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Platelet-Derived Growth Factor Induces Phosphorylation of Multiple JAK Family Kinases and STAT Proteins

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Receptors for interferons and other cytokines signal through the action of associated protein tyrosine kinases of the JAK family and latent cytoplasmic transcription factors of the STAT family. Genetic and biochemical analysis of interferon signaling indicates that activation of STATs by interferons requires two distinct JAK family kinases. Loss of either of the required JAKs prevents activation of the other JAK and extinguishes STAT activation. These observations suggest that JAKs provide interferon receptors with a critical catalytic signaling function and that at least two JAKs must be incorporated into an active receptor complex. JAK and STAT proteins are also activated by ligands such as platelet-derived growth factor (PDGF), which act through receptors that possess intrinsic protein tyrosine kinase activity, raising questions about the role of JAKs in signal transduction by this class of receptors. Here, we show that all three of the ubiquitously expressed JAKs—JAK1, JAK2, and Tyk2—become phosphorylated on tyrosine in both mouse BALB/c 3T3 cells and human fibroblasts engineered to express the PDGF-β receptor. All three proteins are also associated with the activated receptor. Through the use of cell lines each lacking an individual JAK, we find that in contrast to interferon signaling, PDGF-induced JAK phosphorylation and activation of STAT1 and STAT3 is independent of the presence of any other single JAK but does require receptor tyrosine kinase activity. These results suggest that the mechanism of JAK activation and JAK function in signaling differs between receptor tyrosine kinases and interferon receptors.

Receptor tyrosine kinases (RTKs), such as the platelet-derived growth factor (PDGF) receptor (PDGFR), possess intrinsic protein tyrosine kinase activity in their cytoplasmic domains. In contrast, receptors for cytokines have no such activity. Despite this difference, recent data suggest that the two types of receptor have common intracellular signal transduction pathways involving members of the JAK and STAT families (for reviews, see references 4, 7, 15, 16, 21, 37, 43, 44, and 56).

The JAKs, also known as Janus kinases, are nontransmembrane protein tyrosine kinases. The structure of the JAK family members is characterized by the presence of seven highly conserved regions (13), the most C-terminal of which harbors the tyrosine kinase activity. The first evidence for a role for JAKs in cytokine signaling came from studies of cells defective in their response to interferons (IFNs). The genetic approach to the dissection of IFN-α signaling pathway was developed by analyzing mutant cells selected for nonresponsiveness to IFN-α. These mutants were derived from an HT1080 human fibrosarcoma cell clone (2fTGH) that expressed the bacterial guanosine phosphoribosyltransferase gene under the control of an IFN-α-inducible promoter. Mutant group U1 cells fail to respond to IFN-α and are deficient in the expression of the Tyk2 protein tyrosine kinase (45). Another mutant line, U4A, responds to neither IFN-α nor IFN-γ. It expresses a truncated JAK1 mRNA and can be complemented for both IFN-α or IFN-γ responses by a wild-type JAK1 cDNA (25). A similar approach was used to isolate mutants defective in response to IFN-γ. In this case, the IFN-γ-responsive *9-27* promoter was fused upstream of the gene encoding the cell surface protein CD2. Mutagenesis of these cells (2C4) yielded two complementation groups (γ1 and γ2) nonresponsive to IFN-γ (7, 48). Expression of a JAK2 cDNA restored IFN-γ responsiveness to γ1A cells. Together, these experiments showed that at least two JAK family kinases are required for each IFN ligand: both JAK1 and JAK2 are required for IFN-γ signaling, whereas IFN-α signaling requires both JAK1 and Tyk2.

Soon after the discovery that JAKs were essential for signaling by IFN receptors, JAKs were implicated in signaling by a spectrum of cytokines, including growth hormone, interleukin-3 (IL-3), erythropoietin, prolactin, granulocyte colony-stimulating factor, IL-2, IL-4, and IL-6 (2, 5, 8, 12, 17, 27, 40, 41, 49, 50). JAKs were shown to associate with cytokine receptors and to undergo tyrosine phosphorylation upon ligand binding. In addition, protein sequence homologies, termed box 1 and box 2, in the membrane-proximal region of the cytoplasmic domain of cytokine receptors were shown to be required for receptor signaling and JAK association (16). Furthermore, a dominant negative JAK2 mutant was shown to interfere with erythropoietin signaling (55). Together, these data suggest that JAK family kinases perform a critical signaling function for cytokine receptors.

One such function for JAKs is activation of STAT proteins, latent cytoplasmic transcription factors that undergo rapid tyrosine phosphorylation following treatment with cytokines (4, 7, 15, 16, 21, 37, 43, 44, 56). Tyrosine-phosphorylated STAT proteins form dimers, acquire DNA-binding activity, and translocate to the nucleus, where they are presumed to control the expression of target genes. Derivatives of HT1080 cells lacking STAT1 fail to respond to either IFN-α or IFN-γ, and response to both ligands is restored by a STAT1 cDNA (26).

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Current models for cytokine signaling invoke a "JAK/STAT pathway," in which receptor-bound JAK proteins are brought into proximity by ligand-induced receptor dimerization (4, 7, 15, 16, 21, 37, 43, 44, 56). JAK proteins become activated, presumably by cross-phosphorylation, and in turn phosphorylate tyrosine residues on the receptor subunits. Phosphorylation of receptor tyrosine residues by JAKs is thought to create recruitment sites on the receptor for the SH2 domains of the STAT proteins, which are activated by phosphorylation on a single tyrosine residue. One characteristic feature of this pathway is the requirement for a JAK associated with each receptor subunit in the cytokine-receptor complex. In the case of IFN receptors, which consist of two distinct subunits, each subunit associates with a unique JAK. The elimination of either JAK extinguishes signaling completely, and phosphorylation of the other receptor-associated JAK is not observed (25). Thus, the juxtaposition of two JAKs in the ligand-induced receptor complex is critical to signaling.

The protein tyrosine kinase responsible for phosphorylating STAT proteins in the cytokine-receptor complex could be a JAK or another receptor-associated protein tyrosine kinase. Most attention, however, has focused on the likelihood of a direct enzyme-substrate relationship between the JAKs and STATs. Consistent with this hypothesis, cotransfection of either JAK1, JAK2, or Tyk2 cDNAs with a STAT1 or STAT3 cDNA in COS cells leads to phosphorylation of STAT1 or STAT3, respectively (29, 30), and overexpression of JAK1 and JAK2 activates coexpressed STAT4 (51). Furthermore, JAK2 can phosphorylate STAT5 in vitro (11). However, the phosphorylations of specific JAKs and STATs are not systematically correlated. For example, although both IL-4 and IL-9 activate JAK1, IL-9 activates STAT1 (52) while IL-4 activates STAT6 (14), suggesting that factors other than the JAKs alone determine STAT activation. Indeed, recent data suggest that receptor binding of STATs rather than the nature of the JAK bound to the receptor specificies STAT activation (42). Furthermore, there is evidence that the Src kinase may be involved in the activation of STAT3 (53). Therefore, it remains to be formally demonstrated that activated JAKs directly phosphorylate STATs in vivo in response to cytokines.

RTKs such as PDGFR or epidermal growth factor (EGF) receptor (EGFR) also activate STAT proteins (29, 31–33, 35, 39, 47). Furthermore, the STAT target site on the c-*fos* promoter, the *sis*-inducible element (SIE), confers, under certain conditions, PDGF-dependent activation of c-*fos* transcription (47). Thus, STAT proteins are functional mediators of PDGF signaling. However, little is known about the signaling pathway linking STATs to RTKs. Unlike receptors of the cytokine family, RTKs harbor an intrinsic protein tyrosine kinase activity, raising the possibility that STAT activation by these receptors is independent of JAKs. Nevertheless, activation of the EGFR in A431 cells leads to phosphorylation of JAK1, as well as STAT activation (35, 38), suggesting that JAKs may indeed play a direct role in signal transduction by RTKs.

To investigate the possible role of JAKs in RTK signaling, we have examined the phosphorylation of JAKs and STATs in response to PDGF. We find that PDGF stimulates the tyrosine phosphorylation of all three ubiquitously expressed JAKs—JAK1, JAK2, and Tyk2. These kinases are also associated with the activated PDGFR-β. In contrast to the IFN response, we found no interdependence among the JAKs for tyrosine phosphorylation in response to PDGF. Furthermore, none of the JAKs is individually required for activation of STAT1 and STAT3 by PDGF. PDGF-induced phosphorylation of both JAKs and STATs requires the intrinsic kinase activity of the PDGFR, suggesting that both the mechanism of JAK activation and their function, if any, differ for RTKs relative to cytokine receptors.

