Characterization of a Novel Anti-Peptide Antibody That Recognizes a Specific Conformation of the Platelet-Derived Growth Factor Receptor

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Two site-specific anti-peptide antibodies (AbP₁ and AbP₂) were raised against the platelet-derived growth factor (PDGF) receptor. These two sites correspond to amino acid residues 977 through 988 (peptide 1) and 932 through 947 (peptide 2) of the murine PDGF receptor. Both antibodies recognized human and murine PDGF receptors in immunoprecipitation and immunoblotting analyses. None of the antibodies was directed to phosphotyrosine. One of the antibodies (AbP₂) showed unusual antigen recognition specificity. This antibody specifically recognized the tyrosine-phosphorylated PDGF receptor and not the unphosphorylated native receptor, suggesting that recognition by this antibody requires a specific conformation that is induced by PDGF-stimulated autophosphorylation.

The human platelet-derived growth factor (PDGF) consists of a family of glycoproteins (M_r , 28,000 to 35,000) composed of two homologous but nonidentical polypeptides (A and B) of M_r 14,000 to 18,000 linked by disulfide bonds (1, 7, 11, 17); the B chain of PDGF is a product of *c-sis* proto-oncogene (8, 23). PDGF initiates cellular replication and transformation by binding to a specific high-affinity cell surface receptor, a transmembrane glycoprotein of 180 kilodaltons (kDa). The PDGF receptor has recently been purified from various sources (3, 6, 19). The purified receptor displays PDGF-stimulated tyrosine kinase activity, suggesting that tyrosine kinase is an integral part of the receptor molecule (3, 19). This conclusion has recently been confirmed by cloning of cDNA for the murine PDGF receptor; the deduced sequence has considerable homology with known tyrosine kinases (24).

Unlike the well-studied receptors for epidermal growth factor and insulin, the PDGF receptor is relatively uncharacterized. Studies on biosynthesis and processing of this receptor have recently been aided by the availability of anti-PDGF-receptor antibodies (4, 10, 13). In this communication, we report the generation and characterization of a conformation-specific novel anti-PDGF-receptor antibody. It recognizes a peptide epitope (residues 932 through 947 of murine PDGF receptor) that is normally cryptic but becomes accessible upon receptor phosphorylation. This antibody, unlike other reported reagents, may be useful in future structure-function analyses of the receptor.

MATERIALS AND METHODS

Materials. Keyhole limpet hemocyanin (KLH) was purchased from Sigma Chemical Co. (St. Louis, Mo.), and *N*-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) was from Pharmacia Fine Chemicals (Piscataway, N.J.). Wheat germ agglutinin-agarose was from Vector Laboratories (Burlingame, Calif.). Tran³⁵S-label (³⁵S-labeled *Escherichia coli* hydrolysate containing [³⁵S]methionine) and ³²P_i were obtained from ICN Pharmaceuticals Inc. (Irvine, Calif.); Na¹²⁵I was from Amersham Corp. (Arlington Heights, Ill.).

2G8 hybridoma cells (9) producing antiphosphotyrosine monoclonal antibody were obtained from the American Type Culture Collection (Rockville, Md.; catalog no. 8190).

PDGF, ¹²⁵I-PDGF and platelet-poor plasma. PDGF was purified from outdated human platelets (American Red Cross) by the method of Scher et al. (20). Bio-Gel (Bio-Rad Laboratories, Richmond, Calif.)-purified PDGF was iodinated by the chloramine T procedure, and ¹²⁵I-PDGF was purified as described previously (3). Platelet-poor plasma, which lacks PDGF, was prepared as described previously (21).

[γ -³²P]ATP. Labeled ATP was prepared with ³²P_i (ICN) and a γ -prep A kit (Promega Biotec, Madison, Wis.) according to the manufacturers' directions. The [γ -³²P]ATP was diluted with unlabeled ATP to a final specific activity of 40 to 60 Ci/mmol.

