Activation of Stat3 by the Src Family Kinase Hck Requires a Functional SH3 domain

Steven J. Schreiner, Anthony P. Schiavone and Thomas E. Smithgall

Department of Molecular Genetics and Biochemistry
University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA

Address all correspondence to:

Thomas E. Smithgall
University of Pittsburgh School of Medicine
Department of Molecular Genetics and Biochemistry
E1240 Biomedical Science Tower
Pittsburgh, PA 15261

Phone: (412) 648-9495
Fax: (412) 624-1401
e-mail: tsmithga@pitt.edu
Stat3 is a member of a family of transcription factors with SH2 domains that are activated by tyrosine phosphorylation in response to a wide variety of cytokines and growth factors. In this study, we investigated the mechanism of Stat3 activation by the Src family of non-receptor tyrosine kinases, which have been linked to Stat activation in both normal and transformed cell types. Using Sf-9 insect cells, we demonstrate direct Stat3 tyrosine phosphorylation and stimulation of DNA binding activity by five members of the Src kinase family (Src, Hck, Lyn, Fyn, and Fgr). We also observed stable Stat3-Src family kinase complex formation in this system. Recombinant Src family kinase SH3 domains were sufficient for interaction with Stat3, suggesting a mechanistic basis for Src kinase-Stat3 interaction. To test the contribution of Src family kinase SH3 domains to the recruitment and activation of Stat3 in vivo, we used Rat-2 fibroblasts expressing activated mutants of the myeloid Src family member Hck. Transformation of fibroblasts by an activated Hck mutant lacking the negative regulatory tail tyrosine residue (Hck-YF) induced strong DNA-binding activity of endogenous Stat3. Inactivation of Hck SH3 function by Ala substitution of a conserved Trp residue (W93A mutant) completely abolished Stat3 activation by Hck-YF and reduced transforming activity by 50% without affecting Hck kinase activity. Finally, over-expression of Stat3 in Rat-2 cells transiently stimulated Hck and c-Src kinase activity in the absence of extracellular signals, an effect that was dependent upon a putative SH3-binding motif in Stat3. These results support a model in which Src family kinases recruit Stat3 through an SH3-dependent mechanism, resulting in transient kinase activation and Stat3 phosphorylation.
The Src family of protein-tyrosine kinases regulates a diverse array of cellular processes in both normal and transformed cells, including proliferation, survival, differentiation, adhesion and motility (1-5). Of the eight members of the Src kinase family expressed in mammalian cells, several are ubiquitously expressed (Src, Yes, Fyn) while the others are restricted to specific lineages of cells including macrophages (Hck, Fgr) and lymphocytes (Lyn, Lck, Blk). Tissue-specific expression and constitutive activation in multiple tumor types has led to renewed interest in Src family kinases as targets for anti-cancer drug design (6-9).

Src kinases share the same overall structural architecture and regulation (10,11). N-terminal sequences are unique to each family member, and provide targeting signals for myristylation and in some cases palmitylation which target Src kinases to the plasma membrane. Conserved structural features include SH3, SH2 and kinase domains, followed by a short C-terminal tail region containing a conserved tyrosine phosphorylation site. Recent structural analyses of c-Src and Hck demonstrate that the SH2 and SH3 domains contribute to the negative regulation of kinase activity through intramolecular interactions (12-16). The SH2 domain binds to the negative regulatory tail, an interaction that is dependent upon prior phosphorylation by the Src tail kinase, Csk (17). The importance of tail-SH2 interaction to negative regulation is illustrated by the constitutive tyrosine kinase and transforming activities of the v-Src oncogene, which lacks C-terminal tail residues including the conserved tyrosine. The crystal structures also revealed intramolecular contacts between the SH3 domain and a polyproline type II helix formed by the linker connecting the SH2 domain with the N-terminal lobe of the kinase domain. Point mutations of linker prolines as well as some SH3 mutations can also release kinase activity and...
induce a transformed phenotype in fibroblasts, providing evidence for an essential role of this SH3-mediated interaction to overall regulation of kinase activity (18,19). Together, these SH2 and SH3-mediated interactions work through an allostERIC mechanism to push the two lobes of the kinase domain together, thus preventing substrate binding and stabilizing the inactive form of the kinase domain.

The crystal structures of the down-regulated forms of Src family kinases suggest that physiological protein-protein interactions through these domains may induce transient kinase activation, leading to substrate phosphorylation. In this report, we investigated the SH3-dependent interaction of the Stat3 transcription factor with Src family kinases in vitro and in vivo. Stats are latent cytoplasmic transcription factors that become activated by tyrosine phosphorylation, which induces Stat dimerization, nuclear translocation and transcriptional regulation (20,21). A growing body of evidence strongly supports the idea that Stats are substrates for Src family kinases, particularly Stat3 (22). For example, transformation of rodent fibroblasts with constitutively activated Src kinases results in strong activation of Stat3, and activation appears to be essential for transformation (23-26). Here we show that Stat3 is a direct substrate for all members of the Src kinase family tested, and that the SH3 domains of Src kinases are sufficient for Stat3 binding in vitro. Disruption of the SH3 domain of an activated Hck mutant causes a complete loss of Stat3 activation in fibroblasts, indicating a necessary role for Src family kinase SH3 domains in the recognition of Stat3 by Src kinases in vivo. In addition, we show that interaction with Stat3 induces a transient increase in Src kinase activity. Together these results suggest a model in which Stat3 is recruited to Src family kinases via an SH3-dependent mechanism, leading to transient kinase activation and Stat3 tyrosine phosphorylation. SH3-dependent substrate recruitment is sufficient to induce Src activation without
dephosphorylation of the negative regulatory tail, allowing Src family kinases to return to
the inactive state following release of phosphorylated Stat3.
EXPERIMENTAL PROCEDURES

