Platelet-derived growth factor (PDGF)-induced activation of signal transducer and activator of transcription (Stat) 5 is mediated by PDGF β-receptor and is not dependent on c-Src, Fyn, Jak1 or Jak2 kinases

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Several growth factors activate signal transducers and activators of transcription (Stats) but the mechanism of Stat activation in receptor tyrosine kinase signalling has remained elusive. In the present study we have analysed the roles of different platelet-derived growth factor (PDGF)-induced tyrosine kinases in the activation of Stat5. Co-expression experiments in insect and mammalian cells demonstrated that both PDGF β-receptor (PDGF β-R) and Jak1, but not c-Src, induced the activation of Stat5. Furthermore, immune-complex-purified PDGF β-R was able to phosphorylate Stat5 directly. The role of the cytoplasmic tyrosine kinases in the PDGF-induced activation of Stat5 was further investigated by overexpressing kinase-negative (KN) and wild-type Jak and c-Src kinases. Jak1-KN or Jak2-KN had no effect but both Src-KN and wild-type c-Src similarly decreased the PDGF-β-R-induced activation of Stat5. The activation of both Src and Stat5 is dependent on the same tyrosine residues Tyr^{579} and Tyr^{581} in PDGF β-R; thus the observed inhibition by Src might result from competition for binding of Stat5 to the receptor. Finally, fibroblasts derived from $Src^{-/-}$ and $Fyn^{-/-}$ mice showed normal pattern of PDGF-induced tyrosine phosphorylation of Stat5. Taken together, these results indicate that Stat5 is a direct substrate for PDGF β-R and that the activation does not require Jak1, Jak2, c-Src or Fyn tyrosine kinases.

Key words: growth-factor receptor, signal transduction, tyrosine kinase, tyrosine phosphorylation.

INTRODUCTION

Platelet-derived growth factor (PDGF) exists as both homodimers and heterodimers of PDGF-A and PDGF-B polypeptides, which bind in an ordered manner to PDGF α-receptor and PDGF β-receptor (PDGF β-R) tyrosine kinases [1]. Dimerization and subsequent activation of PDGF β-R by ligand binding leads to autophosphorylation of the receptor on tyrosine residues. Receptor phosphorylation triggers the recruitment of several Src homology 2 (SH2) domain-containing signalling proteins, including phospholipase Cγ1, GTPase-acting protein of Ras, phosphoinositide 3-kinase, SHP2, Nck, Grb2, Shc and Src [1,2] to the receptor. These proteins initiate various signalling pathways, leading ultimately to changes in gene expression. One PDGF-induced nuclear pathway uses signal transducers and activators of transcription (Stats) Stat1, Stat3, Stat5 and Stat6 [3–9]. The family of Stat proteins consists of seven mammalian members that are selectively activated by different cytokines and growth factors. On stimulation, Stats are recruited to the tyrosine-phosphorylated receptor complex, become phosphorylated on a conserved C-terminal tyrosine residue, form SH2 domain-mediated homodimers or heterodimers and translocate to the nucleus.

A well-characterized activation mechanism for Stats by cytokine receptors is their phosphorylation by the receptor-associated Jak tyrosine kinases. However, in receptor tyrosine kinase signalling the mechanism of Stat activation has been unclear. Previously we showed that activation of Stat5 was dependent on the catalytic activity of PDGF β-R [9] but the precise activation mechanism of Stat5 remained unknown. In addition to the catalytic activation of the receptor itself, stimulation by PDGF results in the activation of Src and Jak [3,9] family tyrosine kinases, which also could participate in the activation of Stats. The activation of Stat1 by PDGF β-R *in vitro* does not require Jak kinases, whereas the activation of Stat3 is dependent also on Jak activity [10]. So far the role of Jak kinases in the PDGF-induced activation of Stat5 or Stat6 has not been investigated. The role of Src family kinases in the PDGF-mediated activation of Stat5 has not been addressed directly but some indirect evidence suggests that Src could participate in the PDGF β-R-induced activation of Stat5. Activation of Stat5 by PDGF β-R was previously found to require the same receptor tyrosine residues Tyr^{579} and Tyr^{581} that are needed for activation of Src kinases [9]. In addition, the highly active oncogenic v-Src induces the activation of various Stats in different cell models [11–13]. Src family kinases have also been implicated in the activation of Stats in different receptor systems such as interleukin 3 (IL-3) receptor, and epidermal growth factor (EGF) receptor signalling [13,14].

The present studies were undertaken to investigate the role of PDGF β-R, Src and Jak family kinases in the activation of Stat5. The results obtained in mammalian and insect cells as well as *in vitro* indicate that the PDGF-induced activation of Stat5 is mediated by PDGF β-R and is not dependent on c-Src, Fyn, Jak1 or Jak2 tyrosine kinases.

Abbreviations used: βRic, the intracellular part of PDGF β-R; EGF, epidermal growth factor; FBS, fetal bovine serum; HA, haemagglutinin; IL, interleukin; KN, catalytically inactive mutant; PDGF, platelet-derived growth factor; PDGF β-R, PDGF β-receptor; SH2, Src homology 2; Stat, signal transducer and activator of transcription.

