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Reversible Binding of Platelet-derived Growth Factor-AA, -AB, and -BB Isoforms to a Similar Site on the "Slow" and "Fast" Conformations of α_2-Macroglobulin*

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James C. Bonner‡, Audrey L. Goodell, Joseph A. Lasky, and Maureane R. Hoffman§¶

From the Laboratory of Pulmonary Pathobiology, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709 and the §Department of Pathology, University of North Carolina, Chapel Hill, North Carolina 27514

The mechanism by which the platelet-derived growth factor (PDGF)-binding protein, α_2-macroglobulin (α_2M), modulates PDGF bioactivity is unknown, but could involve reversible PDGF-α_2M binding. Herein we report that >70% of ^{125}I-PDGF-BB or -AB complexed to α_2M was dissociated by SDS-denaturation followed by SDS-polyacrylamide gel electrophoresis, *i.e.* most of the binding was noncovalent. Reduction of the PDGF·α_2M complex following denaturation dissociated the cytokine from α_2M by >90%, suggesting covalent disulfide bond formation. Approximately 50% of the growth factor was dissociated by lowering the pH from 7.5 to 4.0. ^{125}I-PDGF-BB bound α_2M in a time-dependent manner ($t_{1/2}$ = ~1 h), reaching equilibrium after 4 h. The ^{125}I-PDGF·BB/α_2M complex dissociated more slowly ($t_{1/2}$ = ~2.5 h). "Slow" and "fast" α_2M bound nearly equal amounts of PDGF-AB or -BB. Trypsin treatment converted PDGF-BB/α_2M complex to the fast conformation but did not release bound ^{125}I-PDGF-BB. All PDGF-isoforms (AA, -AB, and -BB) competed for binding with ^{125}I-PDGF-BB binding to slow α_2M and fast α_2M-methylamine by 65–80%. Other cytokines that bind α_2M (transforming growth factor-β1 and -β2, tumor necrosis factor-α, basic fibroblast growth factor, interleukin-1β, and -6) did not compete for ^{125}I-PDGF-BB binding slow α_2M, but transforming growth factor-β1 and basic fibroblast growth factor inhibited ^{125}I-PDGF-BB binding α_2M-methylamine by 30–50%. The reversible nature of the PDGF·α_2M complex could allow for targeted PDGF release near mesenchymal cells which possess PDGF receptors.

Platelet-derived growth factor (PDGF)[1] and PDGF-like factors secreted by smooth muscle cells (1), endothelial cells (2), monocytes (3, 4), and macrophages (4–6) are potent mitogens and chemoattractants for cells of mesenchymal origin. Numerous studies that implicate PDGF as a key mediator in the normal processes of development, tissue maintenance, and wound healing have been reviewed (7). PDGF has also been proposed as a link in the progression of diseases such as atherosclerosis (8) and pulmonary fibrosis (9, 10). Two different monomeric chains of PDGF (A and B) give rise to three possible dimers (AA, AB, BB), and these dimeric isoforms recognize dimeric cell-surface receptors composed of α and/or β chains (11). PDGF isoforms recognize their receptors according to a receptor subunit model, *i.e.* AA, AB, BB dimers bind to $\alpha\alpha$ receptors; BB, AB dimers to $\alpha\beta$; and the BB dimer to $\beta\beta$ receptors (12). The different subtypes of PDGF and receptors could allow for a fine tuning of cell responsiveness, since different cell types can vary greatly in the ratio of isoforms secreted and in the receptor composition which the target cell possesses (13). Cell responsiveness to PDGF *in vitro* can be further modulated by other growth factors such as TGF-β (14) and by PDGF-binding proteins (15).