*Recombinant protein expression in Sf-9 insect cells* - Sf-9 cells (InVitrogen) were cultured in Grace’s complete insect cell medium containing 10% fetal bovine serum (FBS) and 50 µg/ml gentamycin. Recombinant Stat3, Stat3YF (Stat3 mutant lacking the conserved tyrosine phosphorylation site at Tyr 705), Hck, c-Src, Lyn, Fyn, and Fgr baculoviruses were generated as described previously (27,28). Stat3 proline residues 331 and 334 were substituted with alanines to create the Stat3-2PA mutant using the Gene Editor oligonucleotide-directed mutagenesis kit (Promega). The resulting mutant cDNA was subcloned into the baculovirus transfer vector pVL1392 (Pharmingen). Stat3 was subcloned into the pVL-GST transfer vector (29) to generate the GST-Stat3 baculovirus. The Stat3-2PA and GST-Stat3 baculoviruses were produced using BaculoGold DNA and the manufacturer’s protocol (Pharmingen).

For protein expression, 2.5 x 10^6 Sf-9 cells were infected with high-titer viral stocks for 1 h at room temperature, and lysed 48 h later in 1 ml of Hck lysis buffer containing protease and phosphatase inhibitors (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, 10 mM MgCl₂, 1% Triton X-100, 0.5 mM PMSF, 20 mM NaF, 1 mM Na₃VO₄). Lysates were clarified by centrifugation prior to further analysis.

*Immunoprecipitation, immunoblotting and GSH-agarose precipitation assays* - The antibodies used in this study include anti-Hck (C-30; Santa Cruz Biotechnology), anti-Src phosphospecific (Src pY418; Biosource International), anti-phosphotyrosine (PY99; Santa Cruz), anti-Stat3 (C-20; Santa Cruz), anti-Stat3 phosphospecific (Stat3 Tyr-705; Santa Cruz), anti-cyclin D1 (Ab-3; Calbiochem), anti-actin (MAB1501; Chemicon) and anti-GST
For immunoprecipitation, clarified cell lysates were incubated with 1 \( \mu g \) primary antibody and 20 \( \mu l \) of protein G-Sepharose (50\% slurry, Amersham Biosciences) for 2 h at 4\°C. The immunoprecipitates were collected by centrifugation and washed twice with cold radioimmune precipitation assay (RIPA) buffer (18,27). For immunoblotting, clarified lysates and immunoprecipitates were heated in SDS-PAGE sample buffer and resolved on SDS-polyacrylamide gels and transferred to PVDF membranes. Following incubation with primary antibody, immunoreactive proteins were detected with an appropriate secondary antibody-alkaline phosphatase conjugate and NBT/BCIP colorimetric substrate (Sigma) or CDP-Star chemiluminescent alkaline phosphatase substrate (Perkin-Elmer). For glutathione (GSH)-agarose precipitation experiments, GSH-agarose beads (20 \( \mu l \) of a 50\% w/v slurry; Sigma) were used to precipitate the GST-Stat3/Src kinase protein complexes; beads were washed with RIPA buffer and associated Src kinases were visualized by immunoblotting.

**Expression of GST-SH3 domain fusion proteins and Stat3 binding assay** - For GST-SH3 fusion protein expression, PCR fragments encoding the SH3 domains of Hck, c-Src, Lyn, Fyn and Hck-W93A were subcloned into the bacterial expression vector pGEX-2T (Amersham Biosciences). GST and GST-SH3 fusion proteins were expressed in *E. coli* and immobilized on GSH-agaose beads as described elsewhere (30,31). Clarified lysates from Sf-9 cells expressing recombinant Stat3 were incubated with 10 \( \mu g \) of each GST-SH3 fusion protein or GST alone for 2 h at 4\°C. Stat3-SH3 complexes were precipitated by centrifugation, washed extensively in RIPA buffer, and eluted by heating in SDS-PAGE sample buffer. The samples were resolved by SDS-PAGE and Stat3 was detected by immunoblotting.
Electrophoretic Mobility Shift Assay (EMSA) for Stat3 - The sis-inducible element (SIE) double-stranded oligonucleotide probe for Stat3 binding was $^{32}$P-labeled as described elsewhere (24,28). The Stat3 EMSA was performed on equal amounts of nuclear protein extracts (1-5 µg protein) from Rat-2 fibroblasts prepared as described by Skorski, et al. (32). Binding reactions (20 µl) contained 40,000 cpm of SIE probe in 10 mM HEPES, pH 7.9, 25 mM KCl, 0.5 mM DTT, 0.5 mM EDTA, 1 µg poly dI·dC, and 5 µg bovine serum albumin. Control reactions for binding specificity contained a 100-fold molar excess of unlabeled SIE probe. Binding reactions were incubated at 30°C for 30 min and quenched on ice. Stat3-SIE complexes were resolved on 5% non-denaturing polyacrylamide gels, and radiolabeled bands were visualized by autoradiography.

