The biological effects of platelet-derived growth factor (PDGF), a potent mitogen for cells of mesenchymal origin, has been linked to the etiology of a number of human diseases, including vascular restenosis following angioplasty, atherosclerosis, glomerulonephritis, and cancer (1–6). PDGF is a disulfide-linked dimer of two related polypeptide chains, designated A and B, which are assembled structurally alike but also share the essential functional roles of the extracellular Ig-like domains of the PDGFR, which have an intracellular tyrosine kinase domain and an extracellular region comprising five immunoglobulin-like domains (D1–D5). Using deletion mutagenesis we mapped the PDGF binding site in each PDGFR to the D2–D3 region. In the case of α-PDGFR, 125I-PDGF AA and 125I-PDGF BB bound to the full-length extracellular domain, D1–D5, and D2–D3 with equal affinity (Ka = 0.21–0.42 nM). Identical results were obtained for 125I-PDGF BB binding to β-PDGFR mutants D1–D5 and D2–D3, establishing that D1, D4, and D5 do not contribute to PDGF binding. Monoclonal antibodies (mAb) directed against individual PDGFR Ig-like domains were used to extend these observations. The anti-D1 mAb 1E10E2 and anti-D5 mAb 2D4G10 had no effect on α- or β-PDGFR function, respectively. In contrast, mAb 2H7C5 and 2A1E2 directed against D2 of the α- and β-receptor, respectively, blocked PDGF binding, receptor autophosphorylation and mitogenic signaling with IC50 values of 0.1–3.0 nM. An anti-D4 mAb 1C7D5 blocked β-receptor autophosphorylation and signaling without inhibiting PDGF binding consistent with the observation that D4 is essential for PDGFR dimerization (Omura, T., Heldin, C.-H., and Ostman, A. (1997) J. Biol. Chem. 272, 12676–12682). mAbs identified here act as potent PDGFR antagonists that can be used as research tools and potentially as therapeutic agents for the treatment of diseases involving unwanted PDGFR signaling.

Platelet-derived growth factor (PDGF), a potent mitogen for cells of mesenchymal origin, has been linked to the etiology of a number of human diseases, including vascular restenosis following angioplasty, atherosclerosis, glomerulonephritis, and cancer (1–6). PDGF is a disulfide-linked dimer of two related polypeptide chains, designated A and B, which are assembled structurally similar to PDGFR dimerization, with non-conjugated homologous or heterologous mAbs (0.1–30 nM) in binding sites for physical interactions with a number of proteins that contain Src homology region 2 domains, including phosphatidylinositol 3-kinase, GTPase-activating protein, phospholipase Cγ, Src, Grb2, and Nck (for review, see Ref. 17). These interactions effect the activation of intracellular signaling pathways that mediate calcium and lipid metabolism, gene expression, changes in cell morphology, and cell replication. The PDGFRs belong to a subgroup of receptor tyrosine kinases that includes stem cell factor (SCF) receptor, c-Kit, and colony-stimulating factor 1 (CSF-1) receptor, c-Fms. These type III receptor tyrosine kinases are characterized by an extracellular ligand-binding region comprising five immunoglobulin (Ig)-like domains (D1–D5), a single transmembrane-spanning region, and a split intracellular tyrosine kinase domain (18, 19). The extracellular region of these receptors are not only structurally alike but also share the essential functional roles of the PDGFRs using domain-specific mAbs and receptor deletion mutants.

**EXPERIMENTAL PROCEDURES**

**Antibody Production—**PDGFR extracellular domain was purified from CHO cell conditioned media as described previously (8). Utilizing standard techniques (27), BALB/c mice were immunized with 10 μg of α- or β-PDGFR protein and additional boosts were given every 2 weeks until a high serum titer was achieved. Splenoimhibitory effect was detected by monitoring the rate of product formation at 650 nm with non-conjugated homologous or heterologous mAbs (0.1–30 nM) in 96-well plates with plastic-immobilized PDGFR. After 1 h at 25 °C, wells were washed, ABTS™ was added, and the amount of bound antibody was detected by monitoring the rate of product formation at 650 nm using a plate reader (Molecular Devices).