The major PDGF-binding protein is α_2-macroglobulin (α_2M) and PDGF·α_2M complexes have been isolated from plasma and from macrophage supernatants (16–19). This 725-kDa protein apparently serves mutiple functions as a cytokine-binding protein (20), wide spectrum proteinase inhibitor (21–25), and immune regulator (26, 27). α_2M was first described as a proteinase inhibitor and the mechanism whereby native or electrophoretically "slow" α_2M covalently entraps proteinases has been extensively studied (see Ref. 25 for review). A proteinase cleaves α_2M in its "bait region," and this cleavage induces a conformational change in the α_2M molecule which entraps the proteinase. The conformational change makes the α_2M more compact and hence has greater mobility on nondenaturing gel electrophoresis than the native or slow form of α_2M. The irreversible triggering of the proteinase trap is mimicked by primary amines (28), and the electrophoretically "fast" α_2M-proteinase or α_2M-amine complex is receptor-recognized by fibroblasts (29, 30) and macrophages (31–34). PDGF binds both slow and fast forms of α_2M (15), and PDGF-stimulated fibroblast proliferation (15) and chemotaxis (35) are inhibited by slow α_2M. α_2M inhibits the binding of PDGF to its cell-surface receptor and thus has been suggested to limit the amount of PDGF that is available to bind to these receptors (16). On the other hand, methylamine-modified, fast, α_2M synergistically enhances the growth promoting activity of human PDGF purified from platelets (15). Thus, α_2M modulates the biological activities of PDGF *in vitro*. It has been speculated that α_2M could serve mutiple functions as a PDGF-binding protein *in vivo* (16), including 1) modulation of PDGF biological activity as discussed above, 2) protection of PDGF against proteolytic degradation, and

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‡ To whom correspondence should be addressed: P. O. Box 12233, NIEHS, Research Triangle Park, NC 27709. Tel.: 919-541-4410; Fax: 919-541-4133.

¶ Present address: Veterans Administration Medical Center, Duke University, Durham, NC 27705.

[1] The abbreviations used are: PDGF, platelet-derived growth factor; α_2M, α_2-macroglobulin; SDS, sodium dodecyl sulfate; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; IL, interleukin; FPLC, fast protein liquid chromatography; BAPNA, Na-benzoyl-DL-arginine-*p*-nitroanilide; ELISA, enzyme-linked immunosorbant assay; BSA, bovine serum albumin; PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

3) clearance of PDGF from the circulation.

α_2M also binds and modulates the biological activities of several other growth promoting cytokines, including transforming growth factor-β (TGF-β) (36–42), tumor necrosis factor-α (TNF-α) (43), basic fibroblast growth factor (bFGF) (44), interleukin-1β (IL-1β) (45), interleukin-6 (IL-6) (46), nerve growth factor (47) and human growth hormone (48). The biological activity of some of these cytokines is inhibited when bound to α_2M, as is the case with TGF-β (42). Others such as IL-6 (46) and PDGF (15, 18) retain biological activity when complexed to this binding protein. While it is becoming increasingly apparent that a number of different cytokines utilize α_2M as a binding protein, the sites on the α_2M molecule to which these factors bind could differ. Determining whether or not these different cytokines compete for the same binding site(s) on this protein could be important in discerning the *in vivo* effects of α_2M as a potential modulator of cytokine activity. This is likely since many cell types (*e.g.* macrophages) secrete mixtures of these cytokines and α_2M (49). Also, it has been suggested that these cytokines bind α_2M either covalently, noncovalently, or via a mixture of covalent and noncovalent associations (see Ref. 20 for review).

