Ligand Activation Causes a Phosphorylation-dependent Change in Platelet-derived Growth Factor Receptor Conformation*

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The effect of ligand binding on platelet-derived growth factor (PDGF) receptor conformation was examined using peptide antibodies directed against specific receptor domains. Antibodies 83, which was directed to the receptor's carboxyl terminus (residues 934–951), preferentially immunoprecipitated the ligand-activated form of the PDGF receptor from 35S-labeled BALB/c 3T3 cells. By contrast, two antisera directed against other receptor sequences precipitated unactivated and activated receptors equally well. De-natured receptors were recognized equally by all antisera, even 83. Thus, ligand activation caused a change in PDGF receptor conformation that enhanced accessibility of the antibody to the carboxyl terminus. The activated receptor conformation was induced by three different forms of PDGF (AA and BB homodimers and AB heterodimers) and was reversed by suramin, a polyanionic compound that dissociates PDGF from the receptor. The inhibitory effect of suramin on receptor conformation was abolished by the phosphatase inhibitor, sodium orthovanadate, suggesting that receptor phosphorylation mediated the conformational change. In a cell-free assay, the change in receptor conformation was induced by PDGF only in the presence of ATP and was inhibited by adeny1-5′-yl imidodiphosphate, a nonhydrolyzable analog of ATP. The functional significance of receptor conformation was examined in Chinese hamster ovary fibroblasts transfected with wild-type or mutated forms of the PDGF receptor. When receptor tyrosine kinase activity was abolished by a mutation of the ATP binding site the receptor no longer underwent PDGF-induced conformational change even though 125I-PDGF binding was normal. These findings show that ligand binding elicits a phosphorylation-dependent change in PDGF receptor conformation that may be important for receptor function.

Platelet-derived growth factor (PDGF) is a potent mitogen for mesenchymal cells. The initial step in PDGF-induced mitogenesis is the interaction of ligand with specific receptor sites on the surface of responsive cells. Early cellular events following PDGF binding include activation of receptor tyrosine kinase (1, 2), increased phosphoinositide hydrolysis (3), protein kinase C activation (4), accumulation of intracellular calcium (5), characteristic shifts in intracellular pH (5), dramatic cytoskeletal changes (6), and enhanced expression of the c-myc and c-fos proto-oncogenes (7, 8). Studies of the receptors for epidermal growth factor (9, 10), insulin (11, 12), and PDGF (9-1) have correlated receptor tyrosine kinase activity with mitogenic responsiveness, suggesting that the receptor's phosphotransferase activity is critical for signal transduction by mesenchymal growth factors. It is not clear, however, how receptor phosphorylation mediates PDGF-induced DNA synthesis and cytokinesis. In this report we used peptide antisera directed against specific PDGF receptor domains to study the effect of PDGF on receptor conformation and function. We found that ligand binding induces a change in receptor conformation that depends on receptor tyrosine kinase activity. Using cells that express mutated forms of the receptor we correlated this conformational change with the ability of the receptor to mediate PDGF-induced mitogenesis.

MATERIALS AND METHODS

Receptor antisera were obtained by injecting rabbits with receptor peptides conjugated to keyhole limpet hemocyanin (19). The peptide sequences were deduced from the nucleotide sequence of the PDGF receptor cDNA clone (14). Extracellular domain antisera (Ab 77) was directed against a synthetic peptide located at amino acid residues 425–446. Synthetic peptides containing amino acid residues 738–760 and 934–951 were used to generate antisera against the cytoplasmic domain of the receptor (Ab 88 and Ab 83, respectively). No PDGF activity was detected in antisera prepared by radioreceptor assay. Antibody against phosphotyrosine was provided by J. Y. J. Wang (University of California, San Diego, CA). Heterodimeric PDGF was obtained from outdated platelets as previously described (16–17). Homodimeric AA and BB PDGF were obtained from Lawrence Cousins (Chiron Corp., Emeryville, CA) (18).

