Activation of Stat3 in v-Src-transformed Fibroblasts Requires Cooperation of Jak1 Kinase Activity*

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Signal transducers and activators of transcription (STATs) are latent cytoplasmic transcription factors that transduce signals from the cell membrane to the nucleus upon activation by tyrosine phosphorylation. Several protein-tyrosine kinases can induce phosphorylation of STATs in cells, including Janus kinase (JAK) and Src family kinases. One STAT family member, Stat3, is constitutively activated in Src-transformed NIH3T3 cells and is required for cell transformation. However, it is not entirely clear whether Src kinase can phosphorylate Stat3 directly or through another pathway, such as JAK family kinases. To address this question, we investigated the phosphorylation of STATs in baculovirus-infected Sf-9 insect cells in the presence of Src. Our results show that Src can tyrosine-phosphorylate Stat1 and Stat3 but not Stat5 in this system. The phosphorylated Stat1 and Stat3 proteins are functionally activated, as measured by their abilities to specifically bind DNA oligonucleotide probes. In addition, the Jak family member Jak1 efficiently phosphorylates Stat1 but not Stat3 in Sf-9 cells. By contrast, we observe that AG490, a Jak family-selective inhibitor, and dominant negative Jak1 protein can significantly inhibit Stat3-induced DNA binding activity and as well as Stat3-mediated gene activation in NIH3T3 cells. Furthermore, wild-type or kinase-inactive platelet-derived growth factor receptor enhances Stat3 activation by v-Src, consistent with the receptor serving a scaffolding function for recruitment and activation of Stat3. Our results demonstrate that Src kinase is capable of activating STATs in Sf-9 insect cells without expression of JAK family members; however, Jak1 and platelet-derived growth factor receptor are required for maximal Stat3 activation by Src kinase in mammalian cells. Based on these findings, we propose a model in which Jak1 serves to recruit Stat3 to a receptor complex with Src kinase, which in turn directly phosphorylates and activates Stat3 in Src-transformed fibroblasts.

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1 The abbreviations used are: STAT, signal transducers and activators of transcription; JAK, Janus kinase; PDGF, platelet-derived growth factor; PDGF-R, PDGF receptor; PBS, phosphate-buffered saline; EMSA, electrophoretic mobility shift assay.
tide hormones involves nonreceptor tyrosine kinases in addition to the intrinsic tyrosine kinase of their receptors.

We and others have observed the constitutive activation of Stat3 in v-Src-transformed cells (23–25). Further studies demonstrated that this Stat3 activation results in gene activation and is essential for v-Src transformation (26, 27). Although direct activation of Stat3 by Src has been suggested (24, 28), the mechanism of Stat3 activation is not entirely clear (29) and may employ different mechanisms that are dependent on the cell type. In v-Src-transformed mouse fibroblasts, Jak1 and, to a much lesser extent, Jak2 are also constitutively activated (30). To investigate the mechanism of Stat3 activation by Src, we examined STAT activation by Src and Jak1 expressed from recombinant baculoviruses in Sf-9 insect cells as well as the role of Jak1 in Stat3 activation in mouse fibroblasts transformed by v-Src.

Here we report that Stat1 and Stat3 are tyrosine-phosphorylated in Sf-9 cells by activated Src in the absence of other mammalian tyrosine kinases. The phosphorylated STAT proteins bind to specific DNA sequences in gel shift assays, indicating that this phosphorylation induces functional activation of the STAT proteins. Furthermore, Jak1 enhances activation of Stat1 but not Stat3 when co-expressed with Src in Sf-9 cells, and the phosphorylation level of Jak1 is also increased with the expression of Src. By contrast, in NIH3T3 cells, Jak1 activity is required for maximal Stat3-mediated gene induction. In addition, activation of Stat3 by Src in mammalian cells is enhanced by the PDGF receptor independently of receptor kinase activity, consistent with a scaffolding function for the receptor. Our results indicate that, although Src can directly activate Stat3 in insect cells, Jak1 plays an important role in the activation of Stat3 in Sf-9-cell transformed mouse fibroblasts. These findings support a model in which Src and Jak1 cooperate together with the PDGF receptor and possibly other receptors to activate Stat3 in the context of oncogenesis.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents—**NIH3T3 cells and their Sf-9 transformed counterparts have been described previously (31). Human fibrosarcoma cell, 2TGH (32), was a kind gift from Dr. George Stark (Cleveland Clinic Foundation, Cleveland, OH). Expression vectors for wild-type and kinase-inactive PDGF receptor-β (33, 34) were generously provided by Dr. Andrius Kazlauskas (The Schepens Eye Research Institute, Boston, MA). Anti-Jak1 antibody (HR-785) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-phosphotyrosine 701-Sstat1 and anti-phosphotyrosine 684-Sstat3 were from Zymed Laboratories Inc.; anti-phosphotyrosine 705-Stat3 was from New England Bio-labs; anti-phosphoserine 727-Stat1 and -Stat3 (35) were kind gifts from Dr. David Frank (Dana-Farber Cancer Institute, Boston, MA); anti-Stat1 (E-23), anti-Stat1 (K-15), anti-Stat5α (L-20), and anti-phosphotyrosine (PY-99) for Western blot analyses were from Santa Cruz Biotechnology; anti-Stat1 (E-23) and anti-Stat3 (H-190) for gel supershift assays were also from Santa Cruz Biotechnology. Peroxidase-free Triton X-100 was obtained from Roche Molecular Biochemicals; recombinant protein A/G-agarose beads with rotation. The immunoprecipitates were then washed twice with PBST, the membranes were treated with ECL detection solutions and exposed to films.