PDGF has been reported to bind α_2M covalently (17, 18). However, other studies suggest that at least part of the PDGF is noncovalently bound, since it can be released by 1 M acetic acid treatment (16, 19). Thus, the first objective of the present study was to establish the ratio of covalent/noncovalent binding of PDGF to α_2M slow and fast forms. The issue of reversible binding is of paramount importance if one is to elucidate the mechanism(s) by which this binding protein modulates PDGF activity. Furthermore, such information may reconcile the apparent differences that currently exist in the literature regarding covalent and noncovalent associations between PDGF and α_2M (16–19). Second, because only PDGF purified from platelets has been reported to bind α_2M, it is not known if one or all of the three different PDGF isoforms (AA, AB, BB) bind to a similar site on the α_2M molecule. Thus, we sought to establish the competition of these isoforms for ^{125}I-PDGF-BB binding to α_2M. Third, since several other cytokines bind α_2M, it is of major importance to determine the possible competitive nature of these factors for PDGF binding to α_2M. Herein, we report that the majority of PDGF binding to slow or fast α_2M is reversible or noncovalent, and that all PDGF isoforms compete for a similar site on α_2M.

MATERIALS AND METHODS

Growth Factors and α_2M—Human PDGF purified from platelets, TGF-β1, and TGF-β2 were purchased from R & D Systems (Minneapolis, MN). PDGF-AA, -AB, and -BB isoforms, bFGF, TNF-α, IL-1β, and IL-6 were obtained from UpState Biotechnology (Lake Placid, NY). Human α_2M was purchased from Calbiochem (San Diego, CA) and bovine plasma α_2M was obtained from Boehringer Mannheim.

Conversion of α_2M from Slow to Fast—All α_2M preparations were subjected to dialysis against 100 volumes of distilled water to precipitate fast α_2M, which was present to some extent in all preparations. The native α_2M was tested for trypsin binding activity as described below. Slow α_2M was converted to fast α_2M by incubation with 25 mM methylamine (Tris-HCl, 50 mM, pH 8.0) overnight at 25 °C or by incubation with a 4:1 molar excess of trypsin or plasmin for 30 min at room temperature. Excess methylamine was removed from α_2M-methylamine complexes by dialysis against 100 volumes of 50 mM Tris-HCl, pH 8.2, at 4 °C. Excess trypsin or plasmin was removed from α_2M-trypsin or α_2M-plasmin complexes by gel filtration chromatography (Superose 6 FPLC). Fast α_2M preparations were stored at 4 °C in 50 mM Tris, pH 8.2, to prevent precipitation. Slow α_2M was stored at 4 °C in 20 mM sodium citrate buffer, pH 6.5. α_2M preparations were tested for PDGF contamination as described previously (15).

Trypsin Binding Assay for α_2M—Native α_2M was tested for trypsin binding activity by a modification of a previously described method (50). Increasing concentrations of α_2M were added to 96-well microtiter plates to a final volume of 50 μl/well in 25 mM Tris-HCl, 150 mM NaCl, pH 7.4. 3 μl/well of 1 mg/ml trypsin (Sigma) was then added for 10 min to bind available native α_2M, followed by the addition of 6 μl/well of 1 mg/ml soybean trypsin inhibitor (Sigma), which inhibited all trypsin activity not bound to α_2M. After 10 min, 80 μl/well of 0.1 M Tris-HCl, 10 mM $CaCl_2$ buffer, pH 8.0, was added, followed immediately by 100 μl/well 3 mM Na-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) hydrochloride (Sigma). The colorimetric reaction was stopped by the addition of 10 μl of glacial acetic acid. The increase in the optical density read at 405 nm is proportional to the quantity of active trypsin (covalently trapped within α_2M) available to convert the BAPNA substrate to its product.

Gel Filtration Chromatography—PDGF·α_2M complexes were routinely prepared by incubating 1 ng of human ^{125}I-PDGF-AB or human recombinant ^{125}I-PDGF-BB with 100 μg of α_2M fast or slow form for 24 h at 37 °C. These mixtures were isolated by loading onto a gel filtration, molecular weight exclusion column (Superose 6 FPLC, Pharmacia LKB Biotechnology Inc.) equilibrated in phosphate-buffered saline, pH 7.5, operating at a flow rate of 0.5 ml/min. The column was standardized with the following molecular mass markers: aprotinin (6.5 kDa), cytochrome *c* (12.4 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), β-amylase (200 kDa), apoferritin (440 kDa), thyroglobulin (669 kDa), and blue dextran (V_0). Fractions (1 ml) were counted on a γ-counter to measure radiolabeled PDGF or assayed for α_2M by ELISA as described below. Protein was routinely measured by absorbance (280 nm) to ensure that identical amounts of PDGF/α_2M were loaded onto the column.

