Association and Direct Activation of Signal Transducer and Activator of Transcription1α by Platelet-derived Growth Factor Receptor

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Abstract

PDGF stimulates tyrosine phosphorylation of Janus kinase 1 (JAK1) and the signal transducer and activator of transcription 1 (STAT1α). However, it is not known whether JAKs are required for STAT1α phosphorylation or if the PDGF receptor itself can directly tyrosine phosphorylate and activate STAT1α. In vitro immunecomplex kinase assay of PDGF β receptor (PDGFR) or STAT1α immunoprecipitates from lysates of mesangial cells treated with PDGF showed phosphorylation of a 91- and an 185-kD protein. Incubation of lysates prepared from quiescent mesangial cells with purified PDGFR resulted in STAT1α activation. Immunodepletion of Janus kinases from the cell lysate before incubation with the purified PDGFR showed no effect on STAT1α activation. Moreover, lysates from mesangial cells treated with JAK2 inhibitor, retained significant STAT1α activity. To confirm that STAT1α is a substrate for PDGFR, STAT1α protein was prepared by in vitro transcription and translation. The addition of purified PDGFR to the translated STAT1α resulted in its phosphorylation. This in vitro phosphorylated and activated protein also forms a specific protein–DNA complex. Dimerization of the translated STAT1α protein was also required for its DNA binding. Incubation of pure STAT1α with autophosphorylated PDGFR resulted in physical association of the two proteins. These data indicate that activated PDGFR may be sufficient to tyrosine phosphorylate and thus directly activate STAT1α. (*J. Clin. Invest.* 1998. 101:2751–2760.) Key words: mesangial cells • PDGFR • STAT1α

Introduction

Platelet-derived growth factor binds to specific high affinity receptors commonly present in cells of mesenchymal origin including fibroblasts, smooth muscle, and glomerular mesangial cells, vascular pericyte of the kidney glomerulus essential for filtration (1). PDGF receptor β (PDGFR)[1] activation is essential for mesangial cell development and is an important component of proliferative and fibrotic disorders of the kidney (1–3). Binding of PDGF to its receptor leads to autophosphorylation of the receptor which then recruits a set of SH2 domain–containing enzymes that include the regulatory subunit of phosphatidylinositol 3 kinase (PI 3 K), phospholipase Cγ1 (PLCγ1), GTPase activating protein (GAP), phosphotyrosine phosphatase SHP-2, and the Src family members Src, Fyn, and Yes (4, 5). Phosphorylated PDGFR also recruits another class of adaptor proteins Grb2, Shc and Nck which also contain SH2 domains (6–8). These early signaling pathways converge on transcriptional activation events to regulate the biological activity of PDGF in target cells.

Signal transducers and activators of transcription (STATs) represent a group of transcription factors that plays an important role in signaling by interferons and other cytokines (9–11). Six different members of this group have been identified. In some instances long and short isoforms exist. Thus STAT1 exists in two alternatively spliced forms, α and β. Only STAT1α is able to activate transcription of genes responsive to interferon γ and to confer antiviral effect (12, 13). In quiescent cells, STATs are present in the cell cytoplasm. After interferon or cytokine treatment, members of the Janus kinase (JAK) family of cytoplasmic tyrosine kinases are activated and phosphorylate STATs on a conserved tyrosine residue. Tyrosine-phosphorylated STAT proteins then translocate to the nucleus and bind specific DNA elements to initiate transcription of target genes. Biochemical and genetic analysis of interferon-signaling pathways have indicated the requirement of two distinct JAKs for the activation of STATs. Receptor tyrosine kinases such as epidermal growth factor receptor and PDGFR also activate STAT proteins (14, 15). It has recently been shown that PDGF stimulates tyrosine phosphorylation of members of the JAK family (16). We have previously shown that treatment of primary glomerular mesangial cells with PDGF enhances JAK1 tyrosine kinase activity (17). Furthermore, JAK1 may be a potential candidate to tyrosine phosphorylate and to activate STAT1α in response to PDGF (17). In mouse BALB/c 3T3 cells and human fibroblasts overexpressing PDGFR, all three of the ubiquitously expressed JAKs, JAK1, JAK2, and TYK2, associate with the activated PDGFR (16). However, PDGF-induced JAK tyrosine phosphorylation and activation of STAT1 is independent of the presence of any one of the JAKs. This conclusion arose from the use of cells lacking individual JAKs and overexpressing PDGFR (16). These studies suggest that in the absence of a single JAK, the other family member(s) or PDGFR itself can tyrosine phosphorylate STATα to activate its DNA binding.

In the present study, we investigated the possibility that PDGFR directly activates STAT1α. In glomerular mesangial

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1. *Abbreviations used in this paper:* GMSA, gel mobility shift analysis; JAK, Janus kinase; PDGFR, PDGF receptor β; STAT, signal transducer and activator of transcription; TYK, tyrosine kinase.

cells, PDGFR coprecipitates with the 91-kD STAT1α protein in a PDGF-dependent manner. Immunopurified PDGFR activates STAT1α present in extracts of quiescent glomerular mesangial cells that have no demonstrable intrinsic STAT activity. Immunodepletion of JAK1, JAK2, and TYK2 does not have an effect on PDGFR-phosphorylated STAT1α activation. Furthermore, we show that the in vitro translated purified STAT1α is a direct substrate for PDGFR and that the phosphorylated STAT1α is active in a DNA binding assay. This in vitro activation of STAT1α requires tyrosine phosphorylation and dimerization. Finally, we show that STAT1α can physically interact with tyrosine phosphorylated PDGFR. These data indicate that STAT1α is a substrate for activated PDGFR and that direct phosphorylation by PDGFR is sufficient for STAT1α activation.

Methods

Materials. Tissue culture materials were purchased from Gibco/BRL (Rockville, MD). PDGF BB was provided by Amgen Inc., (South San Francisco, CA). Monoclonal human PDGFRβ-specific antibody was obtained from Genzyme Corp., (Cambridge, MA). JAK1 antibody was purchased from UBI (Lake Placid, NY). JAK2 and TYK2 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). STAT1 antibody was purchased from Transduction Laboratories, (Lexington, KY) and UBI. Anti-HA epitope specific antibody was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). AG490 was obtained from Alexis Corp. (San Diego, CA). Protein A sepharose CL 4B was obtained from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Recombinant PTP1B linked to glutathione agarose was purchased from UBI. Phosphotyrosine was from Sigma Chemical Co. (St. Louis, MO). The TNT coupled reticulocyte lysate system was purchased from Promega Corp. (Madison, WI). Oligonucleotides were synthesized in an Applied Biosystem DNA/RNA synthesizer (Foster City, CA). All other reagents were of analytical grade.

Cell culture. Human mesangial cells have been extensively characterized by electron microscopy and immunohistochemical staining as described (18). The cells represent a homogeneous population of smooth muscle–like cells free of endothelial or epithelial cell contamination. The cells were grown in Waymouth's medium in the presence of 17% fetal calf serum. For serum deprivation, monolayers were washed with PBS three times and incubated in serum-free Waymouth's medium for 2 d, with fresh medium each day.

