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STAT3 Serine Phosphorylation by ERK-Dependent and -Independent Pathways Negatively Modulates Its Tyrosine Phosphorylation

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Recent studies have indicated that serine phosphorylation regulates the activities of STAT1 and STAT3. However, the kinase(s) responsible and the role of serine phosphorylation in STAT function remain unresolved. In the present studies, we examined the growth factor-dependent serine phosphorylation of STAT1 and STAT3. We provide in vitro and in vivo evidence that the ERK family of mitogen-activated protein (MAP) kinases, but not JNK or p38, specifically phosphorylate STAT3 at serine 727 in response to growth factors. Evidence for additional mitogen-regulated serine phosphorylation is also provided. STAT1 is a relatively poor substrate for all MAP kinases tested both in vitro and in vivo. STAT3 serine phosphorylation, not its tyrosine phosphorylation, results in retarded mobility of the STAT3 protein on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Importantly, serine 727 phosphorylation negatively modulates STAT3 tyrosine phosphorylation, which is required for dimer formation, nuclear translocation, and the DNA binding activity of this transcriptional regulator. Interestingly, the cytokine interleukin-6 also stimulates STAT3 serine phosphorylation, but in contrast to growth factors, this occurs by an ERK-independent process.

The JAK/STAT pathways are activated by various cytokines and growth factors such as interleukin-6 (IL-6), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF) (reviewed in references 5 and 15). Once these growth factor or cytokine receptors are occupied by their ligands, tyrosine residues in the cytoplasmic domain of the receptors become phosphorylated by cytokine receptor-associated JAK family tyrosine kinases (19, 32) or by the growth factor-activated receptor tyrosine kinases (16, 26, 34). The phosphorylated tyrosine residue, positioned within specific amino acid sequences, provides docking sites for the recruitment of specific STATs via their SH2 domains (13, 33). STATs associated with the receptors are consequently phosphorylated at conserved tyrosine residues. This induces the STATs to dimerize via their own SH2 domains (29), translocate into the nucleus, and activate STAT target genes (30). To date, six STAT proteins have been characterized at the molecular level (reviewed in reference 15).

EGF and PDGF activate primarily STAT1 and STAT3 (27, 28, 31, 41). STAT1 and STAT3 can form three distinct dimers to activate target genes: STAT1 or STAT3 homodimers and STAT1-STAT3 heterodimers. Although STAT1 and STAT3 are simultaneously activated by various growth factors and cytokines, the results of knockout-mouse experiments suggest a specific role of STAT1 as a target for interferons (10, 21). However, STAT3 is expected to play a broader role. Recent results which show that STAT3 is activated by the expression of Src oncogenes (39) or infection by human T-cell leukemia virus type 1 (22) raise the possibility that STAT3 is involved in tumorigenic cell growth.

Mitogen-activated protein (MAP) kinase pathways play important roles in the regulation of cell growth and differentiation. The MAP kinases, including ERKs, p38 Hog1, and JNKs (SAPKs), are tightly regulated by multicomponent signal transduction cascades consisting of small GTP binding proteins (Ras, Cdc42, and Rac), MAP kinase kinase kinases (Raf and MEKK), and MAP kinase kinases (MEKs, MAPKKs, and SEK) (reviewed in references 7, 20, and 35). The signalling pathways leading to activation of the ERK family of MAP kinases can be stimulated by a variety of growth factors and cytokines. Several downstream targets for MAP kinases have also been characterized. For example, the ERK-MAP kinases and one downstream target, the protein kinase RSK, are transiently activated during the $G_0 \rightarrow G_1$ transition and both translocate into the nucleus (3, 17). Indirect evidence suggests that in the nucleus, these protein kinases may phosphorylate and regulate the activities of several transcription factors, including Jun, Fos, Elk-1, cyclic AMP responsive element binding protein and serum response factor (reviewed in reference 14). Similarly, p38 and JNKs also regulate transcription factors like ATF2 (12), Elk-1 (37), and Jun (8).

However, STATs are latent cytoplasmic transcription factors which are uniquely activated at the plasma membrane by tyrosine phosphorylation and translocate into the nucleus to activate target genes (reviewed in references 5 and 15). Although the essential role of the tyrosine phosphorylation is clear (30), recent reports imply that serine phosphorylation of STATs also regulates their transcriptional activities (36, 40). How serine phosphorylation regulates the transcriptional activities of STATs is still controversial. One group of results suggests that serine phosphorylation affects the homodimer formation of STAT3 and its DNA binding (40). However, others have shown that serine 727 phosphorylation of STAT1 and STAT3 affects their transcriptional activation (36) rather than their DNA binding activity. Although the serine kinase(s) responsible for the phosphorylation of STATs has not been characterized, the proposed MAP kinase phosphorylation site (11), -Pro-X-Ser-Pro-, is conserved among STAT1, STAT3,

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and STAT4 (36, 40), suggesting a role for the MAP kinases in the serine phosphorylation of all these. Physical interaction between ERK2 and STAT1 after stimulation with alpha/beta interferon (6) also supports the notion that there is a functional communication between these signalling molecules when cells are activated.

In the present study, we have shown that the ERKs, but not p38 or JNK, participate in STAT3 serine phosphorylation in growth factor-stimulated cells. STAT3 is a superb substrate for ERK-MAP kinases in vitro and is phosphorylated at serine 727, the same site phosphorylated in vivo. We provide evidence that ERK-MAP kinases are responsible for the phosphorylation of serine 727 in vivo by using a Raf-inducible fibroblast cell line and a specific MEK inhibitor, PD 098059. Using similar approaches, we observed smaller changes in serine 727 phosphorylation of STAT1 in comparison to STAT3. This result is consistent with our data that indicate that STAT1 is a poor substrate for ERK-MAP kinases in vitro. Interestingly, our results also suggest that the serine 727 phosphorylation of STAT3 negatively modulates its tyrosine phosphorylation. In addition to serine 727, the existence of other, regulated serine phosphorylation sites is demonstrated. Finally, we provide evidence that an IL-6-activated, ERK-independent mechanism for regulating STAT3 serine phosphorylation also exists.

MATERIALS AND METHODS

Antibodies and materials. Monoclonal antisera for STAT1 and STAT3 were purchased from Transduction Labs. Polyclonal antiserum for STAT3 was kindly provided by Robert Abraham. Monoclonal antiserum to HA was obtained from Boehringer Mannheim. Anti-EE antiserum was a gift from Raymond Erikson. Antiphosphotyrosine antibodies were either purchased from Upstate Biotechnology or kindly provided by Tom Roberts. Antisera against p38 and JNK1 were kindly provided by Roger Davis. H7 and phorbol ester were purchased from LC Laboratories. PD 098059 was kindly provided by Parke-Davis. IL-6 was provided by Selina Chen-Kiang and Hans-Peter Biemann. EGF and PDGF were purchased from Gibco-BRL. 4-Hydroxytamoxifen (4-HT) and other chemicals were obtained from Sigma. Purified JAK2 enzyme was purchased from Upstate Biotechnology.

