Serine Phosphorylation and Negative Regulation of Stat3 by JNK*

(Received for publication, April 22, 1998, and in revised form, July 7, 1999)

Cheh Peng Lim and Xinmin Cao‡

From the Signal Transduction Laboratory, Institute of Molecular and Cell Biology, National University of Singapore, Singapore 117609

STATs are activated by various cytokines and growth factors via tyrosine phosphorylation, which leads to sequential dimer formation, nuclear translocation, binding to specific DNA sequences, and regulation of gene expression. Recently, serine phosphorylation of Stat3 on Ser-727 by ERK has been identified in response to epidermal growth factor (EGF). Here, we report that Ser-727 phosphorylation of Stat3 can also be induced by JNK and activated either by stress or by its upstream kinase and that various stress treatments induce serine phosphorylation of Stat3 in the absence of tyrosine phosphorylation. Inhibitors of ERK and p38 did not inhibit UV-induced Stat3 serine phosphorylation, suggesting that neither of them is involved. We further demonstrate that JNK1, activated by its upstream kinase MKK7, negatively regulated the tyrosine phosphorylation and DNA binding and transcriptional activities of Stat3 stimulated by EGF. Correspondingly, pretreatment of cells with UV reduced the EGF-stimulated tyrosine phosphorylation and phosphotyrosine-dependent activities of Stat3. The inhibitory effect was not observed for Stat1. Our results suggest that Stat3 is a target of JNK that may regulate Stat3 activity via both Ser-727 phosphorylation-dependent and -independent mechanisms.

Serine phosphorylation of Stat3 has also been demonstrated. A Pro-X-Ser-Pro sequence that is a recognition site of ERKs has been found at the COOH terminus of Stat1, Stat3, and Stat4, suggesting that ERK is involved in the phosphorylation of these STATs (10). ERKs are members of the mitogen-activated protein kinase (MAPK) family that are activated by growth factor stimulation and have been shown to play a role in cell proliferation and differentiation (11–13). It has been reported that ERK2 co-immunoprecipitated with Stat1α in response to interferon-β and was involved in the regulation of interferon-β-induced gene expression (14). Recently, it has also been reported that ERKs phosphorylate Stat3 on Ser-727 in vitro as well as in vivo in response to EGF (15). However, the existence of serine/threonine kinases other than ERKs phosphorylating STATs on serine has also been suggested. For example, Stat1 is a relatively poor substrate for ERKs (15). In addition, although phosphorylation of Stat1 on Ser-727 is induced in response to interferon-γ, ERKs are not activated by interferon-γ and therefore are unlikely to be involved in such phosphorylation (16). Moreover, serine phosphorylation of Stat3 by IL-6 stimulation has been shown to be ERK-independent (15), and the involvement of H-7-sensitive serine kinases has also been reported (17–19).

Two other subtypes of mammalian MAPKs that are activated by environmental stress and pro-inflammatory cytokines have been identified. JNKs, also known as stress-activated protein kinases, are activated by IL-1, tumor necrosis factor (TNF), UV radiation, and anisomycin (20–22). JNKs bind to the amino terminus of c-Jun and phosphorylate it on Ser-63 and Ser-73 (23). The third group of MAPKs, p38, is activated by endotoxin lipopolysaccharide or hyperosmolarity (24). Although ERKs, JNKs, and the p38 kinase families are closely related due to their similar regulatory TXY motif for activity, they are distinguishable by unique (although sometimes overlapped) upstream activators and downstream substrates (25–27). We investigated whether Stat3 can be phosphorylated by stress and pro-inflammatory cytokines and examined which kinases are involved in such phosphorylation. We observed that Stat3 was phosphorylated on Ser-727 by TNF-α and various stress treatments. JNK1 activated either by UV or anisomycin or by its upstream kinase MEKK1 phosphorylated Stat3 in vitro. The major phosphorylation site was identified to be Ser-727. Stat3 can also be phosphorylated by cotransfection of JNK1 with MEKK1 in vivo. We further demonstrate that activation of JNK1 either by its upstream kinases or by UV treatment resulted in negative regulation of its activity.
**EXPERIMENTAL PROCEDURES**