Immunoprecipitation. Immunoprecipitation was carried out essentially as described previously (19). Briefly, the cells were lysed in RIPA buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 1% NP-40, 1 mM PMSF, and 0.1% aprotinin) at 4°C for half an hour, scraped from the plates and centrifuged at 10,000 *g* for 30 min at 4°C. The supernatants were used as cell lysates and total protein content estimated by the Bradford method using the BioRad reagent. Supernatant was incubated with the appropriate antibody for 30 min on ice, followed by incubation with protein A Sepharose beads on a rocking platform at 4°C for 2 h. The beads were washed three times with RIPA buffer, then two times with 50 mM Tris-HCl, pH 7.4. These immunoprecipitates were then used for immunecomplex kinase assays. For immunodepletion of JAK1, JAK2, and TYK2, lysates from quiescent glomerular mesangial cells were immunoprecipitated with all three antibodies. The supernatants from these first immunoprecipitation reactions were then used for two more successive rounds of immunoprecipitation with the same antibodies. The final supernatant was considered to be depleted of JAK1, JAK2, and TYK2. To confirm that the supernatant was depleted of these three kinases, control and immunodepleted supernatants were immunoblotted with anti-JAK1, anti-JAK2, and anti-TYK2 antibodies as previously described (17, 20).

Purification of PDGFR. We have demonstrated earlier that PDGFR immunoprecipitates from extracts of serum-deprived quiescent human mesangial cells do not contain detectable receptor-associated proteins (21). We therefore used serum-deprived mesangial cells to purify PDGFR by immunoprecipitation with a monoclonal antibody specific for human PDGFRβ. For each kinase reaction, PDGFR immunoprecipitated from 500 μg of quiescent cell lysate was used.

Immunecomplex kinase assay. PDGFR and STAT1α were immunoprecipitated with specific antibodies. For JAK2, the cell lysate was precleared with protein A sepharose beads for 1 h before immunoprecipitation. The immunecomplex kinase assay was performed essentially as described previously (19, 21). Briefly, immunoprecipitates were incubated with 20 μCi (3,000 Ci/mmol) γ^{32}P-ATP either in the presence or absence of substrate proteins, in kinase buffer (50 mM Hepes pH 7.4, 10 mM $MnCl_2$) for 15 min. The labeled proteins were then separated on a 7.5% SDS gel and visualized by autoradiography.

Phosphorylation of STAT1α in vitro by PDGFR. PDGFR was purified by immunoprecipitation of 500 μg total protein from quiescent mesangial cell extracts. Extracts from quiescent mesangial cells that lack STAT1α DNA-binding activity or pure STAT1α were incubated with PDGFR in kinase buffer in the presence of unlabeled ATP for 30 min at 30°C. The reaction mixture was centrifuged at 10,000 *g* for 2 min at 4°C and the supernatant was collected as the PDGFR-phosphorylated protein samples.

Gel mobility shift analysis (GMSA). A high affinity SIEm67 (22) DNA probe was labeled by incubating γ^{32}P-ATP and T4 polynucleotide kinase with the annealed oligonucleotides 5′-CATTTCCCG-TAAATC-3′ and 5′-GATTTACGGGAAATG-3′. The mobility shift analysis was essentially performed as described previously (17). 10 μg of extracts or PDGFR-phosphorylated samples were incubated with 1 μg of poly dI/dC and labeled probe (50,000 cpm) in binding buffer (35 mM Hepes, pH 7.8, 0.5 mM EDTA, 0.5 mM DTT, 10% glycerol) for 30 min at 25°C. The DNA–protein complex was separated by electrophoresis in 0.5% TBE (tris-borate-EDTA) buffer through 5% polyacrylamide gel. For super shift analysis, the samples were incubated with STAT1α or a nonspecific antibody (anti-HA epitope from influenza A virus) for 30 min on ice before the binding reaction was performed.

In vitro synthesis of STAT1α protein. One μg of plasmid DNA containing STAT1α cDNA driven by the T3 bacteriophage promoter was used as template in an in vitro transcription coupled to translation reaction. The reagents were purchased as a kit. The reaction was carried out in the presence of T3 RNA polymerase, methionine-depleted amino acid mixture and ^{35}S-methionine as suggested by the vendor. The reaction products were analyzed by SDS gel electrophoresis. For cold STAT1α preparation, ^{35}S-methionine was omitted and a mixture of all amino acids including unlabeled methionine was used in the incubation.

Results

Identification of PDGFR in STAT1α immunoprecipitates. Activated PDGFR is present as a signaling complex in PDGF-treated cells although not all the proteins associated directly or indirectly with the receptor are as yet identified (5, 23, 24). Due to the low abundance of signaling proteins present in primary human mesangial cells, analysis of PDGFR immunoprecipitates by antiphosphotyrosine immunoblotting did not show any tyrosine phosphorylated proteins that associated with the PDGFR. To detect the proteins that associate with PDGFR in human mesangial cells, we performed immunecomplex kinase assay of PDGFR immunoprecipitates from PDGF-treated cells for various periods of time in the presence of γ^{32}P-ATP. The data showed phosphorylation of multiple PDGFR-associated proteins in which PDGFR at 185 kD is the major phos-

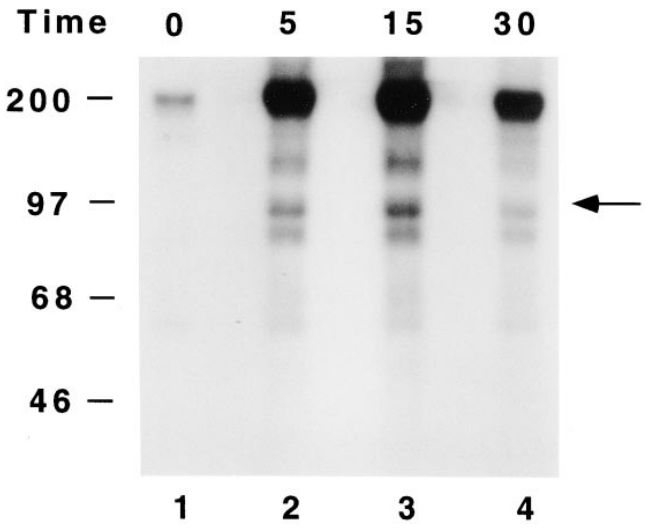

Figure 1. Tyrosine phosphorylation of PDGFR-associated proteins. Quiescent mesangial cells were treated with 10 ng/ml PDGF for indicated periods of time. Cells were lysed in RIPA buffer (see Methods) and the insoluble material was removed by centrifugation. Lysates were then immunoprecipitated with PDGFR antibody. Immunecomplex kinase assay was performed in the presence of γ^{32}P-ATP and the reaction products were separated by SDS gel electrophoresis. The arrow indicates 91 kD tyrosine phosphorylated protein. The heavily phosphorylated protein at 185 kD represents the PDGFR. Molecular weight markers are shown on the left margin in kilodaltons.