Cell culture. COS cells, A431 cells, and HepG2 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum. Swiss 3T3 cells were grown in DMEM with 5% fetal calf serum and 5% bovine serum. 3T3 B-Raf:ER cells (kindly provided by Martin McMahon) were maintained as previously described (25). Before growth factor or cytokine stimulation, nearly confluent cells were deprived of serum for 24 to 48 h by incubation in DMEM containing 0.5% fetal calf serum and 20 mM HEPES buffer (pH 7.4).

Plasmid constructions and preparation of GST fusion proteins. STAT1 and STAT3 mammalian expression plasmids were constructed by inserting their coding sequences into pFJ vector (a gift from J. Jung, Harvard Medical School) at the *Apa*I-*Not*I sites. The EE and HA tag sequences were inserted into the N termini of coding sequences for STAT1 and STAT3, respectively, by PCR (EE 5′ primer, GCTAGCGGCCGCATGTACCCATACGATGTTCCAGATTACGCT CAGTGGAACCAGCTGCAG; HA 5′ primer, GCTAGCGGCCGCATGTAC CCATACGATGTTCCAGATTACGCTCAGTGGAACCAGCTGCAG). Point mutations for conversion of serine 727 of human STAT1 and mouse STAT3 to alanine were also introduced by PCR. Coding sequences for the C termini of human STAT1 (amino acids 680 to 750) and mouse STAT3 (amino acids 677 to 750) were generated by PCR and inserted into the *Bam*HI-*Sal*I and *Bam*HI-*Eco*RI sites, respectively, of GST-4T1 vector in-frame for the glutathione *S*-transferase (GST) coding sequence. GST-STAT constructs were transformed into DH-5α competent cells and expressed as described below. All recombinant DNA constructs described here were sequenced and checked before further experiments were performed.

Column chromatography. Swiss 3T3 cells at 80% confluence were serum starved for 2 days and then stimulated with EGF (50 ng/ml) for 7.5 min. Lysates were prepared by Dounce homogenization with lysis buffer without detergent (3) and clarified by centrifugation for 30 min at 100,000 × *g*. Supernatants were loaded onto a Mono Q column, and cellular proteins were fractionated through a fast protein liquid chromatography system (Pharmacia) using a 0 to 300 mM NaCl linear gradient. All the other fractions were assayed for STAT1 and STAT3 phosphotransferase activities and by ERK immunoblot analysis.

Immunoprecipitations. Cell lysis and immunoprecipitation were performed as previously described (4). Briefly, after lysis, cellular debris was cleared by centrifugation for 10 min at 10,000 × *g*. Supernatants were typically incubated with antisera for 2 h at 4°C and further incubated with protein A-Sepharose (Pharmacia) or inactivated *Staphylococcus aureus* for 1 h. Immune complexes were washed with buffer A and B twice each and analyzed by immunoblot analysis or by immune-complex protein kinase assays.

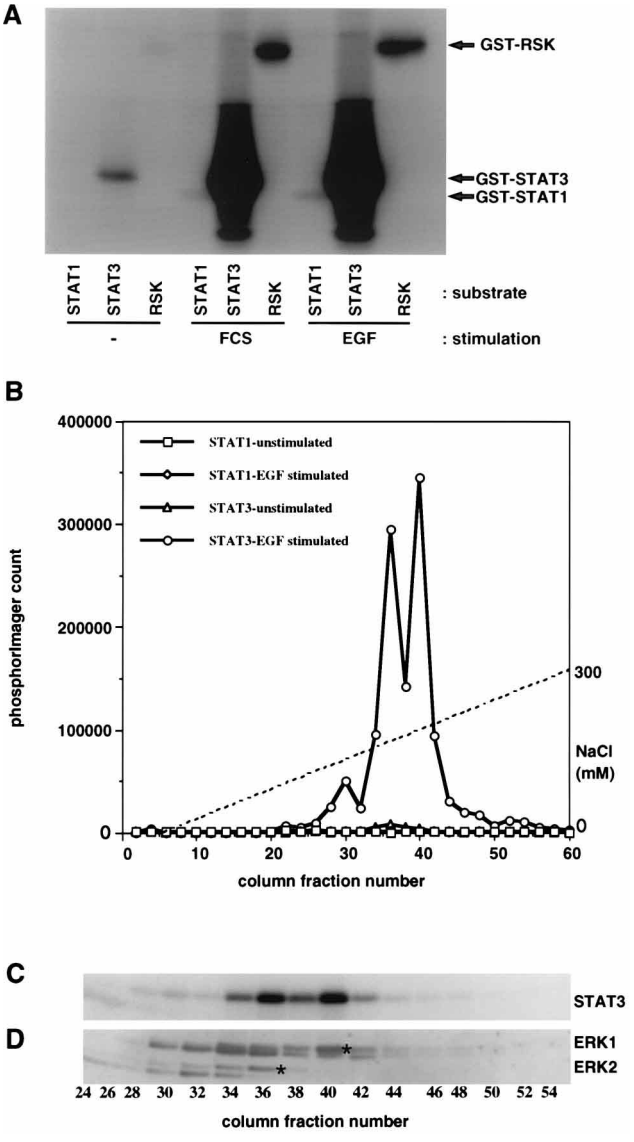

FIG. 1. ERK-MAP kinases phosphorylate GST-STAT fusion proteins in vitro. (A) Quiescent Swiss 3T3 cells were stimulated without (−) or with 10% fetal calf serum (FCS) or 50 ng of EGF per ml for 7.5 min, and lysates were prepared as described in Materials and Methods. Immune-complex kinase assays for equal amounts of ERK1 from various stimulations were performed with GST fusion proteins for STAT1, STAT3, and RSK as described in the text. (B to D) Quiescent Swiss 3T3 cells were stimulated without or with EGF (50 ng/ml) for 7.5 min, and cell lysates were fractionated through a Mono Q column. Phosphotransferase activities for GST fusion proteins of STAT1 and STAT3 were measured from even-numbered fractions (B). Two peaks of ^{32}P phosphorylated GST-STAT3 proteins in the kinase assays (C) were tightly correlated with the activated form (*) of ERK1 and ERK2 by immunoblot analysis (D).

Immunoblot analysis. Immunoblot analyses were performed as previously described (4). Briefly, 20 to 30 μg of total cellular protein or immunoprecipitated proteins was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Schleicher & Schuell). The membranes were incubated with primary antibodies such as anti-ERK (1:2,000), anti-p70-S6 kinase (1:2,000), anti-STAT1 (1:2,500), anti-STAT3 (1:2,500), anti-HA (1:1,000), anti-EE (1:1,000), or antiphosphotyrosine (1:1,000) antibodies and visualized with secondary antibodies coupled to peroxidase (Boehringer Mannheim and Amersham) and enhanced chemiluminescence reagents (Amersham).

