tight regulation of STAT1 activity at the transcriptional level and mediate the fine-tuning of STAT1 signalling in immune responses.

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 is an essential protein in combining the signals from the infectious agents and from the immune modulator cytokines. Our present results that PIAS1 interaction and SUMO-1 modification of STAT1 is regulated in a Ser727 phosphorylation dependent manner, identify another mechanism for coordinated posttranslational regulation of STAT1.

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FIGURE LEGENDS

Figure 1. MKK6 cotransfection enhances SUMO-1 conjugation to STAT1.
COS-7 cells were transiently transfected with STAT1-WT-HA together with SUMO-1 and increasing amounts of MKK6 (0.05, 0.1, 0.3 μg). After 36 hours the cells were treated with IFN-γ and lysed as indicated in Materials and methods. Total cell lysates were immunoblotted with anti-SUMO-1, stripped and blotted with anti-P-S727-STAT1, anti-HA, anti-P-p38MAPK and anti-p38MAPK antibodies.

Figure 2. Inhibition of p38MAPK activation decreases SUMO-1 conjugation to STAT1.
(A) p38MAPKdominant negative (DN) decreases sumoylation of STAT1.
COS-7 cells were transiently transfected with STAT1-WT-HA, SUMO-1 and MKK6b (E) plasmids together with increasing amounts of p38MAPKDN (0.1, 0.3μg) as indicated. After 36 hours the cells were lysed as indicated in Materials and methods. Total cell lysates were analyzed by immunoblotting using anti-HA, anti-P-S727-STAT1, anti-P-p38MAPK and anti-p38MAPK antibodies. **(B)** p38MAPK specific inhibitor SB202190 blocks the enhancement of SUMO-1 conjugation to STAT1. COS-7 cells were transiently transfected with STAT1-WT-HA together with SUMO-1 and MKK6b (E) plasmids. After 36 hours the cells were treated as indicated. Total cell lysates were analyzed as in Fig 2A. **(C)** Osmotic shock enhances SUMO-1 conjugation to STAT1 through p38MAPK activation. COS-7 cells were transiently transfected with STAT1-WT-HA and SUMO-1. After 36 hours the cells were treated as indicated. Cells were lysed and analyzed by immunoblotting as described in 2A.

Figure 3. p38MAPK and ERK1/2 activation induces Ser727 phosphorylation and sumoylation of STAT1.
(A) Expression of MKK6(E) and Raf-1 induces Ser727 phosphorylation and enhanced sumoylation of STAT1. COS-7 cells were transiently transfected with STAT1-WT-HA together with SUMO-1. Constitutively active forms of MKK6b (E), MKK1 and Raf-1 plasmids, were transfected as indicated. After 36 hours the cells were lysed as indicated in Materials and methods and total cell lysates were immunoblotted with anti-P-S727-STAT1, anti-HA, anti-P-p38MAPK and anti-P-ERK1/2 antibodies. **(B)** Enhancement of sumoylation by Raf-1 is counteracted by PD98059 treatment. COS-7 cells were transiently transfected with STAT1-WT-HA together with SUMO-1 and Raf-1 or MKK6b (E) plasmids. After 36

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hours the cells were left untreated or treated for 60 min with PD98059 or SB202190 as indicated. Cells were lysed and total cell lysates were analyzed by immunoblotting as described in Fig. 3A. **(C)** Inhibition of p38MAPKand ERK1/2 pathways reduces the stress induced sumoylation of STAT1. COS-7 cells were transiently transfected with STAT1-WT-HA together with SUMO-1 plasmid. After 36 hours the cells were subjected to sorbitol treatment as indicated. Samples in lanes 5 and 6 were treated for 60 min with PD98059 or SB202190 inhibitors as indicated. Cells were lysed and total cell lysates were analyzed as described in Fig. 3A.