Production of recombinant retroviruses and transformation assays - Retroviral expression vectors for wild-type Hck, the activated tail mutant (Hck-YF), and kinase-defective Hck (Hck-KE) have been described elsewhere (18,33,34). The SH3-inactivating mutation W93A was introduced into full-length Hck using the Gene Editor oligonucleotide-directed mutagenesis kit (Promega). This mutation was combined with Hck-YF by restriction fragment swapping to create Hck-YFW. These Hck clones as well as the coding sequences for GFP, Stat3, and Stat3-2PA were subcloned into the retroviral expression vector pSRαMSVtkneo (35). These vectors were used to generate high-titer retroviral stocks in 293T cells by co-transfection with an ecotropic packaging vector (36).

Rat-2 fibroblasts (ATCC) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS and 50 µg/ml gentamycin. Retroviral stocks were supplemented with Polybrene (hexadimethrine bromide, Sigma) to 4 µg/ml and added to
fibroblasts in 6-well plates (2 x 10^5 cells/well). The plates were centrifuged at 1,000 x g for 3 h at room temperature to enhance infection efficiency. For the focus-forming assay, 2 x 10^4 infected cells were plated in 60 mm culture dishes in the presence of 800 µg/ml G-418. Transformed foci were visualized 10-14 d later by Wright-Giemsa staining. For the soft-agar assay, 1 x 10^4 infected cells were plated in 35 mm culture dishes in DMEM containing 0.3% Seaplaque agarose (FMC). Colonies were stained with iodonitrotetrazolium violet formazan (Sigma) and counted using a BioRad Imaging Densitometer and colony-counting software (37).

*In vitro kinase assay* - Rat-2 fibroblasts stably expressing GFP, Stat3 or Stat3-2PA were infected with c-Src or Hck retroviruses as described above. Kinase activity was assessed 48 h later using the in vitro kinase assay described elsewhere (18,27). This assay employs a 50 kDa GST fusion protein containing residues 331-443 Sam 68 as substrate (38,39) (Santa Cruz Biotechnology) and [γ-^32^P]ATP (New England Nuclear).
RESULTS

Direct phosphorylation of Stat3 on Tyr 705 by Src family kinases - Recent studies have implicated Src family kinases in Stat3 activation, both in normal and transformed cell types (22,40). To investigate whether Stat3 is a direct substrate for the Src kinase family, c-Src, Hck, Lyn, Fyn and Fgr were co-expressed with Stat3 in Sf-9 insect cells using recombinant baculovirus vectors. Sf-9 cells provide a useful system to study direct tyrosine kinase-Stat3 interaction, because they lack homologs of mammalian tyrosine kinases involved in Stat3 activation (28,41). In addition, Src family kinases are constitutively active in Sf-9 cells due to the absence of Csk. Stat3 was immunoprecipitated from co-infected cell lysates and immunoblotted with anti-phosphotyrosine antibodies. As shown in Figure 1, Stat3 was strongly phosphorylated by all of the Src family members tested. Immunoblotting of a duplicate membrane with phosphospecific antibodies that recognize the tyrosine phosphorylation site responsible for Stat3 dimerization and activation (Tyr 705) indicates that Src kinases phosphorylate Stat3 on Tyr 705. To confirm this result, a Stat3 mutant lacking this tyrosine residue (Stat3YF) was co-expressed with Src family kinases and analyzed in the same manner. Anti-phosphotyrosine immunoblotting shows that Tyr 705 mutagenesis substantially reduced or eliminated Stat3 phosphorylation by Src family kinases. Interestingly, c-Src and Hck induced low-level phosphorylation of Stat3YF, suggesting that alternative phosphorylation sites may exist for these two Src family members. Control immunoblots show equal amounts of Stat3 in each lane. In addition, a control immunoblot of cell lysates with an antibody specific for the autophosphorylated Src activation loop reveals approximately equal levels of activated Src family members in each cell lysate.
Induction of Stat3 DNA Binding Activity by Src Family Kinases - We next investigated whether tyrosine phosphorylation of Stat3 by Src family kinases correlated with Stat3 DNA binding activity. Sf-9 insect cells were infected with recombinant Src family kinase baculoviruses in combination with the Stat3 or Stat3YF viruses. Electrophoretic mobility shift assays (EMSA) were then performed using clarified cell lysates and a radiolabeled Stat3-specific DNA probe (SIE) to check for the presence of Stat3 DNA binding activity. As shown in Figure 2, co-expression of Src family kinases with Stat3 induced a strong gel-shift, while co-expression of the kinases with Stat3YF did not. Over-expression of Stat3 alone (Figure 2) or the kinases alone (data not shown) did not result in a shifted band. Stat3 EMSA performed in the presence of a 100-fold molar excess of unlabeled probe resulted in complete inhibition of Stat3/DNA complex formation, indicating the specificity of the DNA binding activity for the probe (data not shown). While all of the Src kinases induced Stat3 DNA binding activity, Fyn and Fgr produced consistently lower levels of Stat3 DNA binding compared to the other kinases tested. However, this effect does not appear to be due to differences in Stat3 protein levels or tyrosine phosphorylation (Figure 1), and may instead relate to less efficient release of Stat3 from these kinases following tyrosine phosphorylation. These results indicate that Stat3 is a common substrate for the Src kinase family, and show that phosphorylation and activation occur via a direct interaction.