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MATERIALS AND METHODS

Cell culture

293T, COS-7 and Swiss 3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal bovine serum (FBS; Gibco) and antibiotics. NIH 3T3, normal mouse fibroblasts, as well as $Src^{-/-}$ and $Fyn^{-/-}$ fibroblasts {derived from $Src^{-/-}$ and $Fyn^{-/-}$ mice [15,16], which were a gift from Dr. P. Soriano (Fred Hutchinson Cancer Research Center, Seattle, WA, U.S.A.)}, were cultured in Dulbecco's modified Eagle's medium with 10 % (v/v) newborn calf serum (Gibco) and antibiotics. *Spodoptera frugiperda* (Sf9) cells were grown in Sf900 medium (Gibco) with 10 % (v/v) FBS and antibiotics. PAE cells expressing wild-type β-receptor (PAE/PDGFR-β) [17] were cultured in Ham's F-12 medium (Gibco), supplemented with 10 % (v/v) FBS and antibiotics. The baculovirus-infected Sf9 cells were lysed 72 h after infection. 293T and COS-7 cells were transfected with a calcium phosphate transfection system (Gibco) in accordance with the manufacturer's instructions, and lysed after 72 h. Before stimulation, COS-7 and 293T cells were starved overnight without serum, followed by stimulation with 50 ng/ml PDGF-BB (Upstate Biotechnology) for 10 min at 37 °C. All fibroblasts were stimulated with 100 ng/ml PDGF-BB for 10 min at 37 °C after starvation overnight in 0.2 % (v/v) newborn calf serum. PAE cells were stimulated with 100 ng/ml PDGF-BB for 10 min at 37 °C.

Antibodies

Monoclonal anti-phosphotyrosine antibody (clone 4G10) and polyclonal anti-(PDGF β-R) antibody were from Upstate Biotechnology. Monoclonal anti-phosphotyrosine (PY20) and anti-Jak1 antibodies were from Transduction Laboratories. Polyclonal anti-Stat5B (recognizing both Stat5A and Stat5B) and c-Src antibodies were from Santa Cruz Biotechnology. Monoclonal anti-Stat5A antibody was from Zymed Laboratories. Anti-haemagglutinin (HA) monoclonal antibody (16B12) was from Berkeley Antibody Company; monoclonal anti-(v-Src) antibody Ab-1 (recognizing both v-Src and c-Src) was from Oncogene Research Products.

DNA constructs

PDGF β-R [18] and PDGF β-R mutants $Tyr^{579} \rightarrow$ Phe and $Tyr^{581} \rightarrow$ Phe [19] were cloned into pCIneo expression vector (Promega). Expression vectors for Jak1 [20], c-Src [20], sheep Stat5A [a gift from Dr. B. Groner (Georg-Speyer-Haus, Frankfurt, Germany)] [21] have been described previously. The catalytically inactive mutant Src-KN ($Lys^{297} \rightarrow$ Met) was a gift from Dr. David O. Morgan (University of California at San Francisco, San Francisco, CA, U.S.A.). Mouse Stat5A and SH2 mutant ($Arg^{618} \rightarrow$ Leu) of mouse Stat5A were gifts from Dr. T. Wood (Pharmacia-Upjohn, Stockholm, Sweden). Jak1-KN was constructed by mutating Lys^{835} to Glu by PCR; Jak2-KN-HA was similar to the previously described Jak2-HA [22] but had a $Lys^{882} \rightarrow$ Glu substitution. The intracellular part of PDGF β-R (βRic) [23] and Jak1 [a gift from Dr. J. Ihle (St. Jude Children's Research Hospital, University of Tennessee, Memphis, TN, U.S.A.)] [24] baculoviruses have been described previously. c-Src baculovirus was a gift from Dr. David O. Morgan; Stat5A baculovirus was a gift from Dr. T. Wood.

Immunoprecipitation and Western blotting

Fibroblast cells were lysed in Nonidet P40 buffer [1 % (v/v) Nonidet P40/10 % (v/v) glycerol/20 mM Hepes (pH 7.5)/150 mM NaCl/0.4 mM Na_3VO_4/50 mM NaF] supplemented with Aprotinin (Sigma), Pepstatin A (Sigma), Leupeptin (Sigma) and PMSF (Sigma). Triton lysis buffer [50 mM Tris/HCl (pH 7.5)/10 % (v/v) glycerol/150 mM NaCl/1 mM EDTA/1 % (v/v) Triton X-100/50 mM NaF/0.1 mM Na_3VO_4 containing protease inhibitors] was used for lysing other mammalian cells. The protein concentrations were determined by using the Bio-Rad protein assay, with BSA (Sigma) as the standard. Cell lysates were incubated with specific antibodies at 4 °C for 2 h, followed by incubation with Protein A–Sepharose (Sigma) or Protein G–Sepharose (Pharmacia) at 4 °C for 1 h. Immunoprecipitates were washed four times with lysis buffer, followed by elution with reducing Laemmli sample buffer. Cell lysates and immunoprecipitates were subjected to SDS/PAGE (the running buffer consisting of 25 mM Tris/HCl, 250 mM glycine and 0.1 % SDS) and transferred to nitrocellulose membrane (Micron Separation or Amersham) by using transfer buffer consisting of 39 mM glycine, 48 mM Tris/HCl, 0.037 % SDS and 20 % (v/v) methanol. Immunodetection was performed with specific primary antibodies, biotinylated secondary antibodies (Dako A/S) and streptavidin–biotin horseradish-peroxidase-conjugated secondary antibodies (Amersham) and enhanced chemiluminescence (ECL®; Amersham) or by using specific primary antibodies and horseradish-peroxidase-conjugated anti-rabbit secondary antibodies (Amersham), followed by detection by enhanced chemiluminescence.

Kinase assay *in vitro* and non-radioactive phosphorylation assay

Cells were lysed in kinase lysis buffer [10 mM Tris/HCl (pH 7.6)/1 % (v/v) Triton X-100/20 % (v/v) glycerol/50 mM NaCl/50 mM NaF/0.1 mM Na_3VO_4/5 mM EDTA containing protease inhibitors]. Immunoprecipitates were washed three times with kinase lysis buffer and then twice with kinase assay buffer [10 mM Hepes (pH 7.4)/50 mM NaCl/5 mM $MgCl_2$/5 mM $MnCl_2$/0.1 mM Na_3VO_4]. After being washed, the immunoprecipitates were suspended in kinase assay buffer. [γ-^{32}P]ATP (10 μCi) and, where indicated, Stat5 peptide (AKAADGYVK-PQIKQVV) corresponding to the tyrosine phosphorylation site of Stat5, was added to peptide kinase reactions at final concentration of 1 mg/ml. For non-radioactive phosphorylation assay, immunoprecipitated Stat5 together with 20 μM ATP were added to the kinase reactions. After incubation for 30 min at room temperature, the reactions were stopped with reducing Laemmli sample buffer. The radioactive kinase assay products were separated by SDS/PAGE followed by autoradiography. The reaction products from non-radioactive reactions were separated in SDS/PAGE and transferred to nitrocellulose membrane, followed by immunodetection.

