Evidence of STAT1 phosphorylation modulated by MAPKs, MEK1 and MSK1

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Phosphorylation at Ser727 in signal transducer and activator of transcription 1 (STAT1) is essential for its activation and signal transduction. However, the upstream kinases responsible for phosphorylating Ser727 are still elusive. Here, we provide evidence showing that UVA-induced mitogen-activated protein kinase (MAPK) signaling pathways lead to STAT1 Ser727 phosphorylation. Our experimental results show that UVA-induced Ser727 phosphorylation of STAT1 was, to different degrees, diminished by PD98059 and U0126, two specific inhibitors of MEKs, or an N-terminal or C-terminal kinase-dead mutant of p38 kinase (p38K), respectively. STAT1 phosphorylation was also blocked by a dominant negative mutant of p38K or JNK1, JNK1- or JNK2-deficiency, or an N-terminal or C-terminal kinase-dead mutant of mitogen- and stress-activated protein kinase 1 (MSK1), a downstream kinase closer to p38 kinase and extracellular signal-regulated kinases (ERKs), respectively. STAT1 phosphorylation was also blocked by a dominant negative mutant of p38K or JNK1, JNK1- or JNK2-deficiency, or an N-terminal or C-terminal kinase-dead mutant of mitogen- and stress-activated protein kinase 1 (MSK1), a downstream kinase closer to p38 kinase and extracellular signal-regulated kinases (ERKs), respectively. STAT1 phosphorylation was also blocked by a dominant negative mutant of p38K or JNK1, JNK1- or JNK2-deficiency, or an N-terminal or C-terminal kinase-dead mutant of mitogen- and stress-activated protein kinase 1 (MSK1), a downstream kinase closer to p38 kinase and extracellular signal-regulated kinases (ERKs), respectively. STAT1 phosphorylation was also blocked by a dominant negative mutant of p38K or JNK1, JNK1- or JNK2-deficiency, or an N-terminal or C-terminal kinase-dead mutant of mitogen- and stress-activated protein kinase 1 (MSK1), a downstream kinase closer to p38 kinase and extracellular signal-regulated kinases (ERKs), respectively. STAT1 phosphorylation was also blocked by a dominant negative mutant of p38K or JNK1, JNK1- or JNK2-deficiency, or an N-terminal or C-terminal kinase-dead mutant of mitogen- and stress-activated protein kinase 1 (MSK1), a downstream kinase closer to p38 kinase and extracellular signal-regulated kinases (ERKs), respectively. STAT1 phosphorylation was also blocked by a dominant negative mutant of p38K or JNK1, JNK1- or JNK2-deficiency, or an N-terminal or C-terminal kinase-dead mutant of mitogen- and stress-activated protein kinase 1 (MSK1), a downstream kinase closer to p38 kinase and extracellular signal-regulated kinases (ERKs), respectively. STAT1 phosphorylation was also blocked by a dominant negative mutant of p38K or JNK1, JNK1- or JNK2-deficiency, or an N-terminal or C-terminal kinase-dead mutant of mitogen- and stress-activated protein kinase 1 (MSK1), a downstream kinase closer to p38 kinase and extracellular signal-regulated kinases (ERKs), respectively.

Introduction

Signal transducer and activator of transcription 1 (STAT1) is highly homologous to STAT3. It was identified as a transcription factor that mediates interferon action, but is now known to be involved in many other signaling pathways that play a role in regulating diverse cellular processes including growth, proliferation, differentiation and transformation, apoptosis, and is even involved in oncogenesis (1–5). When cells are stimulated with cytokines (e.g. interferon) or growth factors (e.g. epidermal growth factor, EGF), STAT1 activation is generally accepted to be initiated by tyrosine phosphorylation at a single site (Tyr701), which is carboxyl to the SH2 domain (1,3). Tyr701 phosphorylation by the Janus kinase (JAK) family members (e.g. JAK1, JAK2 and PYK2) (5–7) or receptor tyrosine kinases (e.g. EGF receptor, EGFR) (8,9) appeared to be all that was required for dimer formation, nuclear translocation and activation of STAT1. However, recent studies demonstrated substantial tyrosine phosphorylation-independent nuclear translocation of STAT1 in some signaling responses (10–12). On the other hand, several interesting observations indicated that STAT1 activation might also require a secondary phosphorylation modification at the serine/threonine residues, possibly by extracellular signal-regulated kinases (ERKs) (13,14) or a H7-sensitive kinase (15,16). Furthermore, phosphorylation of STAT1 at serine 727, in addition to Tyr701 phosphorylation, was shown to be required for its maximal activation induction by cytokines (17,18). Importantly, stimulation of cells by diverse stresses (e.g. short wave ultraviolet irradiation) induced Ser727 phosphorylation of STAT1 resulting in its activation independently of Tyr701 phosphorylation (19–24). These findings therefore indicate that Ser727 phosphorylation of STAT1 allows the integration of signals from multiple pathways, resulting in activation of STAT1-mediated target genes.

