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Activation and Association of Stat3 with Src in v-Src-Transformed Cell Lines

XINMIN CAO,* AGNES TAY, GRAEME R. GUY, AND Y. H. TAN

Signal Transduction Laboratory, Institute of Molecular and Cell Biology, National University of Singapore, Singapore 119260, Singapore

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STAT proteins are a group of latent cytoplasmic transcription factors which function as signal transducers and activators of transcription. Stat1 and -2 were originally identified to function in interferon signaling, and Stat1 was also found to be activated by epidermal growth factor (EGF) and other cytokines. New members of the STAT gene family are identified. Among them, Stat3 has 52.5% amino acid sequence homology with Stat1 and is activated by platelet-derived growth factor (PDGF), colony-stimulating factor 1 (CSF-1), EGF, interleukin-6, and other cytokines. Treatment of cells with EGF activates Stat1 and Stat3, which become phosphorylated on tyrosine residues to form homo- or heterodimers and translocate into the nucleus, binding to the sis-inducible element (SIE) in the c-*fos* promoter. Somatic cell genetic analyses demonstrated that Jaks, a family of nontransmembrane protein tyrosine kinases, are required for the activation of Stat1 and Stat2 in interferon-treated cells. However, little is known about the activation of Stat3 by growth factors. Here we report that in all v-Src-transformed cell lines examined, Stat3 is constitutively activated to bind to DNA and the phosphorylation of tyrosine on Stat3 is enhanced by the induction of v-Src expression. We also report that Src is shown to be associated with Stat3 in vivo, as well as in vitro, and phosphorylates Stat3 in vitro. Stat3 is also activated by CSF-1, possibly through CSF-1 receptor–c-Src association in NIH 3T3 cells overexpressing CSF-1 receptors. Together, the data suggest that Src is involved in activation of Stat3 in growth factor signal transduction.

Binding of interferons (IFNs) to their receptors on the cell surface induces tyrosine phosphorylation of a group of latent cytoplasmic transcription factors (STATs) which act as signal transducers and activators of transcription (9). Treatment of cells with IFN-α induces the phosphorylation of Stat1α (a 91-kDa protein), Stat1β (an 84-kDa form of Stat1α lacking 38 COOH-terminal amino acids), and Stat2 (a 113-kDa protein). These phosphoproteins form a complex with an IFN regulatory factor, p48, which translocates into the nucleus to bind to the IFN-stimulated response element (ISRE) found in IFN-α/β-responsive genes. Unlike IFN-α, IFN-γ only induces the phosphorylation of Stat1 (α and β) at Tyr-701, which leads to the formation of homodimers. The homodimers translocate into the nucleus and bind to the IFN-γ-activated site (GAS sequence), which is unrelated to the ISRE (32, 33). Although Stat1 was originally identified to function in IFN signaling, it was also found to be activated by epidermal growth factor (EGF), interleukin-10, ciliary neurotrophic factor, and other cytokines (14, 21, 35), suggesting that it plays a more universal role in growth factor and cytokine signaling than simply in IFN signaling. To study the specificity of the STATs in the signal transduction pathways triggered by different ligands, new members of the STAT gene family were identified (1, 18, 48, 49). One such gene, the Stat3 gene, was found to be activated in mouse and human cells by platelet-derived growth factor (PDGF), colony-stimulating factor 1 (CSF-1), EGF, interleukin-6, and other cytokines (1, 48). Stat3 and Stat1 have 52.5% amino acid identity and have similar sizes and structures, including Src homology 2 (SH2) and Src homology 3 (SH3) domains and a conserved tyrosine residue at a location carboxy terminal to the SH2 domain (13). After EGF stimulation, they become phosphorylated on tyrosine residues, forming homo- or heterodimers which translocate into the nucleus, where they bind to a GAS-related sequence, hSIE, which is a mutant high-affinity version of the sis-inducible element (SIE) in the c-*fos* promoter (31, 34, 43). Unlike Stat1, however, Stat3 is not activated by IFN-γ; neither does it form a complex with Stat2 or bind to the ISRE after IFN-α treatment.

Somatic cell genetic analyses showed that Jaks, a family of nontransmembrane protein tyrosine kinases, are required in the IFN response pathways (23, 40, 44). In particular, both Jak1 and Tyk2 are essential for the IFN-α/β response, while Jak1 and Jak2 are essential for the IFN-γ response. In general, Jaks associate with cytokine receptors which lack intrinsic tyrosine kinase activity, become activated upon ligand-induced receptor dimerization, and activate STATs by tyrosine phosphorylation (reviewed in reference 38). However, the identity of the kinase(s) involved in the activation of the STAT proteins by growth factors which bind to their receptors with intrinsic protein tyrosine kinase activity is not clear. In this study, we demonstrated that v-Src is involved in the activation of Stat3 in v-Src-transformed cell lines. Furthermore, Stat3 is associated with Src in these cells and can be phosphorylated by Src in vitro. We also observed that the activation of Stat3 by CSF-1 in NIH 3T3 cells transfected with the CSF-1 receptor gene may involve the binding of c-Src to the CSF-1 receptor.

MATERIALS AND METHODS

Cell cultures and nuclear extraction. A-431 cells were from the American Type Culture Collection (Rockville, Md.). Cell lines REF-src and IND-REF-src were provided by C. J. Pallen, and REF-E2F cells were from W. J. Hong of our institute. The NIH 3T3-v-src cell line was a gift from D. Shalloway (Cornell University, Ithaca, N.Y.), and NIH 3T3-CSF-1R and NIH 3T3-CSF-1R (809F) were gifts from M. F. Roussel (St. Jude Children's Research Hospital, Memphis,

* Corresponding author. Mailing address: Institute of Molecular and Cell Biology, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260, Singapore. Phone: (65) 777-3496. Fax: (65) 777-0402.

Tenn.). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. The cells were starved in DMEM with 0.5% fetal bovine serum FBS for 24 to 48 h after they had reached 80 to 90% confluence. EGF (100 ng/ml), PDGF (75 ng/ml), IFN-α (1,000 U/ml), or IFN-γ (1,000 U/ml) was added, and the cultures were incubated for the various times indicated in the figure legends. Cells were then harvested, and nuclear extracts were prepared as previously described by Dignam et al. (12), with some modifications. In brief, the cells from 150-mm-diameter dishes were washed three times with cold phosphate-buffered saline (PBS), harvested with a cell scraper, and transferred to a centrifuge tube. The cells were collected by centrifugation, resuspended with hypotonic buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM phenylmethylsulfonyl fluoride [PMSF], 0.5 mM dithiothreitol [DTT]) equal to approximately three times the packed cell volume, and incubated on ice for 10 min. The cells were lysed with 20 strokes in a Dounce homogenizer, and the nuclei were collected by centrifugation and resuspended with low-salt buffer (20 mM HEPES [pH 7.9], 25% glycerol, 1.5 mM $MgCl_2$, 20 mM KCl, 0.2 mM EDTA, and 0.5 mM DTT) equal to one-half of the packed nuclear volume. Next, high-salt buffer (same composition as low-salt buffer except containing 1.2 M NaCl) equal to one-half of the packed nuclear volume was added. Phosphatase and proteinase inhibitors were added to the above-described buffers at the final concentrations of 5 mM for NaF, 100 μM for sodium orthovanadate, 5 μg/ml for leupeptin, 5 μg/ml for aprotinin, 2 μg/ml for pepstatin A, and 1 mM for PMSF. The nuclear proteins were extracted for 60 min with constant agitation. The pellets were removed by centrifugation, and the supernatant was dialyzed against buffer D (20 mM HEPES [pH 7.9], 20% glycerol, 2 mM $MgCl_2$, 20 mM KCl, 0.2 mM EDTA, 1 mM PMSF, 0.5 mM DTT, 5 mM NaF, and 100 to 500 μM sodium orthovanadate) for 3 h.