phoprotein (Fig. 1). Also a 91-kD protein was phosphorylated in a time-dependent manner peaking at 15 min of PDGF stimulation (Fig. 1, *arrow*). Based on apparent molecular weight, this 91-kD protein likely represents STAT1α. To determine if PDGFR associates with STAT1α as a signaling intermediate, glomerular mesangial cells were treated with PDGF. STAT1α was thereafter immunoprecipitated and the immunebeads were used in an in vitro immunecomplex kinase assay in the presence of γ^{32}P-ATP. The labeled proteins were analyzed by SDS gel and autoradiography as shown in Fig. 2 *A*. Multiple phosphorylated proteins in both control and PDGF-treated cells were observed. However, a 185-kD protein was present only in samples from PDGF-treated cells (Fig. 2 *A*, lane *2*, *arrow*). Note that, in PDGF-treated cells, the heavily phosphorylated proteins at 130 and 91 kD and the phosphoprotein at 65-kD positions migrated more slowly than the same proteins from serum deprived nontreated cells. Thus the data indicate that these proteins are more abundantly phosphorylated in PDGF-treated mesangial cells. To characterize the 185-kD protein in PDGF-treated mesangial cell extracts, we eluted the labeled proteins from the kinased STAT1α immunoprecipitates. The eluted labeled proteins were used in a second immunoprecipitation reaction using a PDGFR antibody and the proteins on the immunebeads were analyzed by SDS gel electrophoresis (Fig. 2 *B*). The data show that in mesangial cells, in response to PDGF, the 185-kD PDGFR is present in a complex in the STAT1α immunoprecipitates.

Activation of STAT1α by PDGFR. We have recently shown that serum-deprived quiescent mesangial cell extracts do not contain any detectable amounts of proteins that associate with PDGFR (21). In such cell extracts, no DNA binding activity representing STATs could be detected (20). This observation prompted us to use quiescent mesangial cell extracts to immunopurify the PDGFR. The same cell extracts can also be used as a source of inactive STAT1α. To test the possibility that PDGFR can activate STAT1α in vitro, we incubated purified PDGFR, in the presence of ATP, with quiescent mesangial cell extracts containing no intrinsic STAT binding activity. The phosphorylated cell extract was then used in a gel mobility shift assay to test the ability of proteins contained in the extract to bind a STAT-specific DNA sequence, SIEm67. The data show that quiescent mesangial cell extracts do not form any specific DNA–protein complex in the absence of PDGFR

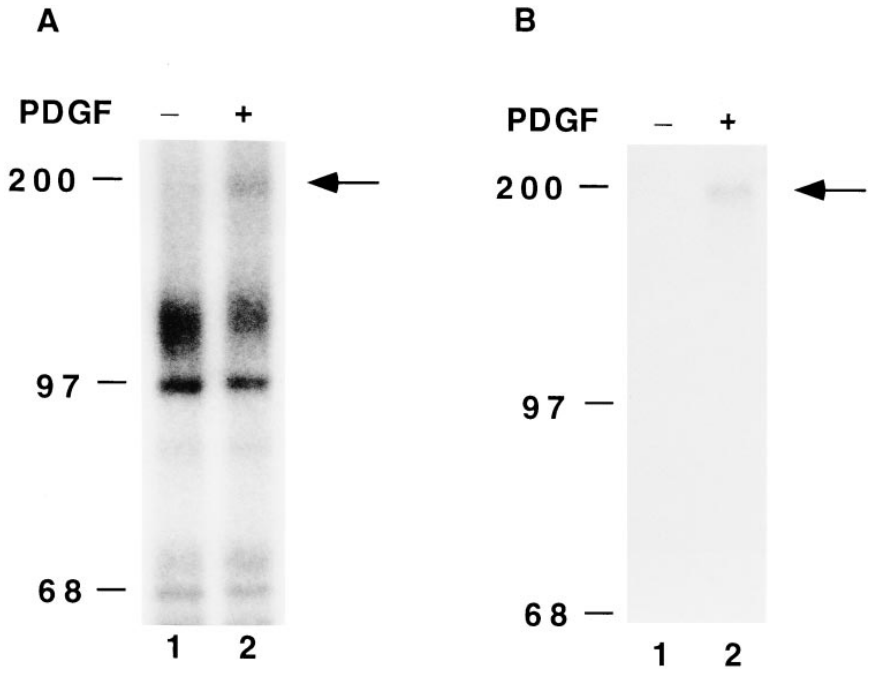

Figure 2. PDGF-induced association of STAT1α with the PDGFR in mesangial cells. Quiescent cells were treated with 10 ng/ml PDGF for 15 min. (*A*) Cell lysates were then immunoprecipitated with STAT1α antibody. Immunecomplex kinase assay was performed in the presence of γ^{32}P-ATP and the reaction products were separated by SDS gel electrophoresis. Arrow indicates the 185-kD tyrosine phosphorylated protein in PDGF-treated cells only (lane *2*). (*B*) Labeled proteins present in the anti-STAT1α immunoprecipitates in *A* were eluted by boiling in 1.5% SDS. The eluted proteins were then diluted and immunoprecipitated with an antibody specific for PDGFR. The arrow indicates the presence of 185 kD PDGFR in PDGF-treated cells only (lane *2*). Molecular weight markers are shown in kilodaltons.

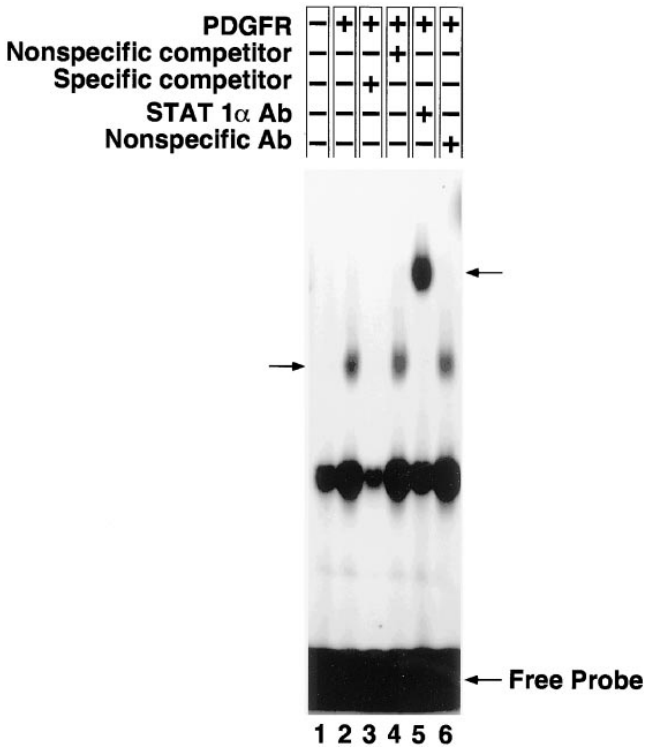

Figure 3. In vitro activation of STAT1α by PDGFR in mesangial cell extracts. PDGFR was purified as described in Methods. Quiescent mesangial cell lysate lacking STAT1α activity was incubated with this PDGFR in the presence of ATP. Phosphorylated cellular proteins were then used in GMSA with ^{32}P-labeled SIEm67 probe. The DNA–protein complexes were then separated on a 5% polyacrylamide gel. Lane *1*, unphosphorylated quiescent cell extract. Lanes *2–6*, PDGFR-phosphorylated mesangial cell extracts. Lanes *3* and *4*, 100-fold molar excess of specific and nonspecific competitor oligonucleotides were added; lanes *5* and *6*, before GMSA, phosphorylated cell extracts were preincubated with STAT1α specific and nonspecific anti-HA epitope antibodies, respectively. Arrow on the left indicates the specific protein–DNA complex. Arrow on the right indicates the supershifted STAT1α–DNA complex.