Stat3 forms stable complexes with Src family kinases - Previous reports have shown that Src can be co-immunoprecipitated with Stat3 from fibroblasts and other cell types, suggesting that these proteins interact in vivo (25,42). To determine if this interaction is dependent upon other mammalian proteins, Src kinases were co-expressed with a
GST-Stat3 fusion protein in Sf-9 insect cells. GST-Stat3 was precipitated from Sf-9 cell lysates with GSH-agarose beads, and Stat3-bound Src kinases were detected by immunoblotting. As shown in Figure 3, all of the Src kinases tested in this assay readily formed complexes with GST-Stat3, while no interaction was observed with GST alone. These results indicate that Src family kinases have the potential to bind directly to Stat3 in vivo. Control blots show that GST and GST-Stat3 were expressed at the same level in the infected cell lysates, and that each culture contained the same amount of each active Src family member. We also investigated the dependence of the interaction on tyrosine phosphorylation using a kinase-defective mutant of Hck. This mutant also formed a stable complex with GST-Stat3, indicating that recruitment of Stat3 is independent of kinase function (data not shown).

**Src family kinase SH3 domains bind Stat3 in vitro** - We next investigated the mechanism of Src family kinase-Stat3 interaction. Inspection of the amino acid sequence of Stat3 revealed an extended PxxP motif identical in spacing to one found in the HIV-I Nef protein, a well-characterized binding protein for the Hck and Lyn SH3 domains (43) (Figure 4A). To determine the binding activity of Src family kinase SH3 domains towards Stat3, the Hck, c-Src, Lyn and Fyn SH3 domains were expressed in bacteria as GST-fusion proteins, immobilized on GSH-agarose beads and used in binding reactions with clarified lysates from Sf-9 cells expressing recombinant Stat3. As shown in Figure 4B, all four recombinant SH3 domains were sufficient for Stat3 binding. To determine if Stat3-SH3 binding involved a typical interaction with the hydrophobic surface of SH3, we substituted Trp 93 in the Hck SH3 domain with Ala, a mutation previously shown to destroy Hck SH3 function (W93A
mutant) (44). Figure 4C shows that the W93A mutation substantially reduces binding of the Hck SH3 domain to Stat3 compared to the wild-type SH3 protein.

The Hck SH3 domain is essential for Stat3 activation in vivo - We next investigated the role of Src family kinase SH3 domains in the recruitment and activation of Stat3 in mammalian cells. For these experiments, we employed Rat-2 fibroblasts, which are readily transformed by activated mutants of Hck (18,33,34). We first investigated whether fibroblast transformation by activated Hck correlates with constitutive activation of endogenous Stat3, as shown previously for Src (24,25). Fibroblasts were infected with recombinant retroviruses carrying either an activated mutant of Hck in which the negative regulatory tail tyrosine has been replaced with phenylalanine (Hck-YF), or with wild-type Hck as a negative control. Wild-type Hck is phosphorylated on the tail tyrosine by Csk, resulting in suppression of its kinase and transforming activities in this cell type (33). Nuclear extracts were prepared from these cells and analyzed for active Stat3 by EMSA using the same SIE probe described above for the Sf-9 cell experiments. As shown in Figure 5A, Rat-2 fibroblasts expressing Hck-YF showed strong Stat3/SIE complex formation, consistent with the transformed phenotype. In contrast, Stat3 DNA binding activity was not observed in Rat-2 cells expressing wild-type Hck. These results suggest that Stat3 is an endogenous substrate for Hck in mammalian cells.

To address whether the SH3 domain of Hck is required for Stat3 activation in fibroblasts, the Hck-YF mutant was combined with the W93A SH3 mutation to create the double mutant, Hck-YFW. The W93A mutation was shown to block binding of the Hck SH3 domain to Stat3 in Figure 4C. The Hck-YFW mutant was expressed in Rat-2 fibroblasts, and Stat3 DNA binding activity was assessed by EMSA. As shown in Figure 5A, the SH3
mutation completely blocked the ability of Hck-YF to activate endogenous Stat3. As a control, the W93A mutation was also introduced into wild-type Hck, and the effect of this SH3 mutation on endogenous Stat3 activation was assessed by gel-shift assay. Figure 5A shows that this mutant was also unable to activate endogenous Stat3, despite release of kinase activity as a result of the mutation (see below). These results provide evidence for an SH3-dependent mechanism of Stat3 recognition by Src family kinases in vivo.

To determine whether loss of Stat3 activation correlates with reduced expression of a Stat3 target gene, we examined cyclin D1 expression in the same panel of Rat-2 fibroblasts. Previous studies have shown that activation of Stat3 is sufficient to induce cyclin D1 expression in this cell type (45). Rat-2 fibroblasts expressing each of the Hck constructs were serum-starved and cyclin D1 levels were determined by immunoblotting. As shown in Figure 5B, Rat-2 fibroblasts expressing the constitutively active Hck-YF mutant show an elevated level of cyclin D1 protein relative to cells expressing wild-type or kinase-defective Hck. However, introduction of the W93A SH3 mutation reduces the induction of cyclin D1 expression by Hck-YF, consistent with the inability of this mutant to activate Stat3. Similarly, introduction of the W93A mutation into wild-type Hck has very little effect on cyclin D1 expression, consistent with the lack of Stat3 activation by this Hck mutant.