**Construction and Expression of α- and β-PDGFR Deletion Mutants—**The α- and β-PDGFR cDNAs were isolated from a human placental cDNA library (28). Standard mutagenesis techniques were used to generate irreversible and intrinsic tyrosine kinase activity (10, 11). The receptor binding specificity for the PDGF isofroms dictates that PDGF AA induces only α/α-receptor dimers, PDGF AB induces α/α- and α/β-receptor dimers, and PDGF BB induces all three receptor dimer combinations (12–16). The next step in PDGFR signaling involves receptor autophosphorylation on tyrosine, which creates the sites for physical interactions with a number of proteins that contain Src homology region 2 domains, including phosphatidylinositol 3-kinase, GTPase-activating protein, phospholipase Cγ, Src, Grb2, and Nck (for review, see Ref. 17). These interactions effect the activation of intracellular signaling pathways that mediate calcium and lipid metabolism, gene expression, changes in cell morphology, and cell replication.

The PDGFRs belong to a subgroup of receptor tyrosine kinases that includes stem cell factor (SCF) receptor, c-Kit, and colony-stimulating factor 1 (CSF-1) receptor, c-Fms. These type III receptor tyrosine kinases are characterized by an extracellular ligand-binding region comprising five immunoglobulin (Ig)-like domains (D1–D5), a single transmembrane-spanning region, and a split intracellular tyrosine kinase domain (18, 19). The extracellular region of these receptors are not only structurally alike but also share the essential functional roles of the PDGFRs using domain-specific mAbs and receptor deletion mutants.
introduce translation termination codons or internal deletions (29). The α-PDGFR mutant that encodes the full-length extracellular region, α(R1–I–D5), was generated by introducing a translation stop at codon 525 (8). Mutants encoding larger carboxyl-terminal deletions, α(R1–D4), α(R1–D3), α(R1–D2), and α(R1–D1) were made by introducing a translational stop at codons 413, 314, 214, and 125, respectively. α(R2–D3) contained an internal deletion made by fusing codon 23 to codon 123 and a translation termination signal at codon 314. β-PDGFR cDNA encoding the extracellular domain, β(R1–D5), was generated by introducing a translation stop at codon 532 (8). Mutant c-DNAs encoding larger carboxyl-terminal deletions, β(R1–D4), β(R1–D3), β(R1–D2), and β(R1–D1) were constructed by introducing a translational stop at codons 415, 315, 214, and 124, respectively. Mutant c-DNAs encoding progressively larger amino-terminal deletions, β(R2–D5), β(R3–D5), β(R4–D5), and β(R5–D5) were made by fusing codon 34 to codons 124, 214, 315, and 415, respectively; each had a stop codon at position 532. The c-DNA encoding β(R2–D3) had codon 34 fused to codon 124 and a translation termination signal at codon 315. Following mutagenesis, each PDGFR cDNA variant was sequenced by automated dye-terminator cycle sequencing using AmpliTaq FS (30) and was inserted into the mammalian expression vector pBl-I (8, 31).

For transient expression, COS-7 cells were transfected with pBl-I PDGFR constructs using standard calcium phosphate precipitation methods and secreted PDGFR proteins were collected for analysis after 48 h. Supernatant lines that stably express PDGFR were used to generate CHO-K cells. CHO-K cells were co-transfected with pBl-I PDGFR constructs and the selectable marker plasmid pSV2neo by the Lipofectin method. After G418 selection, conditioned medium was collected from cells for the analysis of mutant PDGFR proteins.

mAb Inhibition of 125I-PDGFR Binding—Binding of 125I-PDGFR to affinity-purified α- or β-PDGFR full-length extracellular domains was analyzed using a solid-phase binding assay as described previously (28). Anti-α- or anti-β-PDGFR mAbs (0.24–1000 nM) were incubated with immobilized PDGFR for 1 h at 25 °C prior to the addition of 1 nM 125I-PDGFR AA or BB (50,000 cpm/ng), and incubations were continued for 4 h. The wells were washed, and bound 125I-PDGFR was quantitated by γ counting. Nonspecific 125I-PDGFR binding, as determined by coinoculation with a 200-fold excess of unlabeled PDGFR, was <15% of total binding.