Figure 4. Ser727 is required MKK6 and stress induced sumoylation of STAT1.
(A) Ser727 is required for MKK6b (E) induced SUMO-1 conjugation to STAT1. COS-7 cells were transiently transfected with plasmids encoding for STAT1-WT-HA (lanes1-3), STAT1-KR-HA (lanes 4-6), STAT1-YF-HA (lanes 7-9) and STAT1-SA-HA (lanes 10-12) together with SUMO-1 and increasing amounts of MKK6b (E) plasmid. After 36 hours the cells were lysed as described in Materials and methods and immunoblotting was performed using anti-HA, anti-P-S727-STAT1, anti-P-p38MAPK and anti-p38MAPK antibodies. **(B)** Stress induced activation of MAPKs modulates SUMO-1 conjugation differently between STAT1-WT and STAT1-Ser727 mutant. COS-7 cells were transiently transfected with plasmids encoding for STAT1-WT-HA (lanes 1-4) or STAT1-S727A-HA (lanes 5-8) and SUMO-1. After 36 hours the cells were treated with sorbitol for the indicated time points, and left untreated or treated by SB202190 as indicated (lanes 4 and 8). Cells were lysed and immunoblotted as described in Fig. 4A.

Figure 5. Phosphorylation of Ser727 enhances sumoylation of STAT1 and interaction with PIAS1
(A) Phospho-Ser727 mimic mutants STAT1-S727E and STAT1-S727D show enhanced sumoylation. COS-7 cells were transiently transfected with plasmids encoding for STAT1-WT-HA (lanes 1-2), STAT1-S727E-HA (lanes3-4), STAT1-S727D-HA (lanes5-6) or STAT1-S727A-HA (lanes 7-8) and SUMO-1. After 36 hours the cells were treated with sorbitol as indicated. Cells were lysed and immunoblotted as described in Fig. 4A. **(B)** PIAS1 readily binds to Ser phosphorylated STAT1. COS-7 cells were transiently transfected with plasmids encoding for STAT1-WT-HA (lanes 1-2, 7), STAT1-S727D-HA (lanes2-4) or STAT1-S727A-HA (lanes 5-6) together with PIAS1 and SUMO-1 (lanes 1-7). After 36 hours the cells were treated with sorbitol and IFN-γ as indicated, and cells were lysed as described

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in Materials and methods. PIAS1 was immunoprecipitated using anti-Flag antibody. Co-precipitated STAT1 was analyzed using anti-HA blotting, and total cell lysates were immunoblotted using anti-HA and anti-P-S727-STAT1 antibodies.

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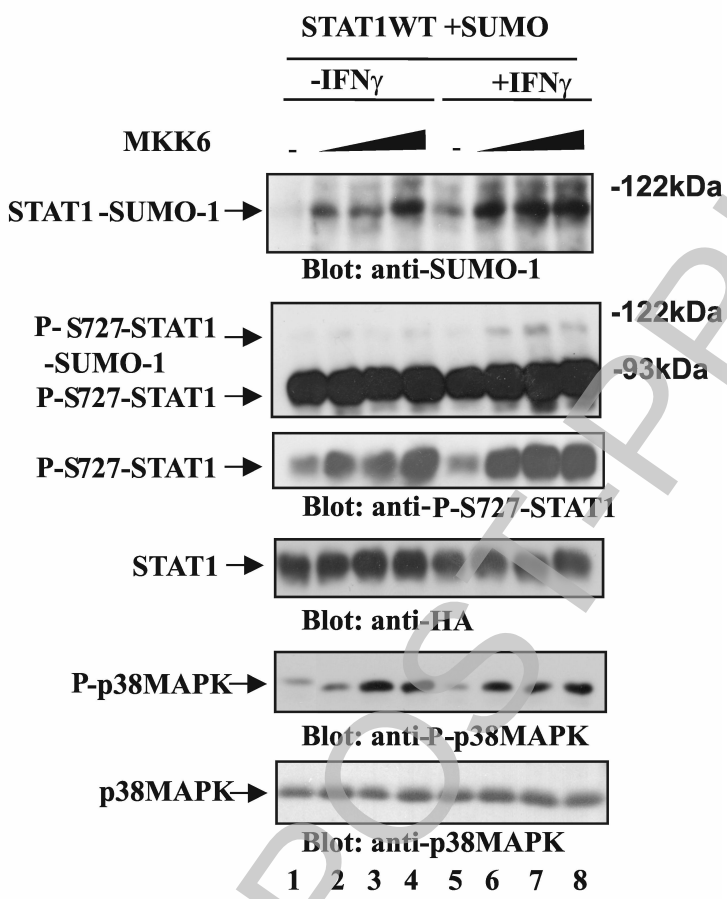
Figure1