Peptide synthesis. The peptides Glu-Gly-Tyr-Lys-Lys-Lys-Tyr-Gln-Val-Asp-Glu-Glu-Phe-Leu-Arg (P₂) and Tyr-Thr-Ala-Val-Gln-Pro-Asn-Glu-Ser-Asp-Asn-Asp (P₁), corresponding to the residues 932 through 947 and 977 through 988, respectively, of the murine PDGF-receptor sequence (24), were synthesized with an additional cysteine residue at their carboxy termini. These peptides were synthesized by the solid-phase method (2) with an automated peptide synthesizer (Applied Biosystems 430A). The peptides were cleaved from the resin by liquid hydrogen fluoride in the presence of anisole and then were purified by liquid chromatography on octadecylsilica. Purified peptides were homogeneous as judged by reversed-phase high-pressure liquid chromatography and amino acid composition. These purified peptides were dried under reduced pressure and stored at -20°C.

Coupling of synthetic peptides to carrier protein. Each peptide was coupled to KLH through the terminal cysteine residue of the peptide by using SPDP as the coupling reagent. Typically, 10 mg of KLH in 1 ml of 0.1 M sodium phosphate buffer (pH 7.5) was functionalized by treatment with SPDP (dissolved in absolute ethanol) at a KLH/SPDP molar ratio of 1:50 with stirring for 25 min at 22°C. The functionalized KLH was separated from excess reagents by Sephadex G-25 chromatography. For conjugation of the

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peptide to the functionalized KLH, the latter was incubated at 22°C for 2 h with 2.5 mg of peptide at pH 7.5. The reaction was followed spectrophotometrically by monitoring at 343 nm the release of pyridine-2-thione. The quantity of peptide conjugated to KLH was calculated by using the extinction coefficient for pyridine-2-thione at 343 nm as $8.08 \times 10^3/\text{M}$ per cm. Generally, 50 to 80 molecules of peptide were coupled per molecule of KLH. At the end of the conjugation reaction, peptide-KLH conjugate was dialyzed overnight against 10 mM sodium phosphate buffer (pH 7.4)–0.15 M NaCl and stored at –20°C.

Production of anti-peptide antibodies. Each peptide-KLH conjugate was injected into two 8-week-old New Zealand White rabbits. After an initial subcutaneous injection of 600 µg of antigen to complete Freund adjuvant, animals were given intramuscular booster injections of 200 µg of antigen in incomplete Freund adjuvant on days 21 and 35. Animals were bled on day 42, and the sera were tested for the presence of antipeptide antibodies by solid-phase radioimmunoassay.

Biosynthetic labeling of the PDGF receptor. MG-63 (2×10^6 cells) and BALB/c-3T3 (2.5×10^5 cells) cells were seeded into 35-mm dishes in Dulbecco modified Eagle medium (DMEM) containing 10% calf serum. After 20 to 24 h, the cultures were washed with methionine-free DMEM and then preincubated at 37°C for 2 h with methionine-free DMEM containing 2% dialyzed platelet-poor plasma. The cells were then incubated at 37°C for 2 h with [^{35}S]methionine (100 µCi/ml, 1,090 Ci/mmol) in 1 ml of the same medium. The labeled cells were rapidly washed and then solubilized with 1% Nonidet P-40 (NP-40) in 20 mM HEPES (*N*-2-hydroxymethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.4)–0.15 M NaCl–10 µg of leupeptin per ml and 1 mM phenylmethylsulfonyl fluoride. The clarified supernatant obtained after centrifugation was used for immunoprecipitation.

To study the effect of PDGF on the stability of the 180-kDa PDGF receptor, both murine BALB/c-3T3 and human MG-63 cells were biosynthetically labeled with [^{35}S]methionine as described above. At 2 h after the addition of [^{35}S]methionine, the cells were either solubilized immediately or washed twice and incubated at 37°C in fresh methionine-supplemented DMEM–2% dialyzed platelet-poor plasma with or without 6 nM PDGF for various times before solubilization. The clarified soluble extracts after centrifugation were immunoprecipitated with antibody to peptide 1 (AbP₁).

Labeling of the receptor by chemical cross-linking with ^{125}I -PDGF. Confluent cultures of MG-63 human osteosarcoma cells and murine BALB/c-3T3 (clone A31) cells in 60-mm dishes were incubated at 4°C for 3.5 h with 100 pM of ^{125}I -PDGF (80,000 cpm/ng) in Earle balanced salt solution containing 10 mM HEPES (pH 7.4). The cultures were washed free of unbound radioactivity and then incubated at 4°C for 10 min with 1 mM bis(sulfosuccinimido)suberate. Under these conditions, ^{125}I -PDGF becomes covalently linked to the receptor. Cells were then washed, solubilized in 0.5% NP-40 containing buffer, and subjected to immunoprecipitation.