PDGFR Autophosphorylation Assays—The α-PDGFR autophosphorylation assay was developed using 32P-ar cells that were characterized previously (32). Cells grown under standard conditions were washed and resuspended at 107 cells/ml in serum-free RPMI medium in the absence or presence of anti-PDGFR mAb (0.24–1000 nM). After 10 min at 37 °C, the cells were placed on ice and stimulated with 0.6 nM PDGF AA for 1.5 h. Cells were pelleted, lysed in 100 μl Tris, pH 7.5, 750 mM NaCl, 0.5% Triton X-100, 10 mM pyrophosphate, 50 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethysulfonyl fluoride, 1 mM sodium orthovanadate, and the lysate was cleared by centrifugation at 15,000 × g for 20 min. Lysates were diluted in binding buffer (0.3% gelatin, 55 mM HEPES, pH 7.6, 100 mM NaCl, 0.01% Tween 20) and transferred to anti-α-PDGFR polyclonal Ig-coated microtiter plate wells (0.5 μg/well). Plates were incubated for 2 h at 25 °C to allow for α-PDGFR capture and then washed three times with 0.02% Tween 20 in PBS. Peroxidase-coupled anti-phosphotyrosine antibody, 4G10 (Upstate Biotechnology, Inc.), was added at 1 μg/ml in binding buffer and incubated at 25 °C for 2 h. Wells were washed prior to adding ABTS™ and the rate of substrate formation was monitored at 650 nm.

For the β-PDGFR autophosphorylation assay, we used HR5, a previously described CHO cell line engineered to overexpress this receptor (28). The cells were grown to confluence under standard conditions, followed by serum-starvation for 16 h. The cells were incubated with mAb (0.1–1000 nM) for 15 min at 25 °C and then stimulated with PDGFR BB (1.2 nM) for 10 min at 37 °C. PDGFR was captured from the lysates with anti-β-PDGFR polyclonal IgG, and receptor autophosphorylation was detected as described above for α-PDGFR.

1H-Thymidine Incorporation Assays—32P-ar and 32D-ar cells, which were engineered to overexpress α- or β-PDGFR, respectively, were used in thymidine incorporation assays as described previously (32). Briefly, cells in log-phase growth were washed, resuspended in serum-free RPMI medium, and incubated at 37 °C in microtiter plates (50,000 cells/well) with either 1.2 nM PDGF AA or PDGF BB in the absence or presence of anti-PDGFR mAb (0.008–600 nM). After 48 h, 0.5 μCi of 3H-thymidine (15 Ci/mmol, Amersham) was added to each well and the incubation was continued for 5 h. Cells were harvested, and incorporated radioactivity was determined by scintillation counting.

| TABLE I Characterization of Anti-PDGFR mAbs |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| mAb Isotype | Antigen | Relative affinity | Antigenic site |
|---------------|---------|------------------|----------------|-----------------|-----------------|
| 2H7C5 | IgG1 | α(R1–I–D5) | 0.33 | A |
| 2B2B11 | IgG1 | α(R1–D1) | 0.13 | B |
| 2F10E12 | IgG1 | α(R1–D5) | 0.26 | C |
| 1E10E2 | IgG1 | α(R1–D1) | 0.16 | D |
| 2A1E2 | IgG1 | β(R1–D1) | 0.14 | A |
| 1B5B11 | IgG1 | β(R1–D1) | 0.15 | A |
| 1C7D5 | IgG2a | β(R1–D1) | 0.17 | B |
| 2D4G10 | IgG1 | β(R1–D5) | 0.58 | C |

- Determined by mAb isotyping kit from Innogenetics.
- Antigen used to immunize mice was purified α- or β-PDGFR extracellular domain.
- Values shown are the concentration of mAb required for half-maximal binding to immobilized PDGFR as determined in a solid-phase ELISA.
- Distinct antigenic sites were identified by determining the ability of mAbs to compete with each other for binding to immobilized PDGFR (see "Experimental Procedures").

