Pyk2 Amplifies Epidermal Growth Factor and c-Src-induced Stat3 Activation*

Received for publication, October 29, 2003, and in revised form, February 11, 2004
Published, JBC Papers in Press, February 12, 2004, DOI 10.1074/jbc.M311875200

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Signal transducers and activators of transcription factors (STATs) mediate many of the cellular responses that occur following cytokine, growth factor, and hormone signaling. STATs are activated by tyrosine and serine phosphorylation, which normally occurs as a tightly regulated process. Dysregulated STAT activity may facilitate oncogenesis, as constitutively activated STATs have been found in many human tumors as well as in v-abl- and v-src-transformed cell lines. Pyk2 is a member of the focal adhesion kinase family and can be activated by c-Src, epidermal growth factor receptor (EGFR), Janus kinase 1, tyrosine kinases, and G-protein-coupled receptor signaling. Although Pyk2 has been implicated in Janus kinase-dependent activation of MAPK and Stat1, no role for Pyk2 in the activation of other STATs has been ascribed. Here, we provide evidence that Pyk2, along with c-Src, facilitates EGFR-mediated Stat3 activation. Pyk2 expression in HeLa cells induces Stat3 reporter gene activation and Stat3 phosphorylation on amino acid residues Tyr-705 and Ser-727. Together Pyk2 and c-Src potently activate Stat3, and Pyk2 enhances Stat3-induced cell proliferation. Moreover, the expression of a dominant negative version of Pyk2 impairs c-Src-induced Stat3 activation and cell proliferation. The treatment of A431 cells with EGF results in the recruitment of c-Src, Pyk2, and Stat3 to the EGFR and the phosphorylation of c-Src, Pyk2, and Stat3. Expression of constructs for dominant negative forms of either Pyk2 or c-Src impair EGF-induced Stat3 phosphorylation. These results indicate that Pyk2 facilitates EGFR- and c-Src-mediated Stat3 activation, thereby implicating Pyk2 activation as a potential co-mediator in triggering Stat3-induced oncogenesis.

Stat3 was first described as an IL-6-inducible DNA binding activity reactive with the acute phase response element (1–3). Molecular characterization led to its identification as a STAT protein and the demonstration that not only IL-6 but also other cytokines (which use gp130 as a signal transducer) potently induced its activity (4). Subsequent studies revealed that other agents, including growth factors, interferons, and oncoproteins, also activate Stat3 (5–7). Ablation of the Stat3 locus in mice led to an early embryonic lethality complicating the assignment of its precise biologic role (8). The analysis of mice in which Stat3 has been disrupted in various adult tissues has led to a recognition that Stat3 participates in a diverse set of cellular responses. These include the migration of keratinocytes (9), the survival of thymic epithelial cells (10), IL-2R expression on T lymphocytes (11), apoptosis in the mammary gland epithelium (12), modulation of inflammation (13), the induction of the acute phase response in the liver (14), and the survival of sensory and motor neurons (15, 16). Despite the lack of a clear molecular understanding of the roles of Stat3 in embryonic and even adult tissues, Stat3 has emerged as a critical mediator in the pathogenesis of a variety of human cancers.

Evidence for the role of Stat3 in human cancer includes the following observations (reviewed in Refs. 17–19). First, constitutively active forms of Stat3 can induce partial cellular transformation. Second, Stat3 is activated by oncogenic tyrosine kinases including v-Src and bcr-abl. Third, dominant negative forms of Stat3 can block cellular transformation induced by these oncogenic tyrosine kinases. Fourth, Stat3 activation leads to the activation of target genes involved in cell proliferation and survival implicating its essential pathways involved in oncogenesis. Fifth, activated Stat3 has been found in human malignancies.

v-Src is a potent oncoprotein, and the activation of Stat3 is critical for its transforming ability (6). In addition, c-Src links IL-5 receptor (21), platelet-derived growth factor receptor (22), epidermal growth factor receptor (EGFR) (3), and angiotensin II AT1 receptor (23) signaling to Stat3 activation. However, the mechanism by which c-Src activation leads to Stat3 activation remains unclear. c-Src family SH3 domains have been reported to directly interact with Stat3, leading to Stat3 tyrosine phosphorylation (24). Another study implicated Etk, a Tec family tyrosine kinase, as an intermediary in v-Src-induced Stat3 activation and transformation (25). Etk is expressed in a variety of tissues including hematopoietic, epithelial, and endothelial cells. Besides linking v-Src to Stat3 activation, Etk participates in IL-6-induced differentiation of prostate cancer cells (26), functions as an intermediary in Ga12/13-induced activation of serum response factor (27), and mediates cell motility in signaling pathways that become activated upon integrin-triggered cell adhesion (28). We have reported previously that the proline-rich tyrosine kinase Pyk2 processes similar upstream information and coordinates the activation of similar downstream signaling pathways, as do the Tec kinases (29). Furthermore, both Tec kinases and Pyk2 participate in cell migration (28, 30, 31).