Gel Electrophoresis—Electrophoresis of the PDGF/α_2M mixtures in a nondenaturing (5% Tris-borate) gel was performed as described previously (51). α_2M that was incubated with ^{125}I-PDGF-AB or -BB as described above for gel filtration chromatography was mixed with Tris-borate buffer (10 μg of α_2M in 40 μl added to 4 μl of 10 × buffer with 10 μl of glycerol) and electrophoresed on a native 5% gel. Native gels were either stained with Coomassie Blue and dried for autoradiography or transferred to nitrocellulose, blocked with 5% BSA for 2 h, then shaken with 1:2000 sheep anti-human α_2M-horseradish peroxidase (Vector Laboratories) in BSA/PBS-Tween for 4 h and developed. For SDS-polyacrylamide gel electrophoresis 80 μl (20 μg) PDGF/α_2M samples were mixed with an equal volume of 10% SDS and heated to 37 °C for 1 h, and half of each resulting sample received 10% 2-mercaptoethanol. Samples were run on a 7.5% SDS gel. The gels were stained with Coomassie Blue and dried for autoradiography and γ-counting.

α_2M ELISA—We developed an enzyme immunoassay for human α_2M using Immulon 2 Removawell flat-bottomed wells (Dynatech, Chantilly, VA) which were coated 100 μl/well with a 1:1000 dilution of rabbit anti-α_2M in PBS (DakoPatts, Santa Barbara, CA) and incubated overnight at 4 °C. The following day, the wells were washed five times with PBS containing 0.05% Tween-20 (PBST) and then 200 μl/well of 1% BSA in PBS was added. After a 5-h incubation at 4 °C, the wells were washed five times with PBST, and 100 μl/well of standard α_2M or unknown sample diluted in 1% BSA-PBS were added and incubated overnight at 4 °C. Zero antigen controls and horseradish peroxidase-antibody blanks received only 1% BSA-PBS. The following day, the plate was washed five times with PBST and 100 μl/well of horseradish peroxidase-conjugated, sheep anti-human α_2M (Serotec, Kidlington, Oxford, United Kingdom) diluted in 1% BSA-PBS (1:5000) was added and incubated at room temperature for 5 h. After washing five times with PBST, the wells were developed for 15–30 min with the diammonium salt of 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (Sigma) containing 0.003% hydrogen peroxide. The absorbance (405 nm) was measured on a Titertek Multiskan 96-well plate reader (Flow Laboratories, Mclean, VA). Doubling dilutions of α_2M (2–1000 ng/ml) were used, and the linear range of the curve was between 5–500 ng/ml.

RESULTS

Isolation of PDGF·α_2M Complexes by Gel Filtration Chromatography—To establish the elution profile of the PDGF·α_2M complex, human ^{125}I-PDGF-AB was incubated with human α_2M in Ham's F-12 buffer with HEPES, $CaCl_2$, and 0.25% BSA (binding buffer) at pH 7.4 and the mixture was loaded onto a Superose 6 FPLC column equilibrated in PBS at the same pH. Later experiments were designed to study the formation of this complex on the same column and the

subsequent dissociation of human PDGF-AB or recombinant PDGF-BB from human or bovine α_2M following a decrease in the pH of the binding buffer. A separate series of experiments addressed the formation of the PDGF·α_2M complex on this column in the absence or presence of the three recombinant human PDGF isoforms or several other cytokines (TGF-β, bFGF, IL-1β, IL-6, TNF-α) that have been reported to bind to α_2M. Human α_2M eluted as single peak as measured by ELISA and the major peak of human ^{125}I-PDGF-AB coeluted with this immunoreactive α_2M (Fig. 1).