Mapping the PDGFR Ig-like Domain Recognized by Anti-PDGFR mAbs—A panel of mAbs directed against the extracellular region of each PDGFR were generated. Four anti-α-PDGFR mAbs (2H7C5, 2B2B11, 2F10E12, 1E10E2) and four anti-β-PDGFR mAbs (2A1E2, 1B5B11, 1C7D5, 2D4G10) were selected for further analysis. These were all specific for the PDGFR subtype to which they were raised; no cross-reactivity against the other receptor was detected at mAb concentrations up to 10 μM. Table I shows the mAb concentration required to obtain half-maximal binding to immobilized PDGFR; all bound with high affinity (0.1–0.6 nM). The mAbs presented in this study all were of the IgG1 subclass, except 1C7D5, which was an IgG2a.

The topographic relationships of PDGFR epitopes recognized by these mAbs were determined by competitive solid-phase binding with horseradish peroxidase-conjugated mAbs. The four mAbs directed against the α-PDGFR evaluated under these conditions showed no competition with each other for binding to α-PDGFR and therefore recognized distinct antigenic sites, designated A–D in Table I. A similar analysis of the mAbs directed against the β-PDGFR revealed that 2A1E2 and 1B5B11 competed with each other for binding to β-receptor but neither competed with 2D4G10 or 1C7D5, which did not com-
pete with each other for β-PDGF binding. Thus, 2A1E2 and 1B5B11 recognized epitopes within the same antigenic site, designated A, and 2D4G10 and 1C7D5 recognized distinct sites, designated B and C, respectively (Table I).

Inhibition of 125I-PDGF Binding by mAb—The ability of these anti-PDGF mAbs to block PDGF binding was examined using the specific, high affinity ($K_d = 0.5–1.0 \text{ nM}$), solid-phase PDGF bindings assays described previously (8). As shown for the α-PDGF in Fig. 1 (A and B), mAb 2H7C5 blocked binding of both 125I-PDGF AA and BB with an IC$_{50}$ of 6 nM, whereas the other anti-α-PDGF mAbs and the anti-β-PDGF mAb 2A1E2, at concentrations up to 250 nM, did not significantly inhibit 125I-PDGF binding.

As shown in Fig. 1C, inhibition of 125I-PDGB BB binding to immobilized β-PDGF was observed with mAb 2A1E2 (IC$_{50} = 1.5 \text{ nM}$) but not with 1C7D5 or 2D4G10. Less than 25% inhibition was seen with 1B5B11, at 250 nM. Anti-α-PDGF mAb 2H7C5, which potently blocked 125I-PDGF AA and BB binding to the α-receptor, did not affect 125I-PDGF BB binding to β-PDGF.

Anti-PDGF mAbs Inhibit Receptor Autophosphorylation—We examined the ability of these mAbs to inhibit PDGF-induced receptor tyrosine autophosphorylation using a sandwich ELISA in which PDGF was captured from cell lysates and detected with an anti-phosphotyrosine mAb (see "Experimental Procedures"). The 32D-αR mouse hematopoietic cell line lacks endogenous PDGFRs, but was engineered to express both α- and β-PDGF autophosphorylation in 32D-αR cells is shown in Fig. 2A. A maximum 200-fold increase in phosphoryrosine on the α-receptor was obtained with 0.6 nM PDGF AA; the EC$_{50}$ was 0.1 nM. Fig. 2B shows a comparable dose-response curve obtained using PDGF BB to induce β-PDGF autophosphorylation in HR5 cells, a previously characterized CHO cell line that overexpresses recombinant human β-PDGF (28). PDGF BB at 4 nM caused a maximal 15-fold increase of β-PDGF autophosphorylation; the EC$_{50}$ was 0.2 nM. These findings were confirmed using anti-phosphotyrosine Western blot analysis.

The ability of anti-α-PDGF mAbs to inhibit receptor autophosphorylation was determined by incubating 32D-αR cells with mAb prior to stimulating with 0.2 nM PDGF AA. As shown in Fig. 3A, mAb 2H7C5, which blocked 125I-PDGF binding to the α-PDGF, was equally effective at blocking PDGF AA-induced α-receptor autophosphorylation with an IC$_{50}$ of 3 nM; complete inhibition was observed at concentrations >30 nM. mAbs 2F10E12 and 1E10E2 displayed no inhibitory activity, and 2B2B11 inhibited α-receptor phosphorylation by 50% only at 200 nM. Anti-β-PDGF mAB 2A1E2 did not inhibit α-receptor phosphorylation.