(Fig. 3, lane *1*). However, in the presence of PDGFR, mesangial cell extracts form a DNA–protein complex (Fig. 3, lane *2*, *left arrow*) with a high degree of specificity as shown by competition with excess cold oligonucleotides (Fig. 3, lane *3*). To confirm the presence of STAT1α in the DNA–protein complex we used a STAT1α-specific antibody in the GMSA. The DNA–protein complex is supershifted and appears as a slower migrating band (Fig. 3, lane *5*, *right arrow*). These data indicate that PDGFR can activate STAT1α which is present in quiescent mesangial cell extracts in vitro. To determine if Janus kinases are necessary for STAT1α activation in vitro, we immunodepleted JAK1, JAK2, and TYK2 from lysates prepared from quiescent mesangial cells. Immunodepleted lysates were then incubated with purified PDGFR in the presence of ATP and used in gel mobility shift assay using SIEm67 as a probe. The data show that the absence of Janus kinases does not influence binding of STAT1α to DNA (Fig. 4 *A*, compare lane *2* with lane *5*). Fig. 4 *B* demonstrates immunodepletion of all three Janus kinases. These data indicate that in the absence of Janus kinases, PDGFR is sufficient to activate STAT1α.

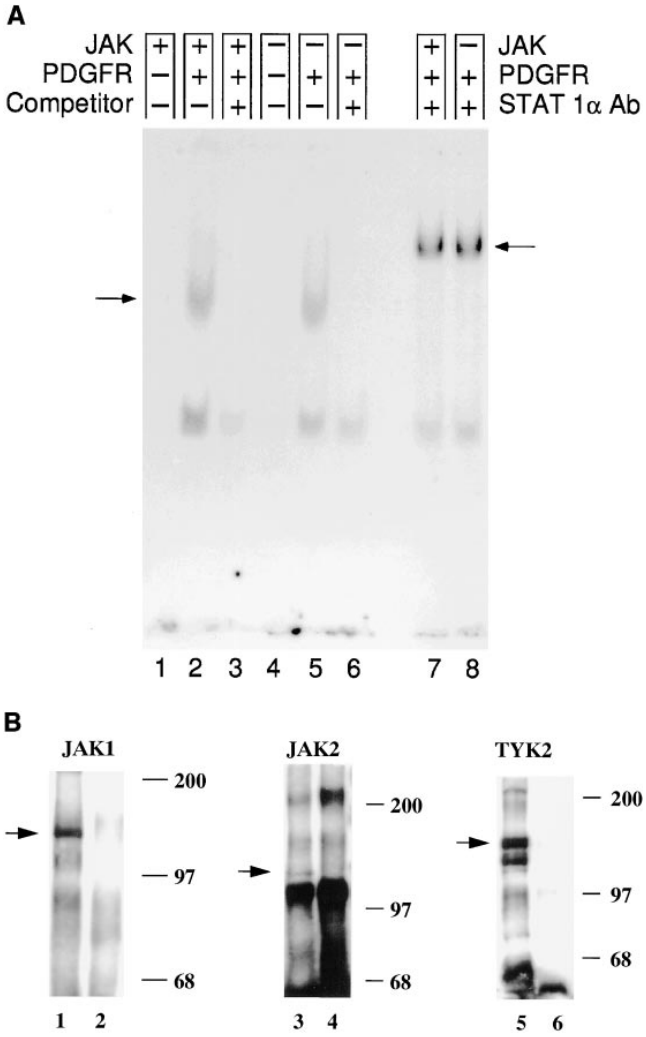

Figure 4. (*A*) Effect of immunodepletion of Janus kinases on PDGFR-phosphorylated STAT1 a activation. Quescent mesangial cell lysate was immunodepleted with JAK1, JAK2, and TYK2 antibodies (lanes *4–6* and lane *8*) as described in Methods. The immunodepleted and undepleted quiescent mesangial cell lysates were then incubated with purified PDGFR in the presence of ATP (lanes *2*, *3*, and *5–8*). Phosphorylated cellular proteins were then used in GMSA with ^{32}P-labeled SIEm67 probe. The DNA–protein complexes were then separated by polyacrylamide gel. Left arrow indicates the protein-DNA complex in lanes *2* and *5.* In lanes *3* and *6*, the samples were incubated with 100-fold excess cold m67SIE before addition of probe. Right arrow indicates the supershifted protein-DNA complex in lanes *7* and *8* in the presence of STAT1α monoclonal antibody. (*B*) Immunodepletion of Janus kinases. Three aliquots of lysates from quiescent mesangial cells were immunoprecipitated with JAK1, JAK2, or TYK2 antibodies. These immunoprecipitates represent the control samples. The remaining supernatants were used for two more rounds of immunoprecipitations with the three respective antibodies and the supernatant was collected. This immunodepleted supernatant was then immunoprecipitated with the corresponding antibody. The final immunoprecipitates represent the immunodepleted samples. Immunoprecipitates from the control and depleted samples were separated by SDS gel electrophoresis and immunoblotted with JAK1 (lanes *1* and *2*), JAK2 (lanes *3* and *4*), and TYK2 (lanes *5* and *6*) antibodies. Lanes *1*, *3*, and *5* are control nonimmunodepleted samples. Lanes *2*, *4*, and *6* represent the immunodepleted lysates. Arrows indicate the Janus kinases. Note the presence of all three JAKs in the control but not in the depleted supernatants.