Binding of PDGF to Slow and Fast α_2M—Isolation of the ^{125}I·PDGF-AB·α_2M complex by nondenaturing gel electrophoresis was performed to demonstrate the purity of slow and fast α_2M preparations and to establish the amount of human plasma-derived ^{125}I-PDGF-AB bound to these forms of α_2M. Both purified forms of slow human α_2M and fast methylamine-modified α_2M bound human ^{125}I-PDGF-AB (Fig. 2*A*). A quantitative analysis of the ^{125}I-PDGF-AB bound to these α_2M forms showed that approximately equivalent amounts of PDGF bound either the fast or the slow form (Fig. 2*B*).

Preferential Noncovalent Binding of PDGF to α_2M—To measure the extent of covalent or noncovalent binding of ^{125}I-PDGF-AB to native α_2M, the ^{125}I·PDGF-AB·α_2M complex was subjected to SDS denaturation or 2-mercaptoethanol reduction prior to SDS-polyacrylamide gel electrophoresis. As expected, SDS treatment dissociated the α_2M molecule into its ~180-kDa subunits as determined by protein staining and autoradiography of ^{125}I-α_2M-methylamine (Fig. 3). No ^{125}I-PDGF-AB was initially detected by autoradiography at the molecular weight of this α_2M subunit (Fig. 3*A*). Instead, the ^{125}I-PDGF-AB was detected at the molecular mass of the ~30-kDa PDGF dimer (SDS treatment) or the ~15-kDa PDGF monomer following reduction. However, a longer exposure of the SDS-denatured ^{125}I-PDGF-AB·α_2M complex revealed a minor autoradiographic signal, indicating that some PDGF remained covalently bound to the α_2M (Fig. 3*B*). The same experiments were performed with recombinant ^{125}I-PDGF-BB. γ-Counting the intact ^{125}I-PDGF-BB·α_2M complex from the nondenaturing gels and the α_2M subunits from denatured or reduced SDS gels (Fig. 4) demonstrated that 20–30% of the ^{125}I-PDGF-BB remained bound to the denatured α_2M subunit and less than 10% of the ^{125}I-PDGF-BB remained bound to the binding protein subunits after reduction (Fig. 4). Similar results were obtained for bovine ^{125}I-PDGF-BB·α_2M complex.

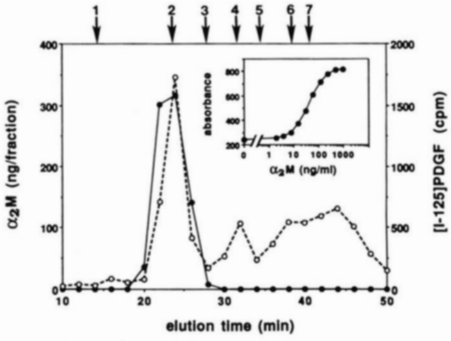

FIG. 1. **Binding of human slow α_2M to human ^{125}I-PDGF purified from platelets.** 100 μg of α_2M was mixed with 1 ng of ^{125}I-PDGF-AB in 500 μl of binding buffer and incubated for 1 h at 37 °C prior to loading on a Superose 6 FPLC column. ^{125}I-PDGF (*open circles*) coeluted with a single peak of α_2M (*closed circles*) that was measured by ELISA. The α_2M was quantified based on a standard curve (*inset*) generated with the same human α_2M that was present in the PDGF-AB·α_2M complex. *Arrows* indicate molecular mass markers, *1*, blue dextran (V_0); *2*, thyroglobulin (669 kDa); *3*, apoferritin (440 kDa); *4*, β-amylase (200 kDa); *5*, BSA (66 kDa); *6*, carbonic anhydrase (29 kDa); and *7*, cytochrome *c* (12.4 kDa).