**Construction of Plasmids**—The glutathione S-transferase (GST)-Stat3 fusion protein, containing an almost full-length Stat3, was constructed as described previously (9). The point mutant GST-S1, in which Ser-727 of GST-Stat3 was replaced by Ala, was prepared using the polymerase chain reaction-based site-directed mutagenesis kit ExSite (Stratagene) following the manufacturer’s instructions. The deletion mutant GST-C2 containing amino acids 480–770 was generated by digesting GST-Stat3 with XmnI/XhoI, isolating the fragment, and inserting it into pGEX-KG. The expression plasmid of Stat3, pRecCMV-Stat3 (6), was obtained from Dr. J. E. Darnell, Jr. (Rockefeller University). Substitution of the phosphorylation site Ser-727 by Ala was performed with the QuikChangeTM site-directed mutagenesis kit (Stratagene). The correct construction was confirmed by sequencing. The activated MEKK1 mutant (28) was provided by Dr. R. Janknecht (Mayo Foundation). pSRo-HA-JNK1 (26) and kinase-deficient mutant JNK1 pcDNA3.FLAG.JNK1(APF) (21) were obtained from Drs. A. Whitmarsh and R. J. Davis (University of Massachusetts). MKK7 plasmid (29) expressing active MKK7 (pcDNA3.MKK7Δ) and the kinase-deficient mutant (pcDNA3.MKK7A) were provided by Dr. J. Han (Scripps Research Institute). The reporter plasmid pSIE-CAT for CAT assays was prepared by inserting three copies of the hSIE consensus sequence (TTCCCGTAA) upstream of a c-fos minimal promoter followed by the CAT gene in plasmid pFOSCATΔ56 (30).

**Immunoprecipitation/Western Blotting and Immune Complex Protein Kinase Assay**—GST-1 cells transfected with expression plasmids were lysed in radioimmune precipitation assay buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.2), 1% deoxycholic acid, 1% Triton X-100, 0.25 mM EDTA (pH 8.0), and protease and phosphatase inhibitors (5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride, 5 mM NaF, and 100 μM sodium orthovanadate)). The cell lysates were incubated with an anti-JNK1 antibody (Santa Cruz Biotechnology) overnight at 4 °C, followed by incubation with protein G PLUS/protein A-agarose (Oncogene Science Inc.) for 1 h. The immunoprecipitates were washed twice with radioimmune precipitation assay buffer and then eluted with sodium dodecyl sulfate buffer and subjected to in vitro kinase assays. The GST-Stat3 fusion proteins were used as substrates and partially purified as described previously (9). Glutathione-Sepharose-bound GST-Stat3 was eluted by vortexing for 15 min at room temperature in an equal volume of 20 mM reduced glutathione, resuspended in 50 mM Tris-HCl (pH 8.0), concentrated using a Centriprep 10 (Amicon, Inc.), washed once in 20 mM Hepes (pH 7.3), and further concentrated using an ULTRA-FREE-SC filter unit (Millipore Corp.). Equal amounts of fusion proteins were used in the kinase assays. The fusion proteins were incubated with immunoprecipitated JNK1 in the kinase assay buffer in the presence of 5 or 10 μCi of [γ-32P]ATP at 30 °C for 15–30 min. The reaction mixture was boiled in Laemmli buffer, separated by 10% SDS-PAGE, transferred to nitrocellulose membrane, and exposed to x-ray film. The blots were also subjected to Amido Black staining to show the equal amount of GST fusion proteins used in each reaction. As for Western analysis of total cell lysates, equal amounts of lysates were separated by SDS-PAGE, transferred to a polyvinylidene difluoride, membrane, and blotted with the respective antibodies, including anti-phospho-Ser-727 Stat3, anti-phospho-Tyr-705 Stat3, or anti-Stat3 antibody as indicated. Western analysis of total cell lysates, equal amounts of lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane, and exposed to x-ray film as described previously (31).

**Orthophosphate Labeling**—[32P]Orthophosphate—[γ-32P]Orthophosphate at a final concentration of 1 μCi/ml for 4 h before harvesting. The cells were lysed, and the lysate was immunoprecipitated with an anti-Stat3 antibody (Santa Cruz Biotechnology). The immunoprecipitates were washed, and the proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane, and exposed to x-ray film as described previously (31).

**[32P]Orthophosphate Labeling**—[32P]Orthophosphate at a final concentration of 1 μCi/ml for 4 h before harvesting. The cells were lysed, and the lysate was immunoprecipitated with an anti-Stat3 antibody (Santa Cruz Biotechnology). The immunoprecipitates were washed, and the proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane, and exposed to x-ray film as described previously (31).