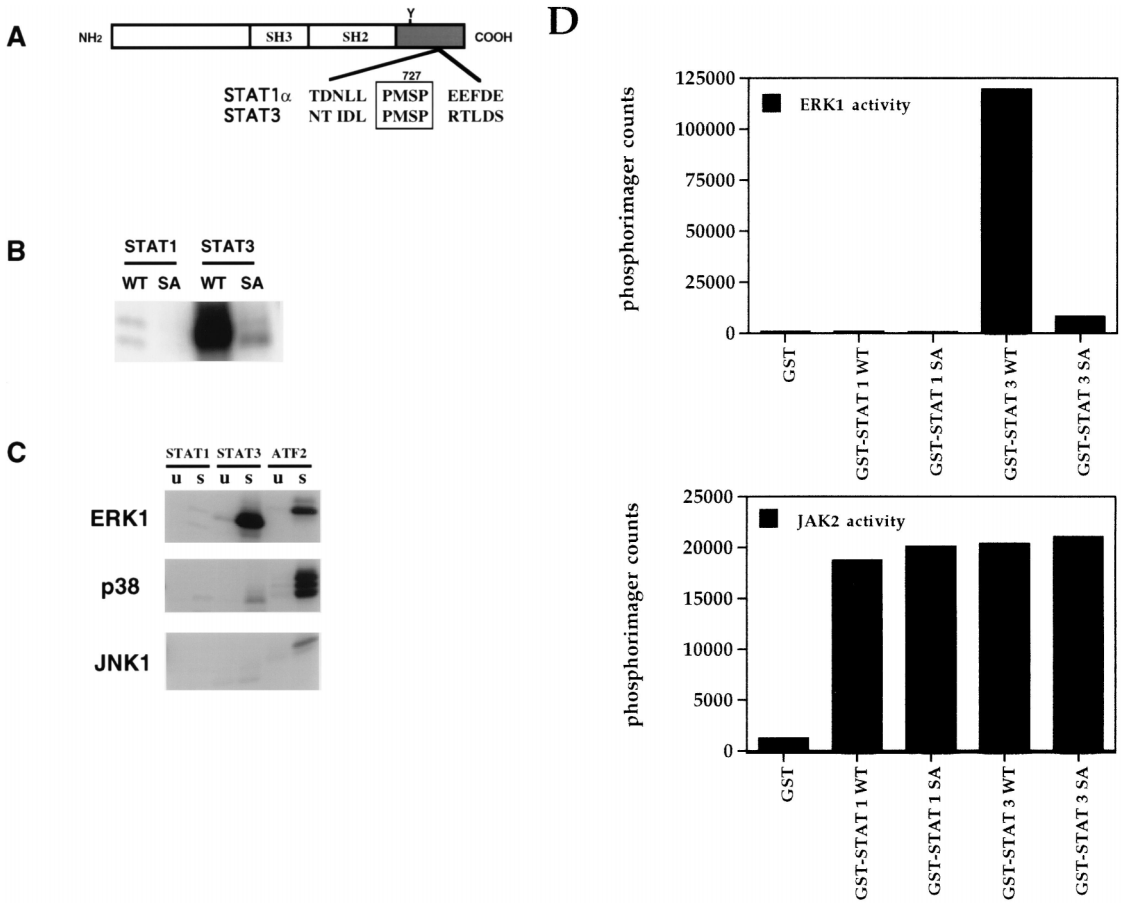

FIG. 2. Serines 727 of STAT1 and STAT3 are phosphorylated by ERK-MAP kinases but not by p38 or JNK. (A) Serine 727 from the conserved serine phosphorylation sites of STAT1 and STAT3 was mutated to alanine. (B) GST fusion proteins for the C terminus of STAT1 and STAT3 from both the wild type (WT) and the mutants (SA) were used as substrates for assays of ERK1 immune complexes from cells stimulated with fetal calf serum. (C) ERK1 and p38 or JNK1 were immunopurified from quiescent Swiss 3T3 cells (u) or cells stimulated (s) with 10% FCS and 600 mM sorbitol, respectively, with an equal amount of cell lysates. Their phosphotransferase activities against GST fusion proteins of STAT1, STAT3, and ATF2 were measured as described in Materials and Methods. (D) GST fusion proteins for the C terminus of STAT1 and STAT3 from both the wild type (WT) and the mutants (SA) were used as substrates for ERK1 immune-complex assays (top) or purified JAK2 kinase assays (bottom). GST itself was also used as a control substrate.

Protein kinase assays. Immune-complex protein kinase assays were performed as described previously (4). GST fusion proteins of recombinant p90 ribosomal S6 kinase (RSK) (4), ATF2 (12), STAT3 C terminus, and STAT1 C terminus were used for their relevant assays. Kinase assays were performed in a 30-μl reaction volume at 30°C for 15 min, and phosphotransferase activities were analyzed by SDS-PAGE and autoradiography. JAK2 kinase assays were performed as specified by the supplier (Upstate Biotechnology Inc.). ^{32}P incorporation into substrate was quantitated by PhosphorImager analysis (Molecular Dynamics).

Transient expression of STATs in COS cells. COS cells were transiently transfected with STAT expression plasmids by the DEAE-dextran method. Cells at 70% confluence, plated 12 to 16 h prior to the transfection, were incubated with DNA-DEAE-dextran for 5 h and subsequently starved for serum for 40 h in fresh DMEM. The cells were stimulated with EGF (50 ng/ml), phorbol ester (100 ng/ml), or PDGF (25 ng/ml), and cell-free lysates were prepared as previously described (4).

In vivo labeling and phosphoamino acid analysis. 3T3 B-Raf:ER cells or COS cells that had been serum starved for 2 days were incubated in phosphate-free DMEM for 1 h and incubated for a further 2 h after the addition of 2 mCi of carrier-free [^{32}P]phosphoric acid (ICN). Cell lysates were prepared as described above. STAT1 and STAT3 were immunoprecipitated from cleared cell lysates either with their specific antisera or with monoclonal antisera against tag sequences. The ^{32}P-labeled proteins were separated from contaminating proteins by SDS-PAGE and recovered by passive elution from the gel as described previously (3). Eluted proteins were hydrolyzed in 6 N HCl at 110°C for 1.5 h, and ^{32}P-phosphoamino acids were separated by two-dimensional electrophoresis at pH 1.9 and 3.5 on thin-layer cellulose plates (Kodak) as described previously (3). ^{35}S labeling for STATs and ERKs was carried out as described previously (3). ^{35}S-labeled protein labeling mix (NEN) was incubated with serum-starved cells for 14 h in methionine- and cysteine-free DMEM.