The inhibition of β-PDGF autophosphorylation by anti-β-PDGF mAbs 2A1E2 and 1B5B11 was half-maximal at 2–3 nM (Fig. 3B). Complete inhibition by 2A1E2 and 70% inhibition by 1B5B11 was attained at concentrations >10 nM. mAb 1C7D5 had an IC$_{50}$ of 100 nM but inhibited only up to 60%.

The other anti-β-receptor mAb tested, 2D4G10, and the potent anti-α-receptor mAb 2H7C5 did not inhibit β-receptor phosphorylation (Fig. 3B). ELISA methods used in these experiments could give an artificially high level of inhibition if preincubation with mAb were to interfere with capture of the receptor. To address this possibility, we used biotinylated anti-α- or anti-β-PDGF polyclonal antibody to detect captured PDGF and found that preincubation of cells with the inhibitory mAbs had no effect on the amount of PDGF captured. Moreover, mAb inhibition of PDGF autophosphorylation was confirmed with anti-phosphotyrosine Western blot analysis.

PDGF Mitogenic Activity Blocked by mAbs—PDGF mitogenic responses in vitro are most commonly studied in fibroblast cell lines that invariably express both α- and β-PDGF (16, 34). We examined mitogenic signaling mediated by each individual receptor subtype in 32D cell transfectants engineered to express only α-PDGF (32D-αR) or β-PDGF (32D-βR) (32). Anti-α-PDGF mAbs were evaluated for their ability to inhibit PDGF AA-induced [3H]thymidine incorporation into
32D-αR cells. As shown in Fig. 4A, mAb 2H7C5 was a potent inhibitor of the PDGF AA mitogenic response; its IC_{50} was 2 nM, and it gave complete inhibition at concentrations >10 nM. The other anti-α-PDGFR mAbs, 2F10E12, 2B2B11, and 1E10E2, showed no inhibition of 32D-αR cell mitogenic response at concentrations up to 100 nM. As expected, the same was true for the potent anti-β-PDGFR mAb 2A1E2.

Fig. 4B shows that three of the β-PDGFR mAbs were potent inhibitors of PDGF BB-induced [3H]thymidine incorporation into 32D-βR cells. mAbs 2A1E2 and 1B5B11 blocked β-PDGFR-mediated mitogenesis with an IC_{50} of 0.08 nM and gave 90% inhibition at 1 nM. Additionally, mAb 1C7D5, which only weakly blocked β-PDGFR phosphorylation (IC_{50} = 100 nM, Fig. 3B), inhibited mitogenesis in 32D-βR cells with an IC_{50} of 1.0 nM. The remaining anti-β-PDGFR mAb, 2D4G10, and the anti-α-receptor mAb 2H7C5 did not significantly inhibit mitogenesis at concentrations up to 200 nM (Fig. 4B).

Mapping of PDGFR Ig-like Domains Recognized by mAbs—To map the PDGFR Ig-like domain required for mAb binding, a deletion mutagenesis strategy was employed as shown schematically in Figs. 5A and 6A. These mutants were expressed either in COS or CHO cells, and conditioned media were collected for Western blot analysis using anti-α- or anti-β-PDGFR polyclonal antibodies. As shown in Figs. 5B and 6B, each PDGFR mutant protein had an electrophoretic mobility consistent with the size predicted by the length of its coding sequence. The apparent molecular masses ranged from ~110 kDa for the full-length extracellular domain to ~20 kDa for the variants comprising a single Ig-like domain.