Purification of ^{32}P -labeled human PDGF receptor. The PDGF receptor was purified from plasma membranes of MG-63 human osteosarcoma cells essentially as described previously (3), except that PDGF-induced phosphorylation of the PDGF receptor was performed after solubilization and WGA-agarose purification. Briefly, the receptor was solubilized from plasma membranes with 0.5% NP-40 in 20 mM HEPES (pH 7.4)–0.15 M NaCl–10% glycerol–10 µg of leu-

peptin per ml and 1 mM phenylmethylsulfonyl fluoride. The PDGF receptor in the clarified extract was partially purified by adsorption to and elution from wheat germ agglutinin-agarose by 0.4 M *N*-acetyl glucosamine. The wheat germ agglutinin-purified PDGF receptor was phosphorylated with [γ - ^{32}P]ATP in the presence of 3 nM PDGF, and then tyrosine-phosphorylated PDGF receptor was purified by affinity chromatography on antiphosphotyrosine-antibody-Sepharose as described previously (3). In our earlier method (3), PDGF-induced phosphorylation of the receptor was carried out with isolated membranes; unlike the previous procedure which showed more than one band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the modified procedure yields a single 180-kDa ^{32}P -labeled band (see Fig. 6, input lane) and is essentially free of the epidermal growth factor receptor (data not shown).

Immunoprecipitation of the PDGF receptor. The reactivity of the two antipeptide sera was determined by their ability to immunoprecipitate radiolabeled PDGF receptor. Receptor was radiolabeled by using one of the following methods: (i) biosynthetic labeling with [^{35}S]methionine as in Fig. 1, (ii) labeling by covalent cross-linking with ^{125}I -PDGF with bis(sulfosuccinimido)suberate as the chemical cross-linker as in Fig. 5, and (iii) labeling with ^{32}P as in Fig. 6. The labeled receptor preparation was incubated for 4 h at 4°C with 5 µl of nonimmune or immune serum in 50 µl of 20 mM HEPES (pH 7.4)–0.15 M NaCl–0.2% NP-40–5 mg of bovine serum albumin per ml and protease inhibitors. The immune complexes were isolated by incubating the mixture at 4°C for 45 min with 5 mg of formaldehyde-fixed *Staphylococcus aureus* (The Enzyme Center, Boston) for ^{125}I -PDGF and ^{32}P -labeled receptors or with 50 µl of packed agarose containing 50 µg of covalently bound goat anti-rabbit immunoglobulin G (Sigma) for ^{35}S -labeled receptors. The bacterial or agarose gel pellets were washed twice with HEPES–NaCl–bovine serum albumin–NP-40 containing protease inhibitors and twice with HEPES–NaCl–NP-40 containing 0.1% SDS. The bound radioactivity was eluted by boiling with SDS sample buffer and then subjected to SDS-PAGE with 5 to 20% gel. The gels containing ^{125}I - or ^{32}P -labeled proteins were dried and subjected to autoradiography at –70°C with Kodak X-Omat AR films and DuPont Cronex Lightning Plus intensifying screens. The gels containing ^{35}S -labeled proteins were prepared for fluorography by immersion in dimethylsulfoxide containing 2,5-diphenyloxazole (15), washed in water, dried, and exposed to X-ray films.

Western blot analysis. Wheat germ agglutinin-agarose-purified proteins from MG-63 cell membranes were subjected to reducing SDS-PAGE with a 5 to 20% linear gradient of acrylamide. Electrophoretically separated proteins were then transferred to nitrocellulose by electrophoresis at 4°C for 5 h and then processed as described previously (12). Briefly, the sheets were soaked overnight at 4°C in 10 mM Tris (pH 7.4)–0.15 M NaCl (TS buffer) containing 5% (wt/vol) nonfat dried milk (TSM buffer) to block nonspecific sites and then incubated at 4°C overnight with a 1:10 dilution of the antiserum or nonimmune serum. The treated sheets were washed three times with TS buffer–0.05% Tween 20–2 M urea and then again three times with TSM buffer. The washed sheets were incubated at 20°C for 2 h with ^{125}I -labeled protein A (75 ng, 10^7 cpm) in TSM buffer. After extensive washing, the sheets were dried and subjected to autoradiography.