MATERIALS AND METHODS

Cells and cell cultures. The cell lines 2fTGH, U1A, U4A, 2C4, γ2A, and γ2A/JAK2 KTE (12, 25, 28, 45, 48), derived from the human fibrosarcoma HT1080 cells, were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). After the initial selection of virus-infected cells in G418 (1 mg/ml), the cells expressing the human PDGFR-β were maintained in DMEM supplemented with 10% FBS. BALB/c 3T3 were grown in DMEM supplemented with 10% calf serum.

Virus infection. PA317 amphotropic packaging cell lines expressing the human PDGFR-β in plasmid LXSN (PA22B cells [19]) were used to infect the HT1080-derived cells. The LXSN vector expresses the PDGFR-β under the control of the retroviral long terminal repeat from Moloney murine sarcoma virus, while the selectable marker (neomycin phosphotransferase) is expressed from the simian virus 40 early promoter (24). PA22B cells were grown to confluence in 6-cm-diameter dishes in DMEM supplemented with FBS (10%) and G418 (1 mg/ml). On day 1, the culture medium was replaced with 2 ml of fresh medium (DMEM supplemented with 10% FBS). On day 2, virus was harvested from the PA22B cells and 2 ml of fresh medium was added back to the PA22B cells. The virus-containing medium was filtered through a 0.45-μm-pore-size Nalgene filter, and Polybrene was added to a final concentration of 3 μg/ml. Cells to be infected, at 30 to 50% confluence, were fed with the filtered virus medium and incubated at 37°C for 24 h. On day 3, the same harvest and infection procedures were performed. On day 4, cells were washed thoroughly in phosphate-buffered saline (PBS), trypsinized, and plated in 10-cm dishes in DMEM containing 10% FBS and 1 mg of G418 per ml. Cells were grown in that medium and passaged twice, at which point G418 was omitted from the medium. For cells already harboring a neomycin resistance gene, the same protocol was used except that G418 was omitted throughout.

Flow cytometry. For fluorescence-activated cell sorting, cells were split into 10-cm dishes the day before analysis to obtain subconfluent cultures. Cells were rinsed twice with ice-cold PBS and harvested in 2.5 ml of prechilled PBS buffer containing 1 mM EDTA and 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA). The cells were sedimented by brief centrifugation and resuspended in PBS-bovine serum albumin (BSA) buffer, which was composed of PBS with 1% BSA (bovine fraction V, heat treated; Sigma). Cells (2×10^6 to 6×10^6 per sort, 8×10^6 cells per ml) were incubated for 20 min at 0°C with an anti-PDGFR-β antibody (40 μg of the anti-porcine PDGFR-β [Ab-2] mouse monoclonal antibody [Oncogene Science] per ml), which recognizes the extracellular portion of the receptor. Cells were washed twice with ice-cold PBS-BSA buffer and stained with fluorescein-conjugated goat anti-mouse immunoglobulin G (5 μg/ml; Southern Biology Associates) for 20 min at 0°C. Excess antibodies were removed by three washes with PBS-BSA buffer, and cells were resuspended in 1 ml of ice-cold PBS-BSA buffer. The stained cells were immediately analyzed and sorted with an EPICS Elite flow cytometer (Coulter Corp.). The collected cells (in 2 to 4 ml of DMEM supplemented with 10% FBS) were immediately incubated at 37°C. For analytical purposes, the same procedure was followed except that 4×10^5 cells were used for each assay.

Immunoprecipitations and Western blots. For analysis of JAK phosphorylation, confluent cultures (15-cm dishes) were starved for 10 to 18 h in DMEM supplemented with 0.5% FBS or 0.5% calf serum. Cells were treated with PDGF-BB (200 ng/ml; Upstate Biotechnology Inc.), IFN-α (500 U/ml; Lee BioMolecular Research Laboratories Inc.), or human IFN-γ (10 ng/ml; Gibco BRL) for 5 min at 37°C. After treatment, the cultures were put on ice and rinsed with ice-cold PBS, and the cells were scraped from the dishes in PBS containing 2 mM Na_3VO_4. Cells were lysed, at 1×10^7 to 2×10^7 cells per ml, by rocking at 4°C for 20 min in either buffer A or buffer B (JAK1 and JAK2 immunoprecipitations in 2fTGH.PS1 cells, respectively). Buffer A contained 50 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 2 mM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 3 μg of aprotinin per ml, 3 μg of leupeptin per ml, and 3 μg of pepstatin per ml. Buffer B contained 50 mM Tris (pH 8.0), 150 mM NaCl, 50 mM NaF, 5 mM $Na_2P_2O_7$, 1% Triton X-100, 2 mM EDTA, 2 mM Na_3VO_4, 5 μg of aprotinin per ml, 5 μg of leupeptin per ml, and 5 μg of pepstatin per ml. Extracts were clarified by centrifugation (20 min at 16,000 × *g*). Protein lysates were precleared with protein A-Sepharose beads, and immunoprecipitations were performed for 6 to 14 h at 4°C with polyclonal antibodies (Upstate Biotechnology Inc.) against JAK1 N-terminal peptide, JAK2 peptide (amino acids 758 to 776), or Tyk2 N- and C-terminal peptides. For the secondary immunoprecipitations, the nonimmunoreactive components of the JAK complex were released by washes with a high-stringency buffer (50 mM Tris [pH 8.0], 50 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.5% deoxycholate, 0.5% SDS, 2 mM Na_3VO_4, 0.05% BSA, 3 μg of aprotinin per ml) and precipitated in the same buffer with polyclonal antibodies against either JAK1 or the human PDGFR-β (Upstate Biotechnology Inc.). Immunoprecipitated proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7.5% polyacrylamide) and transferred either to Immobilon or nitrocellulose. Western blots (immunoblots) were performed with monoclonal anti-phosphotyrosine antibodies (4G10; Up-

state Biotechnology Inc.). Immunoreactive bands were visualized with the epichemiluminescence Western blotting system (Amersham). The nitrocellulose filters were stripped and reprobed with antibodies directed against either JAK1, Tyk2 (Transduction Laboratories), or JAK2 (Upstate Biotechnology Inc.).

For immunoblot analysis of extracts, confluent cultures (6-cm dishes) were starved for 10 to 18 h in DMEM supplemented with 0.5% FBS. Cells were treated with PDGF-BB (200 ng/ml) for 5 min at 37°C. After treatment, the cultures were put on ice and rinsed with ice-cold PBS, and the cells were scraped from the dishes in PBS containing 2 mM Na_3VO_4. Cells were lysed, by rocking at 4°C for 20 min, in 70 μl of a buffer that contained 50 mM Tris (pH 8.0), 200 mM NaCl, 2 mM EDTA, 2 mM EGTA, 50 mM NaF, 30 mM $Na_2P_2O_7$, 2 mM Na_3VO_4, 0.5 mM PMSF, 2 mM DTT, 3 μg each of aprotinin, leupeptin, and pepstatin per ml, 200 nM microcystin, 10% glycerol, and 1% Nonidet P-40. Extracts were clarified by centrifugation (20 min at 16,000 × *g*). Proteins (30 to 45 μg) were denatured in Laemmli buffer, separated by SDS-PAGE (7.5% polyacrylamide), and transferred to nitrocellulose. Western blots were performed with monoclonal antibodies against phosphotyrosine (4G10), STAT1 N-terminal region (Transduction Laboratories), or STAT3 N-terminal region (Transduction Laboratories) or polyclonal antibodies raised against the human PDGFR-β (Upstate Biotechnology Inc.) Immunoreactive bands were visualized by the epichemiluminescence Western blotting system (Amersham).