Ser727 is located within a potential mitogen-activated protein kinase (MAPK) consensus motif of the C-terminal transactivation domain of STAT1 (17,18,24,25) and thereby, is postulated to be phosphorylated through activation of MAPKs, including ERKs, c-Jun N-terminal kinases (JNKs) and p38 kinase (26). However, the kinases responsible for catalyzing Ser727 phosphorylation of STAT1 are still elusive, although involvement of ERKs and p38 kinase in STAT1 phosphorylation induction was proposed previously based on preliminary experimental evidence (13,14,22–24,27,28). Furthermore, contribution of only STAT1 Ser727 phosphorylation to its constitutive activation was detected in some tumor cells (4,5,15). Ser727 phosphorylation and activation of STAT1 was also activated by carcinogens and tumor promoters, including UVC (200–290 nm) and 12-O-tetradecanoylphorbol-13-acetate (15,23,24,28). Therefore, identifying the upstream kinases for serine phosphorylation of STAT1 will provide a clearer understanding of the mechanism of STAT1 signaling activation involved in oncogenesis, possibly leading to the development of novel preventive and therapeutic approaches to intervene in the process. Solar UV irradiation is believed to be one of the most important skin carcinogens. UVA (320–400 nm) constitutes >90% of solar UV, of which all of the UVC and most of the UVB (290–320 nm) are absorbed by the ozone layer of the earth’s atmosphere. Thus, UVA is a major contributor to carcinogenesis. Different spectra of UV (UVA, UVB and UVC) induce different signal transduction pathways (29). However, the UVA-induced signaling pathways leading to Ser727 phosphorylation of

Abbreviations: CMVS, pCMV5-FLAG vector; DMEM, Dulbecco’s modified Eagle’s medium; DNM-JNK1, dominant negative mutant of JNK1; DNM-p38$, dominant negative mutant of p38 kinase; EGF, epidermal growth factor; ERKs, extracellular signal-regulated kinases; FBS, fetal bovine serum; GST, glutathione S-transferase; JAK, Janus kinase; JNKs, c-Jun N-terminal kinases; MAPK, mitogen-activated protein kinases; MSK1, mitogen- and stress-activated protein kinase 1; STAT1, signal transducer and activator of transcription 1.
STAT1 are unknown. Here, we show that a strong phosphorylation of STAT1 at Ser727 is induced in the intracellular response to UVA irradiation and this phosphorylation process occurs through diverse MAPK signaling pathways. Furthermore, we demonstrate that STAT1 is a potential substrate for Ser727 phosphorylation by ERK1, JNK1, p38 kinase or possibly by MEK1 or mitogen- and stress-activated protein kinase 1 (MSK1) in the presence of an unidentified cofactor and/or downstream kinase. In addition, the process is also negatively regulated by a pathway involving EGFR and/or ERKs.

Materials and methods

Amplification of the wild-type STAT1 cDNA

The cDNA fragment of STAT1, including open reading frame (2253 bp), was reverse-transcribed with an oligo-dT primer and SuperScript II RNase H Reverse Transcriptase (GibcoBRL, Grand Island, NY), and then amplified by the polymerase chain reaction with primers 5'-GGA TCC TGT CTC AGT AGC ACG TAC T-3' (BanHI site underlined) and 5'-5'TGG ACG CTT TGC TGC ATA CTG TCA TC-3' (MluI site underlined) and cloned into the PACT vector (Promega, Madison, WI) and BanHI and MluI restriction sites. The sequence of STAT1 was verified by comparison of restriction fragment length and DNA sequence analysis. The primer synthesis and DNA sequencing were performed by Sigma, St Louis, MO.