FIG. 1. Immunoprecipitation of a biosynthetically labeled 180-kDa protein from BALB/c-3T3 and human MG-63 cells. Cells were labeled with [35 S]methionine, and the clarified lysates were subjected to immunoprecipitation with the indicated antiserum (AbP₁ or AbP₂) or with nonimmune serum (Ni) in the absence or presence of 2 μ g of cognate peptide as described in Materials and Methods. MG-63 cell lysate (diluted fivefold with immunoprecipitation buffer) was denatured by heating the lysate at 70°C for 5 min in the presence of 0.1% 2-mercaptoethanol. The heated cell lysate was centrifuged, and the supernatant was subjected to immunoprecipitation. Immunoprecipitates were analyzed by SDS-PAGE and fluorography. Molecular size markers: myosin (205 kDa), β -galactosidase (116 kDa), phosphorylase b (93 kDa), bovine serum albumin (66 kDa), and ovalbumin (43 kDa).

RESULTS

We raised rabbit antibodies against two synthetic peptides corresponding to residues 977 through 988 (P₁) and 932 through 947 (P₂) of the cytoplasmic domain of the murine PDGF receptor. The sequences of these two peptides are unique to the PDGF receptor (24). In initial experiments, the presence of antibodies in immune sera was tested by solid-phase radioimmunoassay with peptides as antigens and 125 I-labeled protein A.

AbP₁ recognizes a 180-kDa protein whose stability is PDGF sensitive. AbP₁ immunoprecipitated a protein of 180 kDa from [35 S]methionine-labeled murine BALB/c-3T3 and human MG-63 cells (Fig. 1); immunoprecipitation of the 180-kDa protein was totally abolished in the presence of P₁ (Fig. 1) but not in the presence of P₂ (data not shown). This suggests that AbP₁ is highly specific. The nonspecific precipitation of other proteins was not blocked by P₁.

To test whether the 180-kDa protein which is immunoprecipitated by AbP₁ is the PDGF receptor, we studied the effect of PDGF on the stability of the 180-kDa protein in intact cells. Since biological half-lives of many receptors are drastically reduced in the presence of their respective ligands (14, 18, 22), reduced stability of the 180-kDa protein in the presence of PDGF will indicate that the 180-kDa protein is the PDGF receptor. To study the effect of PDGF on the turnover rate of the 180-kDa protein, murine BALB/c-3T3 and human MG-63 cells were pulsed for 2 h with

[35 S]methionine and then chased with unlabeled methionine in the presence or absence of 6 nM PDGF for the indicated times up to 24 h. There was a gradual decrease in the amount of immunoprecipitable labeled 180-kDa protein from both BALB/c-3T3 and MG-63 cells with increasing chase time (Fig. 2, inserts). The addition of PDGF to the chase medium accelerated this process of 180-kDa protein loss. A semilog plot of the data revealed that in the absence of PDGF, the immunoprecipitable protein has a half-life of about 2.8 h, whereas in presence of PDGF it is reduced to 0.6 h. An examination of total protein 35 S radioactivity during the chase period indicated a loss of only 10 to 20% after 24 h of chase, and the overall loss was unaffected by PDGF (data not shown). These results suggest that the 180-kDa protein immunoprecipitated by AbP₁ is the PDGF receptor. In addition, our results demonstrate that the PDGF receptor of either human or murine origin is less stable (half-life of <3 h) than the receptors for epidermal growth factor and insulin, which have half-lives of >10 h (14, 18, 22). It should be noted in this connection that the short half-life of the murine PDGF receptor has also been recently reported by Keating and Williams (13).

In addition to the 180-kDa receptor, a protein of 175 kDa was found in immunoprecipitates from samples pretreated with PDGF. The exact nature of this protein is not known; it may represent processed receptor resulting from PDGF-induced proteolysis of the receptor.

AbP₂ recognition of PDGF receptor: dependence on denaturation or phosphorylation. AbP₂ failed to recognize any protein from biosynthetically labeled cells. However, heat denaturation of the labeled lysate resulted in recognition of a 180-kDa protein by AbP₂ (Fig. 1). Like the 180-kDa protein immunoprecipitated by AbP₁, the protein recognized by AbP₂ after heat denaturation had a half-life that was reduced in the presence of PDGF (data not shown). This suggests that the 180-kDa protein immunoprecipitated by AbP₂ is the PDGF receptor. However, unlike AbP₁, which recognizes both native (Fig. 1) and heat-denatured receptors (data not shown), AbP₂ only recognizes the heat-denatured receptor and not the native receptor.