RESULTS

Characterization of STAT1 and STAT3 phosphorylation by ERK-MAP kinases in vitro. Recent reports have indicated that the activities of STAT1 and STAT3 are regulated by phosphorylation at serine 727 (36, 40). This site shows a characteristic ERK-MAP kinase phosphorylation site (see Fig. 2A) (11), -Pro-X-Ser/Thr-Pro-, suggesting that MAP kinases are responsible for the phosphorylation of these STAT proteins. To test this, we generated GST fusion proteins of the carboxyl-terminal regions of STAT1 and STAT3, which includes the serine 727 residue, and used them as substrates for MAP kinase assays. The GST-STAT3 fusion protein was an excellent substrate for ERK1-MAP kinase (Fig. 1A). Indeed, it was a better substrate than any other substrate we have tested for ERK-MAP kinases, including RSK and myelin basic protein (MBP) (Fig. 1A and data not shown). However, the GST-STAT1 fusion protein was a poor substrate (80- to 200-fold less sensitive than STAT3) for ERK1-MAP kinases (Fig. 1A) in spite of having the consensus site for MAP kinase phosphorylation

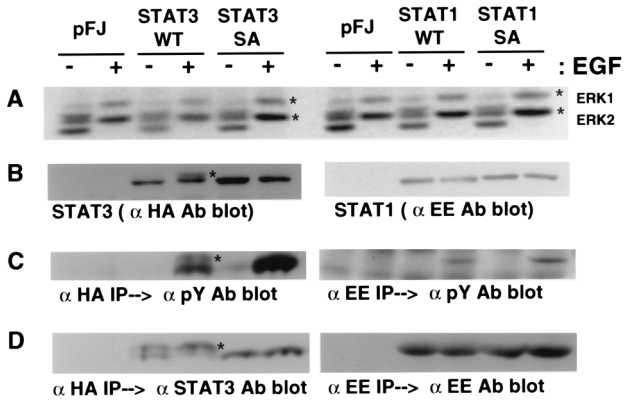

FIG. 3. Serine 727 phosphorylation is required for slow migration of STAT3 in vivo. COS cells transiently expressing vector alone (pFJ), wild type (WT), or the serine-727-to-alanine mutant (SA) of EE-tagged STAT1 and HA-tagged STAT3 were stimulated without (−) or with (+) EGF for 10 min, and lysates were prepared as described in the text. (A) An immunoblot for ERK-MAP kinases showed their activated forms (*) by EGF stimulation. (B) The same lysates were examined for expression of HA-tagged STAT3 and EE-tagged STAT1 by immunoblot analysis. (C) In a similar experiment, transiently expressed STAT1 and STAT3 were immunoprecipitated with anti-EE and anti-HA antibodies, respectively, and their EGF-induced tyrosine phosphorylation was examined by antiphosphotyrosine immunoblot analysis. (D) The same blots used for panel C were reprobed with the antibodies described. The slow-migrating species of STAT3 is highlighted (*) in panels B to D.

(Fig. 2A). Since the sequence surrounding the consensus phosphorylation site for MAP kinase may play important roles in substrate specificity, the differential specificities for ERK-MAP kinase of STAT1 and STAT3 could be explained by their differing surrounding sequences (Fig. 2A). It is worth noting that there is a very acidic stretch immediately following the -Ser-Pro- site in STAT1 that is not present in STAT3. Another possible explanation for the poor phosphorylation of STAT1 by ERK-MAP kinase is that the GST-STAT1 is improperly folded, thus preventing phosphorylation of the GST fusion. However, as shown in Fig. 2D, several GST-STAT1 and GST-STAT3 preparations (including serine-727-to-alanine mutants [see below]) were also tested as substrates for the JAK2 tyrosine kinase and were shown to be similarly phosphorylated, whereas in parallel ERK-MAP kinase selectively phosphorylated GST-STAT3.

Next, we determined whether any STAT1 or STAT3 phosphotransferase activity other than that from the ERK-MAP kinases could be detected by using column fractionated cell lysates. We observed only two peaks of strong STAT3 phosphotransferase activities from EGF-stimulated cell lysates (Fig. 1B and C). The activities were tightly correlated with the activated (slow-migrating) forms of ERK-MAP kinases, p42 ERK2 and p44 ERK1 (Fig. 1D). These results suggest that the ERKs are largely responsible for in vitro phosphorylation of STAT3 protein. Additionally, p44 ERK1 and p42 ERK2 showed almost equal phosphotransferase activities against GST-STAT3 fusion protein (Fig. 1B to D). However, we could not detect any significant STAT1 phosphotransferase activity in these column fractions (Fig. 1B).

To confirm that serine 727 was the site of phosphorylation, we generated serine-727-to-alanine (S727A) mutations for both STATs (Fig. 2A). The poor phosphorylation of the STAT1-GST fusion protein and the strong phosphorylation of the STAT3-GST fusion protein by ERK1 were significantly inhibited by this mutation (Fig. 2B), confirming this serine as the in vitro ERK-MAP kinase phosphorylation site. In addition, both STAT1 and STAT3 serine-to-alanine mutants were phosphorylated by JAK2 to the same extent as observed with the wild-type STAT substrates (Fig. 2D), demonstrating that the mutant STATs are folded properly.

We next determined whether other known MAP kinases could also phosphorylate STAT1 and STAT3 in vitro. As shown in Fig. 2C, p38-Hog1 and JNK1 did not appreciably phosphorylate STAT1 or STAT3 compared to the control substrate ATF2, suggesting that they may not be involved in regulating these proteins in vivo.

Examination of STAT1 and STAT3 serine 727 phosphorylation in cells. We expressed EE-tagged STAT1 and HA-tagged STAT3 proteins in COS cells and determined whether ERK-MAP kinases activated by EGF could phosphorylate STATs in vivo. p44 ERK1 and p42 ERK2 were fully activated by EGF treatment in COS cells transiently transfected with wild-type or S727A mutant STAT1 or STAT3 (Fig. 3A). Coordinately, EGF stimulation also resulted in a slow-migrating species of the transfected wild-type STAT3 protein (Fig. 3B and D). However, EGF did not induce a mobility shift of the STAT3 S727A mutant (Fig. 3B and D), even though tyrosine phosphorylation was intact (Fig. 3C). These results indicate that phosphorylation at serine 727 of STAT3 is required for the slow mobility form of STAT3. Interestingly, we could not detect any slow-migrating species for STAT1 from either wild-type- or mutant-expressing cells in spite of its EGF-induced tyrosine phosphorylation (Fig. 3B and C).

ERK-MAP kinases phosphorylate STAT1 and STAT3 in vivo. We also examined the phosphorylation of STAT3 serine 727 in fibroblasts (3T3 B-Raf:ER) expressing estrogen receptor-Raf fusion proteins (25). Raf and its downstream signal transduction pathway(s) can be quickly activated by treatment of cells with either estrogen receptor agonists or antagonists without stimulating growth factor receptors or JAK family kinases (reference 25 and data not shown). Treatment of these cells with 4-HT, an estrogen receptor antagonist, fully activated ERK1 and ERK2 (Fig. 4A and E) but had no effect on p70-S6 kinase (Fig. 4B), another growth factor-regulated protein serine/threonine kinase (4). As a control, EGF or PDGF treatment activated both ERK-MAP kinases (Fig. 4A and E) and p70-S6 kinases (Fig. 4B) in these cells. Corresponding to the activation of ERKs, STAT3 protein showed a mobility shift in SDS-PAGE after treatment with 4-HT (Fig. 4C), again supporting a role of the ERK-MAP kinases in serine 727 STAT3 phosphorylation and mobility shift. We did not observe a STAT1 mobility shift in response to Raf activation or EGF and PDGF treatments (Fig. 4D).