Mobility shift DNA binding assay. The mobility shift DNA binding assay was carried out as previously described (7), with minor changes. The binding reaction was performed by preincubating 10 μg of nuclear proteins with 2 μg of poly(dI-dC) · poly(dI-dC) in a buffer containing 20 mM HEPES (pH 7.9), 70 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, 0.5 mM EDTA, and 12% glycerol on ice for 10 min. End-labeled probe (0.5 to 2 ng) hSIE (31, 43) was added to the reaction mixture, and the mixture was incubated for 20 min at room temperature. The samples were electrophoresed on a 5% polyacrylamide gel in 0.5× TBE buffer (1× TBE consists of 0.089 M Tris and boric acid and 2 mM EDTA) at 200 V at 4°C. In the supershift assay, anti-Stat3 antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.) was incubated with nuclear extracts for 10 min before the addition of radiolabeled probe hSIE. The same probe was used in all mobility shift assays described.

Antibodies, immunoprecipitation, and Western blotting (immunoblotting) analysis. Antibodies against Stat1, Stat3, and phosphotyrosine (PY20) were purchased from Transduction Laboratories (Lexington, Ky.), Santa Cruz Biotechnology, and Upstate Biotechnology Incorporated (UBI) (Lake Placid, N.Y.). Monoclonal anti-v-Src antibodies Ab-1 and EC10 were purchased from Oncogene Science (Cambridge, Mass.) and UBI, respectively. Rabbit polyclonal antibodies against fusion products Stat3C (antibody 1695) and Stat3N (antibody 1691) containing glutathione *S*-transferase (GST) and either 40 amino acids (amino acids 688 to 727) from the COOH terminus or 158 amino acids (amino acids 2 to 159) from NH_2 terminus of Stat3 were produced as described previously (48). Immunoprecipitations were performed essentially by following the instructions of the manufacturers of the respective antibodies. In brief, the cells were washed three times with cold PBS and lysed in 1 ml of RIPA buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.2], 1% deoxycholic acid, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 0.25 mM EDTA [pH 8.0]) with the protease and phosphatase inhibitors listed above. The cell lysate containing 1 to 2 mg of protein was incubated with the appropriate antibody for 1 to 2 h at 4°C and then with protein A (Sigma, St. Louis, Mo.) or protein G PLUS/protein A-agarose (Oncogene Science) for 1 h. A secondary polyclonal goat-anti-mouse immunoglobulin G (Sigma) was incubated with the lysate for 1 h before addition of protein A when monoclonal antibody was used. This step was omitted when protein G PLUS/protein A-agarose was used. The immunoprecipitates were washed once with RIPA buffer and three times with PBS, separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) after being boiled in Laemmli buffer, and transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with PBS containing 0.1% Tween 20 (TPBS) and 1% bovine serum albumin before it was incubated with the appropriate primary and secondary antibodies. The bound antibodies were visualized by using BM Chemiluminescence (Boehringer GmbH, Mannheim, Germany).

Fusion protein construction and purification. GST fusion GST-Stat3 was made by inserting a 2.6-kb NcoI fragment from Stat3 cDNA (48) into pGEX-KG vector (15). GST-JNK containing GST and full-length c-jun N-terminal kinase JNK (11, 20) was a gift from X. M. Zheng and C. J. Pallen. Bacterial cultures expressing GST fusion proteins were grown in LB (Luria broth) medium containing 50 μg of ampicillin per ml and induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h. The bacterial cultures were pelleted by centrifugation, frozen and thawed, and lysed in PBS with 1% Triton X-100 and protease inhibitors. Fusion proteins were purified by mixing 1 bed volume of glutathione-agarose beads with 5 volumes of bacterial lysate, incubating the mixture overnight at 4°C, and then washing the beads five times with PBS.

In vitro binding and kinase assays. A 500-μg amount of whole-cell extracts was precleared by using glutathione-agarose beads coupled to 20 μg of GST protein. After 1 h at 4°C, the cleared lysate was mixed with beads coupled to 10 μg of GST or GST-Stat3 for 3 h. Beads were then washed three times with ice-cold PBS and resuspended in a kinase assay buffer {10 mM PIPES [piperazine-*N*,N′-bis(2-ethanesulfonic acid)] [pH 7.0], 5 mM $MnCl_2$, 1 mM NaCl, 0.1 mM DTT} containing 5 μCi of [γ-^{32}P]ATP and 10 μM cold ATP at 30°C for 20 min. The beads were extensively washed with PBS, and the bound proteins were eluted and resolved by SDS-PAGE. The dried gel was autoradiographed. The glutathione-agarose bead-coupled GST-Stat3 was also incubated with partially purified recombinant human c-Src (UBI) under the kinase assay conditions described above.

RESULTS

Stat3 is constitutively activated in v-Src-transformed cell lines. Ligand activation of growth factor receptors with intrinsic tyrosine kinase activity often results in the association and activation of a number of cytoplasmic effectors with SH2 domains (reviewed in reference 6). Thus, SH2 domain-containing tyrosine kinases may be necessary for growth factor stimulation of STATs. We decided to examine the role of Src in the activation of STATs. It has been reported that in EGF-treated A-431 cells, both Stat1α and Stat3 are phosphorylated on tyrosine. They form dimers, translocate to the nucleus, and bind to the specific DNA sequence hSIE. Three complexes (SIF-A, SIF-B, and SIF-C) were formed, as detected by mobility shift assays (31, 43). SIF-A and SIF-C consist of Stat3 and Stat1α homodimers, respectively, whereas SIF-B consists of a Stat1α and Stat3 heterodimer, as identified by supershift assays with antibodies specific for Stat1 and Stat3 (30, 31). In our study, the DNA binding activities of Stat1α and Stat3 were tested in a rat embryo fibroblast line (REF-src) overexpressing v-Src (25) by a mobility shift assay using the hSIE probe. We observed that Stat3 was constitutively activated in the serum-starved REF-src cells, forming SIF-A with hSIE (Fig. 1, lane 1). PDGF treatment had no effect on the constitutive activation of Stat3 (Fig. 1, lane 2), whereas both Stat1α and Stat3 are activated, as shown by the increased formation of SIF-B and SIF-C in cells treated with a mixture of rat IFN-α and IFN-γ (rat IFN, lane 3). A trace amount of SIF-B was observed to be present in the serum-starved REF-src cells, suggesting that some Stat1 may also be activated. The amount, however, was much smaller than that of Stat3 in SIF-A (Fig. 1, lane 1). Similar results were obtained with the v-Src-transformed NIH 3T3 cells (3), in which Stat3 was found to be constitutively activated in the serum-starved cells (Fig. 1, lane 9). In these cells, only a slight increase in Stat3 binding was detected in response to PDGF (Fig. 1, lane 10) and IFN-α (lane 11), while the response to IFN-γ (Stat1 activation) was maintained (lane 12). Thus, the activation of Stat3 in response to v-Src expression was not restricted to one cell type. REF-neo cells (REF cells transfected with the vector plasmid) were used as a control. No STATs were detected in the serum-starved cells (Fig. 1, lane 4), while Stat3 binding was strongly activated and Stat1 binding was weakly activated by PDGF treatment (lane 5). These results were similar to those obtained with parental REF and NIH 3T3 cells (data not shown). Similarly, with another control cell line, REF-E2F-1 (REF cells transformed by transcription factor E2F [36]), Stat3 was not activated in the untreated cells (Fig. 1, lane 6) but was activated in response to PDGF or rat IFN (lanes 7 and 8). These results showed that constitutive activation of Stat3 was likely due to v-Src expression and was not likely to be a consequence of cell transformation.