**[32P]Orthophosphate Labeling**—[32P]Orthophosphate at a final concentration of 1 μCi/ml for 4 h before harvesting. The cells were lysed, and the lysate was immunoprecipitated with an anti-Stat3 antibody (Santa Cruz Biotechnology). The immunoprecipitates were washed, and the proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane, and exposed to x-ray film as described previously (31).

**DNase I Protection Assay**—To protect DNA from endogenous RNAse activity, cells were incubated with 10 μg/ml DNase I for 30 min at 37 °C before DNA isolation. DNA was precipitated with ethanol and redissolved in TE buffer. The resulting solution was used for the DNase I protection assay.

**Mobility Shift DNA Binding Assay**—[32P]DNA was purified from yeast using the EconoTm spin column (Bio-Rad) and labeled with [γ-32P]ATP by T4 polynucleotide kinase. The labeled DNA was annealed to an unlabeled competitor to form a double-stranded DNA substrate. The substrate DNA was incubated in a reaction buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM MgCl2, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml bovine serum albumin, and 0.5 μg/ml poly(dI-dC)•poly(dI-dC) (Pharmacia). The reaction was stopped by heating the sample at 95 °C for 5 min and chilled on ice. The DNA was fractionated by polyacrylamide gel electrophoresis, and the DNA binding activity was visualized by autoradiography.

**RESULTS**

**Stress Treatments Induce Serine Phosphorylation of Stat3 in Vivo**—Stat3 is activated by tyrosine phosphorylation on Tyr-705 in response to growth factors and cytokines. In addition, serine phosphorylation of Stat3 has been observed, and the major phosphorylation site is Ser-727. We investigated whether environmental stress or inflammatory cytokines can induce phosphorylation of Stat3. COS-1 cells, which express low levels of endogenous Stat3 (10), were transfected with Stat3 expression plasmid and treated with TNF-α and various stresses. Phosphorylation of Stat3 was examined with antibodies specifically recognizing either Ser-727- or Tyr-705-phosphorylated Stat3 protein in Western blot analysis. Fig. 1A (upper panel) shows that Stat3 Ser-727 phosphorylation was induced by UV, anisomycin, TNF-α, and sodium arsenite and, to a weaker extent, by NaCl, okadaic acid, and lipopolysaccharide. In contrast, Tyr-705 phosphorylation of Stat3 was undetected in cells with these treatments (middle panel). EGF, as a positive control, induced both strong tyrosine and serine phosphorylations.

**Serum Starvation**—Cells were harvested by UV, anisomycin, TNF-α, and sodium arsenite and, to a weaker extent, by NaCl, okadaic acid, and lipopolysaccharide. In contrast, Tyr-705 phosphorylation of Stat3 was undetected in cells with these treatments (middle panel).
cells were either left untreated (U) or treated with UV (60 J/m², 30 min), anisomycin (ANI; 25 ng/ml, 45 min), NaCl (0.2 M, 15 min), or EGF (100 ng/ml, 15 min). The cell lysates were immuno-precipitated with an anti-JNK1 antibody, and an in vitro kinase assay was performed using the GST-Stat3 fusion protein (GST-Stat3) as a substrate. B, COS-1 cells were transfected with empty vector (−) or with JNK1 or JNK1 + MEKK1 plasmids, and the lysates were immunoprecipitated (IP) with an anti-JNK1 antibody. The immunoprecipitates were divided into three portions. Two portions were subjected to in vitro kinase assays using either GST-Stat3 or GST-c-Jun as a substrate. The third portion was subjected to Western blot analysis with either an anti-phospho-JNK1 (Blot: P-JNK1) or anti-JNK1 (Blot: JNK1) antibody. C, COS-1 cells were transfected with JNK1 alone or with MEKK1 as indicated. The cell lysates were immunoprecipitated with an anti-JNK1 antibody, and the immunoprecipitates were subjected to in vitro kinase assay using GST-Stat3, GST-S1, or GST-C2 as a substrate. GST-Stat3 and GST-C2 are indicated by asterisks. The molecular mass markers are indicated in kilodaltons. The lower panel shows the Amido Black staining of the blots to indicate the amounts of fusion proteins used in each reaction. The bands marked with asterisks represent the respective fusion proteins.