**Uncoupling Stat3 activation through SH3 mutation does not affect Hck-YF kinase activity** - Results shown in Figure 5 show that mutation of the Hck SH3 domain causes a loss of Stat3 activation by Hck-YF in Rat-2 cells. To establish that this SH3 mutation affects Stat3 recognition and not kinase function, we investigated the kinase activity of this mutant by immunoblot analysis. As shown in Figure 6, Hck-YF and the corresponding SH3 mutant (Hck-YFW) were autophosphorylated to the same extent in vivo, as determined by
immunoblotting with phosphospecific antibodies for the phosphorylated activation loop. We also examined tyrosine phosphorylation of p40, an endogenous Hck substrate reported previously (18,27,33,34). Figure 6 shows that p40 is phosphorylated on tyrosine by both Hck-YF and Hck-YFW, providing further evidence that Hck-YFW retains kinase activity in vivo despite its inability to activate Stat3. This result also suggests that although the Hck SH3 domain is required for Stat3 activation, it is not required for the tyrosine phosphorylation of p40.

We also investigated the activation status of wild-type Hck, as well as the SH3 domain mutant, W93A, neither of which induced Stat3 DNA-binding activity. Wild-type Hck was not detected by the phosphospecific antibody and did not phosphorylate p40, consistent with earlier work (27,33). Hck W93A, on the other hand, was autophosphorylated and induced p40 phosphorylation, consistent with a loss of intramolecular interaction of the SH2-kinase linker with SH3 as a result of the mutation. Despite the activating effect of the W93A mutation on the kinase domain, this mutant failed to induce Stat3 activation, providing further evidence that an intact SH3 domain is essential for interaction with Stat3 in vivo.

Uncoupling Hck-YF from Stat3 via SH3 mutation reduces its transforming activity - Previous studies have shown that activation of Stat3 is essential for the full transforming activity of Src in fibroblasts (23). Our observation that a point mutation in the Hck SH3 domain uncouples Hck-YF from Stat3 activation suggests that transformation signaling may be affected as well. To test this possibility, we compared the transforming activity of Hck-W93A, Hck-YF, and Hck-YFW with wild-type Hck in focus-forming and soft-agar colony assays, and the results are shown in Figure 7. Wild-type Hck did not demonstrate
transforming activity in either assay, while Hck-YF was strongly transforming in both systems, consistent with earlier results (18,33). Introduction of the SH3 mutation reduced but did not abolish the transforming activity of Hck-YF, consistent with the idea that Stat3 activation is important for the transforming signal of Hck in fibroblasts but is not solely responsible for the transformed phenotype. Hck bearing only the SH3 point mutation also showed lower focus- and colony-forming activities relative to Hck-YF, despite readily detectable autophosphorylation (Figure 6). This is because the SH3 domain mutation has two opposing actions on Hck transforming function. On one hand, the W93A mutation releases the SH3 domain from the SH2-kinase linker, leading to loss of negative regulation. On the other hand, this mutation also uncouples Hck from Stat3 and other substrates that are recruited via SH3. The net effect is the partial transforming phenotype seen in Figure 7. Morphological differences in the foci formed by Rat-2 cells expressing the various Hck mutants were observed as well. Hck-YF produced large spreading foci, while those formed by Hck-W93 and Hck-YFW were smaller with more tightly clustered cells (data not shown). These data clearly show that an intact SH3 domain and Stat3 activation are required for the full transforming activity of Hck in Rat-2 fibroblasts. However, the ability of Hck mutants to transform cells in the absence of Stat3 activation suggests that Stat3 is not absolutely required for transformation to occur (see Discussion).

Co-expression with Stat3 activates c-Src tyrosine kinase activity - Previous work from our laboratory has shown that engagement of Src family kinase SH3 domains by other proteins is sufficient to induce activation of the kinase domain in vivo (18,33,34). The mechanism of activation involves displacement of the negative regulatory interaction between SH3 and the polyproline helix formed by the SH2-kinase linker region (see
Introduction). Our observations that Src family kinase SH3 domains are sufficient for Stat3 binding (Figure 4) and that SH3 function is required for Stat3 recruitment in vivo (Figure 5) suggest that Stat3 binding might activate Src family kinases. Indeed, the β-barrel region of Stat3 has a short PxxP motif with identical spacing to HIV Nef, a well-established SH3-binding protein and activator of Hck (Figures 4 and 8A) (33,34,46). To investigate the effect of Stat3 on Src family kinase activity, Rat-2 fibroblasts stably expressing c-Src or Hck were infected with Stat3 or GFP retroviruses. Forty-eight hours later, c-Src and Hck were immunoprecipitated and assayed in vitro using a Sam-68 fusion protein (p50) as substrate. As shown in Figure 8, co-expression with Stat3 caused an increase in both c-Src and Hck kinase activity toward p50 compared to the cells over-expressing GFP. A reciprocal experiment in which cells stably expressing GFP or Stat3 were infected with Src or Hck retroviruses yielded similar results (data not shown). Interestingly, a mutant form of Stat3 in which the core prolines in the putative SH3-binding sequence have been changed to alanines (Stat3-2PA; Figure 8A) produced a smaller increase in Src and Hck kinase activities (Figure 8B and 8C). These results support the hypothesis that SH3-dependent binding of Stat3 may result in transient activation of Src kinase activity in vivo as part of a Src-Stat3 activation pathway (see Discussion).
DISCUSSION

Stats are latent cytoplasmic transcription factors that require tyrosine phosphorylation for their dimerization and activation (21). Many families of both receptor and non-receptor protein-tyrosine kinases have been implicated in Stat activation (47). In this report, we focused on the molecular mechanisms of Src family kinase interaction with Stat3. A growing body of evidence has implicated the Src kinase family in Stat3 activation, both in normal and transformed cell types (23-25,41,42,48-53). This has led to the question of whether Stat3 is a target for all members of the Src kinase family, and whether Src kinases phosphorylate Stat3 directly. Using the baculovirus/Sf-9 insect cell system, we demonstrate that Stat3 is a substrate for a variety of Src family members, including Hck, Fyn, Lyn, and Fgr in addition to c-Src itself. We also observed that Stat3 stably associates with Src family kinases in this system, supporting a direct relationship between these signaling proteins in vivo.