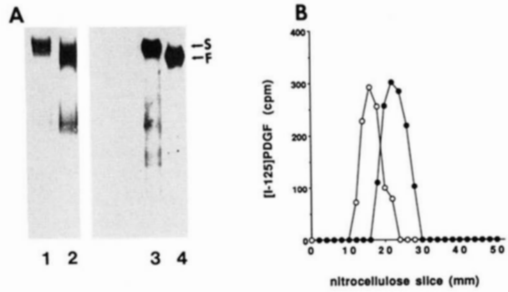

FIG. 2. **Binding of methylamine-treated and untreated slow human α_2M to human ^{125}I-PDGF-AB purified from platelets.** Slow α_2M was modified by methylamine as described under "Materials and Methods." ^{125}I-PDGF-AB (1 ng) was mixed 100 μg of human slow α_2M or α_2M-methylamine in 500 μl of binding buffer. 10 μg of the ^{125}I-PDGF-AB/α_2M mixture was loaded onto a nondenaturing 5% Tris-borate gel and electrophoresed to demonstrate fast (*F*) α_2M-methylamine and slow (*S*) α_2M migration. Complexes of ^{125}I·PDGF·α_2M were transferred to nitrocellulose. *Panel A*, Western blot of slow α_2M (*lane 1*) and methylamine-modified α_2M (*lane 2*) using a rabbit anti-human α_2M horseradish peroxidase-conjugated antibody and the corresponding autoradiograph of ^{125}I-PDGF bound to slow α_2M (*lane 3*) and α_2M-methylamine (*lane 4*). *Panel B*, quantitation of bound ^{125}I-PDGF-AB in slices of nitrocellulose from the same experiment shown in *panel A* as determined by γ-counting. *S* and *F* represent slow α_2M (*open circles*) and α_2M-methylamine (*closed circles*), respectively.

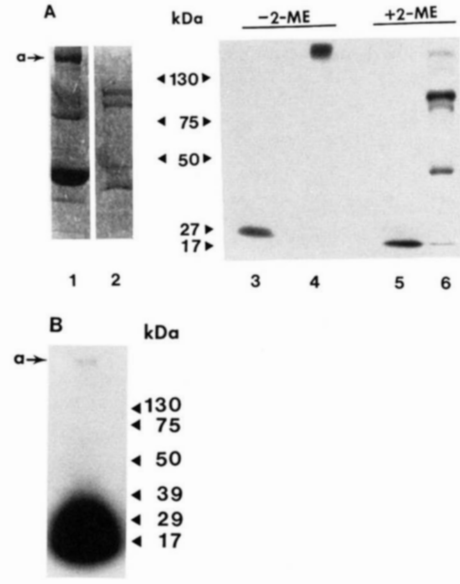

FIG. 3. **Effect of denaturation and reduction on the human ^{125}I·PDGF-AB·α_2M complex and ^{125}I-α_2M-methylamine.** Human slow α_2M (100 μg) was incubated with ^{125}I-PDGF-AB (1 ng) in 500 μl of binding medium, pH 7.4, and incubated for 1 h at 37 °C and then treated with either 7.5% SDS or 10% 2-mercaptoethanol in SDS prior to electrophoresis in a 7.5% SDS gel. *Panel A*, Coomassie Blue-stained protein of ^{125}I-PDGF-AB/α_2M that was denatured (*lane 1*) or reduced (*lane 2*); *arrow* (*a*) indicates α_2M subunit in *lane 1*. Autoradiography of *lane 1* shows that human ^{125}I-PDGF-AB was dissociated from the α_2M subunit upon denaturation by SDS and all visible radioactivity appears at ~30 kDa (*lane 3*). Autoradiography of *lane 2* demonstrates that reduction results in the appearance of a ~15-kDa monomer of ^{125}I-PDGF-AB (*lane 5*). Autoradiography of standard human ^{125}I-α_2M-methylamine that contained no PDGF following denaturation (*lane 4*) or reduction (*lane 6*) was useful in visualizing the subunits of α_2M. *Panel B*, overexposure of *lane 1* from Fig. 3 allowed visualization of some ^{125}I-PDGF-AB bound to the denatured α_2M subunit.