We also immunopurified STAT1 and STAT3 proteins from the ^{32}P-labeled 3T3 B-Raf:ER cell lysates and examined changes in serine 727 phosphorylation (Fig. 5A) by activation of the Raf/MAP kinase pathway (Fig. 5C). In three independent experiments, the phosphorylation of STAT1 and STAT3 increased by 1.1- to 1.5-fold and by 3- to 5-fold, respectively, after 4-HT treatment, although similar levels of both proteins were examined (Fig. 5B). Phosphoamino acid analysis showed that the phosphorylation of STATs in Fig. 5A occurred solely on the serine residues (data not shown). These results support the notion that the Raf-activated protein kinases, presumably ERK-MAP kinases, can phosphorylate STAT3 (and, weakly, STAT1) in vivo.

We next used PD 098059, an inhibitor of MEK (the direct upstream regulator for ERK-MAP kinases) (9), to confirm that ERK-MAP kinases are responsible for phosphorylation of the serine 727 of STAT3. As shown in Fig. 6A and C, both the Raf activation-induced and EGF-stimulated ERK activation was inhibited by pretreatment of PD 098059. Coincident with this, the mobility shift of STAT3 protein was also inhibited (Fig.

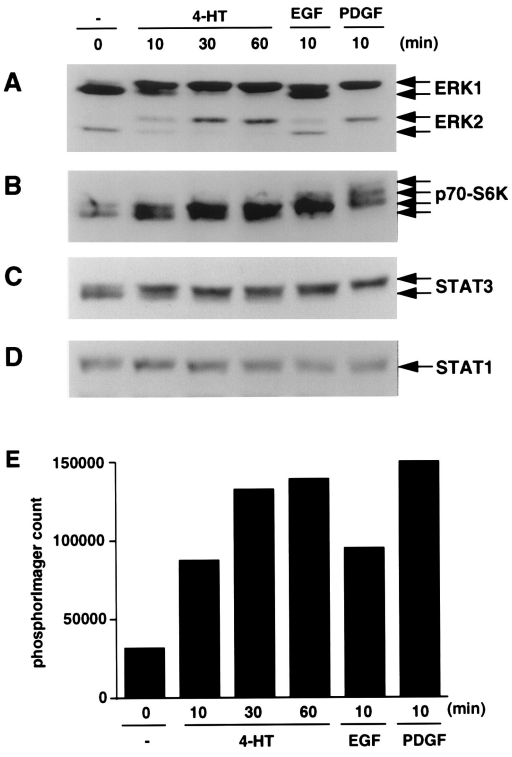

FIG. 4. Serine 727 phosphorylation of STAT3 tightly correlates with the activities of ERK1 and ERK2 in vivo. (A to D) Quiescent 3T3 B-Raf:ER cells were treated with 1 μM 4-HT, 50 ng of EGF per ml, or 20 ng of PDGF per ml for the times indicated. Cell-free lysates were prepared as described in the text and subjected to immunoblot analysis with anti-ERK (A), anti-p70-S6 kinase (B), anti-STAT3 (C), or anti-STAT1 (D) antiserum. Arrows indicate the different phosphorylation statuses of the proteins. (E) ERK1-MAP kinase activities in the same lysates were measured by immune-complex kinase assays with GST-RSK as substrate.

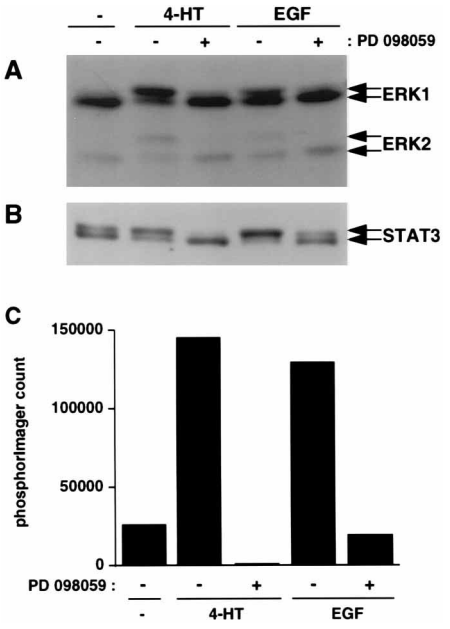

FIG. 6. PD 098059, a MEK inhibitor, blocks serine 727 phosphorylation of STAT3 in vivo. (A and B) Quiescent 3T3 B-Raf:ER cells were preincubated without (−) or with (+) 50 μM PD 098059 for 30 min and stimulated with 1 μM 4-HT or 50 ng of EGF per ml for 10 min. Cell lysates were prepared and subjected to immunoblot analyses with anti-ERK (A) and anti-STAT3 (B). Arrows indicate different phosphorylation statuses. (C) ERK1-MAP kinase activities in the same lysates were measured by immune-complex kinase assays with GST-RSK as the substrate.

6B). This further supports the role of ERK-MAP kinases in the phosphorylation of serine 727 of STAT3.

STAT3 serine 727 phosphorylation negatively modulates its tyrosine phosphorylation. The role of STAT3 serine phosphorylation has been controversial (reviewed in reference 5). One possible mechanism by which serine phosphorylation might regulate STAT3 function is by modulating its tyrosine phosphorylation, which is critical for dimer formation, nuclear translocation, and subsequent transcriptional activity. Therefore, we examined the possibility that serine 727 phosphorylation affects tyrosine phosphorylation of the STAT3 protein. The first hint that this might be occurring is shown in Fig. 3C and D. We initially observed that upon stimulation, although most of the wild-type STAT3 became serine phosphorylated and migrated more slowly, most of the phosphotyrosine was in the faster-migrating species. Subsequently, we measured tyrosine phosphorylation of the S727A mutants of both STAT1 and STAT3. Interestingly, we repeatedly observed that basal and stimulated tyrosine phosphorylation of the STAT3 mutant and to a lesser extent the STAT1 mutant was consistently elevated compared to that of the wild-type protein under conditions of similar protein expression.