To further examine the correlation between v-Src expression and activation of Stat3, the investigation was extended to a REF cell line (IND-REF-src) in which v-Src expression was

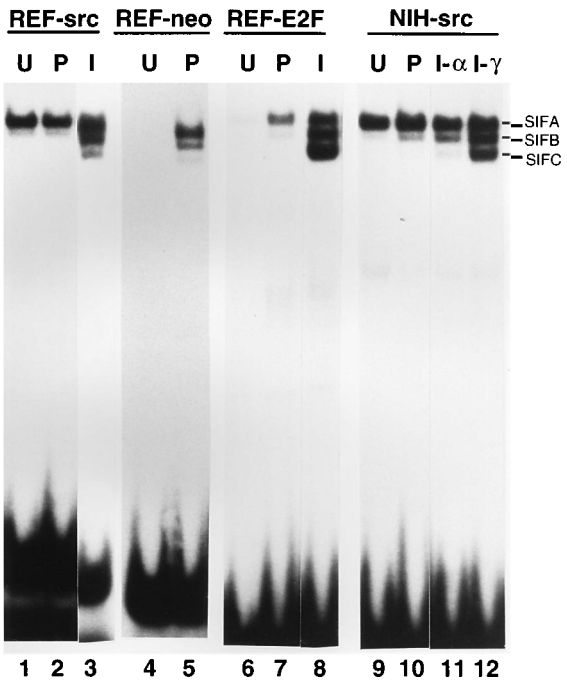


FIG. 1. Constitutive DNA binding of Stat3 in v-Src-transformed cell lines. REF-src, REF-neo, REF-E2F, and NIH 3T3-v-src cells were serum starved for 24 h. The cells were left untreated (lanes 1, 4, 6, and 9) or treated with PDGF (75 ng/ml; Sigma) (lanes 2, 5, 7, and 10), rat IFN (1,000 U/ml; Sigma) (lanes 3 and 8), mouse IFN-α (1,000 U/ml; Sigma) (lane 11), or mouse IFN-γ (1,000 U/ml; Genzyme) (lane 12) for 30 min. Nuclear proteins were extracted, and a mobility shift assay was performed by using hSIE as a probe. U, untreated; P, PDGF; I, rat IFN; I-α, mouse IFN-α; I-γ, mouse IFN-γ.

driven by a dexamethasone-inducible promoter (25). Treatment of these cells with dexamethasone for 16 h induced a time-dependent increase in Stat3 DNA binding activity (Fig. 2A), whereas dexamethasone treatment did not induce Stat3 binding in control REF-neo cells (data not shown). The kinetics of Stat3 DNA binding corresponded with the expression of Src protein upon dexamethasone treatment of the cells, as determined by Western blotting with an anti-Src monoclonal antibody (Fig. 2B, upper panel). Notably, the amount of Stat3 in the whole-cell lysates was unaffected by dexamethasone treatment, whereas the amount of Stat1α decreased slightly (Fig. 2B, middle panels). Overexpression of v-Src by dexamethasone increased the tyrosine phosphorylation of several proteins. Among them, a ~90-kDa protein which has a molecular mass similar to that of Stat3 was strongly phosphorylated on tyrosine in a time-dependent manner (Fig. 2B, bottom panel).

Experiments were performed to show whether the enhanced Stat3 DNA binding activity was due to the phosphorylation of Stat3 on tyrosine. Cell lysates from dexamethasone-treated or untreated IND-REF-src cells were incubated with Stat3 antibodies or preimmune serum. The immunoprecipitated proteins were separated by SDS-PAGE, transferred to PVDF membrane, and blotted with antiphosphotyrosine antibody (anti-P-Tyr) (4G10). A 90-kDa protein immunoprecipitated by antibody 1695 (prepared against the Stat3 C terminus) was detected by 4G10 only in the dexamethasone-treated cells (Fig. 2C, lane 2 [arrow]) and not the control cells (lane 1), and the preimmune serum did not precipitate this protein (lanes 3 and 4). When the 4G10 blot was stripped and reprobed with Stat3 antibody, a 90-kDa protein and its minor form as previously reported (48) were detected (Fig. 2C, lanes 5 and 6). In the same blot, Stat3 was not detected in the cell lysate immunoprecipitated with preimmune serum (Fig. 2C, lanes 7 and 8). Thus, Src overexpression induced by dexamethasone also resulted in the phosphorylation of Stat3 on tyrosine. The phosphorylation of Stat3 on tyrosine was also observed with two other polyclonal Stat3 antibodies (C20 from Santa Cruz Biotechnology and anti-mouse Stat3 from UBI), but to a lesser extent (data not shown). Another protein, of ~80 kDa, was detected to react strongly with the anti-P-Tyr antibody 4G10 in a dexamathesone-inducible manner (Fig. 2C, lanes 1 to 4). This protein is unlikely to be Stat3 because it was also immunoprecipitable by preimmune serum (Fig. 2C, lanes 3 and 4) and was not recognized by Stat3 antibody in the blotting (lanes 7 and 8). Most likely, this is a tyrosine-phosphorylated protein which binds nonspecifically to rabbit immunoglobulin G in the Src-transformed cells.

The distribution of Stat3 in the IND-REF-src cells was then examined by Western blotting. In dexamethasone-induced cells, ~18% of Stat3 is in the nuclear fraction and 82% is in the cytoplasmic fraction (Fig. 2D, lanes 4 and 8), compared with 8 and 92%, respectively, in the uninduced cells. Only a small amount of Stat1 (<3%) was in the nuclear fraction in both induced and uninduced cells (data not shown). Since it is known that Stat3 is also strongly activated by EGF in A-431 cells (30, 43), we also determined the distribution of Stat3 in EGF-induced A-431 cells. We found a similar increase (Fig. 2D, bottom panel) in the amount of Stat3 in the nuclear fraction in EGF-stimulated cells. The maximum amount of Stat3 translocated into the nucleus after EGF treatment (7 min) (Fig. 2D, bottom panel, lane 5) is similar (22%) to that observed to be present in the dexamethasone-induced REF cells.