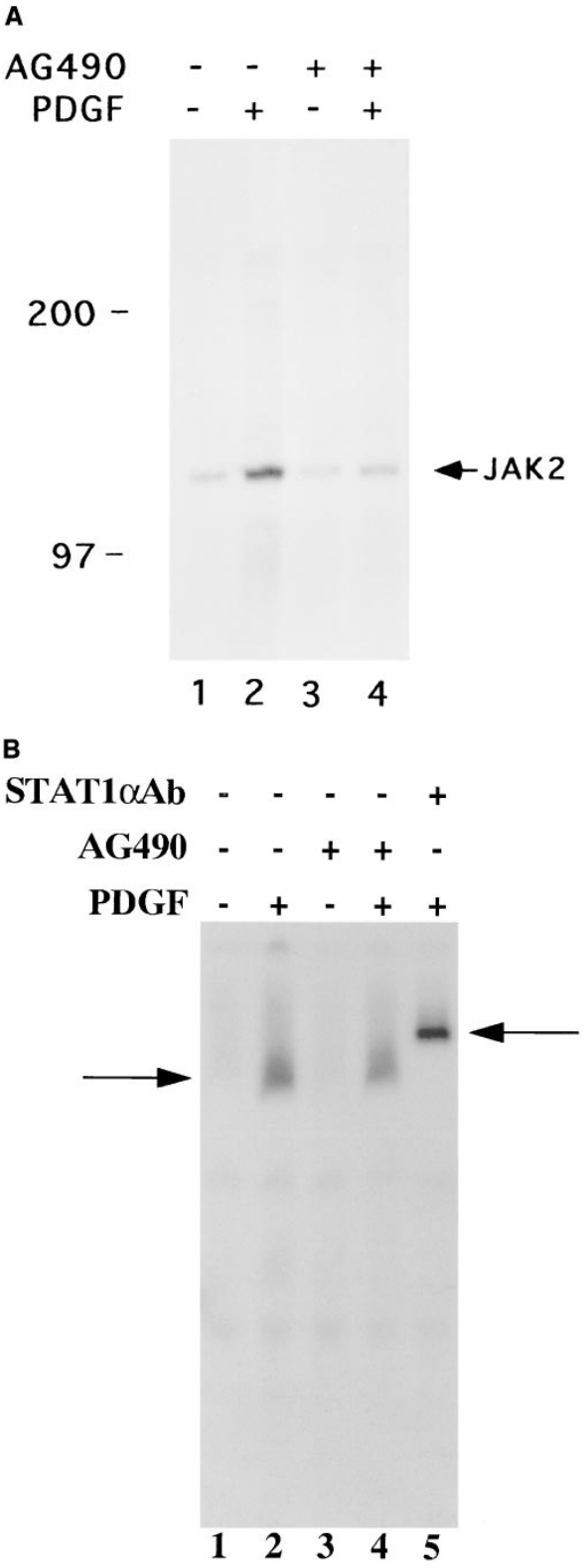

Figure 5. Effect of JAK2 inhibition on PDGF-induced STAT1α activation in mesangial cells. (*A*) Quiescent cells were incubated with specific JAK2 inhibitor, 10 μM AG490 for 17 h before exposure to 10 ng/ml PDGF for 15 min. The cell lysates were immunoprecipitated with JAK2 antibody and immunecomplex kinase assay was performed using γ^{32}P-ATP as described in Methods. Molecular weight markers are indicated in kilodaltons in the left margin. Arrow indicates the phosphorylated JAK2 protein. (*B*) Lysates from control (−) and PDGF-stimulated (+) mesangial cells pretreated with (+) and without (−) 10 μM AG490 were used in GMSA with ^{32}P-labeled SIEm67 probe. The DNA–protein complex was then separated on a 5% polyacrylamide gel. Left arrow indicates the protein–DNA complex in lanes *2* and *4* in the presence of PDGF. Right arrow indicates the supershifted protein–DNA complex in lane *5* in which the PDGF-treated mesangial cell extracts were incubated with a STAT1α specific monoclonal antibody before addition of the probe in GMSA.

It has recently been shown that the ubiquitously expressed JAK1, JAK2, and TYK2 are activated and associate with PDGFR when cells are treated with PDGF (16). We have also shown that in mesangial cells, JAK1 is activated by PDGF (17). Recently, a specific inhibitor of JAK2, AG490, has been identified and used in studying the role of this kinase in various growth factor and cytokine signaling pathways (25–28). To address the involvement of JAK2 in PDGF-induced STAT1α activation, we preincubated mesangial cells with AG490 before the addition of PDGF. Immune complex kinase assay of JAK2 immunoprecipitates showed that PDGF stimulates JAK2 tyrosine kinase activity in mesangial cells (Fig. 5 *A*, compare lane *2* with lane *1*). Treatment of cells with AG490 blocked PDGF-induced JAK2 tyrosine kinase activity. (Fig. 5 *A*, compare lane *4* with lane *2*). In a parallel experiment, the cell extracts were used in DNA binding assay. The data demonstrate that in PDGF-treated mesangial cells, inhibition of JAK2 does not substantially alter STAT1α activation (Fig. 5 *B*, compare lane *4* with lane *2*) (see Discussion).

Phosphorylation of STAT1α by PDGFR. To demonstrate direct involvement of STAT1α and PDGFR, STAT1α protein was synthesized in vitro. For these experiments, in vitro transcription coupled to in vitro translation of STAT1α was performed using a cDNA template coding for full length STAT1α. The ^{35}S-methionine–labeled translated protein was then analyzed by SDS gel. The data show synthesis of a 91-kD protein only when the cDNA template was used (Fig. 6 *A*, lane *2*). This indicates successful synthesis of the 91-kD STAT1α. To confirm that the 91-kD protein synthesized in this experiment is indeed STAT1α, we immunoprecipitated the in vitro translated reaction product with a STAT1α-specific antibody and analyzed the immunoprecipitates by SDS gel (Fig. 6 *B*). The data show that this antibody recognizes the 91-kD protein produced in vitro from the cDNA template and therefore identifies it as STAT1α. Next we determined whether this 91-kD STAT1α could be used as a substrate for PDGFR in vitro. Unlabeled STAT1α protein was synthesized and incubated with PDGFR in the presence of γ^{32}P-ATP. This kinased ^{32}P-labeled reaction product was then compared in an SDS gel with ^{35}S-methionine-labeled STAT1α obtained by in vitro translation. PDGFR-phosphorylated STAT1α migrates slower than the unphosphorylated protein (Fig. 6 *C*, compare lane *2* with lane *1*). These data indicate that STAT1α is a direct substrate for PDGFR.

Activation of STAT1α by PDGFR in vitro. Since tyrosine phosphorylated STAT1α binds to a specific DNA element in a ligand-dependent manner (17, 20), we next sought to test the hypothesis that in vitro PDGFR-phosphorylated STAT1α can bind to a specific DNA sequence. STAT1α was phosphorylated by PDGFR in the presence of cold ATP. GMSA using this reaction product with SIEm67 probe resulted in the for-