**JNK Phosphorylates Stat3 on Ser-727 in Vitro**—Since JNK/ stress-activated protein kinase is activated by stress and JNK1 is a major JNK, we next examined whether JNK1 was able to phosphorylate Stat3 in vitro. COS-1 cells were treated with UV, anisomycin, NaCl, or EGF; and the lysates were immuno-precipitated with an anti-JNK1 antibody, followed by an immuno-complex protein kinase assay using the GST-Stat3 fusion protein as a substrate. As shown in Fig. 2A, UV and anisomycin induced Stat3 phosphorylation by JNK1, whereas NaCl and EGF, which mainly activate p38 kinase and ERKs, respectively, did not stimulate Stat3 phosphorylation significantly.

MEKK1 phosphorylates the JNK upstream kinase SAPK/ ERK kinase-1, which in turn phosphorylates and activates JNK1 (28). To further confirm Stat3 phosphorylation by JNKs, we transfected COS-1 cells with JNK1 in the absence or presence of constitutively activated MEKK1 and performed the immune complex protein kinase assay. We observed that GST-Stat3 was strongly phosphorylated by MEKK1-activated JNK1, but not by JNK1 transfected alone or by endogenous JNK1 (Fig. 2B, upper panel). The activation of JNK1 by MEKK1 was confirmed by the strong phosphorylation of GST-c-Jun (−1–79), a physiological substrate of JNK1 (second panel, lane 3). This was also verified by the strong phosphorylation of JNK1 detected with an anti-phospho-JNK1 antibody that recognizes dual-phosphorylated JNK1 in Western blot analysis (third panel, lane 3). An equal expression of endogenous JNK1 in the presence or absence of MEKK1 was shown (lower panel). These data suggest that JNK1, activated by either stress or by its upstream kinase, phosphorylates Stat3 on Ser-727 in vitro.

We further tested whether Ser-727 was the only phosphorylation site of JNK1. A point mutant (GST-S1) in which Ser-727 was replaced by alanine and a deletion mutant (GST-C2) containing the COOH-terminal portion of Stat3 (amino acids 480–770) were generated, and the phosphorylation by JNK1 was tested. As shown in Fig. 2C, MEKK1-activated JNK1 strongly phosphorylated GST-Stat3 and GST-C2, but failed to phosphorylate GST-S1. The amounts of the fusion proteins GST-Stat3 and GST-S1 used in the kinase assays were comparable (indicated by asterisks in the lower panel). These data suggest that Ser-727 is the only phosphorylation site of JNK1 in vitro.

**JNK Phosphorylates Stat3 in Vivo**—Next, we examined whether JNK1 phosphorylated Stat3 in vivo. COS-1 cells were either transfected with Stat3 expression plasmid alone or co-transfected with JNK1 and MEKK1 and labeled with [32P]orthophosphate. The lysates were immunoprecipitated with an anti-Stat3 antibody. A basal level of Stat3 phosphorylation was observed in cells transfected with Stat3 alone (Fig. 3, lane 1), which was strongly enhanced in cells cotransfected with JNK1 and MEKK1 (lane 2). As a positive control, EGF also stimulated Stat3 phosphorylation (lane 3). These data indicate that Stat3 can be phosphorylated by JNK1 in vivo.

**Effects of ERK and p38 Inhibitors on UV- or EGF-induced Stat3 Serine Phosphorylation**—To ascertain the noninvolvement of other MAPK family members in the stress-induced Ser-727 phosphorylation of Stat3, the inhibitors of MEK1 (PD98059) (33) and p38 kinase (SB203580) (34) were used to pretreat cells, followed by UV or EGF treatment. The Ser-727 phosphorylation was analyzed. UV-induced phosphorylation of Stat3 was not affected by either inhibitor (Fig. 4, middle panel). In contrast, EGF-induced phosphorylation was inhibited by PD98059, but not by SB203580 (right panel). The basal level of phosphorylation in uninduced cells was slightly decreased by both inhibitors (left panel). These results suggest that whereas ERKs phosphorylate Stat3 by EGF stimulation, ERKs and p38
are unlikely to be involved in Stat3 serine phosphorylation induced by UV.