The relative level of binding of each anti-PDGFR mAb to these PDGFR mutants is summarized in Tables II and III. All anti-α-PDGFR mAbs readily detected αR(D1–D5) at the level that was taken to be 100% binding. mAb 2B2B11 did not recognize αR(D1–D4), which was recognized by the remaining mAbs, indicating that 2B2B11 binds D5 (Table II). Removal of D4 resulted in a loss of detection by 2F10E12 to 33% of that observed with αR(D1–D5), demonstrating that its major binding epitope is within D4. mAb 1E10E2 detected αR(D1) and all other mutants that contain D1 but did not detect αR(D2–D3). Finally, the neutralizing α-receptor mAb, 2H7C5, bound to epitopes within D2 because it detected αR(D1–D2) and not αR(D1). Thus, as indicated in Table II, each anti-α-PDGFR mAb recognizes a different Ig-like domain (D1, D2, D4, or D5), which is consistent with the fact that no cross-
competition for binding was observed with these antibodies (Table I).

Table III shows the results of the corresponding analysis of mAbs directed against the \( \beta \)-PDGFR. As expected, \( \beta R(D1–D5) \) was readily detected by each of the mAbs at a level that was determined to be 100% binding. Only mAb 2D4G10 failed to recognize \( \beta R(D1–D4) \), but did recognize \( \beta R(D5) \). The removal of D4 to give mutant \( \beta R(D1–D3) \) eliminated detection by 1C7D5, indicating its epitope(s) is within D4; its recognition of all other mutants containing D4 supports this conclusion. mAbs 2A1E2 and 1B5B11, which competed with each other for binding to \( \beta \)-PDGFR (Table I), bound to \( \beta R(D1–D2) \) but not \( \beta R(D1) \), confirming that both antibodies recognize epitopes in D2. Additional analysis revealed that both mAbs bound \( \beta R(D2–D3) \), as expected, but surprisingly, 1B5B11 also bound \( \beta R(D3–D5) \) at 55% of the level observed with \( \beta R(D1–D5) \). Thus, 1B5B11 recognizes epitopes in D2 and D3, whereas the major binding determinants for 2A1E2 are in D2 alone.

**Localization of the PDGF Binding Domain**—To identify which PDGFR mutants retained the ability to bind PDGF, each mutant was incubated with 0.5 nM 125I-PDGF and the level of specific binding was determined. Consistent with our previous observations (8), the full-length \( \alpha \)-PDGFR extracellular domain, \( \alpha R(D1–D5) \), effectively bound 125I-PDGF AA (Fig. 7A). The same level of specific binding was observed with \( \alpha R(D1–D4) \) and \( \alpha R(D1–D3) \), demonstrating that D4 and D5 were dispensable. In contrast, \( \alpha R(D1–D2) \) had no 125I-PDGF AA binding activity, indicating that D3 was required for ligand binding.
Characterization of PDGFR Extracellular Domain

### Table II

| mAb     | αR(D1–D5)<sup>a</sup> | αR(D1–D4) | αR(D1–D3) | αR(D1–D2) | αR(D1)   | αR(D2–D3) | mAb binding site |
|---------|------------------------|-----------|-----------|-----------|-----------|-----------|-----------------|
| 2H7C5   | 100<sup>b</sup>        | 92        | 82        | 100       | 0         | 100       | D2              |
| 2B2B11  | 100                    | 0         | 15        | 9         | 0         | 0         | D5              |
| 2E10E12 | 100                    | 100       | 33        | 0         | 2         | 2         | D4              |
| 1E10E2  | 100                    | 100       | 100       | 100       | 2         | 2         | D1              |

<sup>a</sup> Structure of each mutant protein is shown in Fig. 5A.

<sup>b</sup> mAb binding of α-PDGFR mutants was determined using a sandwich ELISA (see “Experimental Procedures”). Numerical values are the percent binding as compared to αR(D1–D5), which was defined as 100%.

### Table III

| mAb     | βR(D1–D5)<sup>a</sup> | βR(D1–D4) | βR(D1–D3) | βR(D1–D2) | βR(D1)   | βR(D2–D5) | βR(D3–D5) | βR(D4–D5) | βR(D5)   | βR(D2–D3) | mAb binding site |
|---------|-----------------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------------|
| 2A1E2   | 100<sup>b</sup>       | 88        | 100       | 69        | 0         | 100       | 0         | 0         | 100       | 100       | D2              |
| 1B5B11  | 100                    | 90        | 90        | 94        | 0         | 95        | 55        | 0         | 100       | 0         | D2/D3           |
| 1C7D5   | 100                    | 100       | 0         | 0         | 2         | 2         | 100       | 90        | 0         | 4         | D4              |
| 2D4G10  | 100                    | 0         | 0         | 2         | 2         | 100       | 70        | 100       | 92        | 5         | D5              |

<sup>a</sup> Structure of each mutant protein is shown in Fig. 6A.