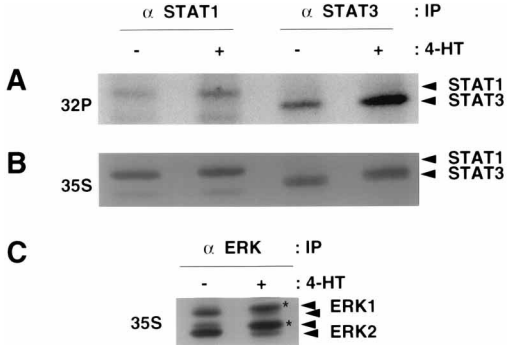

FIG. 5. In vivo phosphorylation of STAT1 and STAT3 induced by activation of the Raf/MAP kinase pathway. (A and B) Quiescent 3T3 B-Raf:ER cells were metabolically labeled with [^{32}P]phosphoric acid or ^{35}S-protein labeling mixture as described in Materials and Methods. By using equal amounts of protein, STAT1 and STAT3 were immunoprecipitated from the ^{32}P-labeled (A) or ^{35}S-labeled (B) lysates by using the same antibodies and procedures. They were separated from contaminating proteins by SDS-PAGE and detected by autoradiography. (C) ERKs were also immunoprecipitated from the same ^{35}S-labeled cell lysates. The activated forms of ERK1 and ERK2 are highlighted (*).

To confirm the observation made with antiphosphotyrosine antibodies, we performed phosphoamino acid analyses of the immunopurified proteins from the ^{32}P-labeled COS cells expressing the wild-type or S727A mutants of STAT1 and STAT3. EGF treatment increased total phosphorylation of both the wild-type and mutant STATs (Fig. 7A). The S727A mutation of both STATs resulted in reduced phosphorylation, consistent with serine 727 being a major phosphorylation site in vivo (Fig. 7A). Additionally, phosphoamino acid analyses of these mutants showed a significant decrease of serine phosphorylation for both STAT3 and STAT1 (Fig. 7B, compare panels 1 and 2 to panels 3 and 4 for STAT3 and panels 5 and 6 to panels 7 and 8 for STAT1). Interestingly, we could still detect EGF-stimulated serine phosphorylation in both mutants (Fig. 7B, compare panels 3 and 4 for STAT3 and panels 7 and 8 for STAT1), suggesting the presence of other mitogenically

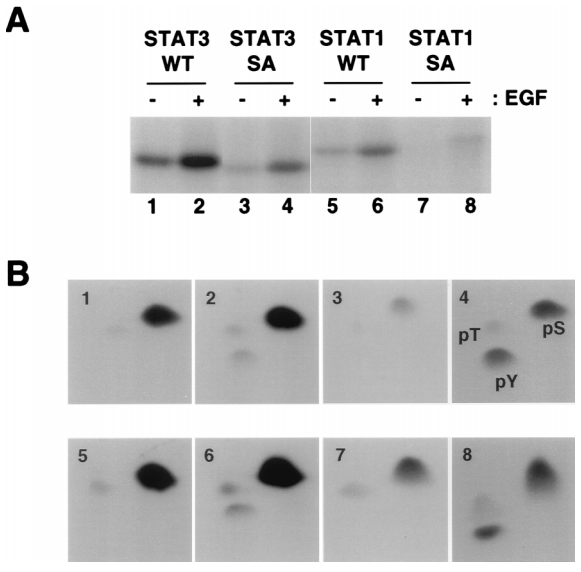

FIG. 7. Phosphoamino acid analysis of STAT1 and STAT3. COS cells transiently expressing the wild-type or the S727A mutant HA-tagged STAT3 and EE-tagged STAT1 were metabolically labeled with [^{32}P]phosphoric acid as described in Materials and Methods and stimulated with EGF (+) for 10 min or left unstimulated (−). STAT3 and STAT1 proteins were immunoprecipitated with anti-HA and anti-EE antibodies and were separated by SDS-PAGE. (A) ^{32}P incorporation of immunoprecipitated STAT proteins was determined by autoradiography. (B) ^{32}P labeled STAT proteins were purified by SDS-PAGE and subjected to phosphoamino acid analyses as described in Materials and Methods. Labeled proteins were quantitatively eluted from the gel, and all the samples were equally treated for analysis. Total eluted proteins were used for thin-layer electrophoresis to reduce error. Sample numbers on the corners are the same as in panel A. pY, phosphotyrosine; pT, phosphothreonine; pS, phosphoserine. Results shown for panels 1 to 4 were exposed on films for 3 days, and those for panels 5 to 8 were exposed for 10 days.

regulated serine phosphorylation sites which are not responsible for the agonist-dependent mobility shift. Threonine phosphorylation was also weakly induced by EGF treatment and was not affected by the mutation (Fig. 7B). However, consistent with the results in Fig. 3C, protein tyrosine phosphorylation was significantly increased by the S727A mutation (Fig. 7B, compare panels 2 and 4 for STAT3 and panels 6 and 8 for STAT1). To visualize the phosphotyrosine signals in panel 1 versus 2 and panel 5 versus 6, the exposure time required resulted in overexposure of the phosphoserine signal. A lighter exposure or quantitation consistently revealed a 2- to 4-fold increase in STAT3 and a 1.5- to 2-fold increase in STAT1 serine phosphorylation, consistent with previously published results (36, 40).

To further investigate the role of serine 727 in STAT3 tyrosine phosphorylation, we examined the tyrosine phosphorylation in cells expressing the wild-type or the S727A STAT3 mutant after treating them with a MEK inhibitor, PD 098059. EGF-stimulated tyrosine phosphorylation of wild-type STAT3 was increased in the MEK inhibitor-treated cells, although it did not match that of the mutant (Fig. 8A and D), probably reflecting incomplete inhibition of serine 727 phosphorylation. As expected, the tyrosine phosphorylation of ERKs (Fig. 8D) and the retarded mobility of ERKs (Fig. 8C) were blocked by treatment with the MEK inhibitor. However, H7, a protein serine/threonine kinase inhibitor, did not inhibit ERK-MAP kinase activity (Fig. 8C), did not affect tyrosine phosphorylation of STAT3, and did not prevent its mobility shift after EGF stimulation (Fig. 8A and D). Additionally, tyrosine phosphorylation of other cellular proteins, including the EGF receptor, was not affected by the expression of exogenous STAT3 proteins or by treatment with PD 098059 or H7 (Fig. 8D). These results are consistent with the hypothesis that serine 727 phosphorylation plays a direct role in regulating the tyrosine phosphorylation of STAT3.