**Western Blot Analyses—**Cytoxic extracts or immunoprecipitated proteins were separated on 8% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dried milk for 1 h and then incubated for 1 h with antibodies against Stat1, Stat3, Stat5, Jak1, or Src at 1 μg/ml in PBS plus 0.1% Tween 20. After incubation, membranes were washed twice with PBST and incubated with anti-phosphotyrosine tyrosine blots, the membranes were blocked with chicken ovalbumin (grade III; Sigma) instead of dried milk for 2 h. The membranes were then washed in PBST and probed with anti-phosphotyrosine antibodies and subsequently with appropriate secondary antibodies for 1 h. After another 15-min wash in PBST, the membranes were treated with ECL detection solutions and exposed to films. The reactions were stopped with 10 mM EDTA, and the agarose beads were then washed with buffer and boiled for 1 min in PAGE sample buffer. Proteins were separated by 8% SDS-PAGE, and phosphorylated Jak1 was visualized by autoradiography. In vitro Src kinase assays were performed as described previously (41, 42). Briefly, whole-cell lysates containing 1 mg of total protein were incubated with 2 μg of anti-Jak1 antibody for 2 h at 4 °C followed by 1-h incubation with 20 μl of protein A/G-agarose. The immunoprecipitates were then washed twice with wash buffer and once with phosphorylation buffer as described previously (30). The kinase reactions were carried out at 30 °C for 40 min in 100 μl of the kinase buffer (100 mM NaCl, 50 mM HEPS, pH 7.6, 0.1% Triton X-100, 0.5 mM dithiothreitol, 6.25 mM MnCl2, 20 μCi of [γ-32P]ATP, 0.1 μM aprotinin, 1 μM leupeptin, and 1 μM antipain). When inhibitors were used, AG490 or PD180970 was added to the reaction mix prior to the addition of the kinase buffer. The reactions were stopped with 10 mM EDTA, and the agarose beads were then washed with buffer and boiled for 1 min in PAGE sample buffer. Proteins were separated by 8% SDS-PAGE, and phosphorylated Jak1 was visualized by autoradiography. In vitro Src kinase assays were performed as described previously (41, 42). Briefly, whole-cell lysates containing 1 mg of total protein were incubated with 2 μg of anti-Src antibody for 4 h at 4 °C followed by 1 h of incubation with protein A/G-agarose beads with rotation. The immunoprecipitates were then washed three times with RIPA-150 buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 10% glycerol, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 100 mM Na3VO4, 0.1 μM aprotinin, 1 μM leupeptin, and 1 μM antipain), two times with RIPA-10 buffer (the same as RIPA-150 buffer except with 10 mM NaCl instead of 150 mM NaCl), and three times with Tris buffer (40 mM Tris, pH 7.4). The immunoprecipitates were then resuspended with 30 μl of kinase reaction buffer (20 mM Tris-HCl, pH 7.4, 5 mM MgCl2) containing 10 μCi of [γ-32P]ATP and 5 μg of acid-denatured enolase as exogenous substrate. The kinase reaction mixtures were incubated 15 min at room temperature and stopped by the addition of loading buffer.

**Electrophoretic Mobility Shift Assays—**The procedures for nuclear extract preparation from mammalian cells and electrophoretic mobility shift assays (EMSAs) were conducted as previously published (23, 43). The 32P-Radiolabeled oligonucleotide probes are (α) hSIE (high affinity sis-inducible element, m67 variant, 5′-AGGCTTATTTCCCGGTTTTACCCCTTA-3′) for Stat1 and Stat3 (43, 44) and (β) MGF (mammary gland factor element from the bovine β-casein gene promoter, 5′-AGATT-TCTGAGGATTCCA-3′) for Stat5 binding (11, 45). For cytosolic extracts from Sf-9 cells, 0.05 μg of probe was used. In the case of competitions, a 100-fold molar excess of unlabeled probes was added to each reaction. The FIRE probe (5′-GGCCGCTCCCGC-CCGGG) was used as nonspecific competitor (23, 43). For supershifts, 1 μl of the antibodies against each specific STAT was preincubated with the extract for 20 min prior to the addition of radiolabeled probes (43). The reactions were incubated at 30 °C for 30 min and then resolved on
5% polyacrylamide gels in 0.25× Tris borate-EDTA buffer. STAT-DNA complexes were detected by autoradiography. For the inhibitor treatment, NIH3T3 cells stably transformed with v-Src were treated with fresh inhibitors, AG490 or PD180970, every 12 h for a total of 24 h.