Pyk2 and focal adhesion kinase (Fak) are members of a distinct family of nonreceptor protein tyrosine kinases that are...
regulated by a variety of extracellular stimuli. Integrins, tumor necrosis factor α, T cell receptor, B cell antigen receptor, G-protein-coupled receptors (GPCRs), vascular endothelial growth factor, EGFR, and platelet-derived growth factor all stimulate Pyk2 activation (reviewed in Refs. 32 and 33). Many GPCRs trigger Pyk2 tyrosine phosphorylation, and the formation of a Pyk2 and c-Src complex leads to activation of MAPK signaling pathways (34). Furthermore, many stress signals utilize Pyk2 activation to link to the Jun kinase pathway. The introduction of Pyk2 expression constructs results in Jun kinase activation, indicating that overexpressed Pyk2 has constitutive activity and is able to trigger downstream pathways without an activating signal (35). Finally, Pyk2 has been reported to be critical for the Janus kinase (JAK)-mediated MAPK and Stat1 activation by interferon-γ but not interferon-α (36). Pyk2 selectively associates with Jak2, resulting in Stat1 activation following interferon-γ treatment.

Based on the overlapping functional roles of the Tec family kinases and Pyk2 and because of the established role of Pyk2 in Stat1 activation and the known role of c-Src in Pyk2 activation, we investigated whether Pyk2 participated in c-Src- and EGFR-mediated Stat3 activation. We report that Pyk2 facilitates c-Src-mediated Stat3 activation and participates in EGFR receptor signaling to Stat3 activation.

EXPERIMENTAL PROCEDURES

Plasmids and Antibodies—The expression constructs pcDNA3-Myc-Pyk2 and pcDNA3-Myc-Pyk2 (K457A and D567A), referred to as Pyk2 KD, were kindly provided by Dr. S. Earp (University of North Carolina). The Stat3 luciferase reporter gene plasmid and Stat3 expression vector were kindly provided by Dr. Geeta Devgan (The Rockefeller University). pUSE-c-Src, activated c-Src, pUSE-c-Src (Y529F), dominant negative c-Src, pUSE-c-Src (K297R), and kinase-dead c-Src, pUSE-c-Src were purchased from Upstate Biotechnology, Lake Placid, NY. Antibodies against the following proteins were purchased: Pyk2 from Transduction Laboratories, San Diego, CA; phospho-Stat3(Y705), phospho-Stat3(Ser727), phospho-Stat3(Tyr416), c-Src, and EGFR receptor from Upstate Biotechnology; Stat3 and ERK from Santa Cruz Biotechnology, Inc., Santa Cruz, CA; and phospho-ERK1/2 (Thr202/Tyr204) antibodies from Cell Signaling, Beverly, MA. Transfections and Reporter Gene Assays—HeLa and A431 cells were obtained from the American Type Culture Collection (Manassas, VA). The cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and transfected using SuperFect (Qiagen Inc., Valencia, CA) in 6-well plates following the manufacturer’s protocol. The collected cells were lysed in 200 μl of reporter lysis buffer (Promega, Madison, WI) for 30 min on ice. After centrifugation, 20 μl of the supernatant was tested for β-galactosidase activity, using galactan chemiluminescent substrate (Tropix, Bedford, MA), or luciferase activity, using a luciferase substrate (Promega). Data from all the transfection assays were normalized using the activity of a control reporter gene.

Immunoblotting and Immunoprecipitations—HeLa cell lysates were prepared using reporter lysis buffer (Promega) for 30 min on ice. The detergent-insoluble material was removed by centrifugation for 10 min at 14,000 rpm, and 4 °C. Equal amounts of protein from each sample were fractionated by SDS-PAGE and transferred to pure nitrocellulose. Membranes were blocked with 5% milk in TTBS (Triton X-100, and 1 m M dithiothreitol) and three with kinase reaction buffer and twice with 500 mM NaCl salt in the same lysis buffer. Subsequently, they were fractionated by SDS-PAGE and analyzed by immunoblotting with the appropriate antibody.