Electrophoretic mobility shift assay. Subconfluent cultures (10-cm dishes) were starved for 15 to 18 h in DMEM supplemented with 0.5% FBS. Cells were treated with PDGF-BB (200 ng/ml), IFN-α (500 U/ml), or IFN-γ (10 ng/ml) for 15 min at 37°C and the incubations were stopped by two rinses with ice-cold PBS. The cells were scraped from the dishes in PBS containing 2 mM Na_3VO_4, rinsed once in hypotonic buffer, and resuspended in a final volume of 100 μl in hypotonic buffer. The protein lysates were prepared as described previously (34). The hypotonic buffer consisted of 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9), 20 mM NaF, 1 mM $Na_2P_2O_7$, 1 mM EDTA, 1 mM EGTA, 0.25 mM ammonium molybdate, 2 mM Na_3VO_4, 3 μg each of leupeptin, aprotinin, and pepstatin per ml, 0.5 mM PMSF, and 1 mM DTT. Cells were lysed by repeated passage through a 25-gauge needle. Crude nuclei and unbroken cells were sedimented by brief centrifugation. The supernatant was supplemented with NaCl to 120 mM and then clarified by centrifugation (20 min at 16,000 × *g*). Glycerol was added to 10%, and the resulting fractions (cytosolic extract) were used for mobility shift assays. The nuclear pellets were resuspended in 40 μl of high-salt buffer and rocked at 4°C for 30 min. The high-salt buffer contained 20 mM HEPES (pH 7.9), 400 mM NaCl, 20 mM NaF, 1 mM $Na_2P_2O_7$, 1 mM EDTA, 1 mM EGTA, 0.25 mM ammonium molybdate, 2 mM Na_3VO_4, 3 μg each of leupeptin, aprotinin, and pepstatin per ml, 0.5 mM PMSF, 1 mM DTT, and 20% glycerol. The nuclear extracts were then clarified by centrifugation (20 min at 16,000 × *g*). High-affinity SIEm67 (47) DNA probe was obtained by incubating Klenow enzyme and [α-^{32}P]deoxynucleotide triphosphates with the annealed oligonucleotides 5′-GTCGACATTTCCCGTAAATC-3′ and 5′-TCGACGATTTACGGGAAATG-3′. The mobility shift assays were performed essentially as described previously (34). Cytosolic (25 to 30 μg) or nuclear (20 μg) extracts were preincubated for 30 min at 0°C with 1 μg of poly(dI-dC) · poly(dI-dC) as nonspecific DNA competitor in the reaction buffer (13 mM HEPES [pH 7.9], 65 mM NaCl, 1 mM DTT, 0.15 mM EDTA, 2% Ficoll 400, 4% glycerol). The labeled probe (50,000 cpm, ~3 fmol) was then added to the reaction mixture, which was incubated for another 20 min at room temperature. The protein-DNA complexes were separated by electrophoresis in 0.5× TBE (Tris-borate-EDTA) buffer through a 5% polyacrylamide gel (39:1 acrylamide-to-bisacrylamide ratio) containing 2.5% glycerol. Gels were dried and subsequently analyzed by autoradiography. Supershift experiments were performed with monoclonal antibodies against the STAT1 N-terminal region, or an irrelevant monoclonal antibody (12CA5 [9]), polyclonal anti-STAT3 antibodies (a gift of J. Darnell, Rockefeller University, New York, N.Y.), or normal rabbit serum.

RESULTS

Tyrosine phosphorylation and receptor association of JAKs in BALB/c 3T3 cells stimulated by PDGF. To test for a possible role of JAK kinases as signaling intermediates for the PDGF receptor, we first investigated whether the JAKs became tyrosine phosphorylated following PDGF treatment. JAK1 was previously found to be tyrosine phosphorylated in EGF-treated A431 cells (38). We treated BALB/c 3T3 fibroblasts with PDGF, immunoprecipitated individual JAKs from cell extracts, and assessed tyrosine phosphorylation by Western blot with an anti-phosphotyrosine antibody. Figure 1 shows that all three of the ubiquitously expressed JAKs—JAK1, JAK2, and Tyk2—displayed an increased level of tyrosine phosphorylation when cells were treated with PDGF (lanes 2, 7, and 9).

In addition, the JAKs coprecipitated with a tyrosine-phosphorylated 190-kDa protein, which comigrates with the phosphorylated PDGFR. This protein was generally not observed in immunoprecipitations with control antibodies (data not shown). To determine if this protein was the PDGFR, we treated the JAK1 immunoprecipitate with a buffer expected to dissociate any proteins bound to JAK1 while sparing the antibody-JAK1 complex. The supernatant solution was immunoprecipitated either with the same JAK1 antibody or with antibody to PDGFR. The JAK1 antibody failed to precipitate detectable amounts of protein (lane 3), confirming that JAK1 remained in the initial immunocomplex (see also lane 5). The PDGFR antibody precipitated the 190-kDa protein (lane 4), suggesting that this protein is directly recognized by the PDGFR antibody and not by the JAK1 antibody. We conclude that the 190-kDa protein observed in the JAK1 precipitates (and, by extension, in the JAK2 and Tyk2 precipitates as well) is the PDGFR. Thus, engagement of the native PDGFR in mouse fibroblasts results in the tyrosine phosphorylation of JAKs and the association of the kinases (in unknown stoichiometry) with the receptor.

Tyrosine phosphorylation of JAKs in PDGF-treated human fibroblasts expressing PDGFR-β. To further explore the hypothesis that JAKs are activated by the PDGFR and to begin to assess the role of JAKs in PDGF signaling, we investigated PDGF-induced signaling events in HT1080-derived human fibrosarcoma cell lines lacking specific JAKs. However, these cell lines harbor very low concentrations of PDGFRs, and PDGF activation of STATs in wild-type (2fTGH) cells was undetectable (Fig. 2A). Therefore, to express higher concentrations of PDGFRs in these cells, we infected them with an amphotrophic retrovirus harboring the PDGFR-β cDNA and sorted pools of stably infected cells by FACS on the basis of PDGFR present at the cell surface, yielding the "PDGFR Sorted" cell line 2fTGH.PS1.

To test whether 2fTGH.PS1 cells acquired the ability to respond to PDGF, we determined whether PDGF treatment of these cells elicited activation of STAT proteins. Cells were incubated with various concentrations of PDGF, cytosolic extracts were prepared, and STAT activation was measured by electrophoretic mobility shift assay with a high-affinity SIE DNA probe (47). Figure 2A shows that whereas uninfected 2fTGH cells did not respond detectably to concentrations of PDGF up to 200 ng/ml (lanes 1 to 4), treatment of 2fTGH.PS1 cells led to a dose-dependent induction of the three complexes, SIF-A, SIF-B, and SIF-C (lanes 6 to 9). Induction of SIF-C and, to a lesser extent, SIF-B by IFN-γ was identical in the two cell lines (lanes 5 and 10).

The SIF-B and SIF-C complexes induced by EGF have been shown previously to contain the STAT1 protein (31, 35), whereas both SIF-A and SIF-B contain STAT3 (33, 54). To identify the components of the three complexes induced by PDGF in 2fTGH.PS1 cells, specific antibodies were added during the incubation of the extracts with the DNA probe. An anti-STAT1 antibody supershifted the SIF-C complex to a more slowly migrating band and also decreased the intensity of the SIF-B band, whereas an unrelated monoclonal antibody had no effect on the three complexes (Fig. 2B, compare lane 3 with lanes 1 and 2). When a polyclonal anti-STAT3 antibody was used, bands of complexes SIF-A and SIF-B disappeared with the concomitant appearance of a slowly migrating band. The intensity of the band of complex SIF-C was unchanged (compare lane 5 with lane 1 and with the control lane 4 obtained with extracts incubated with normal rabbit serum). Together, these results demonstrate that STAT1 is contained in SIF-B and SIF-C complexes while STAT3 (1, 54) is part of the SIF-A and SIF-B complexes induced by PDGF in these cells. PDGF treatment of these cells is also associated with increased tyrosine phosphorylation of STAT3; PDGF-induced tyrosine