**Transfections and Luciferase Reporter Assays—** NIH3T3 cells were grown in Dulbecco’s modified Eagle’s medium containing 5% iron-supplemented bovine calf serum. Transient transfections were performed with calcium phosphate as described previously (26). Briefly, NIH3T3 cells were seeded at 5 × 10^5 cells/100-mm dish in Dulbecco’s modified Eagle’s medium (5% bovine calf serum) at 18 h prior to transfection. 20 μg of total DNA was used for each plate, which contained typically 4 μg of Stat3 reporter construct pLucTKS3, 0.2 μg of β-galactosidase internal control, and the amounts of expression vector described in the figure legends. The plates were washed once with PBS and replenished with fresh Dulbecco’s modified Eagle’s medium at 15 h after transfection. The cells were harvested 48 h after transfection, and whole-cell lysates were assayed for luciferase as well as β-galactosidase activities.

Human fibrosarcoma cell line, 2fTGH, was cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum as described (32). The transfection was performed with LipofectAMINE Plus according to the supplier’s protocol (Life Technologies). Six μg of plasmid DNA expressing wild type or kinase-dead PDGF-Rβ was transfected into 2fTGH culture in a 60-mm dish. Transfected cells were then selected against G418 at 400 μg/ml final concentration. After selection, cells stably transfected with the respective plasmids were pooled and then used for transient transfections with v-Src expression vector using LipofectAMINE Plus as above.

**RESULTS**

**Activation of Stat1 and Stat3 by Src in Sf-9 Cells—** It is now well established that v-Src induces constitutive activation of Stat3 signaling in transformed cells (23–25). However, it is not entirely clear whether Stat3 is activated directly by Src or through the cooperation of JAK family kinases acting downstream of Src. To address this question, we utilized a baculovirus/insect cell system to overexpress STATs and Src in the absence of other mammalian tyrosine kinases. Sf-9 insect cells were infected with recombinant c-Src baculovirus either alone or in combination with Stat3 baculovirus. In this system, c-Src is an activated kinase due to lack of phosphorylation of the negative regulatory Tyr-527 residue (46).

Activation of STAT proteins was examined in cytosolic extracts using EMSA with a specific oligonucleotide probe (hSIE) to detect DNA-binding activities. As shown in Fig. 1, co-expression of activated c-Src kinase and either Stat1, Stat3α, or Stat3β protein induced high levels of hSIE-binding activity (lanes 1–9). As previously observed, Stat3β-hSIE complexes migrate more slowly than Stat3α-hSIE complexes, although the Stat3β splice variant has a C-terminal-deletion relative to full-length Stat3α (26, 47). Expression of either c-Src or Stat1 alone results in no DNA binding activity, while Stat3α or Stat3β expressed individually exhibits very low levels of activity, possibly due to basal levels of phosphorylation. We also expressed c-Src with Stat5A in Sf-9 cells; however, Stat5A co-expressed with c-Src did not result in any detectable MGFe binding activity (Fig. 1, lanes 10–14). These results indicate that Src is capable of efficiently activating Stat1 and Stat3, but not Stat5A, in the absence of additional mammalian tyrosine kinases.

To confirm the identities of each protein-DNA complex, we performed competition and supershift analyses as shown in Fig. 2. Shifted radiolabeled hSIE probe was competed by a 100-fold molar excess of unlabeled hSIE (Fig. 2, lanes 3, 9, and 15) but not the irrelevant FIRE oligonucleotide, demonstrating specificity of DNA binding. Stat1-hSIE complexes were supershifted only with anti-Stat1 antibody (lane 4). Most of the Stat3-hSIE complexes were blocked by anti-Stat3 antibody with some supershifted complexes detected but not affected by antibodies against Stat1 and Stat5A (lanes 10–12 and 16–18). These results suggest that Src is capable of directly activating Stat1 and Stat3 without the presence of any mammalian JAK family kinase or another intermediate protein-tyrosine kinase in Sf-9 insect cells. This finding is consistent with earlier studies of Stat3 activation in Src-transformed cells (23–25), which suggested that Stat3 is directly activated by v-Src. Interestingly, we and others did not observe the activation of Stat1 in v-Src-transformed fibroblasts (23, 24).

**Src Induces Tyrosine Phosphorylation of Stat1 and Stat3 in Sf-9 Cells—** Phosphorylation of tyrosine residues 701 of Stat1 and 705 of Stat3 are required for STAT dimerization and DNA binding (4, 48, 49). To determine whether Stat1 and Stat3 DNA binding activities induced by Src are associated with tyrosine phosphorylation, cytosolic extracts used for EMSA were analyzed by SDS-PAGE and immunoblotting with the anti-phosphotyrosine antibody, PY99. As shown in Fig. 3A, Stat1, Stat3α, and Stat3β were detected with anti-Stat1, anti-Stat3, and anti-Stat5A-specific antibodies. The position of each protein, as determined by comparing the blots in Fig. 3, A and B, is indicated by an arrow in A. In some cases, lower molecular weight forms were detected, which probably represent proteolytic products of the full-length STAT proteins (B). Stat5A did not contain tyrosine phosphorylation, since no band in Fig. 3A aligns with Stat5A protein as detected by anti-Stat5A, consistent with the EMSA results. As further
Activation of Stat3 by Src and Jak1

**Fig. 2. Identification of STAT-DNA complexes activated in Sf-9 cells.** Cytosolic lysates from Sf-9 cells infected with baculoviruses encoding STATs or Src as indicated were incubated with either nonspecific oligonucleotide competitor, FIRE, or the unlabeled hSIE oligonucleotide at a 100-fold molar excess or with anti-Stat1, anti-Stat3, or anti-Stat5A antibodies. The positions of specific STAT-hSIE complexes are indicated as Stat1, Stat3a, and Stat3b. The supershifted STAT-hSIE-antibody complexes are indicated with asterisks.

confirmation of this conclusion, anti-phospho-Stat5A antibody did not detect tyrosine-phosphorylated Stat5A (data not shown), although high levels of Stat5A protein were co-expressed with Src.