Although these results suggest that Src family kinases have the potential to associate directly with Stat3 under physiological conditions, recent work indicates that other kinases may participate in the activation mechanism in mammalian cells. Using kinase-selective pharmacological inhibitors, Zhang et al. have shown that Src and Jak2 may cooperate in the activation of Stat3 in v-Src transformed fibroblasts (41). Their model suggests an essential role for Jak2 in the recruitment of Stat3 to a growth factor receptor such as the PDGF receptor, followed by Src-induced tyrosine phosphorylation of Stat3. Using a related approach, Wang et al. (49) established that Stat3 activation by PDGF also requires c-Src. In both cases, Src is proposed as the kinase responsible for the Stat3 phosphorylation event. Our data suggest that simultaneous recruitment of Stat3 and Src
to the PDGF receptor and possibly other growth factor and cytokine receptors may promote Src-Stat3 interaction through the SH3-dependent mechanism identified here.

Immunoblot analysis with phosphospecific antibodies shows that all Src family members recognize the Stat3 tyrosine residue essential for dimerization and activation (Tyr 705) (54). Using a Stat3 mutant that lacks Tyr 705 (Stat3YF), we investigated if any additional sites of tyrosine phosphorylation were present in Stat3. Unexpectedly, Hck and Src were able to phosphorylate Stat3YF, suggesting the presence of phosphorylation sites in addition to Tyr 705. Although the DNA binding of Stat3YF was not detectable upon co-expression with Hck or Src, these additional phosphorylation sites may be required for other Stat3 functions, such as the recruitment of proteins with SH2 domains to form a signaling complex. An adaptor function for Stat3 has been described for interferon receptor signaling. In this case, tyrosine phosphorylation of interferon receptor-bound Stat3 created a docking site for the p85 regulatory subunit of phosphatidylinositol 3-kinase via its SH2 domain (55). Whether or not the secondary sites for Src and Hck are phosphorylated under physiological conditions and their contribution to the Src-Stat3 signaling pathway will require further investigation.

Several previous reports have demonstrated interactions between Src family kinases and Stat3 (25,42), and work presented here supports a direct association between these proteins. In addition, we show for the first time that Src kinase SH3 domains are sufficient to bind Stat3 in vitro, suggesting a mechanism for Src-Stat3 interaction in vivo. Strong support for this hypothesis is provided by studies of an active mutant of Hck (Hck-YF) in which SH3 function was ablated by substituting a conserved tryptophan residue with alanine (W93A). Previous studies have shown that substitution of this conserved Trp residue inactivates SH3 function both in Hck and Lck (44,56). The Hck W93A mutation
completely blocked Stat3 activation by Hck-YF in fibroblasts without affecting kinase autophosphorylation or tyrosine phosphorylation of the endogenous substrate protein, p40. In a related experiment, we found that the W93A mutation released the Hck kinase domain from negative regulation in vivo, yet this mutant was also unable to recruit and activate Stat3. Together, these findings provide a compelling argument for the SH3-dependent recruitment and activation of Stat3 by Hck and other Src family kinases, at least in this model system. An activating effect of Src family kinase SH3 domain mutations has also been reported for c-Src and Lck (57-59). Whether or not these mutations also affect Stat3 recruitment and phosphorylation has not been reported.

Previous studies have established that Stat3 activation is important for fibroblast transformation by activated mutants of Src. Constitutive Stat3 activation and Src-Stat3 complex formation is readily detected in Src-transformed fibroblasts (24,25,52). More recent studies have shown that Stat3 activation may be required for Src-induced transformation (23,26). These experiments used dominant-negative forms of Stat3, which were found to block Src-mediated transformation of fibroblasts. While our data suggest that Stat3 activation is required for maximal transformation by the Hck tail mutant (Hck-YF), uncoupling Hck-YF from Stat3 activation through SH3 mutation reduced but did not eliminate transforming activity (Figure 7). This finding suggests that Stat3 is important but not absolutely required for fibroblast transformation by Hck-YF.

The finding that the W93A SH3 mutation was able to release Hck from negative regulation in vivo provides further evidence for a dominant role for the SH3 domain in the negative regulation of Hck. The crystal structures of Hck and Src show that the SH3 domain engages the SH2-kinase linker, and that this interaction helps to maintain the kinase in the inactive conformation (see Introduction). Binding of proteins to the SH3
domain, which induces SH3-linker displacement, and mutagenesis of the linker prolines essential for intramolecular SH3 binding both lead to constitutive Hck activation and fibroblast transformation, even in the presence of tail phosphorylation and SH2 engagement (33). Work presented here shows that mutagenesis of SH3 also promotes constitutive Hck autophosphorylation in vivo, presumably by releasing the negative regulatory interaction with the SH2-kinase linker (Figure 6). However, this mutant shows substantially lower transforming activity compared to the Hck SH2-kinase linker mutant (18), presumably because it is uncoupled from Stat3 activation and possibly other SH3-dependent transforming pathways downstream.