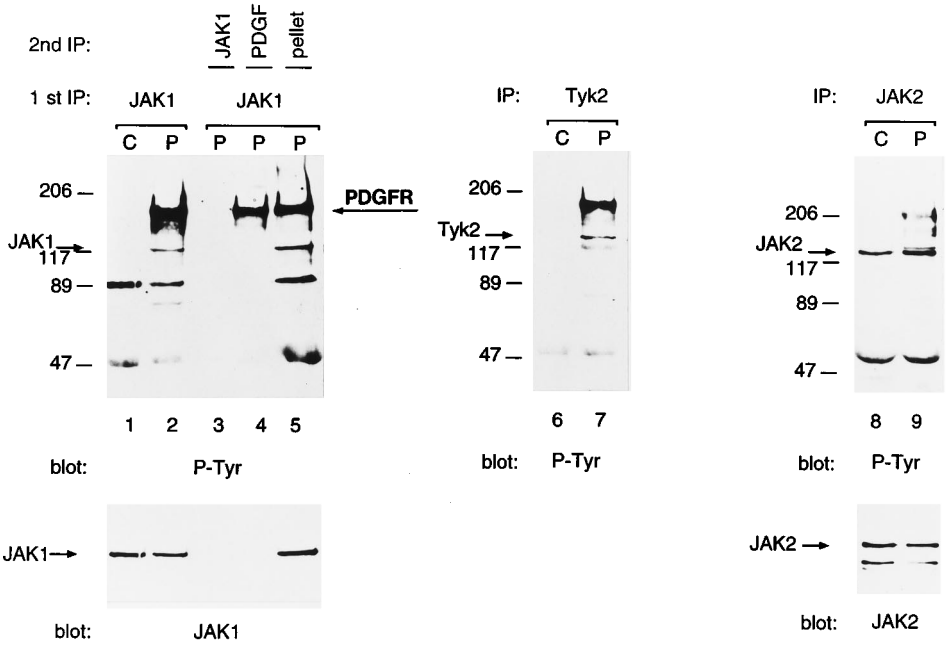

FIG. 1. PDGF-induced tyrosine phosphorylation of JAK1, Tyk2, and JAK2 in BALB/c 3T3 cells. Confluent cultures of BALB/c 3T3 cells were serum starved for 8 to 14 h (0.5% calf serum) and either left untreated or treated with a mixture of PDGF-AA and PDGF-BB (200 ng/ml) for 5 min at 37°C. Cells were lysed in buffer A (see Materials and Methods), and insoluble material was removed by centrifugation. Lysates were precleared with protein A-Sepharose and subjected to immunoprecipitation with antibodies directed against either JAK1 (lanes 1 to 5), Tyk2 (lanes 6 and 7), or JAK2 (lanes 8 and 9). For the secondary immunoprecipitations (lanes 3 to 5), two JAK1 immunoprecipitations were done in parallel with the JAK1 immunoprecipitation shown in lane 2. The beads from these two immunoprecipitations were pooled and washed in high-stringency buffer. The supernatant was divided into two fractions, which were incubated with antibodies against JAK1 (lane 3) or the PDGFR (lane 4). Lane 5 shows proteins remaining bound to the bead pellet after the stringent wash. This lane contains twice as much starting material as lanes 3 and 4. The membranes were probed with anti-phosphotyrosine antibodies (4G10), and antibody-antigen complexes were visualized by enhanced chemiluminescence (ECL). The positions of molecular weight markers are indicated on the left. The blots were stripped and reprobed with anti-JAK1 (lanes 1 to 5) or anti-JAK2 (lanes 8 and 9) antibodies. The murine Tyk2 protein could not be detected by Western blotting with either the monoclonal or polyclonal anti-human Tyk2 antibodies we tested. The 190-kDa protein corresponds to the fully mature PDGFR.

phosphorylation of STAT1 was below the detection limit of our Western blot assay (data not shown). Thus, the addition of the PDGFR to 2fTGH cells is sufficient to restore PDGF-induced STAT activation.

We next investigated whether the JAKs became tyrosine phosphorylated in response to PDGF in the 2fTGH.PS1 cells. Serum-starved cells were left untreated or incubated with PDGF, IFN-α, or IFN-γ. Lysates prepared from these cells were then immunoprecipitated with antibodies against JAK1 (Fig. 3A, lanes 1 to 4), JAK2 (Fig. 3A, lanes 5 to 8), or Tyk2 (Fig. 3B, lanes 1 to 3). The precipitated proteins were resolved by SDS-PAGE, immunoblotted, and probed with an anti-phosphotyrosine antibody. As shown previously (26), JAK1 was tyrosine phosphorylated when cells were incubated with IFN-α or IFN-γ (Fig. 3A, lanes 3 and 4). As we observed previously in BALB/c 3T3 cells, JAK1 was phosphorylated in response to PDGF in 2fTGH.PS1 cells (Fig. 3A, compare lane 2 to the control of unstimulated cells in lane 1). JAK2 was also phosphorylated after both PDGF and IFN-γ treatment, while tyrosine phosphorylation of JAK2 protein was not observed in cells stimulated with IFNα or in unstimulated cells (Fig. 3A, compare lanes 6 and 8 with lane 7 and with the control of unstimulated cells in lane 5). Figure 3B shows the tyrosine phosphorylation of the Tyk2 protein immunoprecipitated from PDGF-stimulated cells (lane 2), compared with a low Tyk2 phosphorylation background in untreated cells (lane 1). As expected, Tyk2 was also phosphorylated in cells incubated with IFN-α (lane 3). These results show that all three ubiquitously expressed JAKs—JAK1, JAK2, and Tyk2—become tyrosine phosphorylated when 2fTGH.PS1 cells are incubated with PDGF, as we observed in BALB/c 3T3 cells. In addition, all three kinases were associated with the PDGFR.

Generation of JAK-deficient cell lines expressing similar concentrations of PDGF receptors. The observation that JAK1, JAK2, and Tyk2 were tyrosine phosphorylated following PDGF treatment of both BALB/c 3T3 and 2fTGH.PS1 cells, in addition to the proposed role of JAKs in the activation of STAT proteins by cytokine receptors, suggested that JAKs could also act as intermediates in signal transduction by PDGFR. In cytokine signal transduction, JAKs are believed to have several direct or indirect substrates, including the receptor itself, other receptor-associated JAKs, and STAT proteins. To determine whether any of these JAKs was required for these events, a set of JAK-deficient cell lines were transduced with the PDGFR-β retrovirus and cells expressing high concentrations of PDGFR at the cell surface were sorted by fluorescence-activated cell sorting as described previously for the 2fTGH cell line. U1A and U4A are derivatives of 2fTGH deficient for Tyk2 and JAK1, respectively (25, 45). The corresponding PDGFR-expressing cell lines were called U1A.PS1 and U4A.PS1. The γ2A cell line is a JAK2$^-$ derivative of the 2C4 cell line (7, 48). The virus-infected 2C4 cell line had to be sorted twice to obtain cells with equivalent amounts of cell surface PDGFR, and the resulting PDGFR-expressing cell line was called 2C4.PS2. γ2A.PS1 is the JAK2$^-$ cell line expressing PDGFR-β.

The cells were checked for PDGFR expression, ligand-induced tyrosine phosphorylation, and STAT1 and STAT3 expression. Whole-cell extracts were prepared from cells that were either untreated or stimulated with PDGF. Proteins from

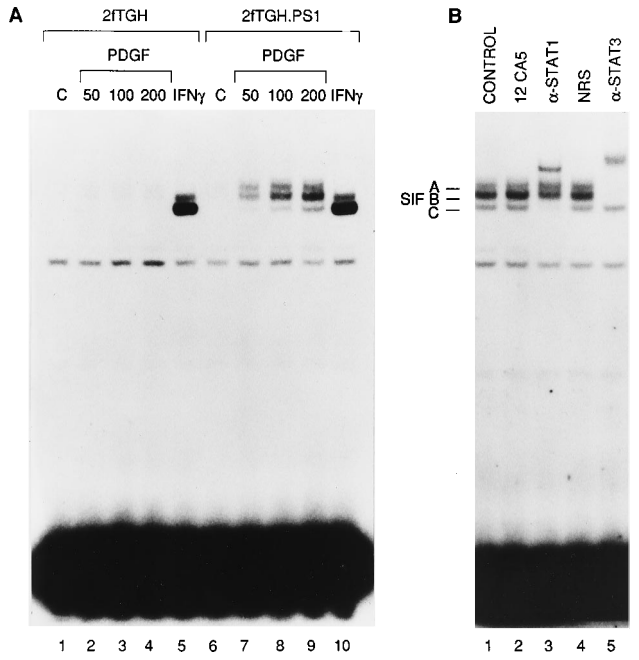

FIG. 2. Activation of STAT1 and STAT3 by PDGF in 2fTGH and 2fTGH.PS1 cells. (A) Titration of PDGF stimulation in 2fTGH.PS1 cells. The noninfected or PDGFR-β-infected 2fTGH cells (2fTGH.PS1) were grown to subconfluence and either left untreated (C), treated for 15 min at 37°C with 10 ng of IFN-γ per ml or with the indicated concentrations of PDGF-BB (50 to 200 ng/ml). Cytosolic extracts prepared from these cells were incubated with the high-affinity SIEm67 ^{32}P-labeled probe and subjected to mobility shift analysis. Lanes: 1 to 5, 2fTGH cells; 6 to 10, 2fTGH.PS1 cells. (B) Evidence that STAT1 and STAT3 are present in SIF complexes. Cytosolic extracts from 2fTGH.PS1 cells stimulated with PDGF-BB (200 ng/ml) were incubated with the high-affinity SIE ^{32}P-labeled probe (control in lane 1) and with the following antibodies: 1 μl of 12CA5 (lane 2), 1 μl of anti-STAT1 monoclonal antibodies (lane 3), 1 μl of normal rabbit serum (lane 4), 1 μl of anti-STAT3 polyclonal antibodies (lane 5). DNA-protein complexes were analyzed by electrophoretic mobility shift assays. The three specific complexes, SIF-A, SIF-B, and SIF-C, are indicated.