To assess the phosphorylation sites of Stat1 and Stat3 further, we reprobed the membrane with the antibodies specifically recognizing either Stat1 phosphorylated at Tyr-701 or Stat3 phosphorylated at Tyr-705. We detected the phosphorylation of these tyrosine residues in Stat1 and Stat3 (Fig. 3, C and F). These results indicate that Src phosphorylates Stat1 and Stat3 (including both Stat3a and Stat3b) at specific tyrosine sites that are required for dimerization. We also observed that Stat1 and Stat3a are phosphorylated at Ser-727 of both proteins, as detected by antibodies against phosphoserine 727-Stat1 (Fig. 3C, lanes 1 and 2) and phosphoserine 727-Stat3 (Fig. 3E, lanes 3 and 4), respectively. Stat3b lacks the Ser-727 residue (47) and did not show this modification as expected (Fig. 3E, lanes 5 and 6). The constitutive phosphorylation of serine residues in Stat1 and Stat3a has been observed previously (38), indicating that a constitutively active, endogenous insect cell serine/threonine kinase is involved in this modification. Tyr-701-phosphorylated Stat1 migrates slower than the non-Tyr-701-phosphorylated Stat1 (Fig. 3, B, C, and D, the top bands in lane 2). Tyr-705-phosphorylated Stat3 did not exhibit any shift in mobility.

**Jak1 Enhances Stat1 Activation Induced by Src—**Since Jak1 is highly activated in Src-transformed NIH3T3 cells (30), we investigated the involvement of Jak1 in the activation of Stat1 and Stat3 induced by v-Src in Sf-9 insect cells. Stat1 was phosphorylated in the presence of either Jak1 or Src in Sf-9 cells (Fig. 4A, lanes 4 and 5), indicating that both Src and Jak1 can use Stat1 as a substrate. Stat1 phosphorylation was enhanced significantly when expressed in combination with both Jak1 and Src (Fig. 4A, lane 6), and the majority of Stat1 shifted to a slower migrating form (compare lanes 4 and 5 with lane 6 in the Stat1 panel). Phosphorylation levels of Jak1 were also increased in the presence of Src (Fig. 4A, lane 6). This enhanced phosphorylation level of Jak1 may result in increased kinase activity toward Stat1, since JAK kinases are activated by transphosphorylation (4). In contrast, Stat3 was weakly activated by Jak1 compared with Src in Sf-9 cells (Fig. 4B). Co-expression of Jak1 and Src did not significantly enhance Stat3 phosphorylation compared with Src alone, although Jak1 was hyperphosphorylated in the presence of Src (Fig. 4B, lanes 10 and 12). These data indicate the differential involvement of Jak1 in phosphorylation of Stat1 and Stat3 in Sf-9 cells. In addition, the EMSA analyses of Stat1 and Stat3 DNA binding activities induced by Jak1 in combination with or without Src are consistent with these phosphorylation results (data not shown).

**Jak1 Is Essential for Stat3 Activation Induced by v-Src In NIH3T3 Cells—**The above results show that Src can activate both Stat1 and Stat3, while Jak1 activates Stat1 efficiently and Stat3 relatively weakly in Sf-9 cells. Although Src is capable of directly activating Stat3, we investigated if the mechanism of Stat3 activation is through direct interaction with Src kinase independent of other protein-tyrosine kinases, such as JAK.
kinases, in mammalian cells. We examined the Stat3 activation by Src in the presence of either a JAK kinase inhibitor, AG490, or a Src kinase inhibitor, PD180970, in NIH3T3 cells. AG490 has been shown to be selective for JAK family kinases and reported to not inhibit Src, Lck, Lyn, Btk, and Syk kinases at the levels tested (36), while PD180970 has been shown to be selective for Src family kinases (37). We first tested AG490 and PD180970 for their effects on Jak1 and Src kinase activities using in vitro kinase assays to which the compounds were directly added. Normal NIH3T3 cells show a minimal basal level of Jak1 kinase activity (Fig. 5A, lane 1), which is greatly increased in v-Src-transformed cells (lane 2) as previously reported (30). As shown in Fig. 5A, AG490 inhibits in vitro Jak1 autophosphorylation in a dose-dependent manner. At 10 \( \mu \text{M} \) of AG490, a majority of Jak1 kinase activity is inhibited (Fig. 5A, lane 4), while at 50 \( \mu \text{M} \) the Jak1 kinase activity is nearly completely abolished (lanes 5 and 6). In contrast, the Src inhibitor, PD180970, has little effect on Jak1 kinase activity even at a high concentration (Fig. 5A, lanes 7–9).