Cell Proliferation Assay—HeLa cells (1 × 10^5/well) were seeded in 24-well plates. After incubating overnight, the cells were transfected as described above with different plasmids as indicated. Before labeling the cells, the cells were cultured in 0.5% serum-containing medium for 24 h. Then the medium was changed with 10% (v/v) Cell Counting Kit-8 labeling solution (Dojindo Molecular Technologies, Inc., Gaithersburg, MD), and the cells were incubated for about 15 min. The medium from the labeled cells was transferred to a 96-well plate, and the absorbance of 450 nm was measured on a plate reader. For the thymidine incorporation assays, HeLa cells (1 × 10^5/well) were seeded in a 96-well plate and cultured for 5 h prior to DNA transfection (40 ng/well) using LipofectAMINE 2000 (Invitrogen). 24 h later, the cells were pulsed for 4 h with 0.2 μCi of [3H]thymidine, after which the cells were harvested. The amount of [3H]thymidine incorporated was measured using a β-counter following the addition of scintillation fluid.

In Vitro Kinase Assay—Pyk2 immunoprecipitates were washed three times with kinase lysis buffer (20 mM Hepes, pH 7.4, 2 mM EGTA, 50 mM a-glycerophosphate, 1% Triton X-100, 1 mM Na_3VO_4, and 10% glycerol) with protease inhibitors. Following six additional washes, three with a LiC wash buffer (500 mM LiCl, 100 mM Tris, pH 7.4, 0.1% Triton X-100), and one with dithiothreitol) and three with kinase reaction buffer (30 mM Hepes, pH 7.5, 10 mM MgCl_2, and 2 mM MnCl_2), the kinase assays were performed. Poly(Glu,Tyr) (4:1) (Sigma) was used as a substrate.

RESULTS

Stat3 Is Activated by Pyk2 Expression—We used two measures of Stat3 activation: the response of a Stat3-sensitive reporter gene and the status of Stat3 phosphorylation using phosphopeptide-specific antibodies. Phosphorylation of Tyr-705 of Stat3 is required for Stat3 dimerization, nuclear translocation, and DNA binding activity (37). Phosphorylation of Ser-727 of Stat3 enhances its transcriptional activity (38). We first determined whether Pyk2 overexpression resulted in enhanced transcription of a Stat3 reporter gene by transfecting HeLa cells with a luciferase reporter gene that contained four copies of the Stat3 binding site fused to a minimal promoter along with different amounts of the Pyk2 expression construct. Pyk2 introduced into cells by transient transfection has constitutive activity, which can be boosted by upstream activating signals (35). In our experiments, expression of Pyk2 resulted in a modest increase in Stat3 reporter gene activity (Fig. 1A). To detect Stat3 phosphorylation, we transfected expression constructs for Pyk2 and Stat3 into HeLa cells and checked the status of Stat3 phosphorylation using phospho-Stat3 antibodies. Pyk2 dramatically induced the phosphorylation of Stat3 on Tyr-705 and Ser-727, whereas a kinase inactive form of Pyk2 (Pyk2 KD) did not induce Stat3 phosphorylation on the same residues (Fig. 1B). Similar to the Pyk2-induced Stat3 reporter gene activity, enhanced expression of Pyk2 resulted in a higher amount of Stat3 phosphorylation on both residues (Fig. 1C). These results indicate that Pyk2 overexpression can lead to Stat3 activation.

Pyk2 Facilitates Src-induced Stat3 Activation—As a first test of our hypothesis that Pyk2 participates in the activation of Stat3 by c-Src, we co-transfected DNA constructs that express Stat3, activated Src, wild type, and the kinase-inactivated form of Pyk2 and measured Stat3 reporter gene activity and the status of Stat3 Tyr-705 and Ser-727 phosphorylation. In these experiments, we used HeLa cells, which express a low level of endogenous Pyk2. The results show that Pyk2 and activated c-Src individually and additively activate the Stat3 reporter gene (Fig. 2A). Expression of Pyk2 tended to result in the preferential phosphorylation of Stat3 on Ser-727, whereas activated c-Src resulted in preferential phosphorylation of Stat3 on Tyr-705. Together they induced a very strong phosphorylation.

null

null
Pyk2 expression is shown. Experiments. Data are reported as -fold induction. An immunoblot of expressing Stat3 (0.25 μg). HeLa cells were transfected constructs expressing Stat3 Tyr-705 and Ser-727. HeLa cells were transfected constructs expressing Stat3 Tyr-705, phospho-Stat3 Ser-727, Stat3, and Pyk2 are shown. C, dose-dependent activation of Stat3 phosphorylation on Tyr-705 and Ser-727. HeLa cells were transfected with different concentrations (0.5, 1.0 μg) of Pyk2 expression vector and a Stat3 expression vector (0.25 μg). The levels of phospho-Stat3 Tyr-705, phospho-Stat3 Ser-727, Stat3, and Pyk2 are shown.