Construction and mutagenesis of glutathione S-transferase (GST)-fusion expression vectors

The STAT1 coding fragment digested with BanHI/EcoRV from the pACT was cloned into BanHI/SmaI sites of the pUC19 vector. To introduce the point mutation in the position of the 701(Y701F) or the 727(T272A) residue, a pair of sense and antisense primers for STAT1(Y701F): 5'-CTA AAG GAA GGT CAT TTA ACA AGA CTG TAT CGT TGC AGT TGA T-3' and 5'-A5'TCA ACT CAG TCT CCG CCT CCC ATG CTT CCT GAG GAG-3' (Statagene, La Jolla, CA) and another pair of the primers for STAT1(T272A): 5'-CCT CTG CTC CCG CAT AGT GGT GAG GAG-3' and 5'-5'CTT CTC CTG ACC AGG AGC CAT GGG GAG CAG G-3 were synthesized (Sigma). Then, the nucleotide substitutions were accomplished using the QuickChange Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and then confirmed by DNA sequence analysis (Sigma). Further, the STAT1 Y701F and STAT1 T272A were ligated into the BanHI/MluI sites of the pGEX-5X-C vector. The above-mentioned restriction endonucleases, ligases and related buffers were purchased from New England Biolabs (Beverly, MA).

GST-fusion STAT1 protein expression and pull down

The pGEX-5X-C plasmids encoding the wild-type full-length STAT1 (STAT1wt) and the point mutant STAT1 (STAT1Y701F or STAT1T272A) as GST-fusion protein sources were used to transform a DH5a competent Escherichia coli strain (Invitrogen Life Technologies, Carlsbad, CA). After induction of protein expression with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (from Sigma) for 4 h, the bacteria were resuspended in a lysis buffer containing 50 mM Tris–HCl pH 8.0, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% (v/v) Triton X-100, 10 μg/ml of aprotinin and leupeptin and 100 μM Na3VO4, and then were further disrupted by addition of 0.1 vol of 10 mg/ml lysozyme (Sigma) and subsequent sonication. After washing twice with the above-mentioned lysis buffer and an additional two times with kinase buffer (described below), the beads were subjected to SDS–PAGE followed by western blotting with a GST or STAT1 antibody to determine expression of the GST-fusion STAT1 proteins. In subsequent in vitro kinase reactions, eluants of the beads with 20 mM reduced glutathione (GSH from Biochrom AG, Berlin, Germany) or 50 mM GSH with 50 mM mercaptoethanol as the reducing agent, with a 100 μM concentration of the respective enzyme as the enzymatic substrates. Control experiments were performed with GST-Sephrose beads generated by expression of GST alone, using the empty pGEX-5X-C vector.

Cell lines and cell culture

Mouse epidermal tumor promotion sensitive JB6 Cl 41 cells and related stable transfectants were cultured in Eagle’s minimum essential medium (EMEM from BioWhittaker, Walkersville, MD) supplemented with 5% heat-deactivated fetal bovine serum (FBS from Gemini Bio-Products, Calabasas, CA), 2 mM l-glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin at 37°C in humidified air with 5% CO2. Mouse wild-type (Egfr+/+ or Jnk1+/+) and knockout (Egfr<sup>−/−</sup> or Jnk1<sup>−/−</sup> or Jnk2<sup>−/−</sup>) embryonic fibroblast lines were generated and identified as described previously (30,31). The cell lines were grown in Dulbecco’s modified Eagle’s medium (DMEM) (GibcoBRL Technolog-ies) containing 10% FBS, 2 mM l-glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin.

Stable transfectants

JB6 Cl 41 cell lines stably transfected with an empty CMV-neo vector (CMV-neo), or a construct containing a dominant negative mutant of ERK2 (DNM-ERK2), INKA (DNM-INKA) or p38 kinase (DNM-p38) was established and identified as reported previously (32–36). Other transfected JB6 Cl 41 cell lines stably expressing pCMV-FLAG vector (CMVS), pCMV-FLAG wild-type MSK1 (MSK1wt), pCMV-FLAG-MSK1-A195/N-terminal kinase-dead (MSK1-ND) or pCMV-FLAG-MSK1-A565/C-terminal kinase-dead (MSK1-Cd) (from Dr D.R.Alessi) were generated and characterized according to previously described methods (29,37). The transfectants were selected in media containing 400 μg/ml of G418 (Gemini Bio-Products). Prior to the experiments performed, we again confirmed that the above-mentioned cells expressed the desired activity by performing related assays for kinase activity or specific phosphorylation.