We then tested the effectiveness and specificity of PD180970 using in vitro Src kinase assays to which inhibitor was directly added with an exogenous substrate, enolase. PD180970 exhibits a dose-dependent inhibition of v-Src kinase activity (Fig. 5B), and Src kinase activity is inhibited nearly completely with 1–2 \( \mu \text{M} \) of PD180970 (Fig. 5B, lanes 6 and 7). In contrast, AG490 has no effect on Src kinase activity at 10 \( \mu \text{M} \) (Fig. 5C, lane 3); however, it does display partial inhibition at 50 \( \mu \text{M} \) and significant inhibitory effects on Src kinase activity at 100 \( \mu \text{M} \) (Fig. 5C, lanes 4 and 5). The PDGF receptor tyrosine kinase inhibitor, AG1296, has no effect on Src kinase even at 100 \( \mu \text{M} \), a concentration at which it potently inhibits PDGF receptor kinase activity (Fig. 5B, lane 8) (42). In summary, AG490 and PD180970 can specifically inhibit Jak1 and Src, respectively, within specified concentration ranges when added directly to in vitro kinase assays.

We next examined whether inhibition of JAK kinases in vivo will affect Stat3 DNA-binding activity in fibroblasts stably transformed v-Src. NIH3T3 cells transformed with v-Src were treated in vivo with various concentrations of AG490 or PD180970. As shown in Fig. 6, nuclear extracts prepared from v-Src-transformed NIH3T3 cells exhibit dose-dependent responses to AG490 and PD180970 treatment. Most of the Stat3 DNA complexes are significantly inhibited by AG490 at 10 \( \mu \text{M} \) and totally abolished at 50 \( \mu \text{M} \) (Fig. 6, lanes 3 and 5). The majority of Stat3 DNA binding ability was decreased with PD180970 at 0.5 \( \mu \text{M} \) (Fig. 6, lane 8), although no additional decrease was observed at 1 \( \mu \text{M} \) (Fig. 6, lane 9). These results demonstrate that Stat3 activation is effectively inhibited by AG490 as well as by PD180970 in a dose-dependent manner. Importantly, the majority of Stat3 inhibition by these kinase inhibitors in vivo is observed at concentrations of the inhibitors that are specific for the JAK or Src kinases in vitro (compare Figs. 5 and 6), particularly taking into consideration that the actual concentrations of the inhibitors are probably lower in vitro than in vivo at any given dosage level. These results suggest that JAK kinases are required for Stat3 activation and that the activation of Stat3 is not mediated exclusively by Src kinase in v-Src-transformed NIH3T3 cells.

**FIG. 4.** Co-expression of Jak1 with Src enhances Stat1 tyrosine phosphorylation in SF-9 cells. Stat1, Stat3a, Jak1, and c-Src were expressed from recombinant baculovirus-infected SF-9 insect cells, either alone or in the combinations indicated at the top. Approximately equal amounts of total Stat1 or Stat3 were used in lanes 4–6 and lanes 10–12, respectively, as estimated by Western blot analysis. The proteins identified by specific antibodies are indicated to the right of the immunoblot. Jak1 was detected with anti-Jak1 antibody, whereas phosphotyrosine-Jak1 (pY-Jak1) was detected after reprobing the membrane with PY-99 anti-phosphotyrosine antibody. Phosphoryrosine-Stat1 (pY-Stat1) and phosphoryrosine-Stat3 (pY-Stat3) were detected with the respective antibodies against Tyr-701-phosphorylated Stat1 and Tyr-705-phosphorylated Stat3. The expression levels of Stat1 and Stat3 were confirmed with antibodies against total Stat1 or Stat3 proteins. Src protein levels were detected with anti-Src antibodies.

**FIG. 5.** AG490 and PD180970 specifically inhibit Jak1 and Src kinases in vitro, respectively. Immunoprecipitated Jak1 and Src proteins from whole-cell lysates of normal and v-Src-transformed NIH3T3 cells were used in the in vitro kinase assays to which the JAK-selective inhibitor, AG490, or Src-selective inhibitor, PD180970, was added directly. A, equal amounts of Jak1 immunoprecipitates were incubated in each reaction and preincubated with various concentrations of the inhibitor as indicated for 5 min prior to the addition of 20 \( \mu \text{Ci} \) of \( \gamma^{32}\text{P} \)ATP. The reaction products were then analyzed by SDS-PAGE and autoradiography. B, Src immunoprecipitates from NIH3T3/ v-Src cells were divided equally and preincubated with or without various concentrations of PD180970. After a 10-min preincubation, 5 \( \mu \text{g} \) of acid-denatured enolase was added to each reaction as an exogenous substrate together with 10 \( \mu \text{Ci} \) of \( \gamma^{32}\text{P} \)ATP. Lane 1 has no enolase added as a control. C, similar to B except that AG490 at various concentrations was used instead of PD180970, and AG1296, a specific inhibitor of PDGF-R, was used as a negative control. The positions of Jak1, Src, and enolase are indicated at the left: DMSO, Me2SO.
Since Jak1 is constitutively phosphorylated in v-Src-transformed NIH3T3 cells (30) and also phosphorylated in the presence of the active Src in Sf-9 insect cells (see Fig. 4, lanes 6 and 12), we examined the tyrosine phosphorylation of Jak1 in v-Src-transformed NIH3T3 cells after treatment in vivo with Src inhibitors. We observed that tyrosine phosphorylation of Jak1 in v-Src-transformed cells is effectively inhibited by the Src inhibitor PD180970 (1 μM) (Fig. 7, lane 4). These results suggest that Jak1 is directly phosphorylated by Src in v-Src-transformed NIH3T3 cells. Jak1 tyrosine phosphorylation in vivo is only inhibited by AG490 at 50 μM, a concentration at which both Jak1 and Src kinase activities may be affected (Fig. 5C, lane 4). Thus, it is possible that both Src and Jak1 contribute to Jak1 tyrosine phosphorylation.