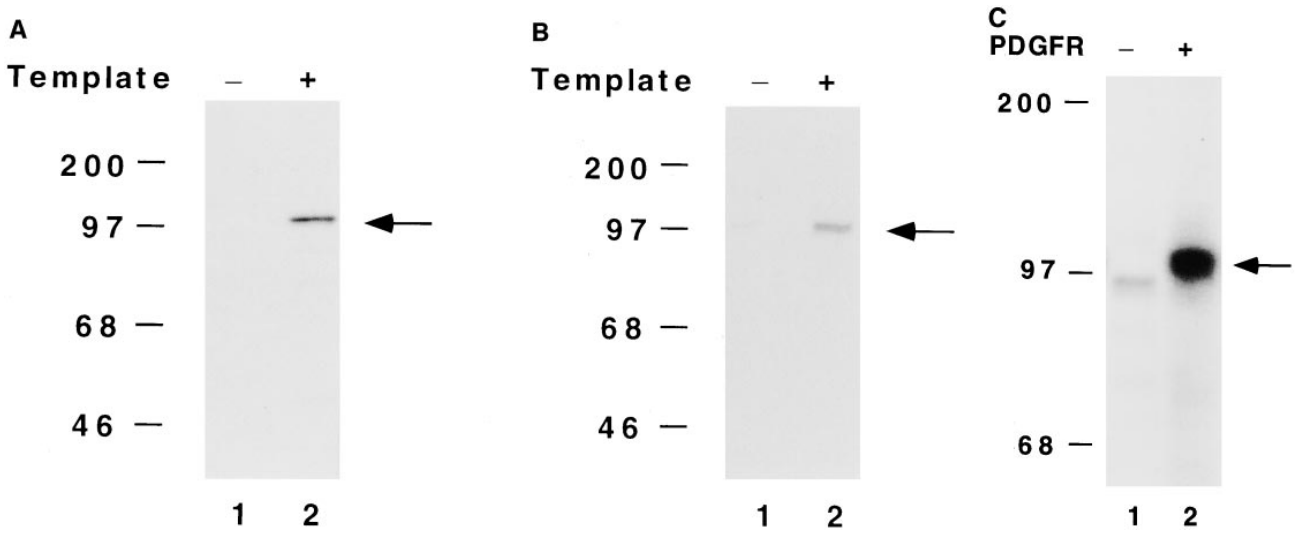

Figure 6. Synthesis of STAT1α protein in vitro. (*A*) Transcription-coupled translation of STAT1α cDNA. Full length STAT1α cDNA driven by T3 bacteriophage promoter was transcribed in the presence of T3 RNA polymerase followed by translation of the RNA in rabbit reticulocyte lysate in the presence of ^{35}S-methionine. The translated protein products were analyzed by SDS gel electrophoresis followed by fluorography. Arrow indicates the 91-kD translated protein. (*B*) Identification of STAT1α. The in vitro synthesized, ^{35}S-methionine labeled protein in panel *A* was immunoprecipitated with a STAT1α antibody. The immunoprecipitates were then analyzed by SDS gel electrophoresis. The arrow indicates the 91-kD STAT1α protein. (*C*) Tyrosine phosphorylation of STAT1α by PDGFR. STAT1α synthesized by in vitro transcription and translation in the presence of unlabeled amino acid mixture was incubated with γ^{32}P-ATP and PDGFR bound to protein A sepharose. The reaction products were centrifuged for 2 min at 10,000 *g.* Phosphorylated supernatant was diluted with sample buffer and analyzed by SDS gel electrophoresis (lane *2*). Unphosphorylated, ^{35}S-methionine-labeled STAT1α was separated in lane *1* as control. Arrow indicates the slower migrating tyrosine phosphorylated STAT1α.

mation of a DNA–protein complex (Fig. 7, lane *2*, *left arrow*). Formation of this complex was highly specific since it was prevented in the presence of excess unlabeled oligonucleotides (Fig. 7, specific competitor, lane *3*). Inclusion of a STAT1α specific antibody in the GMSA confirmed the presence of STAT1α in the protein–DNA complex (Fig. 7, lane *5*, *right arrow*). These data indicate that PDGFR can directly phosphorylate and activate pure STAT1α.

In vitro activation of STAT1α requires dimerization and tyrosine phosphorylation. STATs contain SH2 domains that possess high affinity for phosphotyrosine-containing proteins (29). It has been demonstrated that STAT proteins undergo intermolecular homo- or heterotypic dimerization via their SH2 domains and the conserved phosphotyrosine present in all STAT family members (30). Since dimerization involves the phosphotyrosine residue, it can be inhibited by the presence of excess phosphotyrosine. To investigate whether dimerization is required for the STAT1α binding to SIEm67, we incubated PDGFR-phosphorylated STAT1α with phosphotyrosine and then performed a GMSA in the presence of labeled SIEm67 probe. The data showed that phosphotyrosine abolished the formation of protein–DNA complexes (Fig. 8 *A*, compare lane *3* with lane *2*).

We have shown above that pure STAT1α is a direct substrate for PDGFR (Fig. 6 *C*). To test whether tyrosine phosphorylation is required for its specific binding to DNA, we incubated PDGFR-phosphorylated STAT1α with a recombinant tyrosine-specific phosphatase, PTP1B, then used the reaction mixture in a GMSA with labeled SIEm67. DNA–protein complex formation was inhibited in PTP1B-treated sample (Fig. 8 *B*, compare lane *2* with lane *1* or *3*). This indicates that tyrosine phosphorylation is necessary for PDGFR-phosphorylated STAT1α activity.

Physical association of STAT1α with PDGFR. PDGFR can recruit several signaling proteins via direct and indirect interactions (4–8). These PDGFR-associated proteins initiate the signaling events that lead to the biologic effects of PDGF. We and others have recently shown that STAT1α is a downstream target of PDGFR (16, 17). It is not known, however, whether PDGFR can physically interact with STAT1α, although both these proteins exist in a signaling complex in cells treated with PDGF (Figs. 1 and 2). To address this question, we autophosphorylated PDGFR bound to immunobeads, in the presence of unlabeled ATP, and incubated the reaction mixture with ^{35}S-methionine–labeled STAT1α. The beads were washed extensively and analyzed by SDS gel. Only autophosphorylated PDGFR bound the 91-kD STAT1α (Fig. 9, lane *4*). Unphosphorylated PDGFR did not interact with STAT1α protein (Fig. 9, lane *2*). These data indicate that STAT1α can physically associate with PDGFR.

Discussion

This study demonstrates a direct physical association of autophosphorylated PDGFR and STAT1α protein. Thus PDGFR joins a growing list of growth factors and cytokine receptors that form ligand-induced signaling complexes containing the STAT family of proteins.