Treatment of cells

To diminish a basal level of protein phosphorylation or activity, JB6 Cl 41 cells and related transfectants were starved for 24–48 h in 0.1% FBS EMEM, whereas other above-mentioned cells were starved in serum-free DMEM. Then, the cells were or were not incubated for 1–5 h with specific protein kinase inhibitors, including PD98059, SB202190 (Sigma), PD166316 (Alexis, San Diego, CA) or AG1478 (Calbiochem, San Diego, CA) and subsequently irradiated with UVA, UVB or UVC. A detailed description of UVA, UVB or UVC sources is available in our previous reports (32,33). Non-irradiated cell samples were used as negative controls. In an additional experiment, we treated with EGF (Collaborative Research, Madison, WI).

Western blot analysis

Equal numbers of experimental cells (1 × 10<sup>6</sup> to 1.5 × 10<sup>6</sup>) were cultured for 12–24 h in 100-mm dishes. After 70–80% confluence was reached, the cells were starved for 48 h in serum-free DMEM. At the indicated times after irradiation, the cells were harvested and washed once with ice-cold phosphate-buffered saline (PBS). Then the cell samples were disrupted in 200 μl of RIPA buffer [1% (w/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, and the inhibitors added before use: 10 μg/ml of PMSF, 10 μg/ml of aprotinin and 100 μM Na3VO4] (Sigma). The cell lysates were clarified by microcentrifuge and the supernatant fractions were saved. The samples containing equal amounts of proteins (Modified Lowry’s method, Sigma) in an equal volume of RIPA buffer were diluted with 3 × SDS sample buffer (187.5 mM Tris–HCl pH 6.8, 6% (w/v) SDS, 30% (v/v) glycerol, 150 mM dithiothreitol (DTT) and 0.3% (w/v) bromophenol blue). Then samples were subjected to separation by 8% SDS–PAGE followed by western blot analysis according to the reported methods (32,33). A specific phospho-STAT1 (Ser727) antibody was purchased from Upstate Biotechnology (Lake Placid, NY), and the phospho-specific STAT1 (Ty701) antibody was from Cell Signaling (Beverly, MA). Other antibodies against phospho-ERKs, p38 kinase, JNKs or p90<sup>Erk</sup> (Ser381), and against total STAT1, ERKs or β-actin were from Cell Signaling. Total STAT1 or β-actin was used as an internal control to verify basal level expression and equal protein loading. Net serine phosphorylation was calculated by dividing with total protein and then normalized to non-irradiated control cells and is presented as a fold change (1 of control value). In addition, the intensity of some western blots was quantified as reported previously (29).

Immunoprecipitation (IP) assay

After culturing for 12–24 h, the experimental cells were starved for 24 h in 1% FBS-DMEM. The cells were harvested at the indicated times following irradiation and lysed in 250 μl of IP buffer [20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μM MG132 and 1 μM PMSF]. The clarified supernatant fractions containing equal amounts of protein were subjected to IP followed by western blot analysis or kinase activity assays according to the described methods (29,32,33). The immune complex beads were washed with PBS. An antibody against total STAT1 was used for IP of STAT1 proteins.

Assay for in vitro phosphorylation by protein kinases

After starvation, JB6 Cl 41 cell lysates were prepared as described above and clarified by microcentrifuge. Equal amounts of protein in the supernatant fractions were subjected to IP for STAT1. Samples containing the immune-purified STAT1 proteins, GST-pull down STAT1 proteins, or both combined
together were incubated at 30°C for 60 min with active MSK1, MEK1, ERK1, ERK2, JNK1, JNK2, p38α or p38β kinase (Upstate Biotechnology) in kinase buffer (50 mM Tris–HCl pH 7.5, 10 mM MgCl₂, 1 mM EGTA, 1 mM DTT and 0.01% Brij 35) (Cell Signaling) containing 5 mM ATP or 1 μCi of [γ-32P]ATP. The reactions were stopped by adding 5× SDS sample buffer. Then phosphorylation of STAT1 was analyzed by western blotting with a specific antibody against phospho-STAT1 (Ser727) or autoradiography (38). Total STAT1 was utilized as an internal control to verify equal protein loading.