The presence of Stat3 in SIF-A, Stat1 in SIF-C, and both in SIF-B from EGF-treated A-431 cells was demonstrated by a supershift assay with Stat1 and Stat3 antibodies (31). Similar results were also obtained with the mouse liver cells treated with EGF and lipopolysaccharide (30). The presence of Stat3 in SIF-A in both REF-src and IND-REF-src cells was confirmed by a supershift assay. SIF-A was supershifted, forming two complexes (SS1 and SS2 [Fig. 2E]) after the addition of anti-Stat3 antibody to the nuclear extracts of REF-v-src cells (Fig. 2E, lane 2) and the inducible IND-REF-src cells either before (lane 4) or after (lane 6) dexamethasone treatment. Preimmune serum or Stat1 antibody had no effect on the SIF-A supershift in both REF-src and IND-REF-src cells (data not shown). When the DNA binding reaction was performed in the presence of anti-P-Tyr antibody (4G10), there was a decrease in the amount of Stat3 DNA binding (less than 40%) (Fig. 2F, lanes 2 and 3), whereas preimmune serum had little effect on Stat3 DNA binding (lane 4). Thus, tyrosine phosphorylation is required for Stat3 DNA binding.

Stat3 associates with Src in Src-transformed cell lines. The association of Stat3 and v-Src was examined since both proteins contain SH2 domains and phosphorylated tyrosine residues. Proteins were immunoprecipitated from lysates of untreated or dexamethasone-treated IND-REF-src cells with monoclonal anti-Src antibody. The immunoprecipitated proteins were separated by SDS-PAGE and blotted with Stat3 antibody. In the dexamethasone-treated IND-REF-src cells, a predominant protein of approximately 90 kDa coimmunoprecipitated with Src and was recognized by Stat3 antibody (Fig. 3A, lane 2). A small amount of 90-kDa protein was also found in the uninduced cells (Fig. 3A, lane 1). A similar immunoprecipitation experiment with Src antibody was performed, and then the protein was blotted with anti-P-Tyr antibody. The 90-kDa protein was shown to be strongly phosphorylated on tyrosine in the dexamethasone-treated cells (Fig. 3B lane 2)