Inhibition of Jak1 Blocks Gene Expression Induced by v-Src—We further investigated the role of Jak1 in Stat3-mediated gene regulation induced by v-Src. We transiently transfected NIH3T3 cells with a v-Src expression vector and the Stat3 responsive reporter plasmid, pLucTKS3, as described previously (26). pLucTKS3 contains multiple copies of a Stat3-specific binding site derived from the promoter of the human C-reactive protein gene (26, 50). As shown in Fig. 8A, the JAK inhibitor, AG490, reduced Stat3-mediated reporter gene expression induced by v-Src in a dose-dependent manner, paralleling the inhibition of Stat3 DNA binding activity by AG490 (Fig. 6).

We next examined the effect of dominant negative Jak1 on Stat3-mediated gene expression (Fig. 8B). Transfection of the dominant negative Jak1 (Jak1-dn) gene with the Src gene significantly reduced the pLucTKS3 reporter gene expression. This reduction by Jak1-dn correlated with the inhibition by AG490, suggesting that Jak1 has an important role in regulating Stat3-mediated gene expression induced by Src. Transfection of the wild-type Jak1 gene alone did not substantially stimulate the reporter gene expression, indicating that Jak1 is not sufficient without Src to induce Stat3 activation, consistent with the finding that overexpression of Jak1 does not activate Stat3 in Sf-9 insect cells. Furthermore, co-transfection of wild-type Jak1 gene with the Src gene resulted in only a slight
general phosphatase inhibitor. We treated v-Src-transformed  
animal cells with Na₃VO₄ as indicated for 3 h. Equal  
amounts of protein from nuclear extracts were used for EMSA  
with radiolabeled hSIE probe. The positions of Stat3-Stat3, Stat3-Stat1,  
and Stat1-Stat1 dimers bound to DNA are indicated at the left.

increase of reporter expression compared with Src alone, sug-  
gestory that the endogenous levels of Jak1 are not limiting for  
maximal induction of Stat3 by Src in NIH3T3 cells. Thus, while  
Jak1 plays a key role in regulating Stat3-mediated gene  
activation induced by Src in NIH3T3 cells, the finding that Jak1  
does not directly phosphorylate Stat3 efficiently in SF-9 cells  
suggests an indirect role for Jak1 in enhancing Stat3 phos-  
phorylation by Src.

Inactive of Stat1 Is Not Due to Dephosphorylation—Since  
Stat1 is phosphorylated by Src in SF-9 cells, it is surprising that  
Stat1 activation is not detected in v-Src-transformed NIH3T3  
cells. One possible explanation for this is that the phosphoryl-  
ated Stat1 is rapidly dephosphorylated by a cellular phospha-  
tase. An alternative possibility is that the Stat1 is not phos-  
phorylated because it is not accessible to v-Src or Jak1 (e.g.  
Stat1 and Stat3 may employ different docking sites). To test  
the possibility that the lack of Stat1 activation in v-Src-trans-  
formed cells may be the result of rapid dephosphorylation  
rather than inaccessibility to the kinases, we used the phos-  
phatase inhibitor sodium orthovanadate. We reasoned that if a  
phosphatase is responsible for rapid Stat1 dephosphorylation  
after its activation by Src kinase, we should observe a signifi-  
cant increase of phosphorylated Stat1 in the presence of this  
general phosphatase inhibitor. We treated v-Src-transformed  
NIH3T3 cells with increasing amounts of Na₃VO₄ for extended  
periods of time and then compared the DNA binding activities  
of Stat1 and Stat3 with equal amounts of nuclear extracts (Fig.  
9). We did not observe a significant increase in the level of  
the activated Stat1 relative to Stat3 in v-Src-transformed NIH3T3  
cells (Fig. 9, lane 4 and 8). In contrast, normal NIH3T3 cells  
treated with Na₃VO₄ displayed an equal increase of Stat1 and  
Stat3 activation. Thus, in v-Src-transformed cells, lack of Stat1  
activation is probably due to inaccessibility to Src kinase rather  
than to a rapid dephosphorylation by phosphatases.