Results

A strong phosphorylation of STAT1 at Ser727 is induced by UVA

A recent report indicated that irradiation of human keratinocyte lines with UVA stimulated STAT1 activation through Tyr701 phosphorylation (39). On the other hand, previous experiments with UVC irradiation showed that Ser727 phosphorylation was required in the activation of STAT1 signaling, but the activation was independent of Tyr701 phosphorylation (20,23,24,28). However, whether UVA induces STAT1 Ser727 phosphorylation leading to its mediated signaling activation is not known. This question was investigated herein by using western blot analysis with specific antibodies to detect STAT1 phosphorylation at Ser727 or Tyr701. Our data show that compared with Tyr701 phosphorylation, a stronger phosphorylation of STAT1 at Ser727 was induced in a dose-dependent (Figure 1A) and time-dependent (Figure 1B) manner, following UVA exposure of mouse epidermal tumor promotion sensitive JB6 Cl 41 cells. The Ser727 phosphorylation occurred 5 min after irradiation with UVA (160 kJ/m²), increased to a maximal induction at 30 min, and then gradually decreased back to basal level by 240 min following irradiation (Figure 1A and B). In contrast, hardly any induction of phosphorylation of Tyr701 was detected 15 or 30 min after stimulation with UVA (Figure 1A and B). In the same experiments, a similar induction pattern of STAT1 phosphorylation at Ser727 by UVB or UVC irradiation was observed, but Tyr701 phosphorylation was undetectable (Figure 1A), consistent with earlier reported observations (20,23,24,28).

Fig. 1. A strong dose-dependent and time-dependent phosphorylation of STAT1 (Ser727) induced in UVA-irradiated cells. After starvation for 36 h, JB6 Cl 41 cells were or were not irradiated with UVA, UVB or UVC at the indicated doses (A), or with UVA at 160 kJ/m² (B), or were stimulated with EGF (100 ng/ml) (C). The cells were then harvested at 30 min (A) or the indicated times (B and C) after stimulation. In the cell lysates, phosphorylated STAT1 at Ser727 or Tyr701, as well as total STAT1, were separated by 8% SDS–PAGE followed by western blot analysis with a specific antibody against phosphorylation of STAT1 at Ser727 or Tyr701, or against total STAT1. UVB/UVC- or EGF-stimulated samples were used as positive controls, whereas the non-irradiated samples served as negative controls. These data are representative of at least three independent experiments.
In addition, treatment of cells with EGF was shown previously to stimulate both Tyr701 and Ser727 phosphorylation of STAT1 (1,4,26), but here we determined that stimulation of JB6 Cl 41 cells with EGF induced only Ser727 phosphorylation of STAT1 (Figure 1C). This is consistent with the previous observation that only serine phosphorylation stimulated with EGF (27) or a synergy of interleukin-2 and -12 (22) regulates induction of STAT1 signaling activation. Overall, these findings demonstrate that serine phosphorylation plays a role in stimulus-dependent activation of STAT1 signaling and thus indirectly reflects regulation of STAT1 signaling.

**UVA-induced STAT1 serine phosphorylation is independent of EGFR**

Tyrosine phosphorylation of STAT1 was shown to require EGFR tyrosine kinase activity in response to EGF or other mitogens (8,9,40), but whether EGFR plays a role in modulating serine phosphorylation of STAT1 in the cellular responses to UVA, UVB, UVC, EGF or other mitogens (19–24) is as yet unknown. Initiation of several potential EGFR signaling pathways, including Ras/MAPKs and phosphatidylinositol-3 kinase (PI-3 kinase), occurs after irradiation with UVA, UVB or UVC (41). Recently, we also observed that UVA activation of MAPK cascades occurred by both EGFR-dependent and -independent signaling mechanisms (42). Thus, whether EGFR signaling acts as a functional participant in the regulation of STAT1 Ser727 phosphorylation induced by UVA was further explored in the present study. We performed experiments using an identified EGFR-deficient (Egfr⁻⁻) cell line (31) and a specific EGFR tyrosine kinase inhibitor, AG1478 (31). The results of the experiments showed that UVA-stimulated phosphorylation of STAT1 Ser727 was greater in Egfr⁻⁻ cells (Figure 2A) compared with wild-type (Egfr⁺⁺) cells. Moreover, pre-treatment of JB6 cells with AG1478 also enhanced the UVA stimulation of STAT1 phosphorylation (Figure 2B). Total levels of STAT1 were unaffected in these experimental cells (Figure 2A and B). The data indicate that UVA-stimulated phosphorylation of STAT1 (Ser727) may be triggered through both EGFR-dependent and -independent signaling pathways. Taken together with previous data (42), our study suggests that the EGFR-independent JNKs or p38 kinase signaling pathways may play a positive regulatory role in UVA induction of STAT1 serine phosphorylation. However, another possibility cannot be ruled out that this STAT1 phosphorylation response is also regulated by an identified negative EGFR signaling pathway. Such negative regulation may make a significant contribution to deactivation versus phosphorylation effects.