**JNK1 Activated by MKK7 Negatively Regulates Tyrosine Phosphorylation and DNA Binding and Transcriptional Activities of Stat3**—The tyrosine phosphorylation of Stat3 by growth factors and cytokines is a prerequisite for its dimerization, DNA binding, and transactivation, whereas the Ser-727 phosphorylation alone does not stimulate Stat3 DNA binding and transcriptional activities (6, 35, 36). We studied the role of JNK1 in Stat3 function by testing its effect on the DNA binding and transcriptional activities of Stat3 stimulated by EGF. MKK7 (JNK kinase-2) has recently been identified to be a specific upstream activator of JNK1 without affecting ERKs or p38 (29, 37–40). COS-1 cells were transfected with Stat3 alone or were cotransfected with JNK1 and/or MKK7 expression plasmids and treated with EGF. The DNA binding activity was analyzed using hSIE, a high affinity binding site for Stat3, as a probe. As reported previously, EGF induced the activation of Stat3 and Stat1 to form three complexes with SIE: SIF-A (Stat3 homodimer), SIF-B (Stat1/Stat3 heterodimer), and SIF-C (Stat1 homodimer) (6). The DNA binding activity of Stat3 was not observed in the untreated cells transfected with Stat3 (Fig. 5A, lane 3), but was induced after EGF treatment (lane 4, SIF-A and SIF-B). SIF-A was not affected by constitutively activated MKK7, JNK1, or kinase-deficient mutant JNK1– alone (lanes 5–7), but was almost completely destroyed by cotransfection of MKK7 and JNK1 together (lane 8). The DNA binding activity was largely restored by cotransfection of either mutant MKK7 and wild-type JNK1 (lane 10) or constitutively activated MKK7 and kinase-deficient JNK1 (lane 9). It has been previously reported that the formation of SIF-C by endogenous Stat1 can be detected after EGF stimulation in COS cells (41). As shown in Fig. 5A, SIF-C was also detected upon EGF treatment (lane 4), but was unaffected by cotransfection with wild-type or mutant MKK7 and/or JNK1 (lanes 5–10).

We next examined whether activated JNK1 also affected Stat3 transcriptional activity stimulated by EGF. A reporter plasmid containing three copies of hSIE followed by a CAT gene was cotransfected with Stat3 in the presence or absence of JNK1 and/or MKK7 expression plasmids, and the CAT activities were analyzed. As illustrated in Fig. 5B, CAT activity increased 10.4-fold after EGF stimulation, which slightly decreased in the presence of MKK7 (7.5-fold), but was unaffected by either wild-type or mutant JNK1 (10.3- and 11.8-fold, respectively). However, when Stat3 was cotransfected with MKK7 and JNK1 together, CAT activity was completely inhibited (1.4-fold), whereas such inhibition was not observed with cotransfection of MKK7 and mutant-JNK1 or of mutant MKK7 and JNK1. These results are consistent with the DNA binding data, indicating that activated JNK1 suppresses both the DNA binding and transcriptional activities of Stat3.

**UV Pretreatment Decreases Tyrosine Phosphorylation and DNA Binding and Transcriptional Activities of Stat3**—To investigate a possible physiological role of JNK1 phosphorylation in Stat3 function, we examined whether stress affects Stat3 activity stimulated by EGF. COS-1 cells were transfected with wild-type Stat3 and pretreated with UV for various time points, followed by EGF treatment; and the DNA binding activity was measured. As shown in Fig. 6A, EGF induced the activation of transfected Stat3 and endogenous Stat1 to form SIF-A, SIF-B, and SIF-C (lane 3). Pretreatment of the cells with UV significantly decreased SIF-A formation. SIF-B was also reduced, whereas SIF-C was not significantly affected (lanes 4–6). This indicates that UV specifically decreases the DNA binding activity of Stat3, but not of Stat1. The effect of UV pretreatment on the EGF-induced transcriptional activity of Stat3 was also tested in CAT assays and shown to be inhibitory (Fig. 6B). Finally, we analyzed the effect of UV treatment on Stat3 tyrosine phosphorylation. In agreement with the inhibition of the DNA binding and transcriptional activities, a decrease in the tyrosine phosphorylation of Stat3 by UV pretreatment was detected (Fig. 6C, upper panel, lanes 3–5), whereas the Ser-727 phosphorylation induced by EGF was not affected by UV pretreatment (middle panel), probably due to the strong Ser-727 phosphorylation induced by EGF stimulation. An equal expression of Stat3 was indicated (lower panel). These results suggest that pretreatment of UV negatively affects Stat3 tyrosine phosphorylation and the phosphotyrosine-dependent activities. This inhibitory effect could be due to the Ser-727 phosphorylation by UV-activated JNK1 that occurred prior to the tyrosine phosphorylation stimulated by EGF. Alternatively, the repression could also be independent of Ser-727 phosphorylation. The possible factors include a general toxic effect of UV irradiation or phosphorylation on other serine kinase activities.
site(s) that affects Stat3 tyrosine phosphorylation and activities (see details under “Discussion”).