Pyk2 Associates with Stat3—Because Pyk2 and c-Src individually and together potently activate the Stat3 reporter gene and induce Stat3 phosphorylation and because Pyk2 has been reported to interact with c-Src (34) and c-Src to interact with Stat3 (6), we examined whether we could detect a Pyk2-c-Src complex and a Pyk2-Stat3 complex. Based on the immunoblotting results, each of the proteins expressed well in HeLa cells. Using a lysate from the transfected cells, we immunoprecipitated with anti-Pyk2 or anti-Src antibodies or a hemagglutinin antibody as the negative control and examined for the presence of co-precipitated proteins by Western blotting. We detected Stat3 and activated c-Src in the Pyk2 immunoprecipitates and Stat3 and Pyk2 in the Src immunoprecipitates. Pyk2, Stat3, and activated c-Src could not be detected in the hemagglutinin antibody immunoprecipitate (Fig. 3). Nearly equal levels of activated c-Src and Pyk2 immunoprecipitated with the Pyk2 antibody, whereas the Src immunoprecipitates contained significantly less Pyk2. The amounts of Stat3 in the two immunoprecipitations were similar. Because the Stat3 antibody did not efficiently immunoprecipitate Stat3, we could not examine Stat3 immunoprecipitates for the presence of Pyk2 and Src (data not shown).

c-Src Expression Augments Pyk2 Kinase Activity and Results in Its Phosphorylation on Multiple Tyrosines—Next, we examined the effect of c-Src on Pyk2 kinase activity by using an in vitro kinase assay. HeLa cells were transfected with constructs that express Pyk2 or Pyk2 KD in the presence or absence of c-Src, active c-Src, a dominant negative form (c-Src DN), or a kinase-dead form (c-Src KD). We subjected immunoprecipitated Pyk2 to an in vitro kinase assay using poly(Glu,Tyr) (4:1) as a substrate. Both wild type and activated c-Src strongly enhanced Pyk2 kinase activity but had no effect on Pyk2 KD. c-Src DN and c-Src KD slightly enhanced the activity of Pyk2 when compared with its basal activity (Fig. 4A).

We also compared the effects of c-Src, active c-Src, c-Src DN, and c-Src KD on Pyk2 tyrosine phosphorylation using antibodies specific for various PTY peptides from Pyk2. These antibodies recognize Pyk2 PY402, an autophosphorylation site and a Src-family SH2 domain binding site, which is required for Pyk2

![Image](https://example.com/image1.png)

**Fig. 1.** Pyk2 activates Stat3. A, Pyk2 activates Stat3-dependent transcription. HeLa cells were transfected with a construct that expresses Pyk2 (0.5 and 1.0 μg) in the presence of a Stat3-dependent luciferase reporter (0.05 μg). Data shown are the mean ± S.E. of three experiments. Data are reported as -fold induction. An immunoblot of Pyk2 expression is shown. B, Pyk2 expression leads to Stat3 phosphorylation on Tyr-705 and Ser-727. HeLa cells were transfected constructs expressing Stat3 (0.25 μg) and Pyk2 wild type (0.5 μg) or Pyk2 KD form (0.5 μg). The levels of phospho-Stat3 Tyr-705, phospho-Stat3 Ser-727, Stat3, and Pyk2 are shown. C, dose-dependent activation of Stat3 phosphorylation on Tyr-705 and Ser-727. HeLa cells were transfected with different concentrations (0.5, 1.0 μg) of Pyk2 expression vector and a Stat3 expression vector (0.25 μg). The levels of phospho-Stat3 Tyr-705, phospho-Stat3 Ser-727, Stat3, and Pyk2 are shown.