PDGF Receptor Is Required for Stat3 Activation by v-Src—  
Since our initial observation of the association between Jak1  
and Src (30), more recent studies have shown that PDGF recep-  
tor (PDGF-R), Src, and Stat3 form a multiprotein receptor  
complex in fibroblast cells (53). To examine the requirement for  
PDGF-R in STAT activation induced by v-Src, we used the cell  
line 2TGH, which is derived from the human fibrosarcoma  
HT1080 cell line and lacks PDGF-R expression (17, 19). 2TGH  
cells do not respond to PDGF stimulation unless transfected  
with PDGF-R expression plasmid (17, 19). The 2TGH cells  
were transfected with plasmids expressing either wild-type or  
kinase-inactive PDGF β-receptors (PDGF-Rβ) and subsequently  
selected with G418 for stable transfectants. The trans-  
fected G418-resistant cells were pooled (to eliminate clonal  
variation) and transiently transfected with v-Src expression  
vector. The activation of Stat3 DNA binding activity was meas-  
ured by EMSA. As shown in Fig. 10A, Stat3 is activated by  
v-Src in cells stably transfected with either wild-type (WT) or  
kinase-dead (KD) PDGF-Rβ expression vectors (lanes 2 and 3).  
Stat3 was not activated by v-Src in parental 2TGH cells that  
were not transfected with PDGF-Rβ vectors (lane 1). Cells  
transfected with either wild-type or kinase-dead PDGF-Rβ dis-  
play similar levels of receptor expression and Stat3 activation  
(compare lanes 2 and 3, EMSA and Blot). Thus, the presence  
of PDGF-R protein, independent of receptor kinase activity, plays  
a major role in Stat3 activation induced by v-Src in human  
2TGH cells.

We next investigated the role of PDGF-Rβ in Stat3-mediated  
gene regulation induced by v-Src in NIH3T3 cells. We trans-  
iently transfected NIH3T3 cells with a v-Src expression vec-  
tor, the Stat3-responsive reporter plasmid pLucTK3, and a  
plasmid expressing either wild-type or kinase-dead PDGF-Rβ.  
Consistent with the results obtained in 2TGH cells, transfec-  
tion of both wild-type and kinase-dead PDGF-Rβ further en-  
hances the Stat3-mediated reporter activity induced by v-Src  
(Fig. 10B). Unlike the 2TGH cells, NIH3T3 cells express endo-  
genous levels of PDGF-Rβ, and therefore ectopic expression  
of the receptor is not essential although it enhances Stat3  
activation. This result confirms that maximal activation of  
Stat3 by v-Src requires the expression of PDGF-Rβ protein,  
which may serve a scaffolding function for Stat3 recruitment  
and activation. However, our results do not exclude the possi-  
bility that other receptors may substitute for PDGF-R in pro-  
vinding this scaffolding function.

DISCUSSION

While constitutive activation of Stat3 signaling has previ-  
ously been shown to be required for cell transformation by the  
oncogenic Src tyrosine kinase (26, 27), the mechanism of Stat3  
activation by Src was not entirely clear. Our results presented  
here demonstrate that maximal activation of Stat3 requires  
Jak1 and PDGF-R in v-Src-transformed NIH3T3 cells, indicat-  
ing that the mechanism of Stat3 activation induced by onco-  
genic Src is more complex than a simple interaction between  
Stat3 and Src. However, in SF-9 insect cells, Src is much more  
efficient than Jak1 at phosphorylating Stat3, arguing that  
Jak1 is not acting as an intermediary kinase between Src and  
Stat3.

Furthermore, the role of PDGF-R in Stat3 activation by  
Src in NIH3T3 cells does not require the receptor’s intrinsic  
tyrosine kinase activity. Based on our findings, we propose that  
the oncogenic Src kinase activates Jak1 kinase, which in turn  
phosphorylates tyrosine sites on PDGF-R and possibly other  
receptors that provide docking sites for Stat3 (Fig. 11). In this  
model, activation of Jak1 is required for the recruitment of  
Stat3 proteins into a receptor complex with Src kinase, which  
then directly phosphorylates Stat3 at Tyr-705. Our model is  
consistent with the earlier findings that Stat3 is co-immuno-  
precipitated with Src (24, 25) and that Jak1 is constitutively  
avivated in Src transformed cells (30).

Our data (Fig. 1) demonstrate that Src can efficiently acti-  
ivate Stat1 and Stat3, but not Stat5A, in SF-9 cells in the  
absence of other mammalian kinases, suggesting that Stat1  
and Stat3 are immediate substrates of Src. Although we cannot  
quantitatively compare the phosphorylation levels of Stat1 and  
Stat3 with each other, since different antibodies are used for
each protein, we can determine the phosphorylation status of these STAT proteins (Fig. 3). Either Stat5A is not a substrate of Src or else an additional component is required for its activation. This observation is consistent with our previous findings that c-Fes activates Stat3 but not Stat5A in SF-9 cells (39) and that v-Src does not activate Stat5 in NIH3T3 cells (43). Furthermore, the observation that baculovirus-expressed Ber-Ab1 can activate Stat5A rules out the possibility that Stat5A expressed in insect cells is resistant to activation (39). Although we could not exclude the possible involvement of an insect equivalent of mammalian JAK kinase, the endogenous insect JAK counterpart would probably be expressed at very low levels compared with baculovirus-overexpressed STAT and Src proteins. We did not observe a rate-limiting step in STAT activation by Src in insect cells, suggesting that low levels of endogenous insect kinases are not involved in STAT activation by overexpressed Src. Moreover, co-expression of Jak1 did not significantly increase Stat3 activation by Src in SF-9 cells, indicating that JAK family kinases are not a factor in Stat3 activation by Src in insect cells.