**DISCUSSION**

In addition to tyrosine phosphorylation, Stat1 and Stat3 are also phosphorylated on serine in response to cytokines and growth factors. ERKs, the prototype of MAPKs, were the first identified Ser/Thr kinases that phosphorylate Stat3 on serine by EGF stimulation (10, 15, and 17). In this study, we investigated whether environmental stress can induce phosphorylation of Stat3 and elucidated which kinase(s) is likely to be involved. We demonstrated that various stress treatments...
stimulate serine phosphorylation of both endogenous and exogenous Stat3 (Fig. 1); and JNK, a subtype of MAPKs, mediates the stress-dependent serine phosphorylation of Stat3 (Figs. 2 and 3). The site of phosphorylation of Stat3 by JNK1 was identified to be Ser-727 in vitro. Our data using the inhibitors of ERK and p38 pathways (Fig. 4) further support the specificity of Stat3 serine phosphorylation by JNK. These results demonstrate that JNK is the kinase that phosphorylates Stat3 in response to stress. Since phosphorylation of Stat3 on Ser-727 was also observed upon treatment of cells with sodium chloride, okadaic acid, and lipopolysaccharide, which stimulate p38 activity, we examined if p38 kinase, the other member of the MAPK family, could phosphorylate Stat3. We were not able to detect Ser-727 phosphorylation of the GST-Stat3 fusion protein by p38 activated either by stress or by cotransfection with its upstream kinase MKK3 in vitro (data not shown). However, the possibility of Stat3 phosphorylation by p38 in vivo cannot be excluded, and whether JNK is the only kinase family that is involved in Stat3 serine phosphorylation by various stress treatments remains to be determined. In addition to MAPKs, we recently identified protein kinase C treatments remains to be determined. In addition to MAPKs, involved in Stat3 serine phosphorylation by various stress be excluded, and whether JNK is the only kinase family that is involved in Stat3 serine phosphorylation by MKK3 or by UV irradiation, inhibited the tyrosine phosphorylation and DNA binding and transcriptional activities of Stat3 in both cases (Figs. 5 and 6 and data not shown). Such repression is specific for Stat3 since Stat1 DNA activity was not inhibited (Fig. 5A). These results are in agreement with previous reports showing that ERK2, when activated by MEK1, represses the tyrosine phosphorylation of Stat3 mutant S727 in an IL-6-dependent manner (42). Together, these data indicate that Stat3 is a target for multiple Ser/Thr kinases that are activated by distinct extracellular stimuli and suggest that Stat3 may be functionally involved in diverse cellular processes.

It is generally accepted that the tyrosine phosphorylation of STATs is a prerequisite for their DNA binding and transcriptional activity, although growth factors and cytokines induce phosphorylation of STATs on both tyrosine and serine (1–3). The question arising here is how does serine phosphorylation affect STAT activity? An initial report indicated that serine phosphorylation is required for the DNA binding of Stat3 in certain cell types (17). However, it was demonstrated later that phosphorylation on Ser-727 is not necessary for its DNA binding, but is required for the full transcriptional activity of Stat1 and Stat3 (10, 36). On the other hand, a negative effect of Ser-727 phosphorylation on the tyrosine phosphorylation of Stat3 has also been suggested (15). We examined how JNK affects the DNA binding and transcriptional activities of Stat3 stimulated either by EGF or by Src and observed that JNK1, activated either by its upstream kinase MKK7 or by UV irradiation, inhibited the tyrosine phosphorylation and DNA binding and transcriptional activities of Stat3 in both cases (Figs. 5 and 6 and data not shown). Such repression is specific for Stat3 since Stat1 DNA activity was not inhibited (Fig. 5A). These results are in agreement with previous reports showing that ERK2, when activated by MEK1, represses the tyrosine phosphorylation and tyrosine phosphorylation-dependent activities of Stat3 stimulated by EGF or IL-6 (43, 44). Furthermore, an inhibitory effect of protein kinase Cδ on the activity of Stat3 was also reported (42). These results suggest that activated MAPKs as well as other Ser/Thr kinases may negatively regulate STAT activity. These observations seem to be contradictory to the positive regulatory role of Ser-727 phosphorylation in STAT transcriptional activity (10). We attempted to address this question by further investigating the mechanisms of the repression. From our preliminary results, in agreement with the positive role of Ser-727 phosphorylation, we also observed a reduced transcriptional activity of the Stat3 mutant S727A stimulated by EGF. However, the DNA binding and transcriptional activities of...
S727A were further inhibited by activated ERK or JNK (data not shown), suggesting that the repression is unlikely mediated by Ser-727 phosphorylation. This result is consistent with the report showing that repression of IL-6-stimulated Stat3 activity by ERK is independent of Ser-727 phosphorylation (44). From these data, we propose that ERK and JNK may have dual effects on Stat3 transcriptional activity, i.e. up-regulation by Ser-727 phosphorylation and down-regulation in a Ser-727-independent manner, a concerted contribution to the resultant regulation of Stat3 trans-activation. These results also suggest a critical and complex role of the MAPK pathway in the regulation of STATs. Although the mechanisms of the negative regulation are still unknown, a few possibilities may be considered. First, activation of the MAPK pathways may negatively regulate the activity of the upstream tyrosine kinases such as EGF receptor, Src, and Janus kinases, which are involved in Stat3 tyrosine phosphorylation. Second, although we only detected phosphorylation of Ser-727 by JNK1 and ERK2 in vitro (Fig. 2C) (43), serine/threonine site(s) other than Ser-727 may be phosphorylated by these kinases in vivo (15). It is possible that phosphorylation on serine in DNA-binding domain of Stat3 may inhibit its DNA binding and transcriptional activities. Third, activated ERKs and JNKs may affect the activity of the specific inhibitors of Janus kinase/STAT pathways, namely the recently identified suppressor of cytokine signaling-1 or protein inhibitor of activated Stat3 (reviewed in Ref. 45). Finally, these kinases may modulate Stat3 activity by association since we observed a strong binding of Stat3 with activated ERK2 as well as protein kinase C-δ (42, 43).