**Fig. 2.** Pyk2 amplifies c-Src-induced Stat3 activation. A, Pyk2 KD form blocks c-Src-induced Stat3-dependent reporter gene activation. HeLa cells were transfected with constructs that express a Stat3 luciferase reporter (0.05 μg), Pyk2 (0.3 μg), and activated c-Src (0.3 μg) in the presence (+) or absence (−) of Pyk2 KD form (0.5 μg). Data are mean ± S.E. of three experiments and shown as -fold induction. Levels of Pyk2 and activated c-Src were detected by immunoblots. B, Pyk2 and c-Src strongly induce Stat3 phosphorylation on Tyr-705 and Ser-727. HeLa cells were transfected with constructs that express Stat3 (0.25 μg) and activated c-Src (0.25 μg) in the presence (+) or absence (−) of Pyk2 (0.25 μg). The levels of Stat3 Tyr-705 and Ser-727 phosphorylation are shown. The amounts of Stat3, Pyk2, and activated c-Src expressed are shown as detected by immunoblots. C, Pyk2 KD blocks c-Src-induced Stat3 Tyr-705 and Ser-727 phosphorylation. HeLa cells were transfected with constructs that express Stat3 (0.25 μg) and activated c-Src (0.3 μg) in the presence or absence of Pyk2 KD (0.5 μg). The levels of Stat3 Tyr-705 and Ser-727 phosphorylation are shown. The expressed levels of Stat3, Pyk2 KD, and activated c-Src were detected by immunoblots.

**Fig. 3.** Pyk2 associates with Stat3 and c-Src. Similar amounts of Pyk2, Stat3, and activated c-Src were expressed in the HeLa cells. The cell lysates were immunoprecipitated with anti-FLAG, anti-Pyk2, or anti-Src followed by Western blotting (WB) with anti-Pyk2 (top), anti-Stat3 (middle), and anti-Src (bottom). The levels of Pyk2, Stat3, and activated c-Src in the cell lysate, as determined by immunoblotting, are shown. HA, hemagglutinin; IP Ab, immunoprecipitated antibody.
tially associated with Pyk2 when compared with the others (Fig. 4C). This result suggests that conformational change associated with c-Src activation may facilitate its interaction with Pyk2.

**Pyk2 Enhances Stat3 and c-Src-induced Cell Proliferation**—One of the biologic readouts of Stat3 activation is enhanced cell proliferation. Stat3 target genes involved in cell survival and proliferation include Bcl-x, Mcl-1, Bcl-2, Myc, and cyclin D1 (39–41). We used HeLa cell growth as a readout of Stat3 activation. We transfected HeLa cells with various combinations of constructs that express Pyk2, activated c-Src, and Stat3 in the presence or absence of various dominant negative versions of Pyk2 or Stat3 and monitored cell growth 24–30 h later using a colorimetric assay. In this assay, cellular dehydrogenases produce a colored formazan product, which is directly proportional to the number of living cells. We found that expression of Pyk2-enhanced cell growth was 35% above the basal, whereas activated c-Src nearly doubled it. Although Stat3 alone only raised cell growth ~20%, the addition of Pyk2 enhanced cell growth 3-fold. The expression of a dominant negative form of Stat3-impaired Pyk2 induced cell growth. The expression of the kinase-dead form of Pyk2 nearly attenuated the c-Src-enhanced cell growth to the basal level (Fig. 5A). Together Pyk2 and Stat3 synergistically induced HeLa cell growth, whereas interfering with endogenous Stat3 activity impaired Pyk2-induced cell growth. Furthermore, the Pyk2 kinase-dead form inhibited activated c-Src-induced cell growth. These results are consistent with a role for Pyk2 in c-Src-induced Stat3 activation and implicate Pyk2 in Src-mediated cell transformation.

To complement the colorimetric assay, we also used a traditional thymidine incorporation assay. Although the synergy between Pyk2 and Stat3 was not as evident in this assay, we found that overexpression of Pyk2, activated c-Src, or Stat3 in HeLa cells enhanced the incorporation of [3H]thymidine compared with control cells. The combination of Pyk2 and activated c-Src resulted in the highest level of [3H]thymidine incorporation, whereas the levels observed following the expression of Pyk2 and Stat3 exceeded those observed with either construct alone (Fig. 5B). Because the transfection efficiency of HeLa cells is ~60% (i.e. 40% of the cells in the assay do not express the transfected constructs), these results underrepresent the consequences of overexpressing these proteins.