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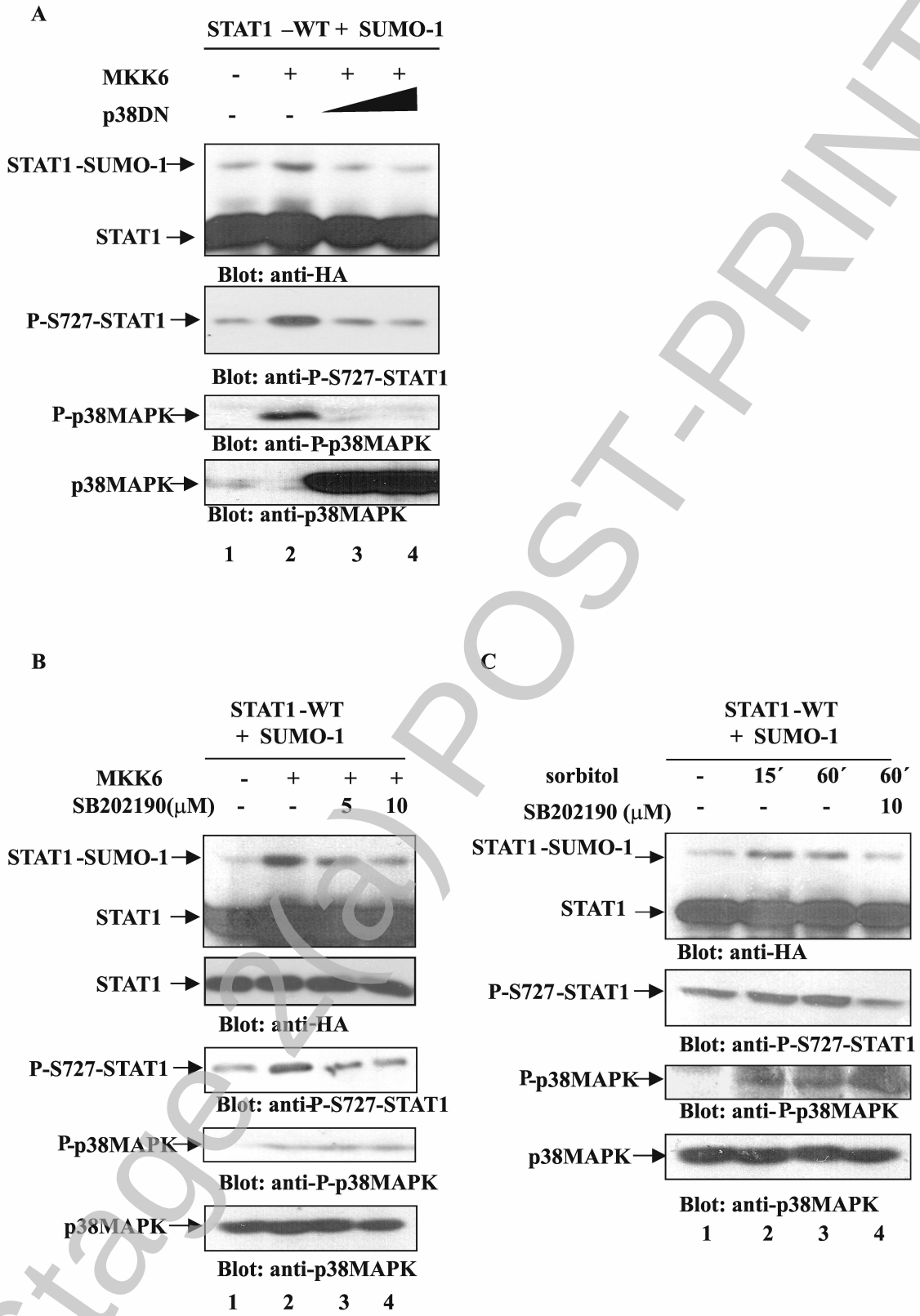