BALB/c 3T3 cells (American Type Culture Collection), passages 65–75, were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum (Colorado Serum Co.), penicillin, and streptomycin on Costar plastic six-well plates and used 5 days after plating. CHO cells, clone K1 (University of California San Francisco Tissue Culture Facility), were grown in Ham's F-12 media supplemented with 10% fetal calf serum, penicillin, and streptomycin.

Preparation of CHO cell transfectants, which express wild-type or mutated forms of the PDGF receptor under the transcriptional control of the SV40 early promoter as well as the gene for neomycin resistance, has been described previously (19). Clone R18 expresses the initiation methionine (amino acid position –31), the signal peptide (–31 to –1), and the full-length PDGF receptor coding sequence (1–1067) (14). Clone K602A (602) expresses a mutated form of the PDGF receptor that lacks the ATP binding site (lysine 602 to alanine substitution). Stable transfectant clones were grown in 6-well plates in Ham's F-12 media with 400 µg/ml G418 and 10% fetal calf serum, and used 3 days after plating.

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1 The abbreviations used are: PDGF, platelet-derived growth factor; Ab, antibody; AMP-PNP, adeny1-5′-yl imidodiphosphate; CHO, Chinese hamster ovary; SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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Metabolic labeling of cells was performed with [35S]methionine (0.25 mCi/ml) for 4 h at 37 °C. Cells were incubated with PDGF at 4 °C to prevent receptor down-regulation. Labeled cells were solubilized in Ripand/Triton/bovine serum albumin buffer as previously described (20). Lysates were cleared by centrifugation for 15 min at 13,000 x g at 4 °C. Immunoprecipitation with antibodies against phosphotyrosine or against the PDGF receptor was performed as described (13). In each experiment, receptor protein was standardized using receptor antisera that detect both unactivated and PDGF-activated receptors equally. Immunoprecipitates were separated on SDS-polyacrylamide gels. Following electrophoresis, gels were treated for 1 h with ENHANCE (Du Pont-New England Nuclear), dried, and exposed to Kodak XAR-5 film for 1–3 days. Methods used in Western blot analysis of PDGF receptors were described previously (13).

To examine receptor phosphorylation and conformation in a cell-free system, cells were solubilized in 20 mM Heps buffer (pH 7.4) containing 0.5% Triton X-100, 10% glycerol, and 1 mg/ml bovine serum albumin. Lysates were cleared by centrifugation and incubated with or without PDGF for 15 min at room temperature. The reaction mixture was cooled and incubated at 4 °C for 15 min with 100 μM ATP (Boehringer Mannheim) and 10 mM MgCl₂. PDGF receptors were immunoprecipitated as described previously (13).

RESULTS AND DISCUSSION

To examine the effect of ligand binding on PDGF receptor conformation, we performed metabolic labeling and immunoprecipitation experiments using a panel of peptide antibodies directed against specific PDGF receptor domains (13). Antiserum 83 (Ab 83), which was directed against the receptor's carboxyl-terminal sequences, preferentially immunoprecipitated the ligand-activated form of the 180-kDa mature receptor protein from [35S]-labeled BALB/c 3T3 cell lysates under non-denaturing conditions (Fig. 1A, lanes 7 and 8). Densitometric analysis of Fig. 1, and seven other representative experiments, indicated that Ab 83 immunoprecipitated 9-fold more receptor protein from lysates of PDGF-activated cells than from comparable lysates of unactivated cells. Iden-
tical results were obtained when immunoprecipitation was performed with either of two antisera that were directed against the same carboxyl-terminal residues as Ab 83 but were generated separately (not shown). By contrast, antisera directed to extracellular (Ab 77) and other intracellular (Ab 88) domains immunoprecipitated the activated and unactivated forms of the receptor equally well (Fig. 1A). These data suggest that ligand binding elicited a change in PDGF receptor conformation, exposing a cryptic carboxyl-terminal epitope that was recognized by Ab 83.