**c-Src Dominant Negative or Pyk2 KD Impairs EGF-induced Stat3 Phosphorylation**—The mechanism by which receptor tyrosine kinases activate Stat3 has been controversial, although the evidence supports a functional role for c-Src activation. Based on our previous experiments showing that Pyk2 enhances c-Src-mediated Stat3 activation, we tested the effects of expressing the Pyk2 KD on EGF-induced Stat3 activation. We first verified that EGF induced the Stat3 reporter construct in HeLa cells and assessed the role of Pyk2 in EGF-induced Stat3 activation in these cells using either the wild type or the KD form of Pyk2 (Fig. 6A). We found that both Pyk2 expression and EGF treatment resulted in a similar low level activation of Stat3-dependent transcription. The addition of Pyk2 significantly enhanced EGF-induced Stat3-dependent transcription, whereas expression of Pyk2 KD impaired EGF-induced Stat3 activation. Next we switched to A431 cells, which express high endogenous levels of Pyk2, Stat3, and c-Src and very high levels of EGFR. Treatment of A431 cells with EGF resulted in the rapid phosphorylation of Stat3 on Tyr-705 and Ser-727, the phosphorylation of Pyk2 on Tyr-402, and Erk activation as assessed by Western blotting of a phosphospecific antibody (Fig. 6B). The expression of either PYK2 KD or c-Src DN resulted in a dramatic decrease in EGF-induced Stat3 phos-
phorylation. c-Src DN had a modest effect on EGF-induced Erk activation, whereas Pyk2 had only a minor effect. The expression of the Pyk2 KD mildly impaired the level of Pyk2 Tyr-402 phosphorylation, whereas c-Src had a much more significant effect. These data argue that EGF-mediated c-Src activation facilitates Pyk2 activation and implicate both c-Src and Pyk2 in EGF-induced Stat3 activation in A431 cells. The effect of Pyk2 KD on Pyk2 Tyr-402 phosphorylation may suggest some role for Pyk2 in amplifying EGF-mediated c-Src activation.

EGF Stimulation Results in the Recruitment of c-Src, Pyk2, and Stat3 to the EGFR—Because we had relied on transfected cells to demonstrate associations between Stat3 and Pyk2 and Src and Pyk2, we attempted to find associations between the endogenous proteins following EGF stimulation. Because both c-Src and Stat3 have been associated with the EGFR, we analyzed both the EGFR immunoprecipitates as well as the Pyk2 immunoprecipitates following stimulation of the A431 cells with different concentrations of EGF. In the absence of EGF treatment, we failed to detect significant levels of Pyk2, Stat3, or c-Src in association with the EGFR. However, following EGF treatment, we detected Pyk2, Stat3, and c-Src in the EGFR immunoprecipitation (Fig. 7A, left panel). In addition, when we examined the Pyk2 immunoprecipitations, we found (following EGF treatment) that we co-immunoprecipitated EGFR, Stat3, and c-Src (Fig. 7A, right panel). We also checked the phosphorylation status of Pyk2, c-Src, Stat3, and Erk following exposure of A431 cells to different concentrations of EGF. EGF stimulation enhanced the levels of phosphorylation of each of the proteins listed above (Fig. 7B). Finally, to get some assessment of the amount of Pyk2 associated with c-Src following EGFR treatment, we extensively immunoprecipitated c-Src and Pyk2 from A431 cell lysates treated with EGF (or not treated) and examined the immunoprecipitates as well as the cell lysates prior to and after immunoprecipitation for the relative...
complexes form containing endogenous Pyk2 along with c-Src, EGFR, or Stat3, arguing that EGF signaling recruits c-Src, Pyk2, and Stat3 to EGFR.

Growth factors apparently activate Stat3 in a manner largely independent of JAKs but dependent upon the activation of Src kinases (42). Recombinant Src family kinase SH3 domains can mediate a direct interaction with Stat3 (24). In Rat-2 fibroblasts, the expression of Hck, a Src family kinase member, with a disrupted SH3 domain resulted in a failure to activate Stat3 and a reduced transforming activity. However, as mentioned previously in studies utilizing WB epithelial cells, Hep3B, and NIH 3T3 cells, the Btk family tyrosine kinase Etk functioned as an intermediary between c-Src and Stat3 activation (25). In co-transfection assays, Etk co-immunoprecipitated with Stat3, suggesting that Etk may directly phosphorylate Stat3. MEKK1 has also been shown to have a role in Stat3 activation (43). Overexpression of MEKK1 led to Stat3 activation, and a kinase-inactive form of MEKK1 inhibited EGF-induced Stat3 activation. In an in vitro kinase assay, MEKK1 phosphorylated Stat3 on Ser-727, and in vivo its expression led to phosphorylation of Stat3 on Tyr-705 through a pathway that involved c-Src and JAKs. Further confusing the issue, activated Rac1 induces Stat3 activation, and a dominant negative form of Rac1 inhibits EGF-induced Stat3 activation as assessed by phosphospecific Stat3 immunoblotting and reporter gene activation (44). In EGF-stimulated COS-1 cells that had previously been transfected with Stat3, a Rac1 immunoprecipitate contained Stat3, implying an interaction between Rac1 and Stat3. Our studies now also implicate Pyk2 in EGF- and c-Src-induced Stat3 activation.