**MEK–ERK cascades have a role in the induction of STAT1 Ser727 phosphorylation**

ERKs are hypothesized to catalyze phosphorylation of Ser727 in STAT1 (17,18). Indeed, serine phosphorylation of STAT1 has been associated with activation of ERK2 (13,14), but no direct evidence was provided for a direct kinase role of ERKs. Here, we further examine a role for the ERKs pathway in the intracellular phosphorylation response. Experimental results showed that pre-treatment of JB6 cells with PD98059 or U0126, two specific inhibitors of MEK1 (MAPK kinase 1) and/or MEK2, significantly blocked UVA-induced phosphorylation of STAT1 (Ser727) (Figure 3A and B). The basal levels of STAT1 were unaffected by these treatments (Figure 3C and D). Recently, Ser727 phosphorylation of STAT3, which is highly homologous to STAT1, was determined to require stress-activated protein kinase/ERK kinase 1 (SEK1), MAPK kinase 4 (MKK4) (43,44) and MEK kinase 1 (MEKK1) (45). These findings, therefore, suggest that the MKK–MEK–ERK cascade may be involved in modulating the STAT1 serine phosphorylation response to UVA irradiation.

**JNKs are required for STAT1 serine phosphorylation**

Both in vivo and in vitro serine phosphorylation of STAT3 were shown to require JNK, a stress-responsive MAPK (46,47). These findings were challenged by the study of
Schuringa et al. (43). However, to date, whether JNKs phosphorylate STAT1 is unknown. Here, to further explore whether JNKs have a role in the intracellular STAT1 phosphorylation response to UVA, we performed experiments using JB6 Cl 41 cell lines stably expressing DNM-JNK1 and embryonic fibroblast lines from Jnk1 or Jnk2 knockout mice. These cell lines were engineered and identified as reported previously (30,32,33). The results showed that UVA-stimulated phosphorylation of STAT1 at Ser727 was significantly prevented by expression of DNM-JNK1 (Figure 4A and B), and was also markedly abolished by deficiency of JNK1 or JNK2 (Figure 4C) compared with their corresponding control cells. On the other hand, expression of total STAT1 was unaffected by DNM-JNK1 (Figure 4A and B) or knockout of Jnk1 or Jnk2 (Figure 4C). In addition, UVA-stimulated STAT1 phosphorylation at Ser727 was also markedly reduced by pre-treatment of cells with PD169316 (Figure 4D), an inhibitor of JNKs and p38 kinase (33). Overall, the data indicate that JNKs may be required for mediating UVA-induced serine phosphorylation of STAT1.

p38 kinase is also required for mediating STAT1 serine phosphorylation

Theoretically, p38 kinase, another stress-responsive MAPK, may phosphorylate STAT1 at Ser727, which lies within a proline-flanked consensus sequence on the C-terminal domain of STAT1 (17,18). In fact, a weaker phosphorylation of a STAT1 C-terminal peptide (aa 711–750) was induced in vitro by activated p38 kinase (23). However, whether p38 kinase is involved in the Ser727 phosphorylation in vivo is still controversial (26). For example, some studies indicated that p38 kinase is involved in the serine phosphorylation response leading to intracellular STAT1 signaling activation (22–24,28), but others showed that p38 kinase was not required for serine phosphorylation (48,49). Here, to assess the role of p38 kinase in mediating serine phosphorylation induction by UVA, we prepared and identified JB6 Cl 41 cell lines stably expressing DNM-p38β as reported previously (32,33). The results showed that UVA-stimulated phosphorylation of STAT1 (Ser727) was inhibited by expression of DNM-p38β (Figure 5A and B) compared with the corresponding control JB6 cells that only expressed the empty CMV-neo vector (CMV-neo). Furthermore, a similar inhibitory effect was also observed after pre-incubation of JB6 cells with SB202190, a specific p38 kinase inhibitor (Figure 5C), or PD169316 (Figure 4D). These observations, therefore, demonstrate that in addition to JNKs, p38 kinase is required for STAT1 serine phosphorylation in the JB6 cellular response to UVA irradiation.