Electrophoretic gel-mobility-shift assay

WCE lysates from transfected cells and from baculovirus-infected insect cells were prepared and mobility-shift assays were done as described previously [22] with [γ-^{32}P]ATP-labelled double-stranded β-casein (5′-AGATTTCTAGGAATTCAAATCC-3′) and GRR (5′-GATCAGCATGTTTCAAGGATTTGAGATG-TATTTCCCAGAAAAG-3′) oligonucleotides.

RESULTS

Activation of Stat5 by PDGF β-R, Jak1 and c-Src in insect cells

We have previously shown that the activation of Stat5 occurs at similar levels after the stimulation of endogenous PDGF and IL-3 receptors in fibroblasts and myeloid 32D cells respectively

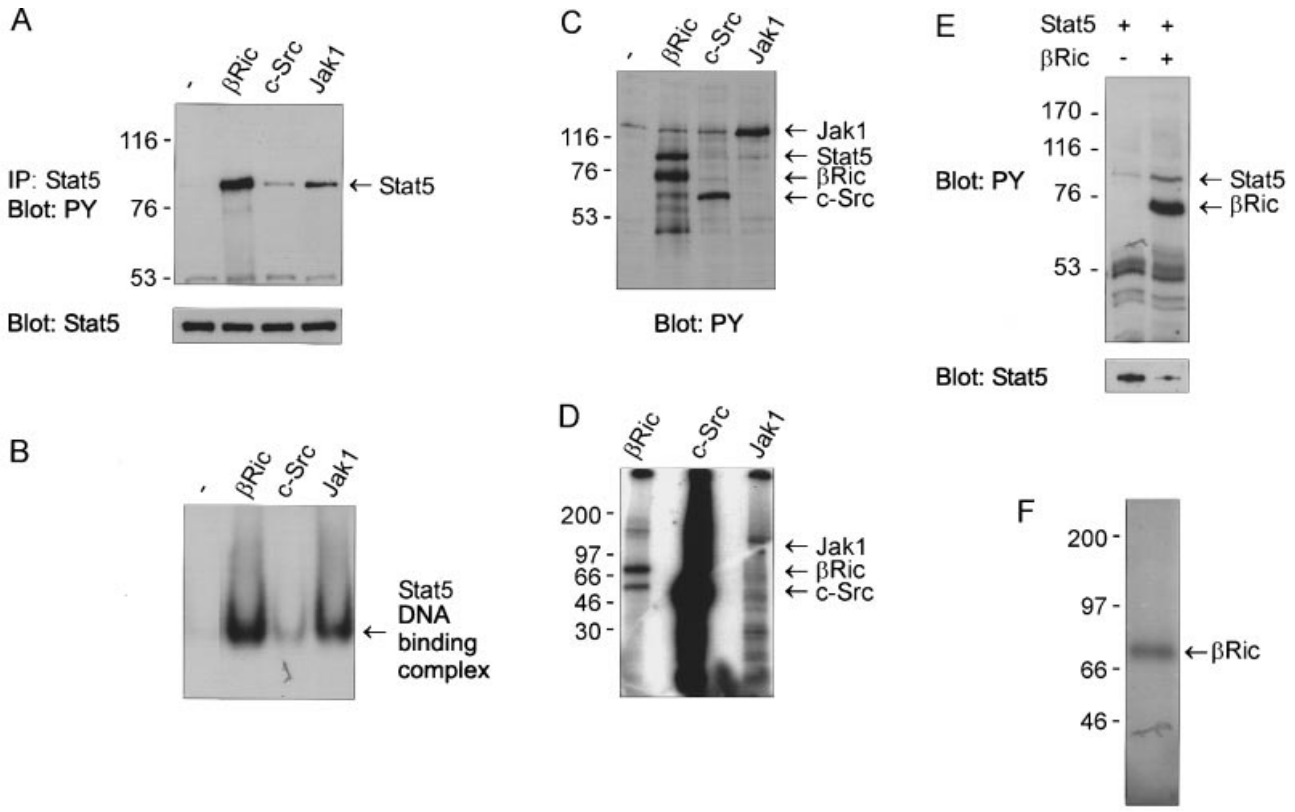

Figure 1 Activation of Stat5 by βRic, Jak1 and c-Src in insect cells

(**A**) Stat5 alone or together with βRic, c-Src or Jak1 was produced by baculoviruses in Sf9 insect cells. Stat5 was immunoprecipitated (IP) from lysates with anti-Stat5A antibody, subjected to SDS/PAGE [7.5% (w/v) gel], transferred to nitrocellulose membrane and immunoblotted with anti-phosphotyrosine (PY, upper panel) and anti-Stat5A (lower panel) antibodies. (**B**) The lysates were also analysed in a mobility-shift assay with ^{32}P-labelled β-casein oligonucleotide. (**C**) Cell lysates (2 μg) were resolved by SDS/PAGE [4–15% (w/v) gel], transferred to nitrocellulose membrane and immunoblotted with anti-phosphotyrosine (PY) antibody. (**D**) Cells were also lysed for kinase assay. βRic, c-Src or Jak1 was immunoprecipitated from lysates and subjected to autokinase assay. Kinase reactions were resolved by SDS/PAGE [4–15% (w/v) gel] followed by autoradiography. (**E**) Stat5 and βRic were produced by baculoviruses in Sf9 insect cells. βRic was immunoprecipitated from cell lysates corresponding to βRic-infected and uninfected cells. Receptor immunoprecipitations were then combined with immunoprecipitated Stat5 in the presence of ATP. Reaction products were subjected to SDS/PAGE [7.5% (w/v) gel] and immunoblotted with anti-phosphotyrosine (PY, upper panel) and anti-Stat5 (lower panel) antibodies. (F) Immunoprecipitated βRic was also subjected to autokinase assay and resolved in 7.5% SDS-PAGE, followed by autoradiography. Molecular masses of the protein standards are indicated at the left (in kDa) in (**A** and **C**–**F**).