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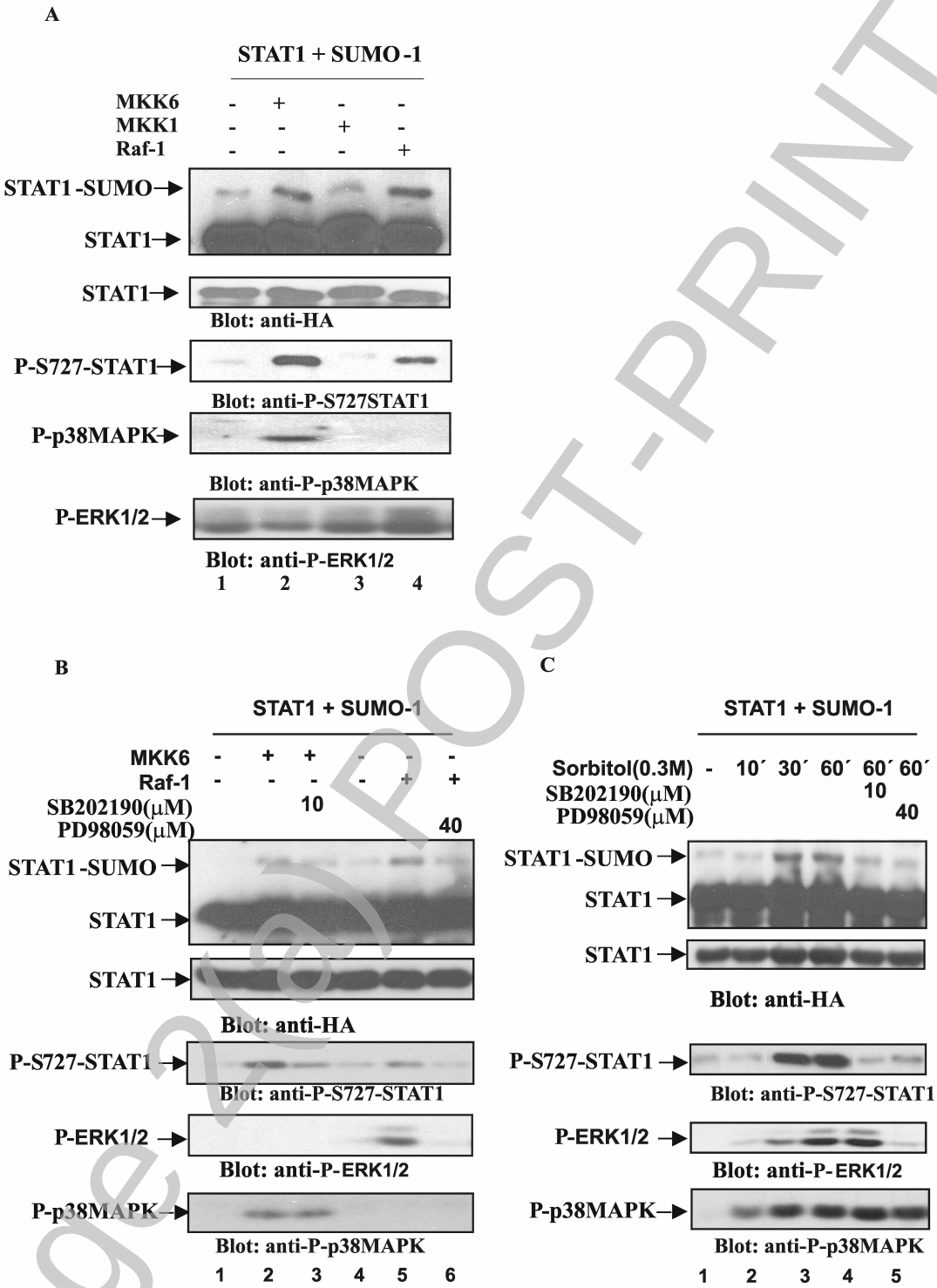