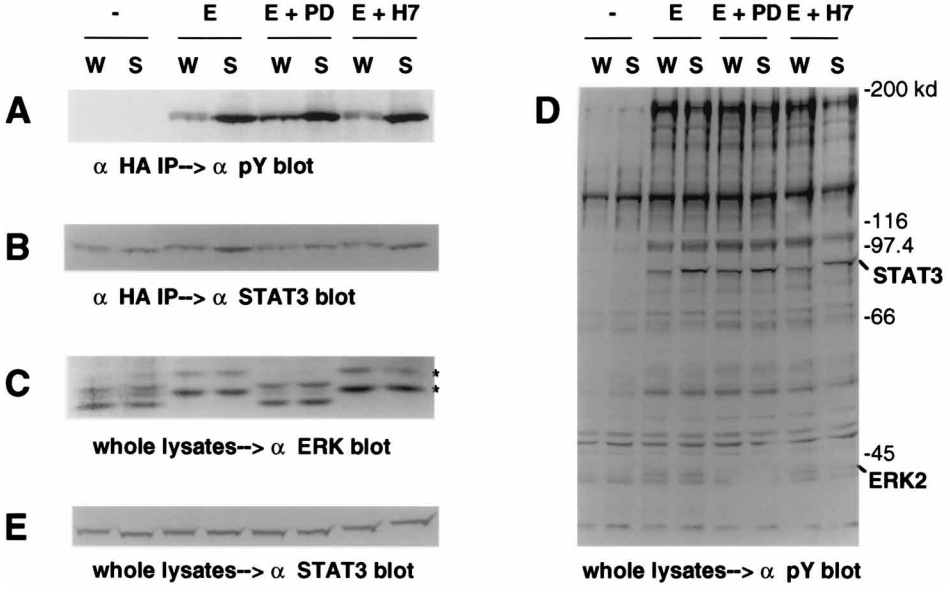

FIG. 8. Serine 727 phosphorylation negatively regulates STAT3 tyrosine phosphorylation. COS cells transiently expressing the wild-type (W) or the S727A mutant (S) HA-tagged STAT3 were stimulated with EGF (E, 50 ng/ml) for 10 min or left unstimulated (−), and lysates were prepared as described in Materials and Methods. Two pairs of plates were pretreated with PD 098059 (PD, 100 μM) or H7 (50 μM) for 30 min before EGF stimulation. (A) Immunoblot with antiphosphotyrosine antiserum for immunoprecipitated STAT3 with a tag antibody (anti-HA). (B) The same blot used for panel A reprobed with anti-STAT3 antibody. (C) Immunoblot for ERK-MAP kinases from the same lysates showing their activated forms (*) by EGF stimulation. (D) The same lysates examined for total protein tyrosine phosphorylation by immunoblot analysis. (E) The same blot used for panel D reprobed with anti-STAT3 antibody.

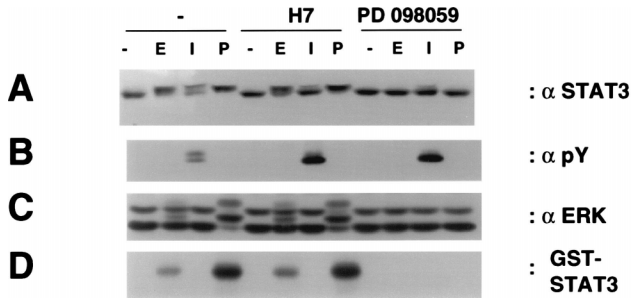

FIG. 9. IL-6 induces H7-sensitive STAT3 phosphorylation by the ERK-MAP kinase-independent pathway. Quiescent HepG2 cells were stimulated (+) with EGF (E, 50 ng/ml), IL-6 (I, 50 U/ml) or PMA (P, 100 ng/ml) for 10 min with (+) or without (−) a 30-min pretreatment with H7 (50 μM) or PD 098059 (100 μM), and cell-free lysates were prepared as described in Materials and Methods. (A) Whole-cell lysates of equal amounts of proteins were used for immunoblot analysis of STAT3. (B) Equal amounts of STAT3 proteins were immunoprecipitated from the same lysates and examined for tyrosine phosphorylation. (C) The same whole-cell lysates were tested for ERK-MAP kinase activation by an immunoblot analysis. (D) ERK-MAP kinases were immunoprecipitated from the same lysates, and the immune-complex kinase assays were performed with GST-STAT3 as the substrate.

IL-6 induces ERK-independent but H7-sensitive STAT3 phosphorylation. In contrast to EGF-dependent STAT3 phosphorylation shown in Fig. 8, IL-6-dependent activation of STAT3 has been shown to be highly sensitive to H7 (2, 18, 24). Therefore, we compared IL-6-dependent activation of STAT3 with its growth factor-dependent activation in HepG2 cells, which express IL-6 receptor as well as EGF receptors. As expected, EGF or other ERK-MAP kinase inducers such as phorbol myristate acetate (PMA) produced the slow-migrating species of STAT3 (Fig. 9A) and its appearance was correlated with the activity of ERK-MAP kinases (Fig. 9C and D). The EGF-dependent signaling was weak compared to that of PMA in these cells or to that of EGF in COS cells. Pretreatment of HepG2 cells with PD 098059 blocked EGF- or PMA-mediated ERK-MAP kinase activation (Fig. 9C and D) and the STAT3 mobility shift (Fig. 9A). However, IL-6 induced the slow-migrating species of STAT3 (Fig. 9A and B) without any detectable stimulation of ERK-MAP kinases (Fig. 9C and D), MEK1, p70-S6 kinases, or other MAP kinases such as p38-Hog1 and JNK1 (data not shown). IL-6-mediated activation of ERK-MAP kinase was not detected in HepG2 cells or HeLa cells (data not shown), treated from 0 to 30 min, during the time when we detected IL-6-dependent STAT3 phosphorylation. This IL-6-induced STAT3 phosphorylation was significantly antagonized by H7 pretreatment (Fig. 9A and B) and was similarly sensitive to PD 098059. However, H7 had no effect on the EGF- or PMA-mediated mobility shift of the STAT3 protein (Fig. 9A) or ERK-MAP kinase activation (Fig. 9C and D). H7 has been described as a nonspecific kinase inhibitor that inhibits protein kinase C in vitro (K_i, ~6.0 μM). In cultured cells, however, 50 μM H7 did not inhibit PMA-mediated ERK-MAP kinase activation while antagonizing IL-6-dependent STAT3 serine phosphorylation. It is not clear if H7 inhibits all PMA-responsive PKC isoforms, and because of its lack of specificity, we cannot conclude that IL-6-dependent STAT3 serine phosphorylation is mediated by PKC isoforms. These results strongly suggest that IL-6 regulates a novel, ERK-independent regulatory pathway(s) for phosphorylation of STAT3 serine 727. Interestingly, experiments similar to that in Fig. 1B have been completed with lysates from IL-6-treated HepG2 cells and no detectable GST-STAT3 phosphotransferase activity has been observed (data not shown). It is possible that we have not identified the proper assay conditions for this activity or that it is removed or inhibited during our lysate preparation.

Tyrosine phosphorylation of STAT3 was strongly induced by IL-6 stimulation in HepG2 cells (Fig. 9B). Inhibition of the serine phosphorylation of STAT3 by either H7 or PD 098059 further increased its tyrosine phosphorylation by IL-6 (Fig. 9B), which supports a general role for serine 727 phosphorylation in negatively regulating tyrosine phosphorylation of STAT3 among different cells and exposed to different agonists. EGF-dependent tyrosine phosphorylation of STAT3 (Fig. 9B) was detectable in HepG2 cells only after a prolonged film exposure.