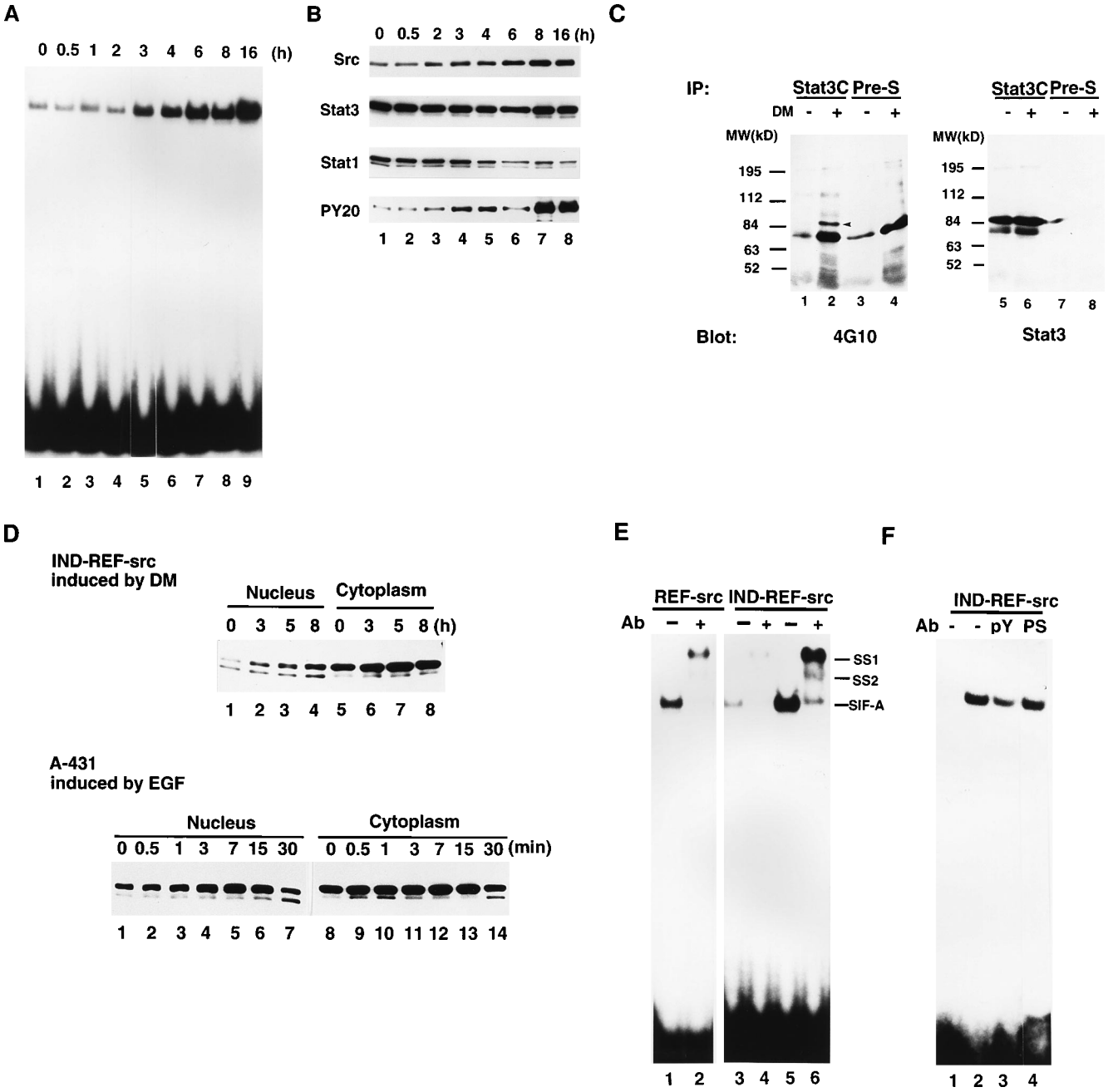


FIG. 2. Stat3 in the v-Src-inducible IND-REF-src cells. (A) Mobility shift assay. Nuclear extracts were prepared from untreated cells (lane 1) or from cells treated with dexamethasone (2 μM) for 0.5, 1, 2, 3, 4, 6, 8, and 16 h (lanes 2 to 9) by using hSIE as a probe. (B) Western blot analysis of Src, Stat3, and Stat1 in IND-REF-src cells. Whole-cell lysates with equal amounts of proteins from untreated cells (lanes 1) or cells treated with dexamethasone for 0.5, 2, 3, 4, 6, 8, and 16 h (lanes 2 to 8) were subjected to SDS-PAGE, transferred to membrane, and probed with anti-Src, anti-Stat3, anti-Stat1, or PY20 antibodies as indicated on the left. (C) Tyrosine phosphorylation of Stat3 in IND-REF-src cells. Cell lysates from untreated (−) or dexamethasone (DM)-treated (+) cells were immunoprecipitated (IP) with the antibodies indicated above the figure. Pre-S, preimmune rabbit serum; Stat3C, rabbit antiserum prepared against the fusion protein GST-Stat3C described in Materials and Methods. Immunocomplexes were resolved by SDS-PAGE, transferred to a membrane, and probed with anti-P-Tyr antibody 4G10 (left panel). The blot was stripped and reprobed with Stat3 antibody (right panel). MW, molecular mass markers. (D) Western blot analysis of nuclear and cytoplasmic Stat3 in IND-REF-src and A-431 cells. Equal amounts of nuclear and cytoplasmic proteins were loaded on an SDS gel, and Western blotting was performed with Stat3 antibody. The percentages of Stat3 in the nuclear and cytoplasmic fractions were estimated by measuring the intensity of the Stat3 band on the blot and the total amounts of proteins in the cytoplasm and nucleus. The top panel shows IND-REF-src cells left untreated or treated with dexamethasone (DM) for the various times indicated before being harvested, whereas the bottom panel shows nuclear and cytoplasmic proteins prepared from A-431 cells left unstimulated or stimulated by EGF for the times indicated. (E) Supershift assay of Stat3 DNA binding. Nuclear proteins were prepared from untreated REF-src cells (lanes 1 and 2) and from IND-REF-src cells left untreated (lanes 3 and 4) or treated with dexamethasone (lanes 5 and 6). The nuclear extracts were incubated with (+) or without (−) anti-Stat3 antibody (Ab) (Santa Cruz) before addition of the probe, hSIE, to the DNA binding reaction mixture. The lowest protein-DNA complex in lanes 1, 3, 5, and 6 is SIF-A, and the two higher bands in lanes 2, 4, and 6 are supershifted SIF-A and antibody complexes indicated as SS1 and SS2. (F) Effect of anti-P-Tyr antibody (Ab) on DNA binding of Stat3. Cells were left untreated (lane 1) or treated with dexamethasone (lanes 2 to 4), and the binding reaction was carried out in the absence of antibody (lane 2) or in the presence of antibody 4G10 (pY, lane 3) or preimmune serum (PS, lane 4) before the mobility shift assay was performed.

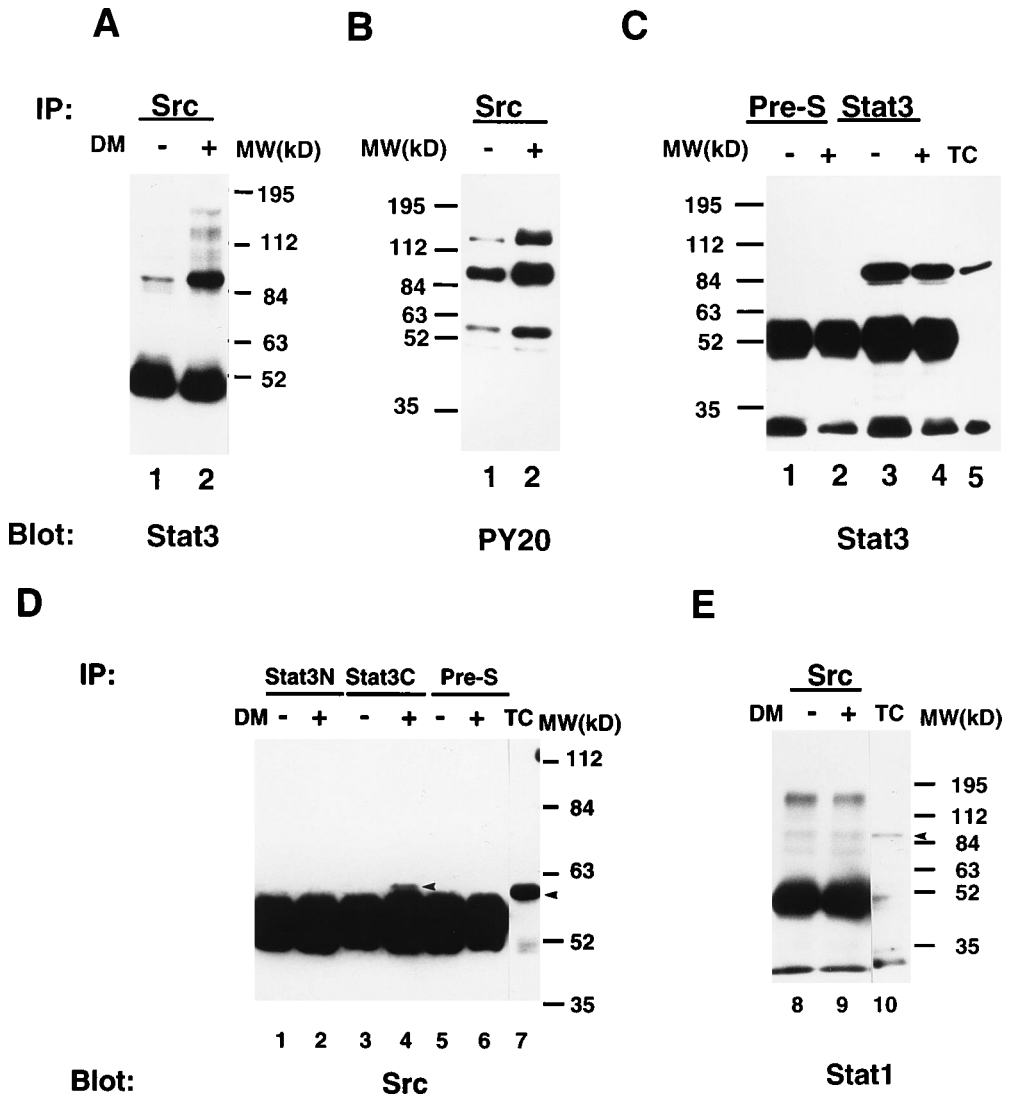


FIG. 3. Association of Stat3 with Src in IND-REF-src cells. Cell lysates from untreated (−) or dexamethasone (DM)-treated (+) cells were prepared as described in Materials and Methods, except that SDS was omitted from the RIPA buffer and proteins were immunoprecipitated (IP) with the antibodies indicated above the different panels. Pre-S, preimmune rabbit serum. Immunocomplexes were resolved by SDS-PAGE, transferred to a membrane, and probed with Stat3, PY20, Src, or Stat1 antibody (indicated below the blots). (C, D, and E) The last lanes contained the total cell lysate (TC) from dexamethasone-induced cells probed with antibodies as indicated. Src and Stat1 are indicated by arrows in panels D and E, respectively. Stat3N and Stat3C (D) refer to the rabbit antisera against GST-Stat3N and GST-Stat3C, respectively. MW, molecular mass markers.