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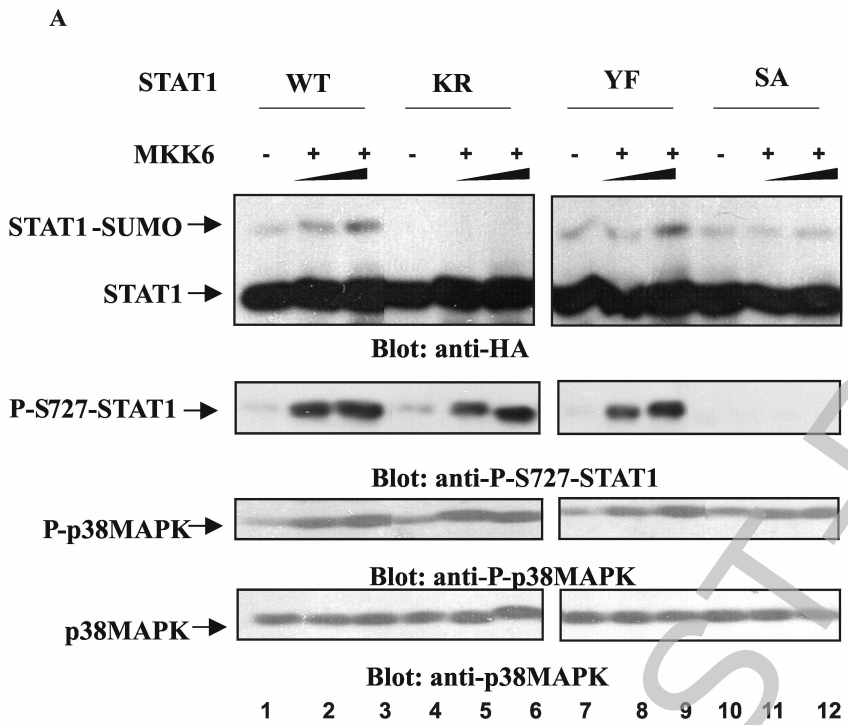