If preferential immunoprecipitation of activated PDGF receptors by Ab 83 was due to a change in receptor conformation, receptor denaturation should abolish preferential recognition of activated receptors by this antiserum. When cell extracts were boiled in SDS, separated by polyacrylamide gel electrophoresis, and transferred to nitrocellulose, Ab 83 (Fig. 1B), as well as Ab 77 and Ab 88 (not shown), recognized activated and unactivated forms of the receptor equally well. By contrast, antibodies against phosphotyrosine recognized only the activated form of the receptor by immunoblot or by immunoprecipitation (Fig. 1, A and B). This result was expected since the PDGF receptor in quiescent fibroblasts is not tyrosine-phosphorylated, and ligand binding induces phosphorylation of the receptor on its own tyrosine residues (1, 2, 21). Preferential immunoprecipitation of activated receptors by Ab 83 was not, however, due to recognition of phosphotyrosine since this antibody recognized activated and unactivated denatured receptors equally by immunoblot analysis (Fig. 1B). Furthermore, Ab 83 was generated using an unphosphorylated synthetic peptide which specifically blocked immunologic detection of ligand-activated and unactivated receptor protein (13). Thus, Ab 83 detects receptor protein, not phosphotyrosine.

PDGF is biologically active in three different forms, AA and BB homodimers and AB heterodimer (22). To determine if the receptor's activated conformation was induced by a particular form of PDGF, cultured fibroblasts were incubated with saturating concentrations of AA, BB, or AB PDGF. All three forms of PDGF resulted in receptor tyrosine phosphorylation which was detected by immunoprecipitation with antibodies to phosphotyrosine (Fig. 2). These activated receptors were also preferentially immunoprecipitated by Ab 83 (Fig. 2). Thus, all three forms of PDGF were capable of activating receptor tyrosine kinase and inducing the receptor's activated conformation.

PDGF is a cationic polypeptide that can be dissociated from

![Fig. 1. Preferential immunoprecipitation of ligand-activated PDGF receptors by Ab 83.](attachment)

A. Immunoprecipitation

Antiserum: APT 77 83

180 kDa 160 kDa

PDGF

B. Immunoblot

Antiserum: APT 83

180 kDa

PDGF

180 kDa

160 kDa

FIG. 1. Effect of AA, BB, or AB PDGF on receptor phosphorylation and conformation. BALB/c 3T3 cells were labeled with [35S]methionine as described in Fig. 1A and then incubated with saturating concentration of AA, BB, or AB PDGF. All three forms of PDGF resulted in receptor tyrosine phosphorylation which was detected by immunoprecipitation with antibodies to phosphotyrosine (Fig. 2). These activated receptors were also preferentially immunoprecipitated by Ab 83 (Fig. 2). Thus, all three forms of PDGF were capable of activating receptor tyrosine kinase and inducing the receptor's activated conformation.

![Fig. 2. Effect of AA, BB, or AB PDGF on receptor phosphorylation and conformation.](attachment)
its high-affinity binding sites by a polyanionic compound, suramin (23). To determine if the ligand-induced change in receptor conformation could be reversed by removing PDGF from activated receptors, [35S]labeled cells were stimulated with PDGF at 4 °C and then incubated with or without suramin. Suramin reduced the number of tyrosine-phosphorylated receptors immunoprecipitated by antibodies to phosphotyrosine (Fig. 3, lane 3), presumably by deactivating receptor kinase without reducing the effect of cellular phosphatases. Suramin also reduced the number of receptors immunoprecipitated by Ab 83, suggesting that the number of receptors in the activated conformation had also been decreased (Fig. 3, lane 7). The inhibitory effect of suramin on receptor phosphorylation and conformation was abolished, however, by pretreatment of cells with the phosphatase inhibitor, sodium orthovanadate (Fig. 3, lanes 4 and 8). These data show that the PDGF-induced change in receptor conformation is reversible and suggest that a direct relationship exists between receptor phosphorylation and receptor conformation.