Data presented here support an SH3-dependent mechanism for Stat3 recruitment in vivo. Because previous studies have shown that SH3 engagement can induce Src family kinase activation, we tested whether Stat3 over-expression affected Src family kinase activity in fibroblasts. Wild-type Stat3 caused a transient activation of c-Src and Hck, while a Stat3 mutant lacking a consensus SH3-binding motif showed a markedly reduced effect (Stat3-2PA; Figure 8). Despite the reduced impact of Stat3-2PA on Src and Hck activity in fibroblasts, this mutant still associated with Hck in the Sf-9 cell co-expression system (data not shown). These results suggest that Src family kinases associate with Stats in a complex manner involving more than one interacting surface, but that the PxxP motif shown in Figure 8 is required for the observed effects on kinase activity. Kinase activation has been reported following association of other proteins with Src family kinase SH3 domains, including HIV Nef (18,33,34), the focal adhesion protein p130 Cas (60,61), and the progesterone receptor (62). These results support a general model in which SH3-dependent recruitment of substrates may induce transient activation of Src family kinases through a linker displacement mechanism (63). In the case of Stat3, phosphorylation
induces release from the kinase, dimerization and nuclear translocation. Once Stat3 is released from SH3, the kinase may return to the inactive state by re-establishing the SH3-linker interaction. Such a model suggests that local concentrations of Stat3 may control the activity of Src kinases. Thus recruitment of Stat3 to cytokine or growth factor receptors may induce the transient activation of receptor-associated Src family kinases through their SH3 domains, leading to Stat activation.

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Fig. 1. **Src family kinases phosphorylate Stat3 on Tyr 705.** Wild-type Stat3 or a Stat3 mutant with a Phe substitution for Tyr 705 (Stat3YF) were expressed either alone (Con) or with the Src family members Hck, Src, Lyn, Fyn, or Fgr in Sf-9 insect cells. Stat3 proteins were immunoprecipitated from clarified cell lysates and immunoblotted with antibodies specific for phosphotyrosine (P-Tyr), Stat3 Tyr 705 phosphorylation (P-Stat3), and the Stat3 protein. Lysates were immunoblotted with a phosphospecific antibody directed against the activation loop to verify Src family kinase expression and activity. This experiment was repeated three times and produced comparable results in each case; a representative example is shown.

Fig. 2. **Direct activation of Stat3-DNA complex formation by Src family kinases.** Wild-type Stat3 or a Stat3 mutant with a Phe substitution for Tyr 705 (Stat3YF) were expressed either alone (Con) or with the Src family members Hck, Src, Lyn, Fyn, or Fgr in Sf-9 insect cells. Clarified cell lysates were prepared and tested for the presence of Stat3 DNA binding activity by EMSA with a radiolabeled SIE probe. The positions of the gel-shifted Stat3-SIE complex and the free probe are indicated by the arrows. This experiment was repeated three times and produced comparable results in each case; a representative example is shown.

Fig. 3. **Association of Src family kinases with Stat3.** A GST-Stat3 fusion protein or GST alone were co-expressed with the Src family members Hck, Src, Lyn, Fyn, or Fgr in Sf-9 insect cells. Protein complexes were isolated from clarified cell lysates with GSH-agarose beads. The beads were washed with RIPA buffer, and associated Src family
kinase proteins were resolved by SDS-PAGE and visualized by immunoblotting with a phosphospecific antibody directed against the autophosphorylated activation loop (Bound: Kinase). Equivalent recovery of GST and GST-Stat3 was verified by immunoblotting with anti-GST antibodies (GST; GST-Stat3). Kinase expression was verified in the cell lysates by immunoblotting with the activation loop-specific antibody (Lysate:Kinase). This experiment was repeated three times and produced comparable results in each case; a representative example is shown.

Fig. 4. **Src family kinase SH3 domains are sufficient to bind Stat3.** A) Alignment of the HIV-1 Nef PxxP motif known to mediate binding to the Hck and Lyn SH3 domains (43) and a putative PxxP sequence in the distal portion of the Stat3 β-barrel region. B) SH3 binding assay. SH3 domains from Hck, Src, Fyn, and Lyn were expressed in bacteria as GST fusion proteins and immobilized on GSH-agarose. Clarified lysates were prepared from Sf-9 cells expressing Stat3, and aliquots of the lysate were incubated with the immobilized GST-SH3 fusion protein or GST alone as a negative control. The reactions were then washed, resolved by SDS-PAGE, and associated Stat3 was detected by immunoblotting. C) Stat3 binding requires Trp 93 on the Hck SH3 domain. An alanine substitution was introduced for Trp 93 on the binding surface of the Hck SH3 domain, and the resulting mutant SH3 (W93A) was expressed in bacteria as a GST fusion protein. The binding activity of this SH3 mutant toward Stat3 was compared to that of the wild-type Hck SH3 domain (WT) as described in Part B. These experiments were repeated three times and produced comparable results in each case; a representative example is shown.
Fig. 5. A functional SH3 domain is essential for Hck-induced activation of Stat3 DNA binding activity and cyclin D1 expression in fibroblasts. A) Gel-shift assay. Rat-2 fibroblasts stably expressing wild-type Hck (WT), an activated tail mutant (YF), an SH3 domain point mutant (W93A), or a double mutant bearing both the SH3 and tail mutations (YFW) were examined for the presence of active Stat3 by EMSA with a radiolabeled SIE probe. The positions of the gel-shifted Stat3-SIE complex and the free probe are indicated by the arrows. This experiment was repeated three times and produced comparable results in each case; a representative example is shown. B) Cyclin D1 expression. Rat-2 fibroblasts stably expressing wild-type Hck (WT), a kinase-defective mutant (KE), an activated tail mutant (YF), an SH3 domain point mutant (W93A), or a double mutant bearing both the SH3 and tail mutations (YFW) were serum-starved for 72 h, and protein extracts were probed for cyclin D1 expression by immunoblotting. Cells expressing GFP were included as an additional negative control (Con). The membranes were stripped and re-probed with an actin antibody as a loading control. Expression of Hck proteins was verified by immunoblotting a replicate membrane with anti-Hck antibodies. The positions of the cyclin D1, Hck and actin bands are indicated by the arrows.