Although we cannot provide a clear synthesis of all these studies, some conclusions can be drawn. First, Src kinases play an essential role in Stat3 activation in all these studies, with the possible exception of the Rac1 study, which implicated Jak2 as the downstream kinase. Second, Etk (and perhaps other Btk kinases) and Pyk2 may play an analogous role; both amplify c-Src-mediated Stat3 activation. The relative importance of these proteins may depend upon their expression levels and intracellular localization. Strong Pyk2 expression is found in the brain and in hematopoietic cells, although many other cell types also express it (45). In addition, Pyk2 is found in many tumors, including glioblastoma, astrocytoma, lymphoma, breast carcinoma, prostate carcinoma, lung carcinoma, and hepatocellular carcinoma. Third, both Rac activation and MEKK1 activation can be tied to Pyk2 activation. For example, in cardiac fibroblasts, angiotensin II induces Pyk2 activation, which leads to Rac1 activation and MEKK1-dependent c-Jun NH2-terminal kinase (JNK) activation (46). In addition, Pyk2 is involved in the control of chemokine- and integrin-mediated Rac activation and associates with the Rac exchange factor, Vav1 (47). In another study, EGF potently activated MEKK1 and resulted in the association of Rac1 with MEKK1 (48). Inhibitory mutants of MEKK1 blocked Rac1-induced JNK activation. Although Pyk2 also couples stress signals to the JNK pathway, an involvement of MEKK1 in those pathways has not been clearly established. Overall, despite these links between Pyk2, Rac1, and MEKK1 activation, their relative importance in growth factor-induced Stat3 activation will require further study.

Fourth, Rac1 has emerged as a mediator of v-Src-induced transformation. In v-Src-transformed cells, Rac1 activity is high, and both Vav2 and Tiam1 (another Rac exchange factor) are phosphorylated on tyrosine residues (49). Although the activation of Tiam1 and Vav2 was attributed to v-Src activity, Pyk2 might function to amplify the activation of the Rac exchange factors as it does in Stat3 activation.

Our data indicate that Pyk2 functions predominantly down-

**Fig. 7.** EGF treatment results in the recruitment of Pyk2, c-Src, and Stat3 to EGFR and triggers Pyk2, c-Src, and Stat3 phosphorylation. A, co-immunoprecipitations. A431 cells were treated with EGF (20 ng/ml, 50 ng/ml, or 100 ng/ml). After 15 min, cell lysates were immunoprecipitated with an antibody against the EGFR (left panel) or Pyk2 (right panel). The immunoprecipitates (IP) were examined by immunoblotting for the levels of EGFR, Pyk2, Stat3, or c-Src. B, EGF-induced Pyk2, Stat3, c-Src, and Erk phosphorylation. The levels of phosphorylated Tyr-402 Pyk2, Tyr-705 Stat3, Ser-727 Stat3, and Tyr-416 c-Src, and Thr-202, Tyr-204 Erk1/2 in the above cell lysates were carried out by immunoblotting with specific antibodies (right panel). The levels of EGFR, Pyk2, Stat3, Erk1/2, and c-Src in the cell lysates were also detected by immunoblotting (right panel). C, relative amounts of endogenous c-Src associated with Pyk2 following EGF signaling. A431 cells treated with EGF (+) or not treated (−) were immunoprecipitated with either a Pyk2-specific antibody or a c-Src-specific antibody. The immunoprecipitates were immunoblotted with the same antibodies (left panel). To reduce the impact of the immunoglobulin heavy chain band (asterisk) on the detection of the c-Src band, the immunoblot was cut just beneath the c-Src band. The cell lysates (Ly), either prior to or following the immunoprecipitations, were immunoblotted for Pyk2 or c-Src (right panel).

amounts of Pyk2 and c-Src (Fig. 7C). Based on this analysis, a significant portion of the Pyk2 in A431 cells becomes associated with c-Src following EGF signaling.