[9]. To gain insight into the individual roles of different PDGF-induced tyrosine kinases in the activation of Stat5, we analysed the abilities of PDGF β-R, c-Src and Jak1 to activate Stat5 in insect cells. Jak1 was chosen for these studies because it has been shown to be the predominantly activated Jak kinase in receptor tyrosine kinase signalling [9,25]. As shown in Figure 1(A), Stat5 became efficiently phosphorylated in cells expressing βRic and to a smaller extent in Jak1-expressing cells. Low levels of tyrosine phosphorylation of Stat5 were also detected in c-Src expressing cells. To examine whether the phosphorylation of Stat5 occurred at the functionally critical C-terminal tyrosine residue (Tyr^{694}), the DNA-binding activity of Stat5 was analysed in electrophoretic mobility-shift assay with oligonucleotides corresponding to the β-casein promoter. Both the intracellular region of PDGF receptor and Jak1 clearly induced the DNA-binding activity of Stat5 (Figure 1B). In contrast, the DNA-binding activity of Stat5 was induced only weakly by c-Src. The kinases were found to be tyrosine phosphorylated to similar degrees (Figure 1C). However, tyrosine phosphorylation is not always correlated with kinase activity; in autokinase assays performed with immunoprecipitated kinases, c-Src showed the highest kinase activity (Figure 1D).

The efficient phosphorylation of Stat5 by βRic in insect cells suggested that PDGF β-R might be the kinase phosphorylating Stat5 in PDGF signalling. However, we wished to confirm that the PDGF β-R-induced activation of Stat5 was mediated by the receptor and not by other receptor-activated kinases. We therefore analysed the ability of βRic to phosphorylate Stat5 *in vitro* directly in a non-radioactive phosphorylation assay. As shown in Figure 1(E), Stat5 was phosphorylated only in the presence of βRic. In an autokinase assay from immunoprecipitated βRic (Figure 1F) we could not detect other phosphorylated proteins even with longer exposures, suggesting that the phosphorylation of Stat5 was mediated directly by PDGF β-R and not by co-immunoprecipitating kinases.

Activation of Stat5 by PDGF β-R, Jak1 and c-Src in mammalian cells

The ability of the PDGF-induced tyrosine kinases to activate Stat5 was also investigated in mammalian cells, in which we were able to analyse the ligand-dependent activation of Stat5. In line with the results from insect cells, both ligand-activated PDGF