but phosphorylated to a lesser extent in untreated cells (lane 1). In the control experiment, preimmune serum or Stat3 antibody was used during immunoprecipitation and the blot was probed with Stat3 antibody. The 90-kDa protein was immunoprecipitated only by Stat3 antibody (Fig. 3C, lanes 3 and 4) and not by preimmune serum (lanes 1 and 2) in both dexamethasone-treated and untreated cells. We also observed a tyrosine-phosphorylated protein migrating between 52 and 63 kDa (Fig. 3B, lanes 1 and 2). This protein is shown to be Src by immunoblotting with Src antibody (data not shown). The association of Stat3 and Src was further examined in a reverse experiment in which Stat3 was immunoprecipitated by Stat3 antibodies and Western blotted by Src antibody (Fig. 3D). Src was detected in the immunoprecipitate of dexamethasone-treated (Fig. 3D, lane 4, arrow) but not control (lane 3) cell lysates by Stat3 antibody 1695 raised against the C-terminal region of Stat3. In contrast, Src was not immunoprecipitated by preimmune serum (Fig. 3D, lanes 5 and 6). The results suggest that the association between Stat3 and Src is specific. Antibody against the N-terminal region of Stat3 (Stat3N) did not immunoprecipitate Src (Fig. 3D, lanes 1 and 2). It is possible that Src binding to Stat3 may block the recognition site of Stat3N antibody at the N terminus. The association of Stat1 and Src was also examined. A small amount of Stat1 is associated with Src in both dexamethasone-treated and untreated cells (Fig. 3E). Together, these results (Fig. 2 and 3) suggest that Stat3 not only associates with Src but also is increasingly phosphorylated on tyrosine with enhanced Src expression.

Presently, it is not possible to exclude the possibility that another tyrosine-phosphorylated protein(s), in addition to Stat3, also associates with v-Src (Fig. 3A and B). Muthuswamy and Muller (24) recently reported an unidentified p89 protein to associate with Src in v-Src-transformed cells. A few weak bands above 90 kDa were also recognized by Stat3 antibody

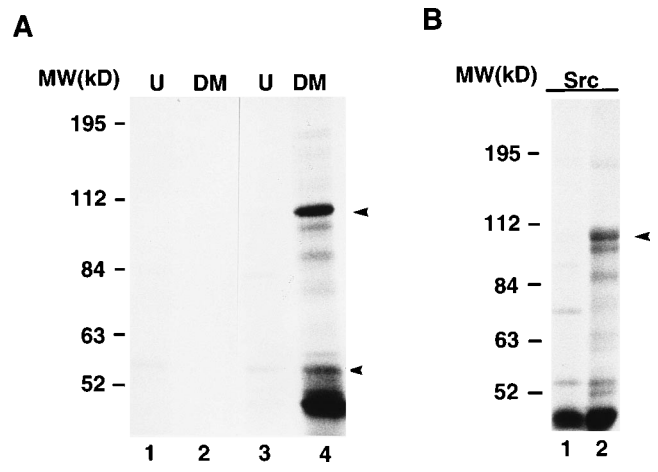
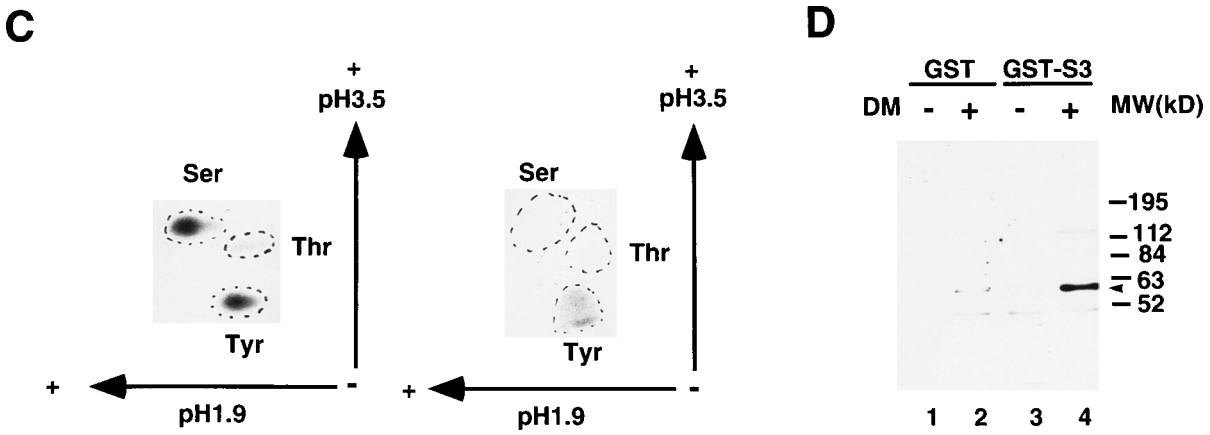


FIG. 4. GST-Stat3 fusion protein associates with and is phosphorylated by v-Src. The fusion proteins were constructed and partially purified by mixing glutathione-agarose beads with bacterial lysate as described in Materials and Methods. (A) In vitro binding and kinase assays were carried out by incubating the whole-cell extracts from untreated (U) cells (lanes 1 and 3) or dexamethasone (DM)-treated IND-REF-src cells (lanes 2 and 4) with beads coupled to GST (lanes 1 and 2) or GST-Stat3 (lanes 3 and 4). Beads were then washed and resuspended in kinase assay buffer with 5 μCi of [γ-^{32}P]ATP. The beads were extensively washed with PBS, and the bound proteins were eluted and resolved by SDS-PAGE. The autoradiograph of the dried gel is shown. (B) The glutathione-agarose bead-coupled GST-Stat3 (lane 2) and GST (lane 1) were incubated with partially purified recombinant human c-Src (2 U per reaction mixture) under the kinase assay conditions described above. The position of GST-Stat3 is indicated by the arrow. (C) Phosphoamino acid analysis of GST-Stat3. GST-Stat3 was phosphorylated by lysate from dexamethasone-induced IND-REF-src cells (left) and by Src (right). The samples were resolved by SDS–7.5% PAGE, transferred to PVDF membrane, and exposed to film. The fusion protein band was excised, and phosphoamino acid analysis was performed as described elsewhere (4). (D) GST (lanes 1 and 2) and GST-Stat3 (lanes 3 and 4) glutathione-agarose beads were incubated with cell lysates prepared from uninduced (−) (lanes 1 and 3) or dexamethasone-induced (+) (lanes 2 and 4) cells. Proteins bound to the beads after washing were subjected to SDS-PAGE. The gel was transferred to membrane and blotted with anti-Src antibody (EC10). The position of Src is indicated by an arrow. MW, molecular mass markers.



(Fig. 3A, lane 2). A 125-kDa protein was also coprecipitated with the Src antibody and shown to be strongly tyrosine phosphorylated in dexamethasone-treated cells (Fig. 3B, lane 2). The molecular mass of this protein compares closely with those of Stat2 (113 kDa) and pp120 (an Src substrate present in the v-Src-transformed cells [26]). However, the 125-kDa protein was not recognized by either of the antibodies against Stat2 and pp120 (data not shown).