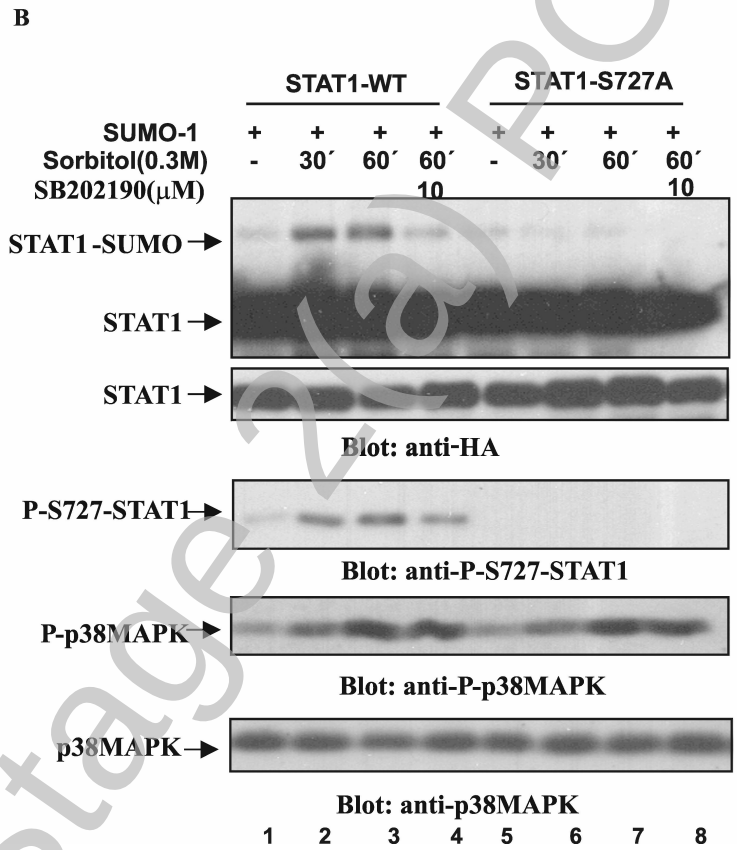

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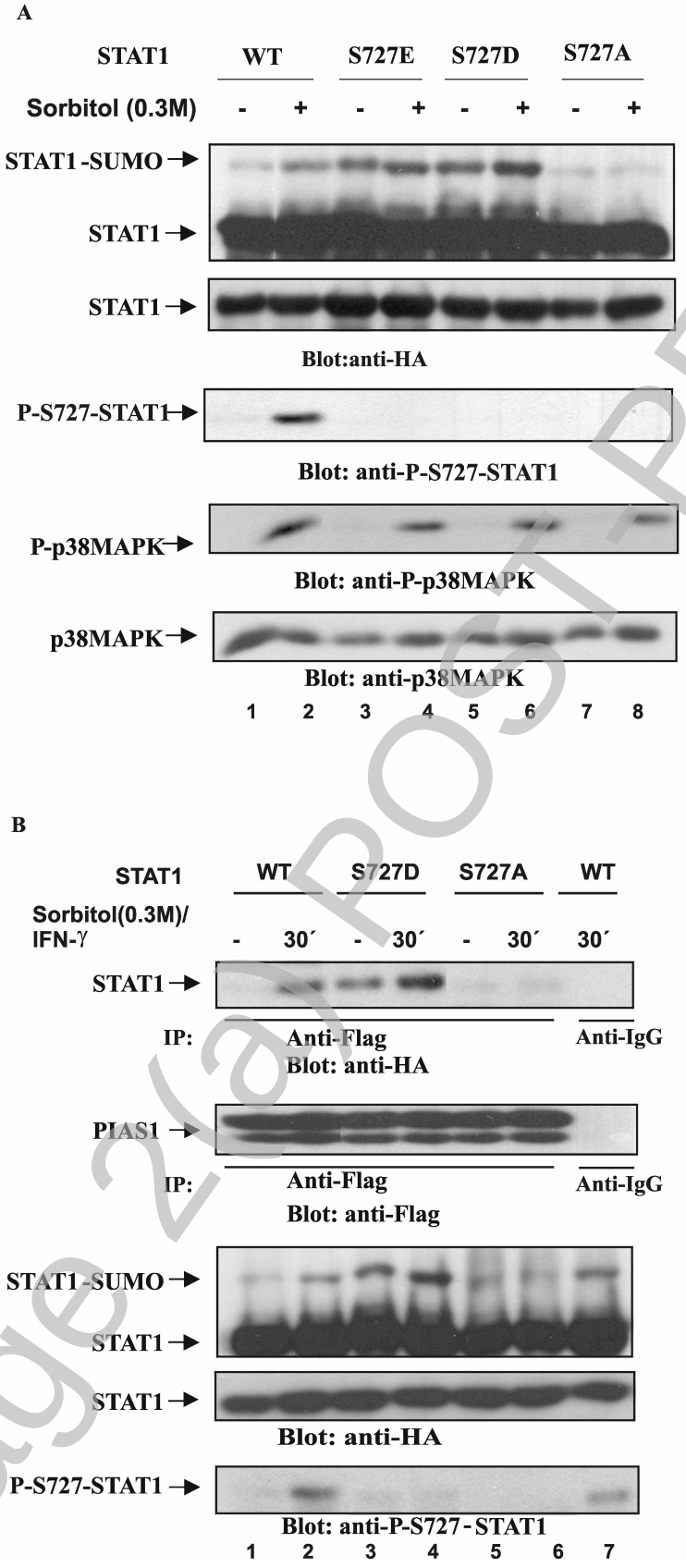

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