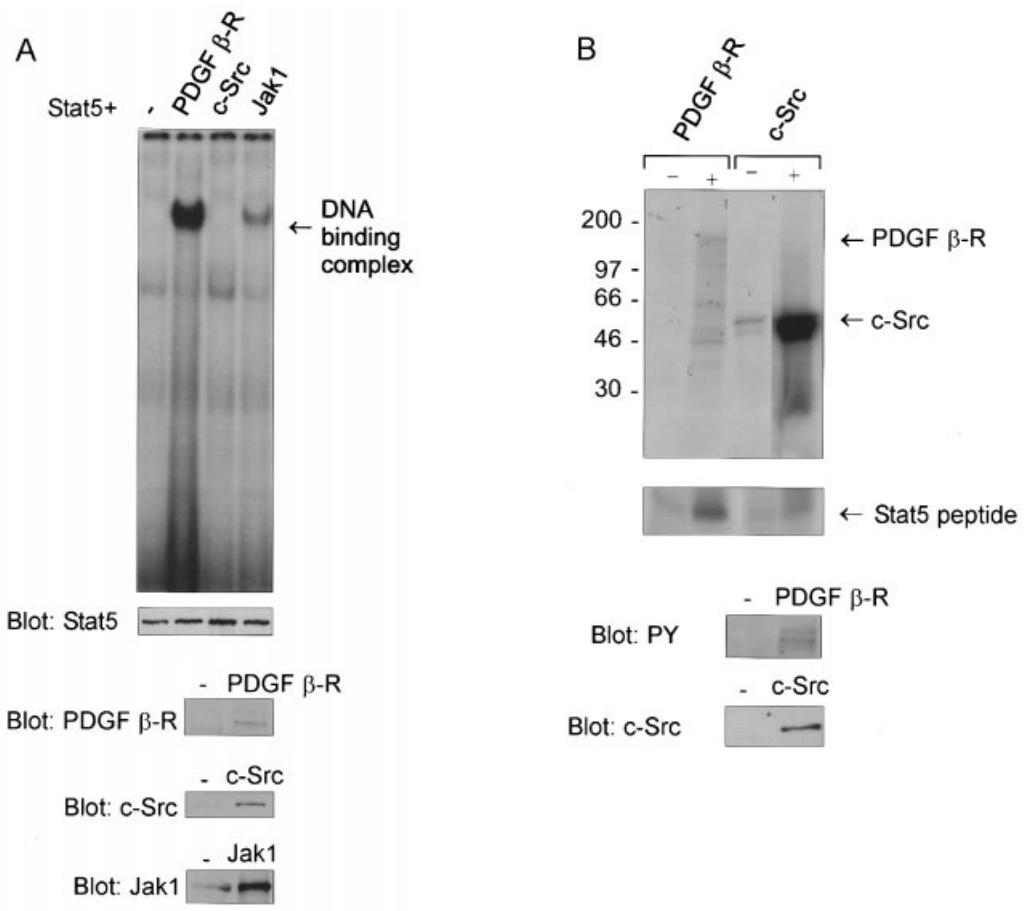

Figure 2 Activation of Stat5 by PDGF β-R, Jak1 and c-Src in 293T cells

(**A**) 293T cells were transfected with expression vectors encoding PDGF β-R, c-Src or Jak1 together with Stat5 expression vector, as indicated. The PDGF β-R-expressing cells were stimulated with PDGF-BB. The lysates were analysed in the mobility-shift assay by using ^{32}P-labelled β-casein oligonucleotide and separated by Tris/borate/EDTA/PAGE [4.5% (w/v) gel], followed by autoradiography. Lysates were also separated by SDS/PAGE and immunoblotted with anti-Stat5A, anti-(PDGF β-R), anti-(c-Src) and anti-Jak1 antibodies. (**B**) 293T cells were left untransfected or transfected with expression vectors encoding PDGF β-R or c-Src, as indicated. The PDGF β-R-expressing cells were stimulated with PDGF-BB. Lysates were subjected to immunoprecipitation with specific anti-(PDGF β-R) or anti-Src antibodies. The immunoprecipitates were subjected to Stat5 peptide kinase assay and to autokinase assay. The peptide kinase reactions were resolved by SDS/PAGE [20% (w/v) gel] followed by autoradiography; the autokinase reactions were resolved by SDS/PAGE [4–15% (w/v) gel] followed by autoradiography. The immunoprecipitates were also separated by SDS/PAGE [4–15% (w/v) gel] and immunoblotted with anti-phosphotyrosine (for receptor detection) or anti-(c-Src) antibodies. Molecular masses of the protein standards are indicated at the left in kDa.

β-R and Jak1 induced the DNA-binding activity of Stat5 when co-expressed in 293T (Figure 2A) and in COS-7 cells (results not shown). The stronger activation of Stat5 induced by PDGF β-R in comparison with Jak1 in mammalian cells is most probably due to the ligand-induced increase in the catalytic activity of the receptor. The mammalian cell-expressed c-Src showed a strong autokinase activity (Figure 2B) but was unable to induce the DNA-binding activity of Stat5. The expression of the kinases was confirmed by Western blots with PDGF β-R, c-Src and Jak1 antibodies.

The catalytic domains of tyrosine kinases have been shown to possess substrate specificity, which is critical for their selective signalling [26]. Next we wished to investigate whether PDGF-activated kinases displayed differences in their ability to phosphorylate Stat5 *in vitro*. PDGF β-R or c-Src was expressed in 293T cells and subjected to immunoprecipitation followed by peptide kinase assays *in vitro* with Stat5 Tyr^{694}-containing peptide as a substrate. The results presented in Figure 2(B) show that the ligand-stimulated PDGF β-R was capable of phosphorylating the Stat5 peptide; c-Src also weakly phosphorylated the peptide. However, taking into account the high autokinase activity of c-Src, the Tyr^{694} site does not seem to be an optimal substrate for c-Src. In several autokinase assay experiments we also studied the ability of Jak1 to phosphorylate Stat5 peptide. In most experiments Jak1 phosphorylated the peptide to a similar degree as PDGF β-R (results not shown).

Activation of Stat5 is dependent on Tyr^{579} and Tyr^{581} in PDGF β-R and on the SH2 domain of Stat5

Src kinases c-Src, Fyn and c-Yes bind directly through their SH2 domains to the autophosphorylation sites Tyr^{579} and Tyr^{581} in the juxtamembrane region of PDGF β-R; the association results in the transient catalytic activation and phosphorylation of Src kinases. In our previous studies PDGF β-R mutants Tyr^{579} → Phe and Tyr^{581} → Phe, which display a decreased ability to activate Src kinases [19], also showed a decreased ability to phosphorylate Stat5 [9]. In agreement with the results obtained in

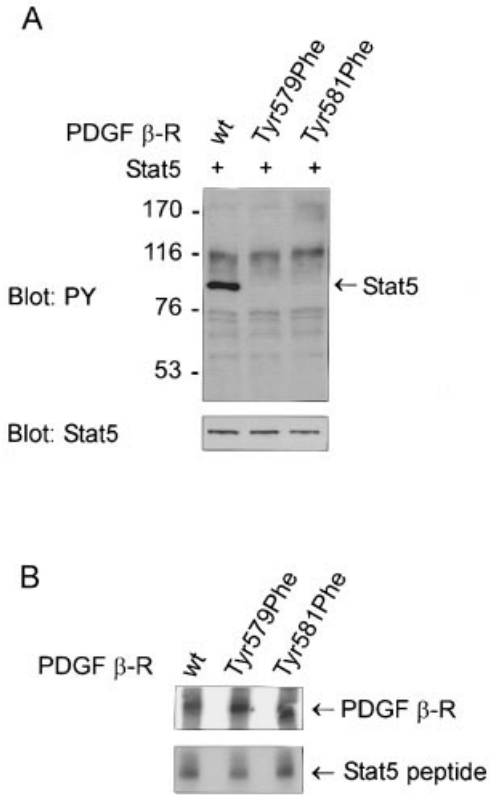

Figure 3 Effect of PDGF β-R mutants on the phosphorylation of Stat5

(**A**) COS cells were transfected with an expression vector encoding Stat5 together with expression vectors encoding wild-type (wt) PDGF β-R or $Tyr^{579} \rightarrow Phe$ or $Tyr^{581} \rightarrow Phe$ mutants of PDGF β-R, as indicated. Cells were stimulated with PDGF-BB. Cell lysates were resolved by SDS/PAGE [7.5% (w/v) gel] and immunoblotted with anti-phosphotyrosine (PY, upper panel) and anti-Stat5A (lower panel) antibodies. Molecular masses of the protein standards are indicated at the left in kDa. (**B**) 293T cells were transfected with expression vectors encoding wild-type or mutant receptors, as indicated, and the cells were stimulated with PDGF-BB. The receptors were immunoprecipitated from cell lysates, subjected to autokinase assay and resolved by SDS/PAGE [4–15% (w/v) gel] followed by autoradiography (upper panel). Immunoprecipitated receptors were also subjected to Stat5 peptide kinase assay. Peptides were resolved in SDS/PAGE [20% (w/v) gel] followed by autoradiography (lower panel).