The observation that both AbP₁ and AbP₂ recognize the denatured PDGF receptor was confirmed by Western blotting. Immunoblot analysis of WGA-agarose-purified MG-63 membrane proteins with either AbP₁ or AbP₂ showed that both antibodies bind specifically only to one polypeptide of 180 kDa (Fig. 3).

To test whether AbP₂ recognizes the native receptor after tyrosine phosphorylation, the following experiment was conducted. WGA-purified PDGF receptor from biosynthetically labeled BALB/c-3T3 cells was phosphorylated with ATP in the presence of PDGF and then subjected to immunoprecipitation with AbP₂ in the presence of phenyl phosphate, an analog of phosphotyrosine. AbP₂ immunoprecipitated a 180-kDa protein only from the receptor preparation that had been incubated with ATP and PDGF and not from the untreated control preparation (Fig. 4A). In addition, the receptor preparation that had been incubated with PDGF or ATP alone was also not recognized by AbP₂ (Fig. 4). The biosynthetically labeled 180-kDa PDGF receptor recognized by AbP₂ is phosphorylated on tyrosine residues as demonstrated by its binding to anti-phosphotyrosine antibody-Sepharose (Fig. 4B). These results indicate that AbP₂ recognizes the nondenatured 35 S-labeled PDGF receptor only after tyrosine phosphorylation and that PDGF-mediated conformational changes alone are insufficient for recognition by AbP₂. However, AbP₂ is not directed to phosphotyro-

FIG. 2. Effect of PDGF on the stability of the 180-kDa protein in BALB/c-3T3 and MG-63 cells. BALB/c-3T3 (A) and MG-63 (B) cells were labeled with [35 S]methionine for 2 h and then chased with unlabeled methionine-supplemented DMEM containing 2% dialyzed platelet-poor plasma in the absence (○) or presence (●) of 6 nM PDGF for the indicated times. The cell lysates were subjected to immunoprecipitation with AbP₁ and then SDS-PAGE and fluorography. The region containing the 180-kDa band was densitometrically scanned. Similar densitometric scans were performed for samples treated with nonimmune serum instead of AbP₁. The plot represents the results of the densitometric scan after subtraction of the nonimmune values.

sine, since the immunoprecipitation by AbP₂ was seen in the presence of phenyl phosphate, an analog of phosphotyrosine.

Recognition of 125 I-PDGF receptor covalent complex by AbP₁ and AbP₂. Additional evidence that the immunoprecipitated 180-kDa protein is the PDGF receptor was obtained by using PDGF receptor covalently cross-linked with 125 I-PDGF (Fig. 5). Nearly 35% of the input 125 I-PDGF receptor covalent complex (210 kDa) from either murine or human cells was immunoprecipitated by AbP₁ (Fig. 5). Furthermore, immunoprecipitation by AbP₁ was inhibited by addition of P₁ but not by the addition of P₂. The extent of immunoprecipitation by AbP₂ (less than 2% of input) was considerably less than that seen with AbP₁. The small extent of immunoprecipitation by AbP₂ may be due to the fact that, under labeling conditions in intact cells with active phosphatases, the majority of the receptors will be in a state of

FIG. 3. Detection of PDGF receptor by Western blot analysis. WGA-agarose-purified MG-63 membrane proteins (5 μ g per lane) were subjected to Western blotting with the indicated antiserum (AbP₁ or AbP₂) or nonimmune serum (Ni) as described in Materials and Methods.

dephosphorylation because of constant dephosphorylation reaction. This conclusion is strengthened by the fact that only a small proportion (~5%) of the total cellular 125 I-PDGF receptor covalent complex is recognized by anti-phosphotyrosine antibody (data not shown); this suggests that the majority (95%) of the covalent complex lacks phosphotyrosine.