**DISCUSSION**

This study provides several lines of evidence supporting a role for Pyk2 in EGFR- and c-Src-induced Stat3 activation. First, the expression of Pyk2 in HeLa cells results in the activation of a Stat3 reporter gene and the phosphorylation of Stat3 on Tyr-705 and Ser-727, and it enhances the growth of Stat3 overexpressing cells. Second, Pyk2 amplifies c-Src-induced activation of the Stat3 reporter gene and augments c-Src-induced phosphorylation of Stat3 on the same residues. Third, the expression of a kinase-inactivated form of Pyk2 interferes with c-Src-induced Stat3 activation, c-Src-induced cell growth, and EGF-induced Stat3 activation. Fourth, c-Src not only phosphorylates Pyk2 on the multiple tyrosine sites but also strongly induces Pyk2 kinase activity. On the contrary, Pyk2 only weakly induces c-Src kinase activation in HeLa cells.2 Fifth, in EGF-treated A431 cells, intracellular protein

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* C.-S. Shi, unpublished observation.
stream of c-Src in EGF receptor signaling. Activated c-Src results in potent Pyk2 activation, whereas (as indicated above) we found that Pyk2 has only a modest effect on c-Src activation. However, other studies have implicated Pyk2 as upstream of c-Src activation (50). Irrespective of its location in c-Src signaling, we would argue that Pyk2 functions as an amplifier to augment c-Src signaling to downstream pathways. Activated Pyk2 may directly or indirectly phosphorylate Stat3 on Tyr-705; however, its induction of Ser-727 phosphorylation must occur indirectly. In our study, the expression of Pyk2 in HeLa cells led to a more prominent phosphorylation of Stat3 on Ser-727 as compared with Tyr-705. The consensus of studies favors a MAPK module as the mediator of Stat3 Ser-727 phosphorylation; p38, Erk, and JNK have all been implicated (43, 51–54). As a known activator of the MAPK modules (55, 56), Ser-727 as compared with Tyr-705. The consensus of studies favors a MAPK module as the mediator of Stat3 Ser-727 phosphorylation; p38, Erk, and JNK have all been implicated (43, 51–54). As a known activator of the MAPK modules (55, 56), ascribed major roles. Possible physical associations have been reported (62). Similar to the growth factor receptors has been reported (62). Similar to the growth factor – 

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54). As a known activator of the MAPK modules (55, 56), favors a MAPK module as the mediator of Stat3 Ser-727 phosphorylation; p38, Erk, and JNK have all been implicated (43, 51–54). As a known activator of the MAPK modules (55, 56), ascribed major roles. Possible physical associations have been reported (62). Similar to the growth factor receptors has been reported (62). Similar to the growth factor

Besides growth factor receptors, signaling through a variety of GPCRs also leads to Stat3 activation (57–59). Predominantly, GPCRs that link to either Gαi or Gαq subfamily members have been associated with Stat3 activation. In the majority of the studies describing GPCR-triggered Stat3 activation, the Gαi family has been ascribed major roles. Possible physical association between the JakS and the angiostatin II AT1 receptor (60), platelet-activating factor receptor (61), and chemokine receptors has been reported (62). Similar to the growth factor receptors, Rac1 activation plays a prominent role in Stat3 activation following the expression of vascular smooth muscle cells to either angiostatin II or thrombin (63). In vascular smooth muscle cells, angiostatin II signaling also leads to prominent Pyk2 activation, and Pyk2 has been found associated with Jak2 constitutively. Furthermore, two distinct Pyk2 dominant negative forms interfered with angiostatin II-induced activation of Jak2 (20). GPCRs may use a number of mechanisms to activate Pyk2, including increases in intracellular Ca2+ triggered by the activation of phospholipase Cβ (Gqα or Gβγ-mediated) and via the activation of G12 (29). Preliminary experiments have supported a role for Pyk2 in GPCR signaling to Stat3 activation. In those experiments, the exposure of HeLa cells (transfected previously with the M1 muscarinic receptor) to carbachol resulted in a weak increase in Stat3 phosphorylation on Tyr-705 and Ser-727. However, the co-transfection of a modest amount of Pyk2 led to a dramatic increase in Stat3 phosphorylation on the same residues, which was much higher than we observed with carbachol alone or following Pyk2 expression. Thus, in those cells that express adequate levels of Pyk2, it may also serve to help link GPCR signaling to Stat3 activation.

In conclusion, Pyk2 plays a significant role in enhancing Stat3 activation following EGF signaling and may be involved in Src-mediated cell transformation and in GPCR signaling leading to Stat3 activation. Also linking c-Src, Pyk2, and Stat3 are their known roles in cell migration. In numerous studies, Pyk2 has emerged as a key mediator linking receptor signaling to critical downstream signaling pathways.

**Acknowledgments**—We appreciate the editorial assistance of Mary Rust and the continued support of Dr. Anthony S. Fau.
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