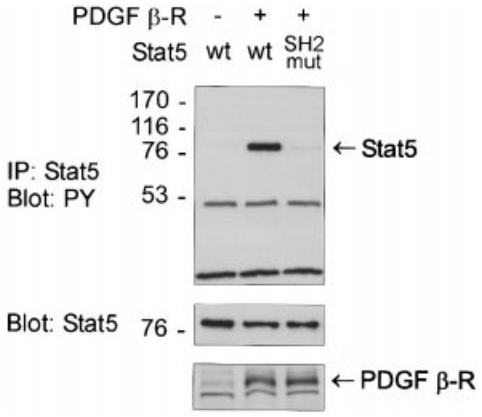

Figure 4 SH2 domain of Stat5 is required for the activation by PDGF β-R

293T cells were transfected with PDGF β-R expression vector together with expression vectors encoding wild-type (wt) Stat5 or the SH2 mutant of Stat5 (SH2 mut), as indicated. Cells were stimulated with PDGF-BB and the lysates were subjected to immunoprecipitation (IP) with anti-Stat5A antibodies. The immunoprecipitates were resolved by SDS/PAGE [4–15% (w/v) gel] and immunoblotted with anti-phosphotyrosine (PY, top panel) and anti-Stat5A (middle panel) antibodies. Total cell lysates (5 μg) were resolved by SDS/PAGE [4–15% (w/v) gel] and immunoblotted with anti-(PDGF β-R) antibody (bottom panel). Molecular masses of the protein standards are indicated at the left in kDa.

PAE cells with stably transfected receptor mutants, PDGF β-R mutants failed to induce the phosphorylation of Stat5 in COS-7 cells too (Figure 3A). The mutants were also impaired in their ability to induce Stat5-dependent transcriptional activation (results not shown). The $Tyr^{579} \rightarrow Phe$ and $Tyr^{581} \rightarrow Phe$ mutations did not affect the autokinase activity of PDGF β-R (Figure 3B, upper panel) and both mutant receptors were able to phosphorylate Stat5 peptide as efficiently as the wild-type receptor (Figure 3B, lower panel). Thus the defect in the activation of Stat5 was not due to the decreased catalytic activity of the mutated receptors towards Stat5.

In cytokine receptor signalling the Stats are recruited through their SH2 domain to the tyrosine-phosphorylated receptor complex; this association is a prerequisite for the C-terminal tyrosine phosphorylation. Phosphorylated peptides containing Tyr^{579} and Tyr^{581} residues were found to bind Stat5, suggesting an important role for the SH2 domain of Stat5 in association to PDGF β-R [9]. As shown in Figure 4, only wild-type Stat5, but not the SH2 mutant ($Arg^{618} \rightarrow Leu$) form, became tyrosine phosphorylated on stimulation by PDGF. These results indicate that the PDGF-induced activation of Stat5 requires the SH2 domain of Stat5. They also suggest that the SH2 domain of Stat5 is required for the association with PDGF β-R and that the mutations interfere with the association of Stat5 to the receptor, leading to a decreased phosphorylation of Stat5.

Effect of kinase negative c-Src, Jak1 and Jak2 on PDGF-induced activation of Stat5

Our results from co-expression studies strongly supported the notion that the PDGF receptor kinase phosphorylates Stat5 after stimulation by PDGF. To explore the possibility that Jak or Src kinases participate in the activation of Stat5 indirectly, we used the catalytically inactive mutants of c-Src, Jak1 and Jak2 kinases. These mutants, when co-expressed, compete with and inhibit wild-type kinases and can also overcome the redundancy of the different Src and Jak family members [27,28]. The effects of Src-KN ($Lys^{297} \rightarrow Met$), Jak1-KN ($Lys^{835} \rightarrow Glu$) and Jak2-KN ($Lys^{882} \rightarrow Glu$) on the PDGF-induced tyrosine phosphorylation and DNA-binding activity of Stat5 were analysed in COS-7 cells by using co-expression. Co-expression of Jak1-KN had no inhibitory effect on the PDGF-induced phosphorylation or DNA-binding activity of Stat5 (Figures 5A and 5B). Identical results were obtained with Jak2-KN (Figures 5A and 5B), whereas Jak2-KN efficiently inhibited the erythropoietin-induced activation of Stat5 (P. Saharinen, unpublished work). Co-expression of Src-KN resulted in a partial inhibition of the PDGF-induced phosphorylation and DNA-binding activity of Stat5 (Figures 5A and 5B). The observed inhibition by Src-KN could either result from the abrogation of Src kinase activity or from competition between Src and Stat5 for binding to the receptor. As shown in Figure 5(C), c-Src inhibited the PDGF-induced activation of Stat5 to the same degree as Src-KN, whereas no inhibition was observed in co-transfected wild-type Jak1 cells. Taken together, these results indicate that the catalytic activity of c-Src, Jak1 or Jak2 is not required for the PDGF-