Neither AbP₁ nor AbP₂ inhibits either basal or PDGF-stimulated receptor tyrosine kinase activity. Since AbP₁ and AbP₂ are directed to two cytoplasmic epitopes of the PDGF receptor, we tested whether these antibodies could inhibit the tyrosine kinase activity of the receptor. In this experiment, WGA-purified PDGF receptor from MG-63 cell membranes was incubated with the immunoglobulin G fraction of either AbP₁ or AbP₂ (purified with protein A-Sepharose) and then phosphorylated with [γ - 32 P]ATP in the absence (basal activity) or presence (PDGF-stimulated activity) of PDGF. The phosphorylated proteins were then analyzed by SDS-PAGE and autoradiography, and the band at 180 kDa was quantitated by densitometric scan. The relative intensities of the 180-kDa bands were as follows: no addition (basal activity), 1; AbP₁, 0.95; AbP₂, 1.1; PDGF, 50; AbP₁ and PDGF, 52; AbP₂ and PDGF, 51. The fact that neither AbP₁ nor AbP₂ had any effect on basal or PDGF-stimulated receptor kinase activity suggested that neither AbP₁ nor AbP₂ inhibits the PDGF receptor autokinase activity.

AbP₂ recognizes only the phosphorylated form of the PDGF receptor. Our studies with 35 S-labeled PDGF receptor suggest that under nondenaturing conditions AbP₂ specifically immunoprecipitates the tyrosine phosphorylated receptor and not the nonphosphorylated form of the receptor (Fig. 4). We conducted the following experiment to demonstrate unequivocally that AbP₂ recognizes only the phosphorylated form of the PDGF receptor under nondenaturing conditions.

FIG. 4. Immunoprecipitation of phosphorylated PDGF receptor from ³⁵S-labeled BALB/c-3T3 cells by AbP₂ and anti-phosphotyrosine antibody. BALB/c-3T3 cells (clone A31) were labeled with [³⁵S]methionine for 4 h. The cells were solubilized, and the PDGF receptor was purified with wheat germ agglutinin-agarose. The purified receptor was either pretreated with 3 nM PDGF as described in the text or left untreated. After 30 min at 4°C, unlabeled ATP (final concentration, 20 μM) was added to indicated samples, and the phosphorylation reaction was continued for 30 min. After the reaction was terminated with EDTA (final concentration, 10 mM), the samples were subjected to immunoprecipitation with AbP₂ or nonimmune serum in the presence of 40 mM phenyl phosphate. The immune complexes were isolated by using anti-rabbit immunoglobulin G-agarose as described in the text. The bound radioactivity was eluted by boiling with SDS sample buffer and then subjected to SDS-PAGE. In immunoprecipitation with nonimmune serum (data not shown), the protein profile was the same as that seen in lane 1 of panel A (no ATP, no PDGF). The receptor preparations were also incubated for 4 h at 4°C with solid-phase anti-phosphotyrosine antibody either in the absence (–) or presence (+) of 40 mM phenyl phosphate. The bound radioactivity was eluted by incubating the gel with 40 mM phenyl phosphate, and samples were subjected to SDS-PAGE. The 180-kDa PDGF receptor is indicated by an arrow.

The 180-kDa ³²P-PDGF-receptor purified from human MG-63 cells was subjected to immunoprecipitation with AbP₁ or AbP₂. AbP₁ and AbP₂ immunoprecipitated 40 and 30%, respectively, of the input ³²P-PDGF receptor after an incubation at 4°C for 4 h (Fig. 6). Thus both antibodies are equally active in recognizing phosphorylated receptors. No immunoprecipitation was seen in the presence of 2 μg of the cognate peptide. P₂ did not block the immunoprecipitation by AbP₁; similarly, P₁ was without effect on immunoprecipitation by AbP₂ (data not shown). These results again indicate that the antisera are highly specific. Moreover, immunoprecipitation by neither antiserum was inhibited by phenyl phosphate (data not shown), suggesting that these antibodies are not directed to phosphotyrosine residues (Fig. 4).

To provide additional evidence that the native nonphosphorylated PDGF receptor is recognized by AbP₁ and not by AbP₂, we conducted the following experiment. The detergent extracts of the MG-63 membranes which contain the nonphosphorylated PDGF receptor were incubated with nonimmune serum, AbP₁, or AbP₂. The immunoprecipitates were then phosphorylated with [γ-³²P]ATP in either the absence or presence of PDGF and analyzed by SDS-PAGE