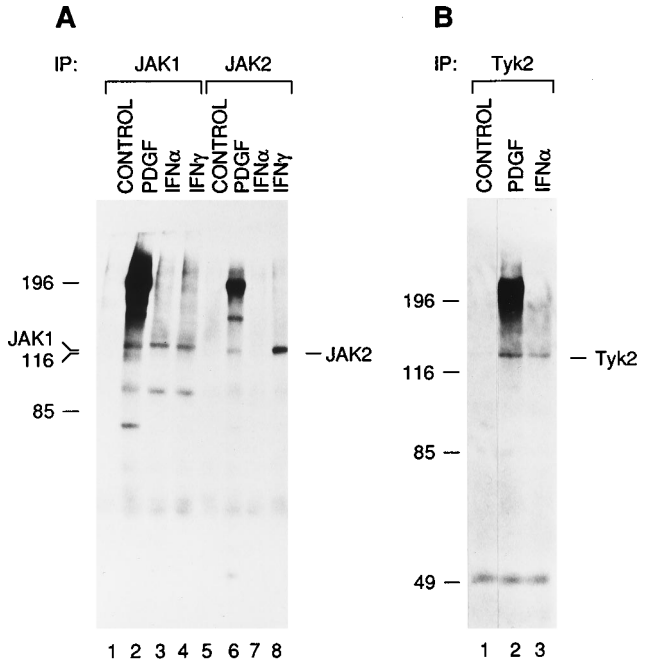

FIG. 3. PDGF induces tyrosine phosphorylation of JAK1, JAK2, and Tyk2 in 2fTGH.PS1 cells. (A) Confluent 2fTGH.PS1 cultures were serum starved for 18 h and either left untreated or treated with PDGF (200 ng/ml), IFN-α (500 U/ml), or IFN-γ (20 ng/ml) for 5 min at 37°C. Cells were lysed in buffer B (see Materials and Methods), and insoluble material was removed by centrifugation. Lysates were subjected to immunoprecipitation with anti-JAK1 agarose or anti-JAK2 agarose. The Immobilon membrane was probed with anti-phosphotyrosine antibody (4G10), and antibody-antigen complexes were visualized by ECL. The molecular weight markers are indicated in thousands. Lanes: 1 to 4, anti-JAK1 immunoprecipitation (IP); 5 to 8, anti-JAK2 immunoprecipitation. (B) Confluent 2fTGH.PS1 cultures were serum starved for 18 h and either left untreated or treated with PDGF (200 ng/ml) or IFN-α (500 U/ml). Lysates were prepared as for panel A, except that buffer A (see Materials and Methods) was used instead of buffer B. Proteins were subjected to immunoprecipitation with anti-Tyk2 polyclonal antibodies. The membrane was probed with anti-phosphotyrosine antibody (4G10), and antibody-antigen complexes were visualized by ECL. Shown is immunoprecipitation with anti-Tyk2 antibodies of protein lysates obtained from cells untreated (control) (lane 1), treated with PDGF (lane 2), or treated with IFN-α (lane 3). The molecular weight markers are indicated in thousands.

these extracts were separated by SDS-PAGE, transferred to membranes, and probed with various antibodies. A PDGFR immunoblot (Fig. 4A) shows that the mature form of PDGFR (190 kDa) was present in similar concentrations in all of the cell lines. In Fig. 4B, cells were either incubated with PDGF (P) or left untreated (C). The blot, probed with anti-phosphotyrosine antibody, showed a strong phosphorylation of the PDGF receptor (compare lanes 2, 4, 6, 8, and 10 with lanes 1, 3, 5, 7, and 9), as well as other proteins likely to correspond to receptor substrates. In contrast, the pattern of tyrosine-phosphorylated proteins in uninfected 2fTGH cells incubated with PDGF was indistinguishable from the pattern obtained with untreated cells (data not shown). The overall patterns of tyrosine-phosphorylated proteins were identical in the different cell lines, indicative of equivalent expression and activity of the PDGF receptors in these cells. Immunoblots for STAT1 and STAT3 (Fig. 4C and D) showed that the concentrations of STAT1 in 2C4.PS2 and γ2A.PS1 cells were slightly higher than in 2fTGH.PS1, U1A.PS1, and U4A.PS1 cells whereas STAT3 concentrations were equivalent in all the cell lines analyzed.

Analysis of PDGF-induced signaling events in the absence of individual JAKs. We first examined the effect of the absence of a given JAK on the PDGF-induced phosphorylation of the remaining JAKs. In the case of IFN signaling, loss of one JAK eliminates the ligand-induced phosphorylation of the other receptor-associated JAK (25). Figure 5A shows that in contrast to the IFN system, PDGF-induced tyrosine phosphorylation of JAK1 and Tyk2 was not abolished by loss of JAK2. Similarly, Fig. 5B shows that in the absence of JAK1 and Tyk2, respectively, PDGF-induced tyrosine phosphorylation of the remaining JAKs was not abolished. Thus, JAKs are phosphorylated independently of one another in response to PDGF treatment.

JAK phosphorylation in response to PDGF could result from true autophosphorylation, from phosphorylation in *trans* by the PDGFR kinase, or from phosphorylation in *trans* by receptor-associated kinase brought into proximity by ligand-induced receptor dimerization. To rule out true autophosphorylation, we examined a JAK2-deficient cell line that expressed a kinase-inactive JAK2 mutant (12). Figure 5C shows that the catalytically inactive JAK2 protein was phosphorylated on tyrosine following PDGF treatment. Although we have not determined that the mutant protein is phosphorylated on the same site(s) as wild-type protein, this observation suggests that at least one component of PDGF-induced JAK phosphorylation is due to phosphorylation in *trans.*

PDGF-dependent tyrosine phosphorylation of the JAKs requires the kinase activity of the PDGFR. Current models for cytokine signaling hold that ligand-induced dimerization of the

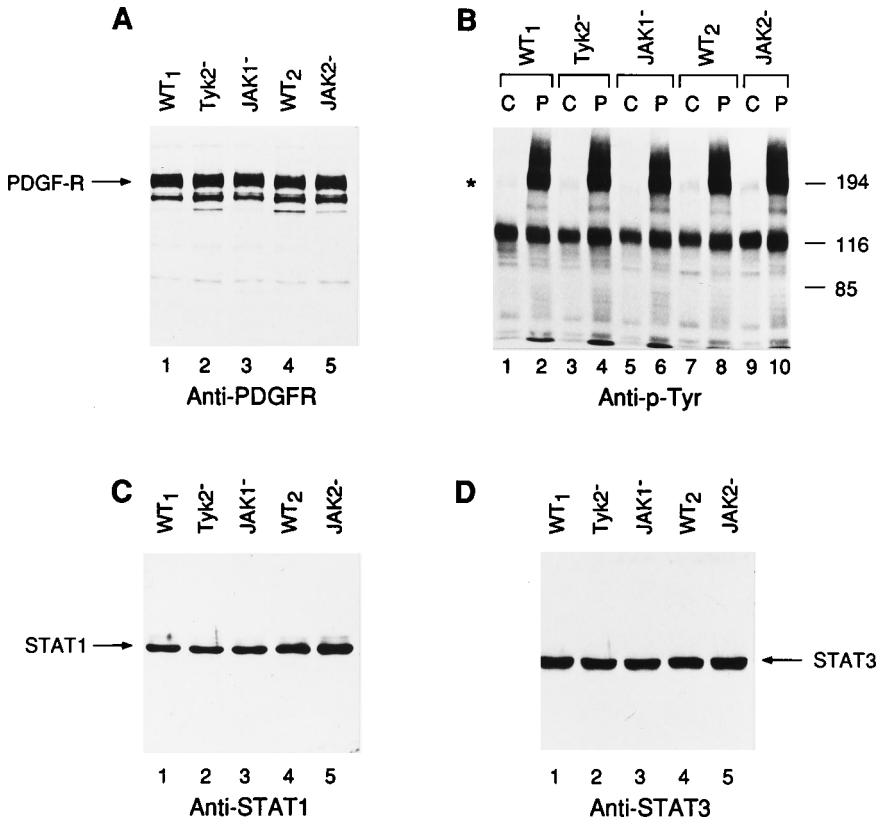

FIG. 4. Characterization of JAK-deficient cell lines expressing the PDGFR-β. Wild-type, Tyk2$^-$, JAK1$^-$, and JAK2$^-$ cell lines were grown in DMEM–10% FBS. Cells were serum starved for 18 h (0.5% FBS) and either left untreated or treated with PDGF (200 ng/ml) for 5 min at 37°C. Cell lysates were prepared in the presence of 1% Nonidet P-40. (A) PDGFR immunoblot analysis. Protein lysates (30 μg) from untreated cells were separated on an SDS–7.5% polyacrylamide gel, transferred to nitrocellulose, and probed with an anti-PDGFR polyclonal antibody. The 190-kDa protein corresponds to the fully mature PDGFR. (B) Phosphotyrosine (p-Tyr) immunoblot analysis. Protein lysates from either untreated (lanes C) or PDGF-treated (lanes P) cells were separated by SDS-PAGE and transferred to nitrocellulose. The membranes were probed with anti-phosphotyrosine 4G10 antibody. The molecular weight markers are indicated in thousands. (C and D) Extracts from untreated cells were prepared and separated on an SDS–7.5% polyacrylamide gel as in panel A. The membrane was incubated with monoclonal anti-STAT3 antibodies (D). The membrane was then stripped and incubated with monoclonal anti-STAT1 antibodies (C). All immunocomplexes were visualized by ECL. WT_1 refers to the 2fTGH.PS1 cell line. Tyk2$^-$ and JAK1$^-$ refer to U1A.PS1 and U4A.PS1 cells, respectively, which are isogenic with 2fTGH.PS1. WT_2 and JAK2$^-$ refer to the isogenic 2C4.PS2 and γ2A.PS1 cell lines, respectively.

receptor brings receptor-associated JAKs into proximity, permitting cross-phosphorylation of JAKs and of receptor subunits. If this were also the case for PDGFR signaling, JAK phosphorylation should not be affected by a mutation that inactivates the catalytic activity of the receptor. To test this hypothesis, we examined JAK phosphorylation in canine kidney epithelial cells expressing a mutant PDGFR bearing an amino acid substitution in the catalytic lysine residue of the kinase domain (19, 20). Figure 6 shows that in fact none of the JAKs were phosphorylated in PDGF-treated cells carrying the mutant receptor. Thus, in contrast to the cytokine system, JAK phosphorylation requires the catalytic activity of the PDGFR, either because JAKs are direct substrates for the receptor tyrosine kinase or because their association with the receptor requires receptor autophosphorylation.