In addition to stress, emerging evidence has shown that STATS are phosphorylated exclusively on serine in the absence of tyrosine phosphorylation. Examples include insulin-induced serine phosphorylation of Stat3 in 3T3-L1 adipocytes (46) and steel factor-induced phosphorylation of Stat3 in human growth factor-dependent myeloid cell lines (47). Activation of protein kinase C by phorbol esters is also reported to induce phosphorylation of Stat3 on Ser-727 in T lymphocytes (19). These data indicate that serine phosphorylation alone may play a role in cellular regulation. Although the physiological role of serine phosphorylation in STAT function is still obscure, reports suggest that Stat3 may be involved in the regulation of differentiation in pathogenesis of chronic lymphocytic leukemia (48, 49). A recent report indicated that Stat1 regulates apoptosis induced by TNF-α by a novel signaling pathway in which phosphorylation on serine, but not tyrosine, may be involved (50). A challenge for further studies is to determine the physiological role of serine phosphorylation in STAT function.

JNK binds and phosphorylates some transcriptional activators. The best studied example is the c-Jun transactivator. The phosphorylation of c-Jun at Ser-63 and Ser-73 in the activation mechanism involves Ser-727 phosphorylation-dependent and -independent mechanisms.

Acknowledgments—We are grateful to Drs. J. E. Darnell, Jr. for Stat3; A. Whitmarsh and R. J. Davis for HA-JNK1, FLAG-JNK1, and MEK1; R. Janknecht for MEKK1; M. Cardone for MEKK1; J. Han for p38, MKK3, and MKK7; and S. A. Courtneidge for Src plasmids. We thank R. Thom and S. Y. Oh for photography.