FIG. 5. Immunoprecipitation of the ¹²⁵I-PDGF receptor covalent complex from murine BALB/c-3T3 and human MG-63 cells. The receptors of BALB/c-3T3 and MG-63 cells were covalently linked with ¹²⁵I-PDGF as described in Materials and Methods. The NP-40-solubilized, clarified lysates (7,000 and 9,000 cpm for the BALB/c-3T3 and MG-63 lysates, respectively) were then subjected to immunoprecipitation with the indicated immune serum (AbP₁ or AbP₂) either in the absence or presence of 2 μg of peptide (P₁ or P₂) as described in the legend for Fig. 1. The immunoprecipitates were then analyzed by nonreducing SDS-PAGE and autoradiography. A densitometric scan of the autoradiogram revealed that nearly 35% and less than 2% of input murine and human PDGF receptors were immunoprecipitated by AbP₁ and AbP₂, respectively.

and autoradiography. If the antibody recognized the non-phosphorylated receptor, there would be the appearance of a ³²P-labeled 180-kDa protein in the immunoprecipitates that could be stimulated by PDGF. If there were no such recognition, no labeled band should be observed. A 180-kDa protein whose phosphorylation was PDGF dependent was immunoprecipitated by AbP₁ (Fig. 7). However, no such PDGF-stimulatable protein was immunoprecipitated either by AbP₂ or by nonimmune serum (Fig. 7). The absence of PDGF-stimulatable protein in immunoprecipitates of AbP₂ is not due to the inhibition of receptor kinase by AbP₂, since neither AbP₁ nor AbP₂ inhibited the basal or PDGF-stimu-

FIG. 6. Immunoprecipitation of the 180-kDa ³²P-labeled PDGF receptor from human MG-63 cells by AbP₁ and AbP₂. Tyrosine-phosphorylated, purified ³²P-labeled PDGF receptor (1,500 cpm) was immunoprecipitated with the indicated antiserum (AbP₁ or AbP₂) or with nonimmune serum (Ni) in the absence or presence of 2 μg of the indicated peptide (P₁ or P₂). The immunoprecipitates were then analyzed by SDS-PAGE (5 to 20% polyacrylamide) followed by autoradiography as described in Materials and Methods.

FIG. 7. Demonstration of PDGF-stimulated tyrosine kinase activity in the immunoprecipitates as measured by receptor autophosphorylation. An NP-40-solubilized extract of MG-63 membranes (100 μ g of membrane protein) was subjected to the following successive treatments. (i) Detergent extract was incubated at 4°C for 4 h with 5 μ l of the indicated antiserum (AbP₁ or AbP₂) or with nonimmune serum (Ni) in 50 μ l of 20 mM HEPES (pH 7.4)–0.15 M NaCl–10% glycerol–0.2% NP-40–5 mg of bovine serum albumin per ml and protease inhibitors. (ii) The immune complexes were isolated with goat anti-rabbit immunoglobulin G-agarose. (iii) The gel beads containing the receptor were incubated at 4°C for 30 min with or without 3 nM PDGF in 90 μ l of 20 mM HEPES (pH 7.4)–0.15 M NaCl–10% glycerol–0.2% NP-40–0.1 mM vanadate–2 mM MgCl₂–0.5 mM MnCl₂. Phosphorylation was initiated by addition of 10 μ l of 250 μ M [γ -³²P]ATP. (iv) After 30 min at 4°C, the reaction was terminated, and the ³²P-labeled receptor was dissociated from gel with 0.1 M glycine (pH 3.0)–0.2% NP-40. (v) The eluted receptor was precipitated with trichloroacetic acid and analyzed by SDS-PAGE followed by autoradiography.

latable receptor kinase activity (see previous section). The simple explanation for the absence of this 180-kDa protein in immunoprecipitates of AbP₂ is that the nonphosphorylated form of the native PDGF-receptor is not recognized by AbP₂.

DISCUSSION

The murine PDGF receptor is a 180-kDa transmembrane glycoprotein of 1,067 amino acids (24). A hydrophobic 25-amino-acid membrane-spanning region separates the N-terminal extracellular domain of 500 amino acids from the C-terminal cytoplasmic segment of 542 amino acids. Unlike other known tyrosine kinases (e.g., pp60^{v-src} and receptors for epidermal growth factor, insulin, and insulin-like growth factor 1), the intracellular tyrosine kinase domain of the PDGF receptor is interrupted by a 104-amino-acid sequence. In this respect, the PDGF receptor is similar to *v-kit* protein and the colony-stimulating factor 1 receptor; these three proteins share considerable sequence homology. However, the carboxy terminus of the murine PDGF receptor contains a region (residues 919 through 1,067) of 148 amino acids which has apparently no sequence homology with any known protein. These unique amino acid sequences in the carboxy-terminal region of the murine PDGF receptor prompted us to select P₁ and P₂, corresponding to residues 977 through 988 and 932 through 947, respectively, as immunogens for the generation of antibodies.