DISCUSSION

We have provided strong evidence that ERK-MAP kinases regulate STAT3 serine phosphorylation in response to growth factors but not the cytokine IL-6. ERK-MAP kinases phosphorylated STAT3 significantly better than they phosphorylated STAT1 in vivo (Fig. 5) as well as in vitro (Fig. 1 and 2). It is possible that STAT1 serine phosphorylation is regulated by an ERK-independent pathway involving a serine kinase not detectable by our phosphotransferase assays. In addition, phosphoamino acid analysis of STAT1 and STAT3 S727A mutants expressed in COS cells with and without EGF treatment revealed the existence of additional mitogen-regulated as well as basally phosphorylated serine phosphorylation sites (Fig. 7). The role of these phosphorylations in STAT1 and STAT3 function will be the subject of future studies. Both ERK1 and ERK2 phosphorylated STAT3 Ser 727 equally in vitro, and we could not detect any other significant STAT3 serine 727 kinase activity from the EGF-stimulated cell lysates under our assay conditions (Fig. 1B).

Since STAT1 has a conserved MAP kinase phosphorylation site and its phosphorylation has been reported previously (36), the result that ERK-MAP kinases only poorly phosphorylated STAT1 was unexpected. The amino acids sequences surrounding the MAP kinase phosphorylation site of STAT1 are acidic, whereas the equivalent sequence of STAT3 is not (Fig. 2A). Although -Pro-X-Ser/Thr-Pro- has been accepted as a phosphorylation site for MAP kinases (11), our results support the idea that the surrounding sequences also play a determining role in specifying substrate recognition. In support of this idea, the stress-activated MAP kinases such as JNK and p38 Hog1 did not significantly phosphorylate either STAT1 or STAT3 in vitro (Fig. 4). It will be of interest to determine if ERK-MAP kinases can also phosphorylate STAT4, which has the conserved MAP kinase phosphorylation site but with a unique surrounding sequence (38, 42).

Our results also show that the serine phosphorylation and the tyrosine phosphorylation of STAT3 can be induced and regulated independently. By activating Raf and its downstream MAP kinase pathway (Fig. 4) or by activating PKCs with PMA (Fig. 9), we induced serine 727 phosphorylation of STAT3 without tyrosine phosphorylation. This suggests that STAT3 tyrosine phosphorylation or dimerization is not a prerequisite for the serine phosphorylation. We have also observed STAT3 tyrosine phosphorylation without serine 727 phosphorylation by treatment of growth factor-stimulated cells with PD 098059 (Fig. 6 and 8) or by transient expression of the S727A mutant (Fig. 3). These results support the notion that at least two independent signalling pathways can converge on STAT3 to regulate its function in the cell and, with evidence for other serine phosphorylation sites, indicate the possibility of additional converging STAT3 regulatory signaling paths.

We have shown that basal tyrosine phosphorylation of the S727A mutant was detectable whereas it was not observed with wild-type STAT3 in quiescent cells. In addition, the EGF-stimulated tyrosine phosphorylation of the S727A mutant was higher than that of the wild-type STAT3 protein (Fig. 3, 7, and 8). Treatment of cells with the MEK inhibitor PD 098059, which antagonized the phosphorylation at serine 727 of STAT3 in response to EGF, also resulted in increased tyrosine phosphorylation of the STAT3 wild-type protein (Fig. 8). Since EGF-stimulated tyrosine phosphorylation of STAT3 after treatment of PD 098059 was increased in cells expressing the wild type but not in those expressing the S727A mutant, the effect of the drug is not due to an effect on the EGF receptor or other STAT3 tyrosine kinases or phosphatases. Additionally, results presented in Fig. 9 showed that inhibition of the serine 727 phosphorylation by either PD 098059 or H7 increased IL-6-induced STAT3 tyrosine phosphorylation in HepG2 cells. These results support the hypothesis that negative regulation of tyrosine phosphorylation by serine 727 phosphorylation is a general phenomenon among different cells and by various agonists. In addition, cell line differences and different agonists may affect the extent, the timing, and therefore the consequences of serine phosphorylation of the STAT proteins. For example, differences in the strength and/or timing of MAP kinase activation by various mitogens and cytokines could yield a variety of outcomes. Then, how does the serine phosphorylation regulate the tyrosine phosphorylation of STAT3? It is possible that serine phosphorylation targets the tyrosine-phosphorylated STAT for dephosphorylation and/or that phosphorylation of serine 727 regulates the accessibility of STAT3 to its tyrosine kinases, JAKs and/or the EGF receptor. Future experiments are aimed at addressing these and other possibilities.

Our preliminary data suggest that IL-6 activates a novel serine/threonine protein kinase(s) which can phosphorylate the STAT3 protein. Interestingly, IL-6-activated STAT3 kinase, unlike the ERKs, is highly sensitive to H7 protein kinase inhibitor (Fig. 9). The results in Fig. 9 strongly support the notion that this H7-sensitive kinase(s) phosphorylates serine 727 of STAT3 since the phosphorylation induced slow migration of the protein in SDS-PAGE. This might explain how H7 antagonizes IL-6-dependent expression of STAT3 regulated genes as shown by others (18, 24). This H7-sensitive phosphorylation was also sensitive to the PD 098059. Since MEK1 is not activated in response to IL-6 (data not shown), there may be other MEK-like kinases sensitive to this inhibitor that activate novel MAP kinase family members. Interestingly, IL-2 is also a relatively poor agonist for MAP kinase activation (1, 23) and IL-2 uses ERK-independent paths for STAT5 serine phosphorylation (1).

It is clear from this discussion that different cell types and different cellular agonists can utilize very complex signalling processes to carefully and differentially regulate transcription. We have provided evidence that the ERK-MAP kinases can engage in cross talk with the JAK/STAT pathway, and based on our data and that of others (36), we propose that this can have two effects, i.e., to antagonize the tyrosine phosphorylation and to stimulate STAT3 transcriptional activity. These are not mutually exclusive, since in one case rapid serine phosphorylation might prevent tyrosine phosphorylation or induce tyrosine dephosphorylation whereas in the case where the serine phosphorylation is delayed and/or mostly nuclear, the result might be enhanced transcription upon serine 727 phosphorylation followed by serine phosphorylation-dependent downregulation by the subsequent tyrosine dephosphorylation. Future studies, carefully taking into account the differences in the timing and amount of serine and tyrosine phosphorylation of the STATs, must now be completed to determine the effects of serine 727 phosphorylation on nuclear translocation, nuclear localization, DNA binding, and transcriptional activity. In addition, serine phosphorylation at other sites may participate in the regulation of some of these processes. These sites must be identified, and their role in STAT function must be defined. Interestingly, with regard to the immune system, where the JAK/STAT systems play a predominant role in hematopoietic differentiation and function, the cytokines IL-2 and IL-6 appear to regulate STAT serine phosphorylation in a MAP kinase-independent fashion. Given the growing number of uncharacterized "MAP kinase" signalling systems, it will also be of interest to identify and characterize the serine/threonine kinases activated by IL-2 and IL-6 that are responsible for the serine phosphorylation of the STATs.

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