Activation of STAT1 and STAT3 by PDGFR-β does not require JAK1, JAK2, or Tyk2 but does require receptor kinase activity. The requirement for individual JAKs in the activation of STATs by various cytokine receptors is clearly documented (12, 25, 48). To test whether individual JAKs have similarly unique roles in STAT activation by PDGF, we performed mobility shift assays on extracts from PDGF-treated JAK-deficient cell lines. Incubation of extracts from PDGF-stimulated 2fTGH.PS1 cells (WT_1) with the SIE probe yielded the three complexes, SIF-A, SIF-B, and SIF-C, as shown previously (Fig. 7A, lane 2, and Fig. 2). PDGF treatment of 2C4.PS2 cells (WT_2) also led to the activation of STAT1 and STAT3 (Fig. 7A, lane 14). The larger amount of SIF-C complex (STAT1 homodimer) observed in 2C4.PS2 cells compared with 2fTGH.PS1 (compare lanes 14 and 2) presumably reflects the higher STAT1 concentration in 2C4.PS2 cells (Fig. 4C). In parallel with PDGF stimulation, cells were treated with either IFN-α or IFN-γ to demonstrate that the wild-type and JAK-disrupted cell lines had not undergone major mutations during the process of virus infection and cell sorting. STAT1 and STAT3 are activated in IFN-α-treated T cells (3). Likewise, incubation of 2fTGH.PS1 (WT_1) or 2C4.PS2 (WT_2) cells with IFN-α induced the activation of these STATs, giving rise to the three complexes SIF-A, SIF-B, and SIF-C (Fig. 7A, lanes 3 and 15). IFN-γ stimulated mostly STAT1 in 2fTGH.PS1 and 2C4.PS2 cells, giving complex SIF-C (lanes 4 and 16). When the JAK$^-$ cell lines were analyzed for their response to IFN-α and IFN-γ, the expected patterns of DNA-protein complexes were obtained. Tyk2$^-$ and JAK2$^-$ cells were unresponsive to IFN-α and IFN-γ, respectively (lanes 7 and 20), while JAK1$^-$ cells were unresponsive to both ligands (lanes 11 and 12). Thus, all of the engineered cell lines displayed the expected responses to IFN treatment.

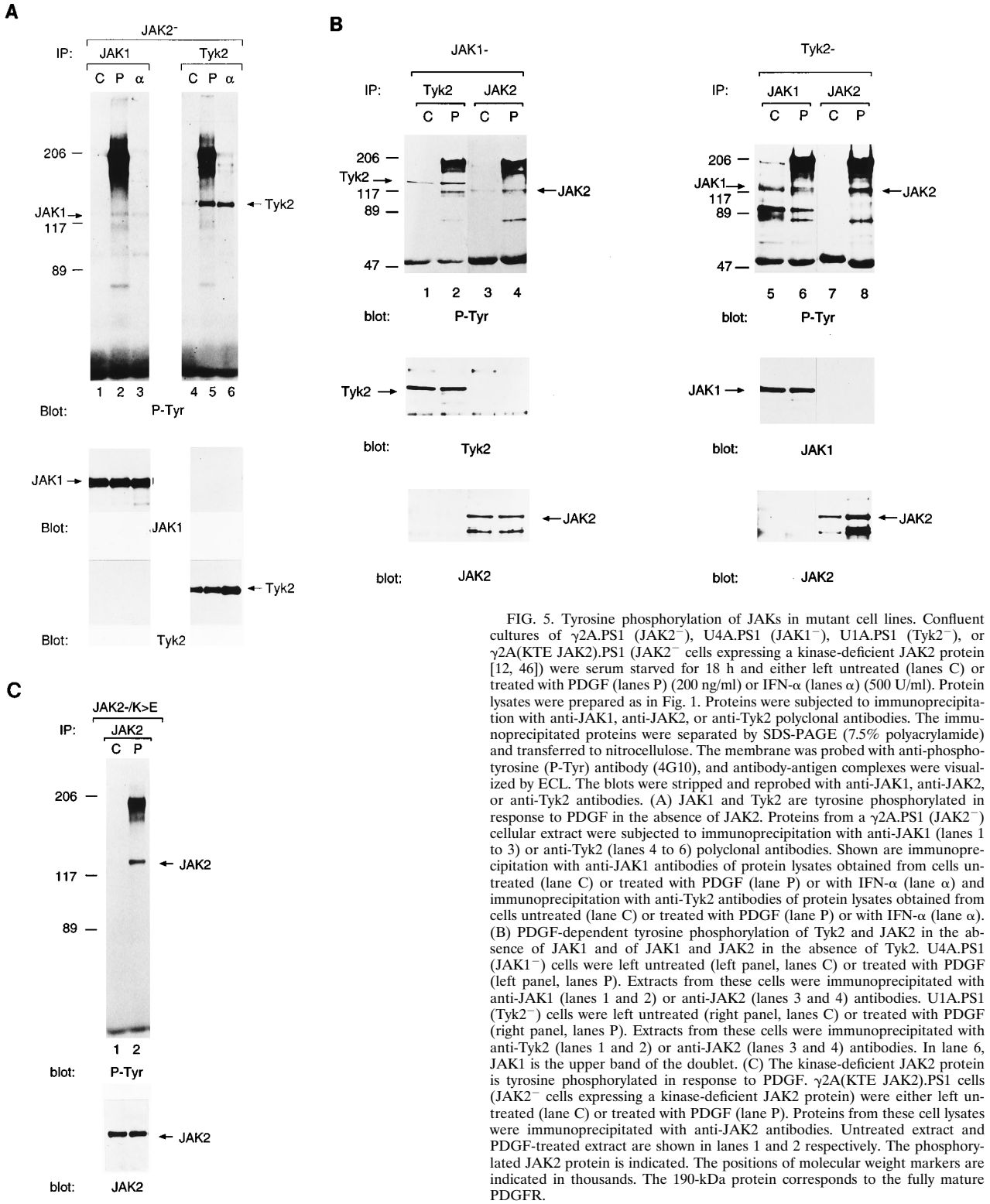

FIG. 5. Tyrosine phosphorylation of JAKs in mutant cell lines. Confluent cultures of γ2A.PS1 (JAK2⁻), U4A.PS1 (JAK1⁻), U1A.PS1 (Tyk2⁻), or γ2A(KTE JAK2).PS1 (JAK2⁻ cells expressing a kinase-deficient JAK2 protein [12, 46]) were serum starved for 18 h and either left untreated (lanes C) or treated with PDGF (lanes P) (200 ng/ml) or IFN-α (lanes α) (500 U/ml). Protein lysates were prepared as in Fig. 1. Proteins were subjected to immunoprecipitation with anti-JAK1, anti-JAK2, or anti-Tyk2 polyclonal antibodies. The immunoprecipitated proteins were separated by SDS-PAGE (7.5% polyacrylamide) and transferred to nitrocellulose. The membrane was probed with anti-phosphotyrosine (P-Tyr) antibody (4G10), and antibody-antigen complexes were visualized by ECL. The blots were stripped and reprobed with anti-JAK1, anti-JAK2, or anti-Tyk2 antibodies. (A) JAK1 and Tyk2 are tyrosine phosphorylated in response to PDGF in the absence of JAK2. Proteins from a γ2A.PS1 (JAK2⁻) cellular extract were subjected to immunoprecipitation with anti-JAK1 (lanes 1 to 3) or anti-Tyk2 (lanes 4 to 6) polyclonal antibodies. Shown are immunoprecipitation with anti-JAK1 antibodies of protein lysates obtained from cells untreated (lane C) or treated with PDGF (lane P) or with IFN-α (lane α) and immunoprecipitation with anti-Tyk2 antibodies of protein lysates obtained from cells untreated (lane C) or treated with PDGF (lane P) or with IFN-α (lane α). (B) PDGF-dependent tyrosine phosphorylation of Tyk2 and JAK2 in the absence of JAK1 and of JAK1 and JAK2 in the absence of Tyk2. U4A.PS1 (JAK1⁻) cells were left untreated (left panel, lanes C) or treated with PDGF (left panel, lanes P). Extracts from these cells were immunoprecipitated with anti-JAK1 (lanes 1 and 2) or anti-JAK2 (lanes 3 and 4) antibodies. U1A.PS1 (Tyk2⁻) cells were left untreated (right panel, lanes C) or treated with PDGF (right panel, lanes P). Extracts from these cells were immunoprecipitated with anti-Tyk2 (lanes 1 and 2) or anti-JAK2 (lanes 3 and 4) antibodies. In lane 6, JAK1 is the upper band of the doublet. (C) The kinase-deficient JAK2 protein is tyrosine phosphorylated in response to PDGF. γ2A(KTE JAK2).PS1 cells (JAK2⁻ cells expressing a kinase-deficient JAK2 protein) were either left untreated (lane C) or treated with PDGF (lane P). Proteins from these cell lysates were immunoprecipitated with anti-JAK2 antibodies. Untreated extract and PDGF-treated extract are shown in lanes 1 and 2 respectively. The phosphorylated JAK2 protein is indicated. The positions of molecular weight markers are indicated in thousands. The 190-kDa protein corresponds to the fully mature PDGFR.