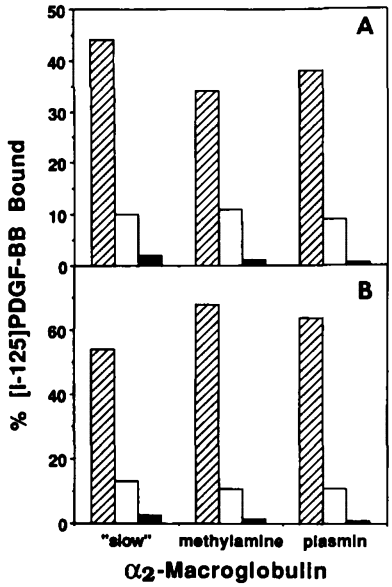

FIG. 4. **Quantitation of ^{125}I-PDGF-BB bound to slow and methylamine or plasmin-treated fast α_2M under nondenaturing, denaturing, and reducing conditions.** 100 μg of human (*panel A*) or bovine (*panel B*) α_2M, α_2M-methylamine, or α_2M-plasmin was incubated with 1 ng ^{125}I-PDGF-BB in 500 μl of binding buffer for 24 h at 37 °C, and then equivalent aliquots containing 10 μg of α_2M were electrophoresed in a nondenaturing (Tris-borate) gel or were pretreated with either SDS or SDS-containing 2-mercaptoethanol prior to electrophoresis in a SDS gel. The amount of ^{125}I-PDGF-BB bound to either the intact (720 kDa) α_2M on a nondenaturing gel (*hatched bars*) or bound to ~180-kDa α_2M subunits on a denatured gel (*open bars*) or a reduced gel (*solid bars*) was measured on a γ-counter. See Fig. 3*A*, *lanes 4* and *6* for visualization of the ^{125}I-α_2M-methylamine subunits by autoradiography. The data are expressed as the percentage of α_2M-bound radioactivity relative to the total amount of radioactivity excised from the gel (*i.e.* α_2M-bound and free ^{125}I-PDGF-BB).

pH-dependent Release of PDGF from α_2M—Denaturation of the PDGF·α_2M complex by treatment with SDS or 1 M acetic acid was shown to release the majority of PDGF bound to α_2M, and these harsh conditions are known to dissociate the α_2M molecule into its subunits. Thus, we studied the possible release of PDGF from α_2M over a range of "physiological" pH (7.5–4.0) where the α_2M molecule remains intact. Recombinant human ^{125}I-PDGF-BB incubated with bovine slow α_2M at pH 7.5 at 37 °C for 24 h formed a high molecular weight complex that eluted on a Superose 6 FPLC column at the same position as the plasma-derived human ^{125}I-PDGF-α_2M (see Fig. 1). By lowering the pH of solutions containing PDGF·α_2M complexes with acetic acid for 1 h prior to loading onto the Superose 6 column, a pH-dependent decrease in ^{125}I-PDGF-BB bound to bovine α_2M was demonstrated (Fig. 5). The α_2M retained its tetrameric (~725 kDa) structure across this pH range, and the amounts of α_2M that eluted at this high molecular mass were equivalent as determined by protein absorbance (280 nm). The quantity of ^{125}I-PDGF-BB bound to α_2M, *i.e.* isolated from the PDGF·α_2M complex on the gel filtration column, decreased ~50% as the pH was decreased from 7.5 to 4.0 (Fig. 5). At pH less than 4.0, the α_2M was denatured and migrated as subunits with molecular mass >440 kDa.