REFERENCES
1. Darnell, J. E., Jr., Kerr, I. M., and Stark, G. R. (1994) Science 264, 1415–1421
2. Ihe, J. N. (1996) Cell 84, 331–334
3. Darnell, J. E., Jr. (1997) Science 277, 1630–1635
4. Larner, A. C., David, M., Feldman, G. M., Igarashi, K., Hackett, R. H., Webb, D. S. A., Sweitzer, S. M., Petricoin, E. F., III, and Finnbloom, D. S. (1993) Science 261, 1730–1733
5. Silvennoinen, O., Schindler, C., Schlessinger, J., and Levy, D. E. (1993) Science 261, 1736–1739
6. Zhong, Z., Wen, Z., and Darnell, J. E., Jr. (1994) Science 264, 95–98
7. Leaman, D. W., Pisarsky, S., Flickinger, T. W., Commane, M. A., Schlossinger, J., Kerr, I. M., Levy, D. E., and Stark, G. R. (1996) Mol. Cell. Biol. 16, 369–375
8. Yu, C. L., Meyer, D. J., Campbell, G. S., Larner, A. C., Carter-Su, C., Schwartz, J., and Jove, R. (1995) Science 269, 81–83
9. Cao, X., Tey, A., Guy, G. R., and Tan, Y. H. (1996) Mol. Cell. Biol. 16, 1505–1603
10. Chen, Y., Zhong, Z., and Darnell, J. E., Jr. (1995) Cell 82, 241–250
11. Marshall, C. J. (1994) Curr. Opin. Genet. Dev. 4, 82–89
12. Manour, S. J., Matten, W. T., Hermann, A. S., Candia, J. M., Rong, S., Fukasawa, K., Yande Wouda, G. F., and Ahn, N. G. (1994) Science 265, 966–970
13. Seger, R., and Krebs, E. G. (1995) FASEB J. 9, 726–735
14. David, M., Petricoin, E. III, Benjamin, C., Pine, R., Weber, M. J., and Larner, A. C. (1995) Science 269, 1721–1723
15. Chung, J., Uchida, E., Grammer, T. C., and Blenis, J. (1997) Mol. Cell. Biol. 17, 6508–6516
16. Zhu, X., Wen, Z., Xu, Z. L., and Darnell, J. E., Jr. (1997) Mol. Cell. Biol. 17, 6618–6623
17. Zhang, X., Blenis, J., Li, H. C., Schindler, C., and Chen-Kiang, S. (1995) Science 267, 1990–1994
18. Boullon, T. G., Zhong, Z., Wen, Z., Darnell, J. E., Jr., Stahl, N., and Yancopoulos, G. D. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 6915–6919
19. Ng, J., and Cantrell, D. (1997) J. Biol. Chem. 272, 24542–24549
20. Kyriakis, J. M., Banerjee, P., Nakakukacii, E., Dai, T., Rubie, E. B., Ahmad, K., Avruch, J., and Watanabe, M. F. (1999) Nature 396, 156–160
21. Derijard, B., Hiroi, M., Wu, I. H., Barnett, T., Su, B., Deng, T., Karin, M., and Davis, R. J. (1994) Cell 76, 1025–1037
22. Cano, E., Hazzalin, C. A., and Mahadevan, L. C. (1994) Mol. Cell. Biol. 14, 7052–7062
23. Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993) Genes Dev. 7, 2135–2148
24. Han, J., Lee, J. D., Bibb, L., and Ulevitch, R. J. (1994) Science 265, 808–811
25. Derijard, B., Raingeaud, J., Barrett, T. W., Su, H., Han, J., Ulevitch, R. J., and Davis, R. J. (1995) Science 267, 682–685
26. Minden, A., Lin, A., McMahon, M., Lange-Carter, C., Derijard, B., Davis, R. J., Johnson, L. G., and Karin, M. (1994) Science 260, 1718–1723
27. Cano, E., and Mahadevan, L. C. (1995) Trends Biochem. Sci. 20, 117–122
28. Xu, S., Robbins, D., Frost, J., Dang, J., Lange-Carter, C., and Cobb, M. H. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 6808–6812
29. Wang, Y., Su, B., Sah, V. P., Brown, J. H., Han, J., and Chien, K. R. (1998) J. Biol. Chem. 273, 5423–5426
30. Gilman, M. Z., Wilson, R. N., and Weinberg, R. A. (1986) Mol. Cell. Biol. 6, 4305–4316
31. Jain, N., Mahendran, R., Wilson, R., Guy, G. R., Tan, Y. H., and Cao, X. (1996) J. Biol. Chem. 271, 15350–15356
32. Gorman, C. M., Moffat, L. F., and Howard, B. M. (1982) Mol. Cell. Biol. 2, 1044–1051
33. Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J., and Saltiel, A. R. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7686–7689
34. Cuenda, A., Roose, J., Doza, Y. N., Meier, R., Cohen, P., Gallagher, T. F., Younger, P. R., and Lee, J. C. (1995) FEBS Lett. 364, 229–233
35. Akira, S., Nishiy, Y., Nishiy, X. J., Wei, S., Matsuoka, T., Yoshida, K., Sudo, T., Naruto, M., and Kishimoto, T. (1994) Cell 77, 63–71
36. Wen, Z., and Darnell, J. E., Jr. (1997) Nuclear Acids Res. 25, 2062–2067
37. Tournier, C., Whitmarsh, A. J., Cavanagh, J., Barrett, T., and Davis, R. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 7337–7342