When examined for STAT activation by PDGF, however, the mutant cell lines lacking either Tyk2 or JAK1 responded similarly to parental 2fTGH.PS1, showing activation of both STAT1 and STAT3 (Fig. 7A, compare lanes 6 and 10 with lane 2). Another independently derived JAK1⁻ clone, U4C, gave similar results (lane 22). Likewise, STAT1 and STAT3 were activated in cells lacking JAK2 to the same extent as in the parental 2C4.PS2 cells (compare lanes 18 and 14). These results show that both STAT1 and STAT3 are activated by PDGF in the cells lacking individual JAKs and therefore dem-

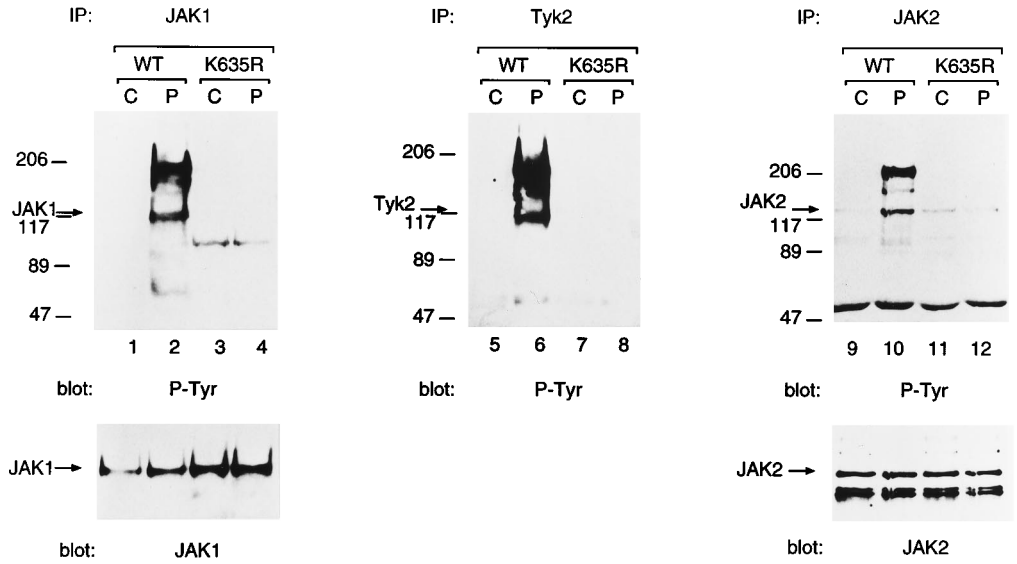

FIG. 6. The tyrosine kinase activity of the PDGF receptor is required for the PDGF-dependent tyrosine phosphorylation of the JAK proteins. Confluent cultures of TRMP cells expressing either a wild-type or a tyrosine kinase-deficient (K635R) mutant PDGF-β receptor (19, 20) were serum starved for 18 h and either left untreated (lanes C) or treated with PDGF (lanes P) (200 ng/ml). Protein lysates were prepared as in Fig. 1. Proteins were subjected to immunoprecipitation with polyclonal antibodies directed against JAK1 (lanes 1 to 4), Tyk2 (lanes 5 to 8), or JAK2 (lanes 9 to 12). The immunoprecipitated proteins were separated by SDS-PAGE (7.5% polyacrylamide) and transferred to nitrocellulose. The membrane was probed with anti-phosphotyrosine antibody (4G10), and antibody-antigen complexes were visualized by ECL. The blots were stripped and reprobed with anti-JAK1 (lanes 1 to 4) or anti-JAK2 (lanes 9 to 12). The canine Tyk2 protein could not be detected by Western blotting with the monoclonal or polyclonal anti-Tyk2 antibodies we tested.

onstrate that JAK1, JAK2, and Tyk2 are not individually required for STAT activation by PDGF.

Figure 7B shows that activation of STAT1 and STAT3 by PDGF is abolished by the receptor mutation that eliminates RTK activity but not by a significant deletion that eliminates the entire kinase insert region of the receptor, which encodes three known receptor autophosphorylation sites (19, 20). Thus, the catalytic activity of the RTK is required for STAT activation by PDGF but recruitment sites within the kinase insert region are not.

DISCUSSION

It is now clear that JAK and STAT family proteins are critical components of the signal transduction cascades triggered by cytokine receptors (reviewed in references 4, 7, 15, 16, 21, 37, 43, 44, and 56). The dissection of the IFN signaling pathway showed that specific individual members of the JAK family are required for ligand-induced activation of STAT proteins and of IFN target genes (25, 45, 48). The more general importance of JAKs in cytokine signaling came from the observation that JAK phosphorylation accompanies receptor activation in a variety of cases (2, 5, 8, 17, 27, 40, 41, 49, 50). Presumably, members of the JAK family are utilized by cytokine receptors in lieu of intrinsic protein tyrosine kinase activity.

Surprisingly, however, recent data suggest that receptors with intrinsic tyrosine kinase activity, such as EGFR or PDGFR, share components of this signal transduction pathway. Both EGF and PDGF induce activation of STAT proteins (29, 31–33, 35, 39, 47). Furthermore, EGF treatment of A431 cells induces the tyrosine phosphorylation of JAK1 (38). Our data suggest that JAK activation may be a general consequence of engagement of this class of receptors. We find that all three ubiquitously expressed JAKs—JAK1, JAK2, and Tyk2—are associated with the activated PDGFR-β and become tyrosine phosphorylated upon treatment with PDGF. JAK phosphorylation was observed following engagement of the native PDGFR population in BALB/c 3T3 cells and in human fibroblasts and canine kidney epithelial cells engineered to express human PDGFR-β by retroviral transduction.

To investigate the mechanism of JAK activation by PDGFR and the role of JAKs in STAT activation by PDGFR, we used a retroviral vector to express PDGFR-β in human fibroblast cell lines that each lacked one of the JAKs. The use of a retrovirus to obtain efficient gene transfer coupled with fluorescence-activated cell sorting enabled us to obtain matched populations of cells with similar numbers of cell surface receptors, permitting direct comparisons among different populations. Our results with these cell lines suggest several potentially significant differences between the mechanism of JAK activation by RTKs and cytokine receptors.

For the best studied of the cytokine receptors, the receptors for IFN-α and IFN-γ, receptor signaling and STAT activation require two distinct JAKs. Elimination of either JAK prevents phosphorylation of the second JAK and all subsequent signaling events. This interdependence of JAK phosphorylation is thought to reflect a requirement for *trans* phosphorylation of one JAK by another upon ligand-induced clustering of receptor subunits. Similar observations have been reported for other cytokine receptors complexes composed of distinct receptor subunits that interact with different JAKs (16, 21, 44, 46).

No such interdependence was observed following activation of PDGFR. Despite the observation that a kinase-deficient JAK2 protein is efficiently phosphorylated in response to PDGF—suggesting that at least a portion of PDGF-induced phosphorylation of JAK2 must occur in *trans*—in the absence of any single JAK, phosphorylation of the remaining JAKs was not significantly affected. Thus, in contrast to interferon signaling, phosphorylation of any given JAK in response to PDGF is not strictly dependent upon any other JAK.