Src associates with and phosphorylates Stat3 in vitro. The association of Stat3 and Src protein was examined in vitro. GST-Stat3 fusion protein was produced in *Escherichia coli* and partially purified by using glutathione-agarose beads. The GST-Stat3-bound beads were incubated with lysates derived from dexamethasone-treated or untreated IND-REF-src cells. The beads were washed and used as a substrate in the Src kinase assay with [γ-^{32}P]ATP. The ^{32}P-labeled protein(s) was subjected to SDS-PAGE, and the gel was dried and autoradiographed. GST-Stat3 was phosphorylated only when incubated with cell lysate from dexamethasone-treated cells (Fig. 4A, lane 4) and not when incubated with lysate from untreated cells (lane 3). No phosphoprotein was detected when GST alone was incubated with either cell lysate (Fig. 4A, lanes 1 and 2). A protein (indicated by an arrow in Fig. 4A) migrating between the 63- and 52-kDa molecular mass markers is likely autophosphorylated Src, since it runs to the same position as does autophosphorylated recombinant Src protein (data not shown). In the Src-transformed cells, Src enzymatic activity is increased significantly in comparison with levels for control cells. We showed that bacterially expressed GST-Stat3 can be phosphorylated only when Src is overexpressed (Fig. 4A), suggesting that Src is likely to be the major tyrosine kinase phosphorylating Stat3, although phosphorylation by other tyrosine kinases cannot be excluded. GST-Stat3 is also phosphorylated by recombinant human c-Src in vitro (Fig. 4B, lane 2). Other tyrosine kinases were also tested for their ability to phosphorylate Stat3 in vitro. Fyn phosphorylated GST-JNK, which contains a full-length c-jun amino-terminal kinase, JNK (11, 20), with 2 U of the enzyme per reaction but did not phosphorylate GST-Stat3 even at 6 U per reaction, whereas Lyn phosphorylated both fusion proteins at a low concentration (2 U per reaction; data not shown). Hence, there is some degree of specificity in Stat3 phosphorylation by the different tyrosine kinases. Phosphoamino acid analysis of GST-Stat3 showed that Src phosphorylated this protein on a tyrosine residue in vitro (Fig. 4C, right panel). However, both serine and tyrosine residues were strongly phosphorylated, with a trace amount of phosphorylation on threonine, when GST-Stat3-bound beads were incubated with cell lysate prepared from dexamethasone-treated IND-REF-src cells (Fig. 4C, left panel). In agreement with this, equal amounts of phosphorylation on serine and tyrosine were observed when GST-Stat3 beads were incubated with cell lysate derived from NIH 3T3-v-Src cells (data not shown). This indicates that a serine/threonine kinase(s) may also be involved in the phosphorylation of Stat3 in these cells.

These results are consistent with the recent reports showing that both serine and tyrosine phosphorylations of Stat1 and Stat3 are required for their DNA binding and transcriptional activation (45, 47).

In a parallel experiment, glutathione-agarose beads coupled with GST-Stat3 were incubated with cell lysates, washed with PBS, and then subjected to SDS-PAGE and blotted with Src antibody. Src was detected only when GST-Stat3 was incubated with lysates prepared from dexamethasone-treated cells (Fig. 4D, lane 4), and not when it was incubated with lysates from untreated cells (lane 3). A very weak band was observed in a position similar to that of Src in the GST sample incubated with cell lysate from induced cells, possibly as a result of nonspecific binding of Src to the GST beads (Fig. 4D, lane 2). In order to confirm the tyrosine phosphorylation of GST-Stat3, a similar experiment in which the GST-Stat3-bound beads were incubated with the cell lysates, washed a few times with PBS, and then incubated with Src kinase reaction buffer as described previously, except without addition of [γ-^{32}P]ATP, was performed. The proteins were subjected to SDS-PAGE, transferred to membrane, and blotted with anti-P-Tyr. The results showed that GST-Stat3 and its degradation products, as well as the associated Src, were phosphorylated on tyrosine in the dexamethasone-treated sample (data not shown). Hence, not only does Stat3 associate with v-Src, but also association resulted in the phosphorylation of Stat3 on tyrosine.

Inhibition of Stat3 activation by CSF-1 in mutant CSF-1 receptor-transfected NIH 3T3 cells. The effect of the cellular counterpart of v-Src, c-Src, in Stat3 activation in normal cells was also investigated. NIH 3T3 cells were transfected with wild-type or mutant human macrophage CSF-1 receptor (28). The CSF-1 receptor is a transmembrane glycoprotein with an intrinsic, ligand-responsive protein tyrosine kinase activity. It has been shown that three Src family kinases, Src, Fyn, and Yes, bind to and are activated by ligand-stimulated CSF-1 receptor in the cells transfected with the wild-type receptor. The association of Src family kinases with the CSF-1 receptor is impaired when one of the autophosphorylation sites (Tyr-809) is replaced by phenylalanine (Phe) (8). In addition, the ability to induce cell proliferation in response to CSF-1 is lost (27, 28). To study STAT activation by CSF-1, nuclear extracts were prepared from wild-type (CSF-1R) and mutant (809F) cell lines and the DNA binding activities of Stat1 and Stat3 were examined in mobility shift assays. The level of Stat3 binding was very low in wild-type CSF-1R cells not treated with CSF-1 (Fig. 5, lane 1) but increased after CSF-1 treatment (10 [lane 2] or 100 [lane 3] ng/ml). In contrast, Stat3 DNA binding activity was not observed in the mutant 809F cells in response to CSF-1 treatment (100 ng/ml) or in the untreated cells (Fig. 5, lanes 5 and 6). This suggests that the activation of Src family kinases, through their association with the CSF-1 receptor, may be involved in the induction of Stat3 DNA binding by CSF-1. No Stat1 activation was detected in either of the cell lines after CSF-1 treatment. In both wild-type and mutant cell lines, the activation of Stat3 and Stat1α in response to PDGF treatment (Fig. 5, lanes 4 and 7) was maintained. In addition, activation of Stat3 or Stat1 by IFN-α or IFN-γ was also observed to occur in both cell lines (data not shown), indicating that loss of Stat3 activation by CSF-1 in the mutant 809F cells was not due to a nonspecific effect of mutant CSF-1 receptor overexpression.

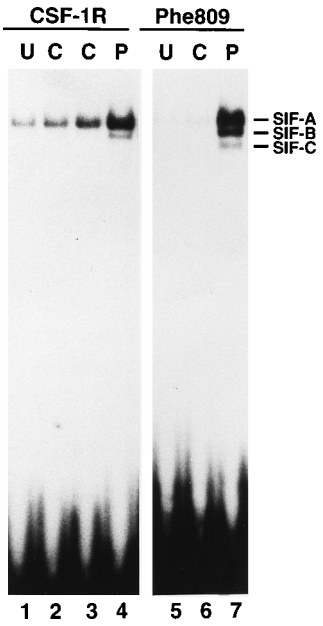


FIG. 5. Mobility shift assays of Stat3 and Stat1 in CSF-1R and CSF-1R (Phe-809) cell lines. CSF-1R or CSF-1R (Phe-809) cells were left untreated (lanes 1 and 5) or treated with CSF-1 (Genzyme) at 10 ng/ml (lane 2) and 100 ng/ml (lanes 3 and 6), or PDGF (75 ng/ml) (lanes 4 and 7). Nuclear extracts were prepared and subjected to mobility shift assays with the hSIE probe. U, untreated; C, CSF-1 treated; P, PDGF treated.