In cultured cells, STAT1 is activated by several cytokines and growth factors including PDGF (14, 16, 17). Our recent

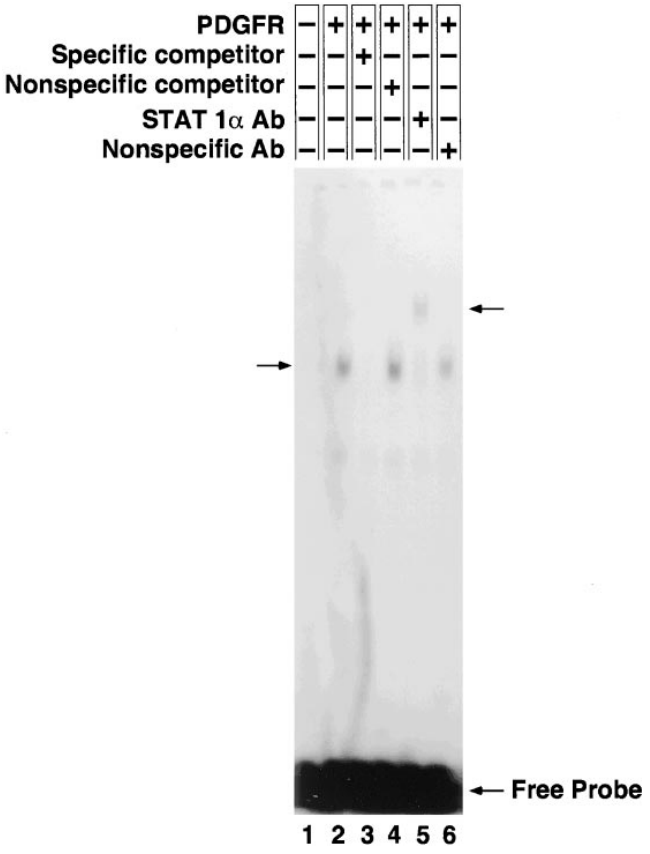

Figure 7. PDGFR stimulates STAT1α DNA binding in vitro. In vitro synthesized STAT1α was incubated with PDGFR in the presence of cold ATP as described in Methods. The tyrosine phosphorylated STAT1α was then used in GMSA in the presence of ^{32}P-labeled m67SIE as probe. Lane *1*, unphosphorylated STAT1α. Lanes *2–6*, STAT1α phosphorylated by PDGFR. Lanes *3* and *4*, competition analysis in the presence of specific and nonspecific competitor oligonucleotides, respectively. Lanes *5* and *6*, supershift analysis in the presence of specific STAT1α and anti-HA antibodies, respectively. Arrow on the left indicates the DNA–STAT1α complex. Arrow on the right indicates the supershifted band in lane *5*.

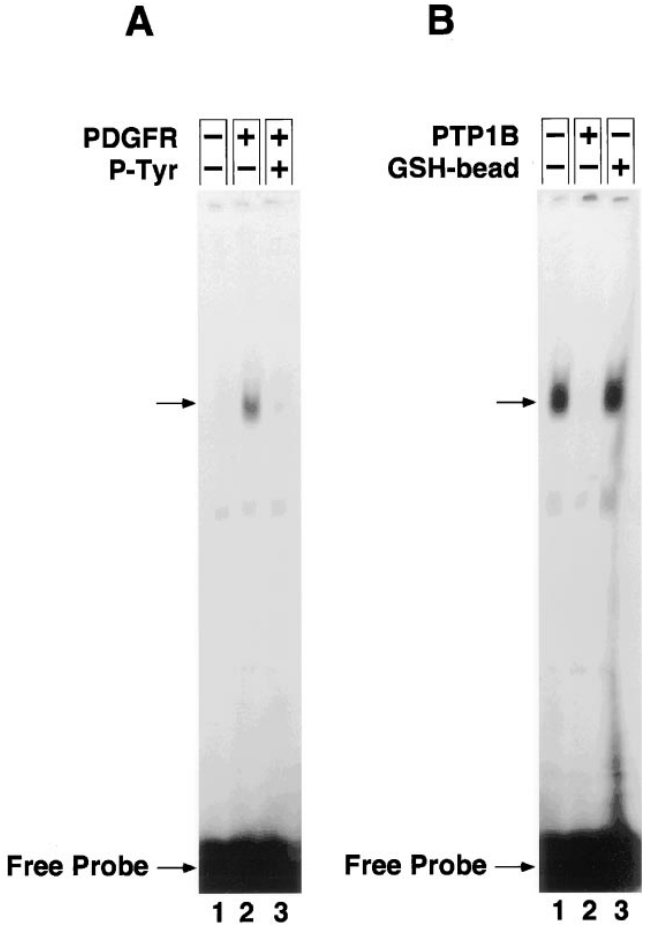

Figure 8. (*A*) PDGFR-phosphorylated STAT1α forms dimers. In vitro synthesized STAT1α was incubated with PDGFR and ATP. The phosphorylated STAT1α was incubated with 0.25 mM phosphotyrosine (*P-Tyr*) on ice for 30 min before performing GMSA in the presence of the m67SIE probe. Arrow indicates the STAT1α–DNA complex. (*B*) PDGFR-phosphorylated STAT1α is sensitive to tyrosine phosphatase. PDGFR-phosphorylated STAT1α was incubated with PTP1B linked to glutathione agarose (lane *2*) or with only glutathione agarose beads (lane *3*) at 30°C for 30 min. The reaction mixture was centrifuged and the supernatants were used in GMSA as described in panel *A*. Arrow indicates the STAT1α–DNA complex.

work has implicated a role for STAT1α in interferon γ-mediated synergistic effect on PDGF-induced DNA synthesis in glomerular mesangial cells and liver fat storing cells (20). One possibility for the increased activation of STAT1α in these cells is an increase in tyrosine phosphorylation of STAT1α protein followed by recruitment of this transcription factor to the receptor. PDGF-mediated signal transduction is initiated on the plasma membrane by transtyrosine phosphorylation of its dimerized receptor that binds multiple SH2 and SH3 domain-containing enzymes and adaptor proteins (5–8, 24). One group of enzymes that is recruited to the PDGFR is the Src family of tyrosine kinases (31, 32). It has been shown that activation of Src is necessary for PDGF-induced mitogenesis (33). This indicates that although PDGFR possesses intrinsic tyrosine kinase activity, activation of other tyrosine kinases are also important for PDGFR signaling in target cells. We and others have recently shown that the JAK family of tyrosine kinases is also a down stream target in the PDGF-induced signal transduction pathway (16, 17). Vignais et al. showed that JAK1, JAK2, and TYK2 associate with PDGFR in a PDGF-dependent manner (16). In response to various hormones and cytokines, these tyrosine kinases are involved in STAT-mediated transcriptional events (9–11). Chao-Lan et al. reported that STAT3, a family member of the same group of transcription factors, is constitutively activated in v-src–transformed cells (34). These data indicate that activation of STAT family members can potentially bypass the JAK pathway. The studies described here address the question of whether PDGFR can directly interact and activate STAT1α. We demonstrate that the 185-kD PDGFR associates with STAT1α in human glomerular mesangial cells when treated with PDGF (Figs. 1 and 2). These data indicate that STAT1α is present in a complex with the receptor in PDGF-treated cells. Furthermore, phosphorylation of other proteins migrating at 130 to 65 kD in