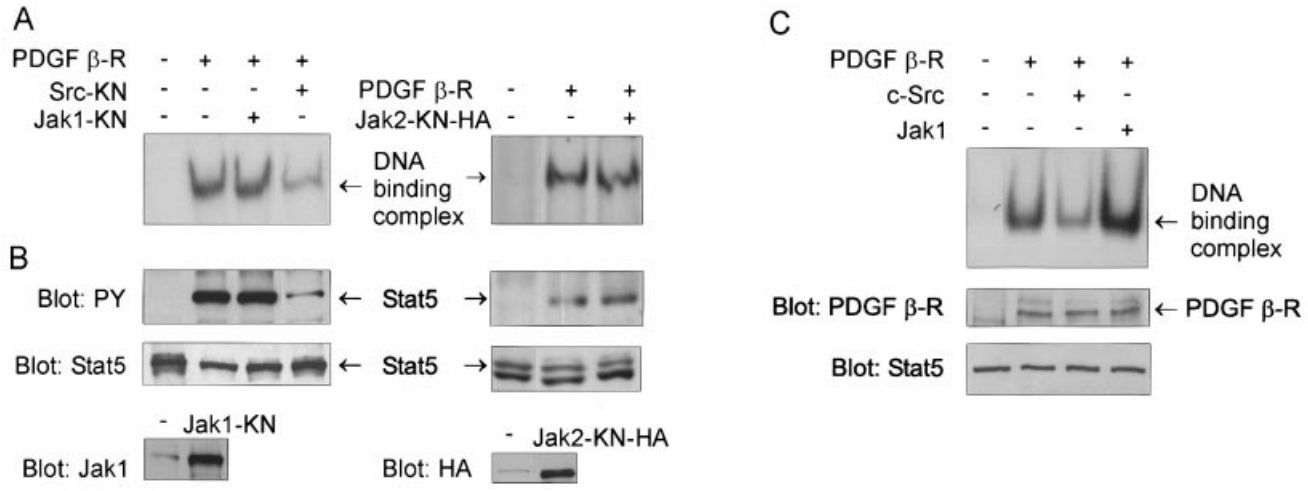

Figure 5 Overexpression of both Src-KN and c-Src inhibit PDGF-induced DNA binding of Stat5

(**A**) COS cells were transfected with expression vectors encoding Stat5 alone or together with expression vectors encoding PDGF β-R and Src-KN or Jak1-KN or Jak2-KN-HA, as indicated. Cells were stimulated with PDGF-BB and the lysates were analysed in the mobility-shift assay by using ^{32}P-labelled GRR oligonucleotide. (**B**) The same lysates were subjected to SDS/PAGE [7.5% (w/v) gel] and immunoblotted with anti-phosphotyrosine (PY, upper panel) and anti-Stat5A (middle left panel), anti-Stat5B (middle right panel, where the upper band corresponds to Stat5 and the lower band is a constitutive band blotted from COS cells), anti-Jak1 (lower left panel) and anti-HA (lower right panel) antibodies. (**C**) COS cells were transfected with expression vectors encoding Stat5 alone or together with PDGF β-R and c-Src or Jak1 expression vectors, as indicated. Cells were stimulated with PDGF-BB and the lysates were analysed in the mobility-shift assay by using ^{32}P-labelled β-casein oligonucleotide. The same lysates were subjected to SDS/PAGE [7.5% (w/v) gel] and immunoblotted with anti-(PDGF β-R) and anti-Stat5A antibodies (lower two panels).

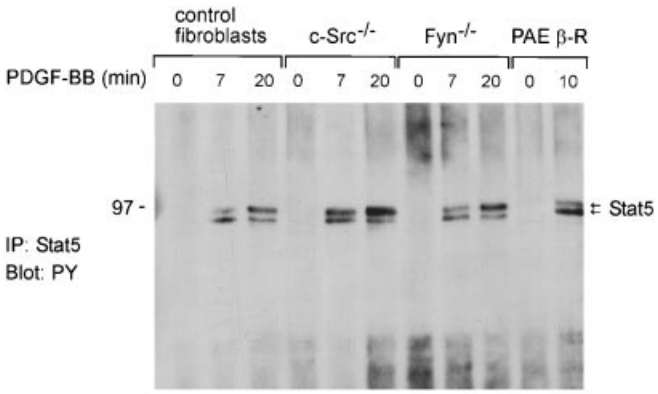

Figure 6 PDGF-induced Stat5 phosphorylation in Src$^{-/-}$ and Fyn$^{-/-}$ cells

Fibroblasts derived from normal mouse and Src$^{-/-}$ and Fyn$^{-/-}$ knock-out mice, as well as PAE/PDGFR-β cells, were stimulated for the indicated durations with PDGF-BB. Stat5 was immunoprecipitated (IP) from cell lysates with anti-Stat5B antibody, resolved by SDS/PAGE [7.5% (w/v) gel] and subjected to anti-phosphotyrosine (PY) immunoblotting. Two phosphorylated bands correspond to Stat5A and Stat5B, as indicated.

induced activation of Stat5, and suggest that Stat5 and c-Src compete for the same receptor-binding sites.

PDGF induces the tyrosine phosphorylation of Stat5 in Src$^{-/-}$ and Fyn$^{-/-}$ cells

We used the previously characterized Src$^{-/-}$ and Fyn$^{-/-}$ cells [15,16] to analyse further whether the PDGF-induced activation of Stat5 was dependent on the PDGF β-R-activated Src kinases. Fibroblasts derived from Src$^{-/-}$ and Fyn$^{-/-}$ knock-out mice and normal control mice, as well as PDGF β-R-expressing PAE cells (PAE/PDGFR-β), were stimulated for different periods with PDGF. Stat5 was immunoprecipitated from cell lysates by using anti-Stat5B antibody (which recognizes both Stat5A and Stat5B) and subjected to anti-phosphotyrosine immunoblotting. Because both Stat5A and Stat5B are activated by stimulation with PDGF in mouse fibroblast cells [9], the two phosphorylated bands in Figure 6 correspond to Stat5A and Stat5B. The tyrosine phosphorylation of Stat5 occurred at similar levels and kinetics in Src$^{-/-}$ and Fyn$^{-/-}$ cells to those in normal control fibroblasts and in PAE/PDGFR-β cells, thus indicating that both c-Src and Fyn are dispensable for the PDGF-induced activation of Stat5.