To further examine the relationship between receptor phosphorylation and conformation, receptor tyrosine kinase was stimulated in a cell-free system and assayed by immunoprecipitation with antibodies to phosphotyrosine. [35S]-Labeled cell lysates were incubated with PDGF in the absence or presence of ATP. Tyrosine phosphorylation of the 180-kDa mature receptor protein was detected only in the presence of ATP and ligand (Fig. 4A, lane 4). Although preferential immunoprecipitation of activated receptor protein by Ab 83 was less pronounced in this cell-free assay, analysis of five representative experiments by densitometry showed that Ab 83 detected 3-fold more receptor protein in the presence of ATP and PDGF (Fig. 4A, lane 8). PDGF, by itself, was not sufficient to cause the change in receptor conformation. The specificity of this reaction was confirmed by incubating cell lysates with AMP-PNP. In the presence of this nonhydrolyzable analog of ATP (24) receptor phosphorylation was inhibited (Fig. 4B, lane 3). AMP-PNP also inhibited preferential immunoprecipitation of receptor protein by Ab 83, even in the presence of ATP and PDGF (Fig. 4B, lane 7). These data suggest that PDGF receptor tyrosine kinase activity is required for the ligand-induced change in receptor conformation.

Interestingly, in this cell-free system, a 165-kDa tyrosine phosphoprotein was immunoprecipitated by antibodies to phosphotyrosine (Fig. 4, A and B, lane 4). This protein is likely to represent the phosphorylated form of the 160-kDa PDGF receptor precursor which is normally located in intracellular compartments and not exposed to PDGF. In the presence of ATP and PDGF, and the absence of AMP-PNP, Ab 83 immunoprecipitated approximately 2-fold more 160-kDa receptor precursor protein (Fig. 4, A and B, lane 8). These data provide direct evidence that the 160-kDa receptor precursor, like the mature form of the receptor, can respond enzymatically and conformationally to PDGF.

In recent experiments, we have examined the functional significance of specific PDGF receptor domains using CHO cells transfected with wild-type and mutated forms of the receptor (19). To study the functional significance of changes in receptor conformation, immunoprecipitation experiments were repeated using these transfected cells. Untransfected CHO cells do not express PDGF receptor and do not respond mitogenically to PDGF. In cells transfected with the wild-type receptor cDNA (R18), PDGF-induced tyrosine phosphorylation of the expressed receptor was detectable by immunoprecipitation with antibodies to phosphotyrosine (Fig. 5A). Preferential immunoprecipitation of ligand-activated receptor protein by Ab 83 was also noted in these cells (Fig. 5B). Thus, wild-type receptors in transfected R18 cells undergo similar ligand-induced tyrosine phosphorylation and conformational changes seen in the native receptor in 3T3 cells. We have shown that R18 cells also respond mitogenically to PDGF as measured by incorporation of thymidine into DNA and cell count (19). Thus, CHO cells expressing wild-type receptors respond to PDGF in an apparently normal fashion.

Immunoprecipitation experiments were performed using CHO cells expressing a mutated form of the PDGF receptor (K602A) that lacked the ATP binding site and had no PDGF-stimulated tyrosine kinase activity. On average, expression of receptor protein, as measured by 125I-PDGFB binding, Western blot analysis, and immunoprecipitation with Ab 77 and Ab 88, was 20% greater in K602A than in R18 cells. Although

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**Fig. 3. Effect of suramin and orthovanadate on PDGF receptor phosphorylation and conformation.** BALB/c 3T3 cells were metabolically labeled with [35S]methionine as described in Fig. 1A in the presence or absence of 37.5 μM sodium orthovanadate. Cells were incubated at 4 °C with or without a saturating concentration of PDGF for 1 h and then with or without 140 μM suramin for 1 h. PDGF receptors were immunoprecipitated with antibody to phosphotyrosine (APT) or Ab 83. Receptor and tyrosine phosphoproteins are shown.