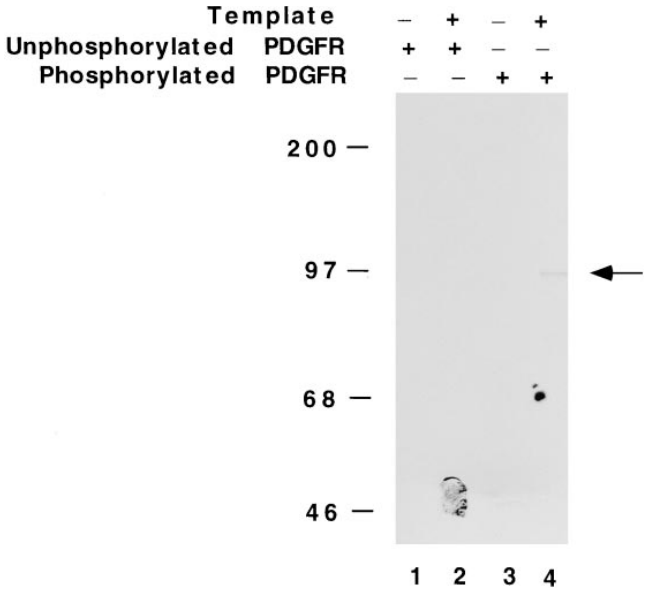

Figure 9. Physical association of STAT1α with activated PDGFR. In vitro transcription-coupled translation reaction was carried out with ^{35}S-methionine in the presence and absence of STAT1α cDNA template as described in Methods. The reaction products were then incubated with unphosphorylated or phosphorylated PDGFR bound to protein A sepharose in RIPA buffer for 2 h at 4°C. After incubation, the beads were washed three times with RIPA buffer and twice with 50 mM Tris-HCl, pH 7.5. The bound proteins were analyzed by SDS gel followed by fluorography. Arrow indicates the PDGFR-associated STAT1α protein in lane *4*.

STAT1α immunoprecipitates seen in the control-untreated mesangial cells suggest that STAT1α is present in a complex with other kinases that may include the JAK family of tyrosine kinases represented by the proteins at 130 kD (Fig. 2 *A*).

We have recently shown that serum-deprived quiescent human mesangial cell extracts do not posses detectable STAT binding activity when tested by GMSA using SIEm67 as probe (20). However, incubation of this cell lysate with PDGFR in vitro stimulates STAT binding activity (Fig. 3). Src is a downstream target of PDGFR and is necessary for its biological activity (31, 32). In addition Cao et al. reported that Src associates with and phosphorylates STAT3 in v-Src–overexpressing cells (35). These observations raised the possibility that Src kinase, present in the quiescent mesangial cell extract, is a potential candidate for activation of STAT1α. However, our data demonstrate that the in vitro translated STAT1α is a direct substrate of PDGFR (Fig. 6 *C*) indicating that similar to STAT3 phosphorylation by v-Src, STAT1α can be directly phosphorylated by PDGFR.

All STAT family members including STAT1α contain an SH2 domain, which is known to interact with a specific phosphotyrosine in the cytoplasmic domain of interferon receptors upon stimulation of target cells (9–11). This specific binding of STAT proteins with the receptor facilitates tyrosine phosphorylation of the STAT proteins by the JAKs which are constitutively associated with the cytoplasmic domain of interferon receptors. In the present study, we have demonstrated direct physical association between autophosphorylated PDGFR and STAT1α (Fig. 9). Whether this interaction occurs through the SH2 domain or other region of STAT1α is yet to be determined. However, the association between STAT1α and PDGFR may serve as the intermediate for the phosphorylation reaction demonstrated in Fig. 6 *C*.

Activation of same JAKs by multiple cytokines raises questions concerning specificity of activation of STATs. However, recent data suggest that specificity is not controlled by JAKs but rather by the ability of individual activated receptors to recruit specific members of the STAT family. For example, Stahl et al. have reported that modular tyrosine-based motifs representing STAT3-activating docking motifs, when expressed in a receptor, can activate STAT3 (36). Similarly by swapping the SH2 domain of one STAT protein with another, Heim et al. demonstrated that the specificity of STAT activation is determined by the physical interaction of specific STAT proteins with the receptor complex (37). In the present study, our demonstration of direct interaction of PDGFR with STAT1α and consequent STAT1α activation confirmed these previous observations in another receptor system. It should be noted that EGFR, a member of a different receptor tyrosine kinase family, also associates with STAT1 in an EGF-dependent manner (38). The tyrosine kinase activity of EGFR is necessary for STAT1 activation (39). Although EGFR stimulates tyrosine phosphorylation of JAK1, the activity of this tyrosine kinase is not required for STAT1 activation in EGF-stimulated cells. Unlike PDGF, EGF stimulation of cells does not induce tyrosine phosphorylation of JAK2 and TYK2 (39). More recently it has been shown that immunopurified EGFR is capable of tyrosine phosphorylating purified STAT in vitro (40).

Accumulating evidence suggests that tyrosine-phosphorylated STATs undergo intermolecular dimerization through their SH2 domain and the conserved phosphotyrosine present in the COOH-terminal of the SH2 domain (30). This dimerization is required for subsequent release of STATs from the receptor complex and for translocation to the nucleus for DNA binding (11). Specific receptor interaction of STAT1α also dictates dimerization (37).We now demonstrate that PDGFR-phosphorylated STAT1α undergoes dimerization, a process required for its binding to DNA in vitro (Fig. 8 *A*). Thus our data indicate that dimerization follows tyrosine phosphorylation and that both processes are required for successful DNA binding of the transcription factor in vitro (Fig. 8).

The relevance of our in vitro observations to in vivo intact mesangial cells remain to be determined. PDGFR from specific JAK-deficient cell lines is capable of activating STATs (16), however, generalizations concerning STAT function in the presence of other redundant family members are difficult to draw. Our studies using the JAK2 inhibitor in intact mesangial cells indicate that substantial activation of STAT1α by PDGF is not dependent on this kinase (Fig. 5 *B*). At this point it is not clear how JAK2 may modulate the STAT pathway. Serine phosphorylation of STATs is a requirement for their activity. Specifically, MAPK has been implicated in this phosphorylation and activation process (41, 42). Marrero et al. recently demonstrated that in vascular smooth muscle cells, inhibition of JAK2 blocks PDGF-induced MAPK activity suggesting a cross-talk between these two pathways (28). The slight reduction of STAT1-DNA complex formation in the presence of JAK2 inhibition in intact mesangial cells (Fig. 5 *B*) may be due to modulation of serine phosphorylation of STAT1α. In mesangial cells, we have recently detected the

presence of serine-phosphorylated STAT1α in extracts prepared from cells treated with PDGF (data not shown). In the in vitro STAT1α DNA binding study, we have used mesangial cell lysates immunodepleted of JAK1, JAK2, and TYK2 to show that JAKs have no effect on the activation of STAT1α by PDGFR (Fig. 4). This observation does not exclude the potential involvement of undetectable amounts of JAK kinases in STAT1α activation in vitro. The presence of a minute amount of these kinases might still have contributed to the activation of STAT1α. Indeed, recent evidence suggests that STAT1α activation even by JAK kinases may not involve only tyrosine phosphorylation (28).

Activation of the PDGFR is essential for development of mesangial cell lineage and for changes in the phenotype of these cells during glomerular injury (1–3, 43). Identification of the precise signaling pathways that mediate the biologic activities that result from PDGFR activation may allow selective therapeutic targeting of this receptor and help delineate its role in mesangial cell development and injury.

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