MSK1 is involved in UVA-stimulated phosphorylation of STAT1

To further explore the role of ERK/p38 kinase-dependent serine/threonine kinases in serine 727 phosphorylation of STAT1, we established and identified JB6 Cl 41 cell lines stably expressing an N-terminal or C-terminal ‘kinase-dead’ mutant (Nd or Cd) of MSK1, as well as wild-type MSK1 (MSK1wt) (37). These cells were exposed to UVA and the results showed that the UVA-induced serine phosphorylation of STAT1 (Ser727) was enhanced by expression of MSK1wt compared with the induction in control cell lines expressing an empty vector pCMV5-neo (CMVS). However, expression of MSK1-Cd or MSK1-Nd markedly prevented the UVA-stimulated serine phosphorylation of STAT1 (Figure 5D), in contrast to the response induced in MSK1wt or CMVS cells. No change in total STAT1 expression was observed in the same experiments. Together, these findings suggest that ERK/p38-mediated MSK1 may also be involved in mediating UVA-induced STAT1 Ser727 phosphorylation.

In vitro identification of potential protein kinases responsible for phosphorylating STAT1 proteins

To date, the upstream kinases responsible for phosphorylating Ser727 in STAT1 are still elusive. For example, some studies showed that STAT1 was a relatively poor substrate for activated ERKs obtained from IP (27). Additionally, a weaker phosphorylation of a STAT1 C-terminal peptide (aa 711–750) was induced by activated p38 kinase in vitro (23). Here,
we further examined whether protein kinases in the MAPK cascades are responsible for phosphorylating STAT1 proteins using the immune-purified or GST-pull down STAT1 proteins as kinase substrates. Our results of western blot analysis for the in vitro kinase reactions with STAT1 immunoprecipitates showed that active JNK1, MEK1 and MSK1 stimulated phosphorylation of the STAT1 proteins (Ser727) (Figure 6A and B), but the phosphorylation was undetectable in the ERKs or p38 kinase reactions (data not shown). Furthermore, GST-pull down STAT1 proteins, including GST-fusion wild-type full-length STAT1 (GST±STAT1wt) and point-mutant STAT1 Y701F (GST±STAT1Y701F) and S727A (GST±STAT1S727A) proteins, were identified as shown in Figure 7A. Subsequently, using a phospho-STAT1 (Ser727) antibody or autoradiography, the in vitro kinase reactions showed that none of the experimental kinases phosphorylated the GST-pull down STAT1 proteins (data not shown). Interestingly, additional kinase experiments utilizing combined substrates of the STAT1 proteins from GST-pull down and IP followed by autoradiographic analysis revealed that the immunoprecipitated STAT1 proteins from cell lysates, were to different degrees, phosphorylated by active ERK1, JNK1, p38α kinase, p38β kinase, MEK1 or MSK1, but not ERK2 or JNK2 (Figure 7B). At the same time, GST-fusion wild-type full-length STAT1 proteins in the presence of IP-STAT1 proteins were also, to different extents, phosphorylated by JNK1, JNK2, p38α kinase or p38β kinase, but not ERK1, ERK2, MEK1 or MSK1 (Figure 7B). The data suggest that ERKs, JNKs and p38 kinases, as well as MEK1 or MSK1, may participate in the phosphorylation of STAT1 (Ser727) in the presence of an unidentified cofactor or downstream kinase.

Moreover, further studies showed that a strong phosphorylation of GST±STAT1wt or GST±STAT1Y701F combined with IP-STAT1 proteins was stimulated by either JNK1 (Figure 7C, upper panel) or p38β kinase (Figure 7C, lower panel), whereas a significantly lower phosphorylation level in the GST±STAT1S727A proteins was also observed (Figure 7C). These results suggest that JNK1 and p38β kinase may mediate
phosphorylation of STAT1 mainly at the serine 727 residue, as well as possible non-Ser727 residues. In addition, phosphorylation of IP-STAT1 by JNK1 or p38 kinase was blocked markedly by addition of GST±STAT1S727A (Figure 7C), suggesting that STAT1S727A may be used as a dominant negative mutant form of the STAT1 protein. Taken together, our study suggests that the EGFR-independent JNKs or p38 kinase signaling pathways play a positive regulatory role in UVA induction of STAT1 serine phosphorylation, and the phosphorylation induction is differentially regulated by the EGFR-mediated MEK1/ERKs signaling pathways (Figure 8). Further, phosphorylation of STAT1 (Ser727) catalyzed by ERKs, JNKs and p38 kinases, as well as MEK1 or MSK1 may occur in the presence of an unidentified cofactor or downstream kinase.