Cytokine receptor signaling is thought to be initiated by ligand-induced dimerization of receptor subunits, bringing re-

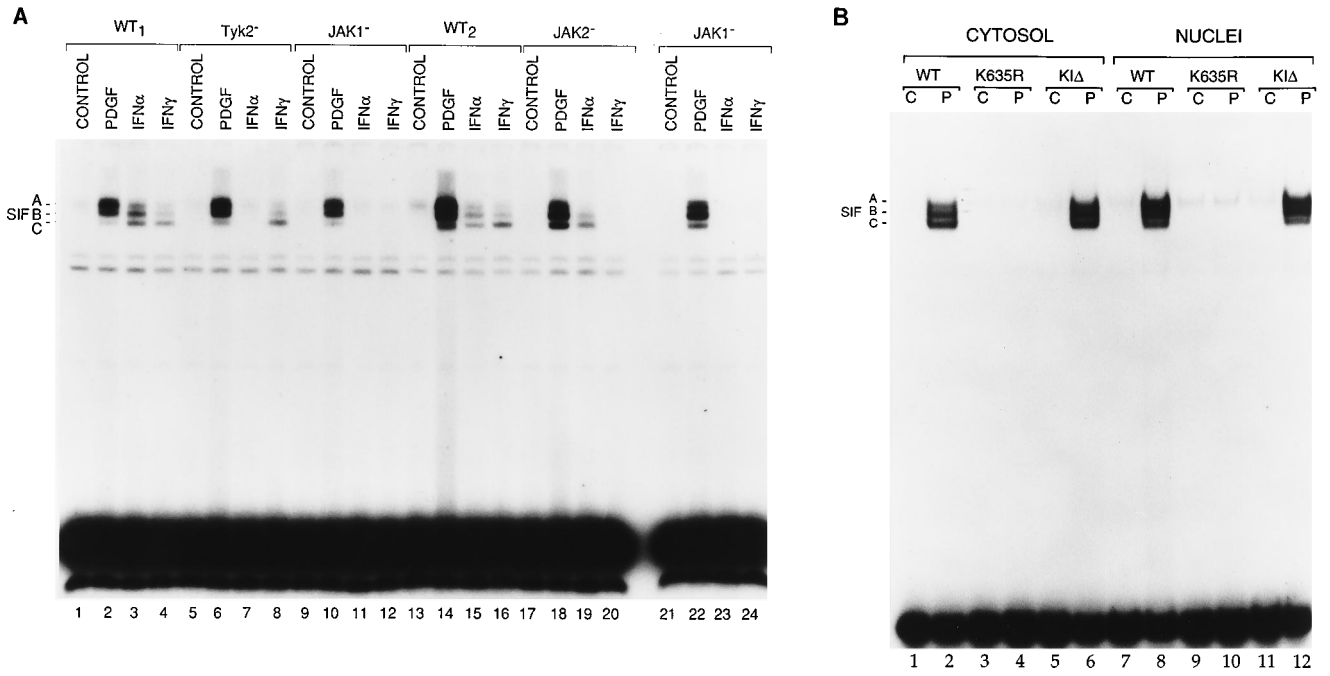

FIG. 7. (A) Activation of STAT1 and STAT3 by PDGFR-β does not require Tyk2, JAK1, or JAK2 kinases. Subconfluent cultures of WT, Tyk2$^-$, JAK1$^-$, or JAK2$^-$ cell lines were serum starved for 16 h (0.5% FBS) and either left untreated (control) or treated with PDGF (200 ng/ml), IFN-α (500 U/ml) or IFN-γ (10 ng/ml) for 15 min at 37°C. Cytosolic extracts (25 μg) from these cells were incubated with the high-affinity SIEm67 ^{32}P-labeled probe and subjected to mobility shift analysis. The three specific complexes SIF-A, SIF-B, and SIF-C are indicated. Lanes: 1 to 4, cell line 2fTGH.PS1; 5 to 8, U1A.PS1; 9 to 12, U4A.PS1 (U1A and U4A cells are, respectively Tyk2$^-$ and JAK1$^-$ variants of the parental 2fTGH cell line); 13 to 16, 2C4.PS2 cells; 17 to 20, γ2A.PS1 cells (γ2A cells are a JAK2$^-$ derivative of the parental wild-type 2C4 cell line); 21 to 24, U4C.PS2 cells that correspond to another JAK1$^-$ variant of the parental 2fTGH cell line. (B) Activation of STAT1 and STAT3 by PDGFR-β requires the tyrosine kinase activity of the receptor. Confluent cultures of TRMP cells expressing either a wild-type (WT), a tyrosine kinase-deficient (K635R) PDGFR-β, or a mutant lacking the kinase insert domain (KIΔ) (amino acids 708 to 793) (18) were serum starved for 18 h and either left untreated (lanes C) or treated with PDGF (lanes P) (200 ng/ml). Cytosolic (25 μg) (lanes 1 to 6) or nuclear (20 μg) (lanes 7 to 12) extracts from these cells were incubated with the high-affinity SIEm67 ^{32}P-labeled probe and subjected to mobility shift analysis. The three specific complexes SIF-A, SIF-B, and SIF-C are indicated.

ceptor-associated JAKs into proximity and permitting phosphorylation of the apposed receptor subunit and its associated JAK. If JAKs were functioning similarly in association with the PDGFR, JAK activation and perhaps certain aspects of PDGFR signaling would be independent of the catalytic activity of the receptor tyrosine kinase itself. Indeed, there is evidence that a catalytically inactive EGFR undergoes tyrosine phosphorylation and activates mitogen-activated protein kinase, apparently because of the activity of a receptor-associated tyrosine kinase (6, 36). However, we observed that a catalytically inactive PDGFR was unable to promote either JAK or STAT activation. This observation contrasts with the scenario in cytokine signaling, in which one JAK is believe to directly phosphorylate another. Rather, it is consistent with JAKs being direct substrates for the RTK or another receptor-associated enzyme. An alternative hypothesis, however, is that association of the JAKs with the PDGFR is dependent upon receptor autophosphorylation. We cannot distinguish between these hypotheses, because the amount of PDGFR present in JAK immunoprecipitates is below the limit of detection of our receptor antibodies. Because JAKs lack an evident SH2 or phosphotyrosine-binding (PTB) domain, phosphotyrosine-dependent association of JAKs with the PDGFR would probably be mediated by an adaptor molecule.

STAT activation by PDGF was not significantly affected by the absence of any single JAK. This again differs from the case of IFN receptors, which exhibit absolute requirements for specific JAKs. Similar results have been obtained with EGF. Leaman et al. (22) have performed a similar study to the one we describe here in which they have transferred the EGFR into 2fTGH cells and its JAK-deficient derivatives. In contrast to our observations, they detected phosphorylation of only JAK1 in EGF-treated cells. The absence of either JAK1, JAK2, or Tyk2 does not affect STAT activation by EGF. We have also examined HeLa cell lines deficient in JAK1 (23) and found that STAT activation by EGF is indistinguishable from controls (data not shown). Thus, for both PDGFR and EGFR, it appears that these JAKs are not individually required for STAT activation.

What is the basis for the differences we observe between RTK and cytokine signaling? One possibility is that JAKs are fully redundant for RTKs but not for IFN receptors. However, our data indicate that in the absence of any one JAK, there is no apparent compensatory increase in the phosphorylation of the others. In a further attempt to uncover a possible redundancy within the JAK family or the involvement of an as yet uncharacterized JAK in STAT activation by the PDGFR, we used a cell line that lacked endogenous JAK2 and overexpressed a JAK2 protein deficient in its kinase activity. This mutant protein might be expected to act as a *trans*-dominant inhibitor of other JAKs by competing either for receptor-docking sites or for substrates. Indeed, such an effect was observed in cells treated with IL-6 (reference 12 and data not shown). However, activation of the STAT proteins by PDGF (and phosphorylation of the remaining active JAKs) was not affected in these cells (data not shown), suggesting that the protein did not exert a dominant-negative effect on STAT activation by PDGF. Together with the results obtained with the EGFR, these data argue against simple JAK redundancy in STAT activation by these RTKs.

Which kinase is responsible for phosphorylating STATs following PDGF treatment? One possibility is the receptor kinase itself. Such a model of STAT activation by receptor tyrosine kinases was proposed for the EGFR (10). Consistent with this hypothesis is the observation that STAT1 is efficiently phosphorylated by the EGFR kinase in vitro (29). In addition, we observed efficient activation of recombinant STAT1 in immune complexes containing PDGFR (46). In the case of STAT3, there is evidence for a role for Src family kinases (53). However, it remains possible that RTK activation promotes association of JAKs with the receptor and that it is the JAKs that phosphorylate the STATs. Our ability to reconstitute EGF- and PDGF-dependent STAT activation in cell-free assays should allow us to address all these issues directly (34, 46).

Finally, what is the function, if any, of JAKs in RTK signal transduction? In cytokine signaling, JAKs are believed to phosphorylate receptor subunits to generate recruitment sites for signaling proteins and perhaps to phosphorylate the signaling proteins themselves. By analogy, JAKs could play a similar role in RTK signaling, phosphorylating receptor tyrosine residues not accessible to the receptor kinase or phosphorylating proteins recruited to the activated receptor. It is worth noting, however, that although JAK phosphorylation and receptor association were reproducibly observed in three different cell lines, the observed stoichiometry of these reactions appears to be low. Therefore, the functional significance of these events is not yet clear.

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