DISCUSSION

The nuclear signalling pathway through Stat transcription factors is used both by cytokine receptors lacking intrinsic catalytic activity and by tyrosine kinase receptors. Our results indicate that PDGF β-R readily activates Stat5 in mammalian and insect cells. The results with immune-complex-purified βRic, which was devoid of detectable amounts of co-precipitating kinases, confirmed that PDGF β-R is able to activate Stat5 directly. The results also showed that the PDGF-induced activation of Stat5 was independent of Jak and Src family kinases. PDGF β-R has previously been shown to activate Stat1 directly *in vitro* [29] and also EGF receptor and insulin receptor have been shown to activate Stat factors *in vitro* [24,30,31]. Thus several tyrosine kinase receptors have the ability to activate Stat proteins directly.

In cytokine receptor signalling a wealth of evidence supports a crucial role for Jak kinases in activation of Stats. In addition, in PDGF signalling the activation of Stat3 has been shown to be dependent on Jak kinases because the immunodepletion of Jak1, Jak2 and Tyk2 inhibited the PDGF-induced activation of Stat3 [10]. In contrast, the PDGF-induced activation of Stat1 was not affected by the depletion of Jaks [10,29]. Our results suggested that Jaks are not essential to the PDGF-induced activation of Stat5. Although Jak1 phosphorylated and activated Stat5, expression of the kinase negative mutants of Jak1 or Jak2 did not have any effect on the PDGF-induced activation of Stat5.

In addition to Jaks and tyrosine kinase receptors, v-Src has been implicated in Stat signalling. In v-Src-transformed cells, Stat1, Stat3 and Stat5 have been found to be constitutively active [11–13]. We found that c-Src was a poor activator of Stat5 in comparison with PDGF β-R and Jak1, although c-Src was very active in the autokinase assay. The current results do not support a direct c-Src-mediated activation mechanism of Stat5. c-Src, even when highly overexpressed, has been shown to be a considerably less efficient activator of Stat3 than v-Src [12]. In at least some cells v-Src induces the activation of Jak kinases [32], which could also be responsible for the Src-induced activation of Stats. In addition, in conditions under which Stat3 co-immunoprecipitated with v-Src, no interaction between v-Src and Stat5 could be demonstrated [13].

Even if Src was not responsible for the PDGF-induced phosphorylation of Stat5, it could indirectly influence the ability of PDGF β-R to phosphorylate Stat5. Mutation of the c-Src phosphorylation site at Tyr^{934} in PDGF β-R [23] did not affect the activation of Stat5 (S. Valgeirsdóttir, unpublished work). In addition, our results obtained with fibroblasts deficient in either c-Src or Fyn showed that the PDGF-induced activation of Stat5 is independent of these Src kinases. In these experiments we cannot completely exclude the possibility of redundancy in the use of different Src kinases by PDGF β-R; triple knock-out mice deficient in c-Src, Fyn and c-Yes would be needed to address this question. Recently, Sachsenmaier et al. [33] showed by using various PDGF β-R mutants that the PDGF-induced activation of Stat1, Stat3 and Stat6 does not require the activation of Src. Our previous studies showed that the activation of Stat5 by PDGF β-R required the same receptor tyrosine residues Tyr^{579} and Tyr^{581} as those needed for the activation of Src kinases [9]. The mutation of either Tyr^{579} or Tyr^{581} in the receptor did not affect the ability of the receptor to phosphorylate the Stat5 Tyr^{694} site *in vitro* or the catalytic activity of PDGF β-R. Our experiments revealed that the SH2 domain of Stat5 was critical for the activation of Stat5 by PDGF β-R. These results suggest that inhibition of the phosphorylation of Stat5 by $Tyr^{579} \rightarrow$ Phe and $Tyr^{581} \rightarrow$ Phe mutations is most probably due to the decreased association of the SH2 domain of Stat5 to these mutant receptors. A recent study with Src kinase inhibitors and a Src-KN mutant suggested a role for Src in the EGF-induced activation of Stats [14]. We found that Src kinase inhibitor PP1 also decreased the PDGF-induced activation of Stat5. However, the same concentrations of PP1 inhibited the catalytic activity of PDGF β-R; PP1 therefore does not seem to be suitable for studies related to PDGF signalling. The expression of Src-KN resulted in decreased phosphorylation and DNA binding of Stat5 in response to PDGF; however, similar inhibition was also seen by overexpression of the wild-type c-Src, thus the inhibition by Src might result from competition between Src and Stat5 for the same receptor-binding sites.

The precise role of Stat5 in PDGF signalling remains to be elucidated. The different Stat knock-out mice have clearly demonstrated that the non-redundant functions of different Stats are highly specific for particular cytokines; the phenotypes are different from those of the PDGF-deficient mice [34–41]. Surprisingly, the Stat5A/B double knock-out mice show normal responses to all haemopoietic cytokines except IL-2, although normally they activate Stat5 efficiently. It is therefore likely that Stats have other, redundant functions in cytokine receptor signalling as well as in PDGF β-R signalling. Such an example in PDGF signalling is the regulation of *fos* expression through the *sis*-inducible element ('SIE') by Stat1 and Stat3 [4–6]. Both Stat5 and PDGF have been implicated in the induction of adipocyte differentiation in various models [39,42]; it is possible that the PDGF-mediated stimulation of Stat5 is involved in this and similar biological processes.

Taken together, our results demonstrate that PDGF β-R activate Stat5 directly and that this activation does not require Jak1, Jak2, Fyn or c-Src kinases. However, the activation mechanisms and requirements in the PDGF β-R system differ between Stats. For example, the PDGF-induced activation of Stat3 requires Jak kinase activity, whereas the activation of Stat1 is independent of Jak activity in the same experimental setting [10]. It is also possible that Jak kinases participate in the regulation of other PDGF-mediated signalling events than activation of the Stats; the functional role of cytoplasmic kinases in PDGF signalling remains an important topic for future studies.

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