**Discussion**

Most information regarding STAT1 signaling regulation is focused heavily on phosphorylation of Tyr701 catalyzed by JAKs or related protein tyrosine kinases. This resulted in the assumption that Tyr701 phosphorylation was indispensable for the STAT1 activation process (1,3). However, this idea is challenged by recent findings revealing the existence of Tyr701 phosphorylation-independent STAT1 activation mechanisms (11,12). Here, we demonstrate a stronger Ser727 phosphorylation and a relatively lower or no Tyr701 phosphorylation induced by stimulation of JB6 cells with UVA, UVB or UVC, as well as EGF. These observations, together with previous findings showing that Ser727 phosphorylation results in a significant increase in STAT1 activity (4,17–24,26), indicate...
that Ser727 phosphorylation plays a pivotal role in activation of STAT1 signaling. But so far, the kinase directly responsible for the Ser727 phosphorylation involved in this activation process has been not fully identified. In the present report, using pharmacological and genetic approaches, we provide a possible mechanism for signal transduction towards Ser727 phosphorylation. A proposed model of the signaling pathways is presented in Figure 8.

Ser727 is located in a proline-directed consensus motif of the C-terminal domain of STAT1 and the motif makes it a potential target for phosphorylation by MAPKs (17,18). Indeed, its phosphorylation has been shown to be correlated with activation of ERKs (27), p38 kinase (22–24) or JNKs (22). However, except for one report indicating that a C-terminal peptide was weakly phosphorylated by p38 kinase (23), no substantial direct evidence has been presented showing that any of the MAPKs act as a direct kinase for STAT1. Here, we performed a series of in vitro kinase reactions using the immunoprecipitated or GST-pull down STAT1 proteins, or combined together as substrates followed by autoradiography or western blotting with a phospho-specific STAT1. The results showed that Ser727 phosphorylation in intact immunoprecipitated STAT1 proteins by active purified JNK1 was detected, but the phosphorylation by active ERK1, ERK2, JNK2 or p38α kinase was not detected by western blotting. However, the more sensitive autoradiography demonstrated that the STAT1 immunoprecipitates are, to different degrees, phosphorylated by JNK1, JNK2, p38α or p38β kinases, suggesting that a conformational change in GST–STAT1wt may be triggered by a yet-to-be unidentified cofactor in STAT1 immunoprecipitates. Furthermore, the phosphorylation of GST–STAT1 by JNK1 or p38β kinase was unaffected by a point mutation at the tyrosine 701 residue (GST–STAT1Y701F), but was significantly decreased by the point mutation at the serine 727 residue (GST–STAT1S727A). Taken together, these observations indicate that ERKs, JNKs and p38 kinases may catalyze phosphorylation of STAT1 at Ser727 in the presence of an unidentified factor. However, a weaker phosphorylation in GST–STAT1S727A proteins suggests that the experimental kinases may be also involved in catalysis of STAT1 phosphorylation at non-Ser727 residues.

Further evidence is presented that UVA-stimulation of Ser727 phosphorylation of STAT1 in JB6 cells was blocked by a dominant negative mutant of JNK1 (DMN-JNK1) or p38β kinase (DNM-p38β), or by biochemical inhibition of JNKs...
and/or p38 kinase, and also abolished by deficiency of Jnk1 or Jnk2. These data, together with in vitro kinase reactions, indicate that JNKs and p38 kinases are required for mediating serine phosphorylation of STAT1 stimulated by UVA.

Interestingly, our results revealed that the presence of MEK1/2 inhibitors, PD98059 and U0126, significantly reduced STAT1 phosphorylation induced by UVA, suggesting an involvement of MEK1/ERK1 in the process. In addition,
SEK1/MEK4, MKK6 and/or MEKK1 have been shown to participate directly or indirectly in regulating Ser727 phosphorylation of STAT1 (Ser727), suggesting that both EGFR-dependent and -independent MAPK signaling pathways lead towards STAT1 Ser727 phosphorylation in the UVA response.

In summary (Figure 8), STAT1 (Ser727) phosphorylation appears to be a point of convergence and is eventually stimulated following the integration of signals from multiple pathways. These signals from EGFR-independent MAPK-mediated pathways to STAT1 are triggered and transduced in the cellular response to UVA. Further, the phosphorylation process by MAPKs, including ERKs, JNKs and p38 kinases, as potential direct kinases for STAT1 (Ser727) phosphorylation, may occur in the presence of an unidentified factor. Additionally, unlike MAPKs, MEK1, an upstream kinase closer to ERKs, and MSK1, a downstream kinase of ERKs/p38 kinase, may also play a role in modulating STAT1 phosphorylation indirectly via an unidentified downstream kinase or other signaling mechanisms. Overall, STAT1 phosphorylation at Ser727 and/or possibly at non-ser727 sites is differentially mediated by diverse MAPK cascades in the UVA response.

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References


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