Several lines of evidence support our model shown in Fig. 11. First, we show in insect cells that Src is able to activate Stat1 and Stat3 selectively, consistent with Src being the immediate upstream kinase for phosphorylation of Stat1 and Stat3. Second, Jak1 is unable to phosphorylate Stat3 efficiently, which is in striking contrast to the result that it phosphorylates Stat1 equally as well as Src does. This finding suggests that Jak1 is unlikely to be the kinase for Stat3 activation acting downstream of Src kinase in v-Src-transformed cells. Third, the JAK-selective inhibitor, AG490, and the Src-selective inhibitor, PD180970, significantly inhibit Stat3 DNA binding activity in v-Src-transformed NIH3T3 cells, which indicates a requirement for both Src and JAKs in Stat3 activation. Fourth, Jak1 is hyperphosphorylated in the presence of active Src in insect cells and NIH3T3 fibroblasts. In addition, inhibition of Src kinase activity by PD180970 abolishes Jak1 tyrosine phosphorylation in v-Src-transformed NIH3T3 cells. Since the tyrosine phosphorylation level of Jak1 has been found to correlate with its kinase activity (30), these results suggest that Jak1 is directly activated by Src in v-Src transformed NIH3T3 cells. Fifth, both AG490 and dominant negative Jak1 inhibit Stat3-mediated gene regulation, further establishing a requirement of Jak1 for Stat3 activation by Src in NIH3T3 cells. Sixth, the lack of Stat1 activation in v-Src-transformed fibroblast cells may be due to the inaccessibility (possibly resulting from the lack of Stat1-specific docking sites) to the kinases, which is consistent with the notion that membrane-bound receptors contribute to the specificity of STAT signaling (4). Previous studies (24) have shown that Stat1 can be activated by interferon-γ stimulation of v-Src-transformed NIH3T3 cells, indicating that the normal Jak1-Stat1 pathway is intact in v-Src-transformed fibroblasts. These findings point to different mechanisms for activation of Stat1 and Stat3. One plausible reason why Stat1 signaling is down-regulated in v-Src-transformed cells may be that Stat1 is involved in growth-inhibitory and proapoptosis functions (51, 52).

In myeloid cells stimulated with interleukin-3, c-Src but not JAKs is required for activation of Stat3 (28), consistent with a direct role for c-Src in Stat3 activation. Normal c-Src has been shown to be activated in epidermal growth factor- and PDGF-stimulated cells and to interact with epidermal growth factor and PDGF receptors (17, 21, 29, 53). Furthermore, one recent study (54) reported that the activation of STAT proteins induced by epidermal growth factor receptor is mediated by c-Src. Using a cell-free system, another recent study provided evidence for differences between Stat1 and Stat3 activation by PDGF (19). In particular, JAK kinases are indispensable for Stat3 activation induced by PDGF but not for Stat1 activation; however, these studies did not address the involvement of c-Src in activation of Stat1 and Stat3 in response to PDGF stimulation (19). Other studies have also suggested that c-Src activates Stat1 and Stat3 in PDGF-stimulated NIH3T3 cells (22, 53), and a multiprotein complex containing PDGF-R, c-Src, and STAT proteins has been detected (55). Moreover, there is evidence that Src and JAK family kinases are both required for PDGF-mediated Stat3 signaling in normal NIH3T3 cells (Ref...
v-Ab1 has been observed, and this interaction is essential for Stat3 activation (57). In v-Ab1-transformed cells, Jak1 is required for proliferation in BAF3 cells (57). Direct interaction of Jak1 and v-Ab1 has been observed, and this interaction is essential for Stat3 activation (57). In v-Ab1-transformed NIH3T3 cells, Jak1, but not Jak2 or Tyk2, is important in Stat3 activation by v-Ab1, since dominant negative Jak2 and Tyk2 did not affect Stat3-mediated gene expression (57).

The requirement of Jak1 kinases for STAT activation has been previously observed in other oncogenic signaling events (55). The inhibitor of JAK family kinases, AG490, blocks IL-6-dependent Stat3 activation in human multiple myeloma tumor cells (8). In human mycosis fungoides tumor cell lines, Jak3 and Tyk2 are in a complex with Stat3 and are required for Stat3 activation as well as for cell growth (56). In the case of v-Ab1-transformed cells, Jak1 is required for proliferation in BAF3 cells (57). The requirement of Jak family kinases is dependent on the specific cell type as well as the particular oncogenic signals involved.

Consistent with the results presented here, recent studies demonstrate that both Src and Jak tyrosine kinases are required for constitutive Stat3 activation in human breast cancer cell lines. The inhibition of Src or Jak1 by PD180970 or AG490, respectively, results in inactivation of Stat3 DNA binding activity and growth inhibition of these breast cancer cells. Thus, the cooperation between Src and JAK tyrosine kinases is important for the constitutive Stat3 activation in various cell types, including human tumor cell lines. Our findings provide evidence for a novel mechanism of Stat3 activation that requires cooperation of Src and Jak1 kinase in v-Src-transformed mouse fibroblasts. In this model, Jak1 has a critical role in recruiting Stat3 to a receptor complex with Src kinase, which in turn directly phosphorylates Stat3. This model may be relevant not only to oncogenic signaling by tyrosine kinases but also to normal growth factor receptor signaling.

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