DISCUSSION

Although the Jak-STAT pathway is reported as the major IFN signal transduction pathway, it is unclear how STATs are activated in growth factor signaling through receptors with intrinsic protein tyrosine kinase activity. Our results suggest that the Src tyrosine kinase activates Stat3. We found that Stat3 is constitutively activated in v-Src-transformed REF as well as in v-Src-transformed NIH 3T3 cells (Fig. 1). The induction of v-Src expression by dexamethasone in the IND-REF-src cells led to an increase in DNA binding of Stat3 but not of Stat1 (Fig. 2A). During the revision of the manuscript of this article, similar results showing that Stat3 is constitutively activated by the v-Src oncoprotein in v-Src-transformed rodent fibroblasts were reported by Yu et al. (46). Therefore, Src is likely involved in the activation of Stat3 in cell growth and transformation. Our results also show that Stat3 is associated with Src (Fig. 3A and D). Furthermore, we also observed that c-Src is associated with Stat3 in EGF-stimulated A-431 cells (data not shown). Hence the Src-Stat3 association may play a role in growth factor signaling, and details of the Src-Stat3 interaction are currently under investigation.

Interestingly, we observed that GST-Stat3 was phosphorylated by Src in vitro, only on tyrosine, but Stat3 was also phosphorylated on serine when incubated with cell lysate prepared from IND-REF-src or NIH 3T3-v-Src cells (Fig. 4C and data not shown). These data suggest that a serine kinase may also associate with and activate Stat3. Phosphoamino acid analysis of Stat3 indicated that serine, in addition to tyrosine, was strongly phosphorylated in REF-src, NIH 3T3-v-Src, and IND-REF-src cells (data not shown). Hence, a serine kinase may also be involved in the phosphorylation of Stat3 in these cells. This is in agreement with the report that serine phosphorylation is required for the formation of a Stat3-DNA complex (47). Recently, Ser-727, within a mitogen-activated protein (MAP) kinase recognition site of Stat1α, and an analogous site (Ser-728) in Stat3 were described to be the major serine kinase targets (45). MAP kinase and Stat1 were coimmunoprecipitated in IFN-β-induced cells, suggesting association of

the two (10). Our results showed that when P-Tyr antibody (4G10) was added to the DNA binding reaction mixture, it reduced but did not abolish Stat3 binding (Fig. 2F, lane 3). We also observed that okadaic acid, an inhibitor of a serine/threonine phosphatase, enhanced Stat3 DNA binding (data not shown). Together, these data point to the requirement of serine as well as tyrosine phosphorylation in the activation of Stat3.

The role of the cellular counterpart of v-Src, c-Src, was also examined in the mutant CSF-1 receptor-transfected cell line (CSF-1R 809F) because unlike the wild-type CSF-1 receptor, the mutant receptor cannot associate with c-Src in response to CSF-1 stimulation (8). The activation of Stat3 by CSF-1 was observed to occur in the wild-type but not the mutant cells (Fig. 5), suggesting a role for c-Src in the activation of Stat3 in CSF-1 signaling. It was recently reported that Tyr-561 of the CSF-1 receptor is a major site for the association of Fyn and other Src family members (2). Whether the mutation in Tyr-809 affected Src association with Tyr-561 is debatable. Although the mutation at Tyr-809 did not affect the association of phosphatidylinositol 3-kinase with the CSF-1 receptor in the mutant 809F cells (28, 39), the mutation in the CSF-1 receptor may affect the other autophosphorylation sites in the CSF-1 receptor (29, 41). The lack of Src association in CSF-1-treated 809F cells may have resulted from a loss of Src binding at Tyr-561. The mutation at Tyr-809 could also interfere with other pathways besides Src association. It is known that the CSF-1 receptor is closely related to the α and β receptors of PDGF (16), and the Src family kinases associated with the PDGF receptor upon PDGF induction (19), indicating that Src participated in the activation of Stat3 by PDGF. Vignais et al. (42) showed that the autophosphorylation site Tyr-579 of the PDGF receptor involved in Src-receptor association is required for Stat3 activation, whereas none of the other autophosphorylation sites involved in association with other signaling molecules are essential for Stat3 activation. Therefore, the activation of Stat3 by growth factor receptors with intrinsic protein tyrosine kinase activity may, in general, require the association of Src with the respective growth factor receptors. In addition, as mentioned above, our results also showed an association of Stat3 with c-Src after EGF stimulation of A-431 cells (unpublished data). Hence, we cautiously suggest that the results shown in Fig. 5 may mean that Tyr-809 mutation affected the binding of the CSF-1 receptor to Src.

Although the role of Stat3 in cell growth and transformation is not fully established, others recently have reported that the Jak-STAT pathway is constitutively activated in human T-cell lymphotropic virus type 1-transformed T cells (22). Stat3 is also reported to activate transcription of c-*fos* by binding to the SIE (43). The mutant CSF-1R cell line 809F, which is defective in Stat3 activation by CSF-1, does not proliferate in response to CSF-1 stimulation (28, 29). Together, these results suggest a possible role of Stat3 in cell growth and transformation.

Stat3 is also activated by various cytokines which bind to receptors lacking intrinsic protein tyrosine kinase activity. Many cytokine receptor complexes consist of more than two subunits. These receptor complexes interact with nonreceptor protein tyrosine kinases like members of the Jak and Src families (5, 38). Somatic genetic analyses of the IFN-α and -γ system provided the evidence for the involvement of Jak family members in their signaling processes (23, 40). It is not clear whether Jaks are involved in pathways associated with cell growth and transformation. Overexpression of Src does not activate Stat1 (Fig. 1 and 2), and it does not affect Stat1 activation by Jak1 and Jak2 after IFN-γ treatment in the v-Src-transformed cell lines. Stat3 is not activated by IFN-γ. These data suggest that Stat1 and Stat3 activations may proceed through different pathways. It will be useful to investigate whether the Src family is also involved in the activation of STATs in cytokine signaling. Recently, Heim et al. (17) showed that SH2 domains in Stat1 and Stat2 play key roles in determining the specificity of binding to IFN-α or IFN-γ receptors, whereas Jak kinases are relatively nonspecific. Stahl et al. (37) reported that Stat3 activation by ciliary neurotrophic factor requires a specific tyrosine-based motif, YXXQ, in receptors where Stat3 is phosphorylated by receptor-associated Jaks. However, the consensus sequence YXXQ was not found in Src, CSF-1, and PDGF receptors. Thus, Stat3 activation by growth factors may be mediated through different pathways and/or by additional kinases, such as Src, other than Jaks. While Stat3 activation by cytokines requires the association of Jaks and their receptors, Stat3 activation by growth factors could instead be mediated by the association of Src with growth factor receptors.

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