Our results with AbP₁ and AbP₂ strongly suggest that AbP₁ recognizes both phosphorylated and nonphosphorylat-

ed PDGF receptors, whereas AbP₂ recognizes only the tyrosine-phosphorylated receptor. However, both antibodies recognize denatured receptor. These conclusions are based on the following observations. (i) Both AbP₁ and AbP₂ recognized the denatured PDGF receptor as shown by Western blot analysis (Fig. 3) and by immunoprecipitation of heat-denatured, ³⁵S-labeled 180-kDa PDGF receptor (Fig. 1). (ii) Both antibodies recognized the tyrosine phosphorylated purified PDGF receptor of 180 kDa (Fig. 6), but only one (AbP₁) recognized the receptor before phosphorylation (Fig. 7). (iii) AbP₁ recognized the biosynthetically labeled native PDGF receptor (Fig. 1), whereas AbP₂ failed to immunoprecipitate the native receptor unless it was phosphorylated on tyrosine residues (Fig. 4).

Although AbP₂ recognized only the tyrosine-phosphorylated and not the unphosphorylated PDGF receptor, the antibody is not directed to phosphotyrosine. This is based on the finding that AbP₂ is capable of immunoprecipitating the tyrosine-phosphorylated PDGF receptor in the presence of phenyl phosphate, an analog of phosphotyrosine (Fig. 4 and 6).

In immunoprecipitation of ¹²⁵I-PDGF receptor covalent complex, AbP₁ and AbP₂ also showed marked difference in antigen recognition ability (Fig. 5). The small extent of immunoprecipitation by AbP₂ may be explained on the basis that the majority of the ¹²⁵I-PDGF receptor covalent complex is not in a phosphorylated state, probably due to the prevalence of cellular phosphatases.

The unusual specificity of AbP₂, an antibody raised against a peptide epitope of the PDGF receptor, can be explained on the basis that this epitope is cryptic and inaccessible to the antibody in the native unphosphorylated receptor but becomes accessible upon PDGF-induced tyrosine phosphorylation. Such unfolding of epitope 2 due to autophosphorylation of the receptor suggests that tyrosine phosphorylation of the PDGF receptor causes an alteration of the receptor conformation. It should be noted in this connection that PDGF-induced conformational changes of the receptor alone are not sufficient for its recognition by AbP₂ (Fig. 4). Since the phosphorylation sites of the receptor have not yet been determined, it is not known whether the unfolding of the P₂ site is due to phosphorylation of the tyrosine residues present in P₂ or due to phosphorylation of other tyrosine residues in the receptor. Whatever may be the case, our studies provide direct evidence that the PDGF receptor may exist at least in two different conformations—one corresponding to the nonphosphorylated form and the other corresponding to the phosphorylated form. In addition, we have identified the P₂ region (residues 932 through 947) as the site whose conformation is highly susceptible to the phosphorylation state of the receptor. Such dependency of the conformation of the P₂ site on the receptor phosphorylation implies that epitope 2 of the PDGF receptor might be important in PDGF-induced biological message transmission.

Growth factor receptors play a central role in cell proliferation. This realization has prompted considerable studies on the molecular structures of receptors, such as, the epidermal growth factor and insulin receptors (5, 16). However, due to lack of specific reagents, similar studies were not possible with the PDGF receptor until recently. The availability of anti-PDGF receptor antibodies (4, 10, 13), particularly anti-peptide antibodies such as those described by Keating and Williams (13) and by us in this communication, will be useful in elucidating the structure-function relationships of the PDGF receptor as well as in analyzing

the receptor in tumor cells. Moreover, AbP₂, which recognizes only the tyrosine-phosphorylated native PDGF receptor, will be useful in studying the migratory behavior as well as intracellular localization of the tyrosine-phosphorylated receptor in intact cells. Such studies are important in determining the role of tyrosine autophosphorylation in the mechanism of PDGF action.

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