**Fig. 4. Effect of ATP and AMP-PNP on PDGF receptor phosphorylation and conformation.** BALB/c 3T3 cells were metabolically labeled with [35S]methionine as described in Fig. 1A, solubilized in Triton X-100, and incubated with or without PDGF at room temperature for 15 min. Cell extracts were incubated at 4 °C with or without 100 μM ATP and 10 mM MgCl2 for 15 min. Extracts were immunoprecipitated with antibodies to phosphotyrosine (APT) or Ab 83. The 160-kDa precursor and 180-kDa mature receptor proteins and 180-kDa tyrosine phosphoproteins are shown. In this cell-free assay, 165-kDa tyrosine phosphoprotein was also immunoprecipitated by antibodies to phosphotyrosine. B, cell monolayers were metabolically labeled, solubilized, and incubated with or without PDGF as described in Fig. 3. Cell extracts were then incubated with 100 μM ATP and 10 mM MgCl2 in the presence or absence of 10 mM AMP-PNP at 4 °C for 15 min. Extracts were immunoprecipitated with antibodies to phosphotyrosine or Ab 83.
Fig. 5. Receptor phosphorylation and conformation in cells expressing mutated forms of the PDGF receptor. CHO cells transfected with wild-type (R18) and mutated (K602A) forms of the PDGF receptor (see “Materials and Methods”) were labeled with \(^{35}S\)methionine as described in Fig. 1A. Intact cells were incubated with (+) or without (−) PDGF at 4 °C for 2 h, solubilized, and immunoprecipitated with antibodies to phosphotyrosine (A) or Ab 83 (B). 160-kDa precursor and 180-kDa mature receptor proteins and 180-kDa tyrosine phosphorylated proteins are shown. In this experiment, expression of receptor protein, as measured by immunoprecipitation with Ab 88 and Ab 77, was approximately 50% greater in K602A cells than in R18 cells.

\(^{125}I\)-PDGF binding was normal in cells expressing the mutated receptor, tyrosine phosphorylation of the receptor was not detected, even in the presence of PDGF (Fig. 5A). Ligand-induced preferential immunoprecipitation of expressed receptors by Ab 83 was also not detected in these cells (Fig. 5B). These data confirm that the ligand-mediated change in PDGF receptor conformation depended upon intact receptor tyrosine kinase activity. In recent experiments we have shown that K602A cells do not respond mitogenically to PDGF. Thus, a direct relationship exists between receptor phosphorylation, conformation, and mitogenic signal transduction.

These studies provide the first direct evidence for a ligand-induced change in PDGF receptor conformation. In intact cells, only the 180-kDa mature cell surface receptor was detected in the activated conformation (Fig. 1A). In a cell-free assay, however, tyrosine phosphorylation and conformational change were also noted in the 160-kDa receptor precursor (Fig. 4). PDGF receptor precursors, therefore, can bind to and be activated by PDGF. These findings were especially interesting in light of recent data showing that autocrine activation of intracellular incompletely processed PDGF receptors may be the mechanism of transformation by the \(v\)-c-sis oncogene (25).

In experiments with cells expressing mutated receptors that lack the ATP binding site (Fig. 5) and in a cell-free assay (Fig. 4) we found that the ligand-induced change in receptor conformation was dependent on receptor tyrosine kinase activity; ligand binding, by itself, was not sufficient to induce the conformational change. A similar phosphorylation-dependent change in conformation has also been reported for the insulin receptor (26). Since sodium orthovanadate abolished suramin’s ability to reverse the ligand-induced change in PDGF receptor conformation (Fig. 3), it is likely that the activated conformation resulted from the transfer of phosphate to one or more of the receptor’s tyrosine residues. Alternatively, the association of the receptor with ATP may induce the conformational change. Future experiments on other mutated forms of the PDGF receptor should distinguish between these two mechanisms and should help define the importance of receptor conformation for receptor function.

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