<sup>b</sup> mAb binding of β-PDGFR mutants was determined using a sandwich ELISA (see “Experimental Procedures”). Numerical values are the percent binding as compared to βR(D1–D5), which was defined as 100%.

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D1 is dispensable since αR(D2–D3) bound labeled ligand as well as αR(D1–D5) (Fig. 7A).

As shown in Fig. 7B, the pattern of 125I-PDGF BB binding to β-PDGFR mutants was identical to that observed for 125I-PDGF AA binding to the α-PDGFR mutants. The same level of specific binding of 125I-PDGF BB was observed for βR(D1–D3), βR(D1–D4), and βR(D1–D5), whereas βR(D1–D2) was inactive, demonstrating that D3 is necessary but that D4 and D5 were not required for PDGF binding. Furthermore, βR(D2–D5) retained a high level of specific 125I-PDGF BB binding activity, whereas βR(D3–D5) was inactive, demonstrating the importance of D2 but not D1 for binding. Finally, efficient binding of 125I-PDGF BB to βR(D2–D3) confirmed that D2 and D3 of the β-PDGFR contain the PDGF BB binding site.

To compare the PDGF binding affinity of D2–D3 to that of the full-length PDGFR extracellular domain, αR(D1–D5), αR(D2–D3), βR(D1–D5), and βR(D2–D3) were each immobilized and incubated with increasing concentrations of 125I-PDGF AA or BB in the presence or absence of excess unlabeled ligand. Specific binding was saturable and was >85% of total binding for each of the ligand concentrations tested. Scatchard plots of the data that best represent the relative affinities of each interaction are shown in Fig. 8. In all cases, a single line fit the data best with correlation coefficients >0.95 indicating a single-affinity class of binding sites. As shown in Fig. 8 (A–D), αR(D1–5) and αR(D2–D3) bound both PDGF isoforms with equal affinity (Kₐ = 0.2–0.4 nM). Similarly, βR(D2–D3) bound PDGF BB with the same affinity (Kₐ = 0.5 nM) as βR(D1–D5) (Fig. 8, E and F). These results demonstrate that D1, D4, and D5 are not involved in PDGF binding to purified PDGFR.

**DISCUSSION**

In this study, we systematically characterized the α- and β-PDGFR extracellular region using receptor deletion mutants and mAbs directed against their constituent Ig-like domains. These domains comprise β-sheet-rich repeats of about 100 amino acids, designated D1–D5, and all but D4 have characteristically spaced cysteine residues predicted to form disulfide bridges (19). Based on the number of β-strands in the predicted structure of D1 and D2, they most closely resemble an immunoglobulin constant domain, whereas D5 resembles a variable immunoglobulin domain in length; D3 and D4 have an inter-
Characterization of PDGFR Extracellular Domain

It has been shown for each of the PDGFR family members that D1–D3 contain the determinants required for high affinity growth factor/cytokine binding (20–24). A structural role for D1 of the α-PDGFR was proposed in a study by Mahadevan et al. (35), wherein a bacterially expressed α-PDGFR D1–D3 protein with weak PDGF binding activity (Kd = 33–74 nM) gave further reduced binding of PDGF AA but not of PDGF BB upon the additional removal of D1. In contrast, our α- and β-PDGFR mutants containing only D2–D3 bound PDGF with the same binding affinity (Kd = 0.2–0.5 nM) as the full-length extracellular region (Fig. 8). This difference may stem from the fact that our α-PDGFR mutants were expressed in mammalian cells, were glycosylated, and had a 50–200-fold higher PDGF binding affinity than the correspondingly bacterially expressed receptor. Consistent with the results obtained using our deletion mutants, mAb 1E10E2 directed against D1 of the α-PDGFR had no effect on PDGF AA or BB binding, receptor autophosphorylation or mitogenic signaling (Figs. 1A, 1B, 3A, and 4A). Thus, our data support the conclusion that D1 of the PDGFR does not play a role in PDGF binding.