Fig. 6. Analysis of kinase activity of Hck SH3 domain mutants. Rat-2 fibroblasts stably expressing wild-type Hck (WT), a kinase-defective mutant (KE), an activated tail mutant (YF), an SH3 domain point mutant (W93A), a double mutant bearing both the SH3 and tail mutations (YFW) or GFP alone as a negative control (Con) were lysed in RIPA buffer and clarified protein extracts were resolved by SDS-PAGE and transferred to PVDF membranes. Replicate membranes were probed for autophosphorylated Hck with an antibody specific for phosphorylated Tyr 390 in the activation loop (PY390), for the
endogenous Hck substrate p40 with anti-phosphotyrosine antibodies (p40), and Hck and Stat3 protein levels.

Fig. 7. **Transforming activity of Hck SH3 domain mutants.** Rat-2 fibroblasts were infected with recombinant retroviruses carrying wild-type Hck (WT), an activated tail mutant (YF), an SH3 domain point mutant (W93A), or a double mutant bearing both the SH3 and tail mutations (YFW). A) Focus assay. Infected cells were plated in 60 mm culture plates and incubated under G-418 selection for two weeks. Foci were visualized by Wright-Giemsa staining, and scanned images of the dishes are shown. This experiment was repeated three times with similar results. B) Soft-agar colony assay. Infected cells were plated in triplicate 35 mm culture plates in DMEM containing 0.3% agarose and transformed colonies were allowed to develop for three weeks. Colonies were stained with iodonitrotetrazolium violet formazan and counted using a BioRad Imaging Densitometer and colony-counting software. Results show the mean colony counts ± S.D. Three independent experiments produced the same pattern of colony formation; a representative example is shown.

Fig. 8. **Co-expression with Stat3 stimulates c-Src and Hck kinase activity.** A) Schematic diagram of Stat3, showing the position of the putative PxxP motif within the overall Stat3 structure. Prolines residues 331 and 334 were mutated to alanine to create the Stat3-2PA mutant. Structural features of Stat3 include an N-terminal region involved in dimer-dimer interaction along DNA (N), a four-helix bundle (4HB), a β-barrel (βBBL), an SH2 domain, a conserved tyrosine phosphorylation site required for dimerization (Yp), and a C-terminal transcriptional transactivation domain (AD) (54). B,C) Kinase activity assay.
Rat-2 fibroblasts stably expressing c-Src (Part B) or Hck (Part C) were infected with recombinant retroviruses carrying GFP, Stat3 or Stat3-2PA. Forty-eight hours later, Src and Hck were immunoprecipitated from clarified cell lysates and incubated with $[^\gamma-32P]ATP$ and a 50 kDa GST-Sam 68 fusion protein (p50). Following incubation, p50 was resolved by SDS-PAGE, transferred to PVDF membranes and labeled proteins were visualized by autoradiography ($^{32}P$; top). The membranes were subsequently immunoblotted for c-Src or Hck to control for equal loading (middle). Expression of Stat3 and Stat3-2PA were verified by immunoblotting of the infected cell lysates (bottom).
## Fig. 1

|        | Stat3 | Stat3YF |
|--------|-------|---------|
| Con    | ![Image](image1) | ![Image](image2) |
| Hck    | ![Image](image3) | ![Image](image4) |
| Src    | ![Image](image5) | ![Image](image6) |
| Lyn    | ![Image](image7) | ![Image](image8) |
| Fyn    | ![Image](image9) | ![Image](image10) |
| Fgr    | ![Image](image11) | ![Image](image12) |

- **P-Tyr**
- **P-Stat3**
- **Stat3**
- **Kinase**
Fig. 3

| GST        | GST-Stat3 |
|------------|-----------|
| Bound: Kinase | GST-Stat3 |
| GST-Stat3  | GST-Stat3 |
| Lysate: Kinase | GST-Stat3 |

(Hck, Src, Lyn, Fyn, Fgr)
Fig. 5

A

WT  YF  W93A  YFW

Stat3 + SIE

Free Probe

B

Con  WT  KE  YF  W93A  YFW

Cyclin D

Hck

Actin
Fig. 6

|       | Con | WT | KE | YF | W93A | YFW |
|-------|-----|----|----|----|------|-----|
| PY390 |     |    |    |    |      |     |
| p40   |     |    |    |    |      |     |
| Hck   |     |    |    |    |      |     |
| Stat3 |     |    |    |    |      |     |
Fig. 7

A

Colony images for WT, YF, W93A, and YFW.

B

Bar graph showing colonies for WT, YF, W93A, and YFW.

Colonies

WT  YF  W93A  YFW
Activation of Stat3 by the Src family kinase Hck requires a functional SH3 domain

Steven J. Schreiner, Anthony P. Schiavone and Thomas E. Smithgall

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