Previous studies using α/β-PDGFR chimeras have provided evidence that the ability of the α-PDGFR to bind PDGF AA is determined by D2 (20). Furthermore, a 39-amino acid deletion in D2 selectively reduced the binding of PDGF AA as compared with that of PDGF BB (36). We have extended these studies by demonstrating that D2 is also required for PDGF BB binding to the β-PDGFR (Fig. 7). The functional importance of D2 was further supported by our observation that mAbs 1H7C5 and 2A1E2 directed against this domain of the α- and β-PDGFR, respectively, completely blocked PDGF binding, receptor phosphorylation, and mitogenic signaling (Figs. 1, 3, and 4). These results are consistent with the previous characterization of an antibody directed against D2 of the α-PDGFR (37).

All PDGFR family members require D3 for growth factor/cytokine binding, as demonstrated by this study and others (Fig. 7; see also Refs. 20, 22, and 24). We observed that mAb 1B5B11, which recognizes epitopes in D2 and D3 of the β-PDGFR, potently blocked receptor phosphorylation and mitogenic signaling, but surprisingly had only a slight effect on PDGF binding (Figs. 1B, 3B, and 4B). Therefore, mAb directed against D2–D3, the PDGF binding site, can block PDGFR activation by means other than inhibition of PDGF binding, possibly by preventing receptor dimerization.

Because dimerization appears to be required for the activation of all transmembrane receptor tyrosine kinases, the mechanisms that mediate it are of great interest (10). Studies utilizing receptor mutants have demonstrated that D4 of the PDGFR and SCF receptor, although not required for ligand binding, mediates receptor dimerization and is required for signal transduction (25, 26). Therefore, the ability of our mAb 1C7D5 directed against D4 of the β-PDGFR, to inhibit receptor phosphorylation and mitogenic signaling without any effect on PDGF binding (Figs. 1B, 3B, and 4B) is likely due to inhibition of receptor dimerization. Inhibition of β-PDGFR and SCF receptor dimerization by anti-D4 mAbs have been reported recently (26, 38). In contrast, binding of mAb 2F10E12 to D4 of the α-PDGFR did not inhibit receptor signaling (Figs. 3A and 4A), indicating that not all mAbs directed against D4 are able to block dimerization.

Within the PDGFR family, a functional role for D5 has not been identified. As shown in Fig. 7 and reported elsewhere, deletion of D5 had no effect on PDGF, CSF-1, or SCF binding or on receptor dimerization in the case of the SCF receptor (20, 21, 24, 26, 34, 37). In addition, anti-D5 mAbs 2B2B11 and 2D4G10 directed the α- or β-PDGFR, respectively, showed no significant inhibition of PDGF binding, phosphorylation, or mitogenic signaling (Figs. 1, 3, and 4). It was reported previously that mAb 2A1E2 blocks β-PDGFR function by binding to D5 (39). This conclusion was based on the observation that mAb 2A1E2 did not immunoprecipitate βR(D1–D4). In the present study, 2A1E2 readily detected this mutant, as well as βR(D1–D3), βR(D1–D2), and βR(D2–D3), all of which lack D5 (Table III). These results were determined by two-site ELISA (Table III) and confirmed by immunoblotting using 2A1E2. Therefore, the previous failure of 2A1E2 to immunoprecipitate βR(D1–D4) was most likely due to limitations with the technique.

In conclusion, through the combined use of mAbs with defined antigenic sites and deletion mutagenesis, we have delineated the functional importance of Ig-like domains within the PDGFR extracellular region. The D2–D3 region of both PDGFRs contain all of the information required for high affinity ligand binding and represent the smallest PDGF binding site so far identified. More importantly, mAbs directed against the PDGF binding site of the α- and β-PDGFR proved to be potent and specific antagonists of receptor function (Figs. 1, 3, and 4). Using mAb 2A1E2, we have recently shown that β-PDGFR blockade inhibits vascular lesion formation in baboons following balloon angioplasty, endarterectomy, and stent placement.
indicating that anti-PDGFR mAbs may have therapeutic potential for the treatment of restenosis after coronary revascularization.  

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