Time Course of ^{125}I-PDGF-BB·α_2M Association and Dissociation—The time course of association of ^{125}I-PDGF-BB with α_2M and the subsequent dissociation of ^{125}I-PDGF-BB from the purified ^{125}I·PDGF-BB·α_2M complex was investigated at pH 7.4. Identical aliquots of slow α_2M (200 μg/ml) and ^{125}I-PDGF-BB (2 ng/ml) were incubated at various time points (30 min to 24 h) at 37 °C in 0.5 ml of binding buffer prior to loading onto a Superose 6 FPLC column. The α_2M peak zone (20–28 min) was pooled and bound ^{125}I-PDGF-BB quantitated by γ-counting as demonstrated in Fig. 1. The association time course showed that PDGF-BB bound α_2M with a $t_{1/2}$ = ~1 h, and equilibrium was reached at ~4 h (Fig. 6*A*). Of the total amount of free ^{125}I-PDGF-BB added to the binding buffer (1 ng = 25,000–27,000 cpm), approximately half of this radioligand bound to α_2M (~12,000–14,000 cpm) after 24 h of incubation. Identical aliquots of ^{125}I-PDGF-BB·α_2M complex isolated by FPLC after 24 h of incubation (the end point on Fig. 6*A*) were allowed to further incubate for various time points (0–24 h) at 37 °C to measure the extent of ^{125}I-PDGF-

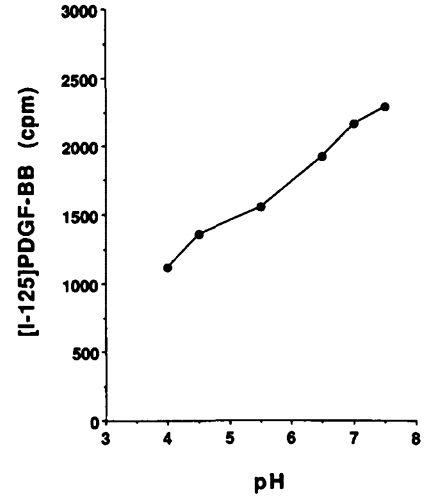

FIG. 5. **pH-dependent dissociation of ^{125}I-PDGF-BB from α_2M.** After bovine slow α_2M (100 μg) was incubated with 1 ng of ^{125}I-PDGF-BB in 500 μl of binding buffer for 24 h (37 °C, pH 7.4), the pH was lowered by titrating parallel incubations with 1 M acetic acid. After reaching the desired pH, the samples were left at 25 °C for 1 h and then loaded onto a Superose 6 FPLC and the ^{125}I·PDGF·α_2M complexes isolated and counted for radioactivity.

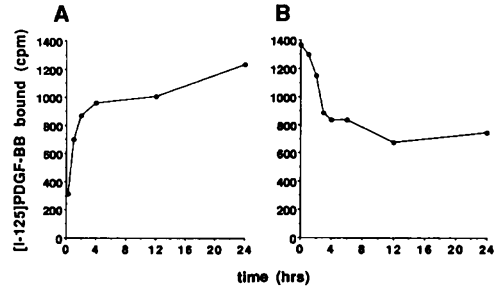

FIG. 6. **Time course of association and dissociation between ^{125}I-PDGF-BB and α_2M.** *Panel A*, to measure association of radiolabeled PDGF with its binding protein, slow bovine α_2M (100 μg) was incubated at increasing time points (30 min to 24 h) at 37 °C in 0.5 ml of binding buffer, pH 7.4, with ^{125}I-PDGF-BB (1 ng = ~26,000 cpm) prior to isolating ^{125}I·PDGF-BB·α_2M complex by FPLC gel filtration chromatography as described under "Materials and Methods." The amount of radioactivity bound to α_2M in the 20–28-min elution zone of the gel filtration column (see Fig. 1) was pooled for each time point. At equilibrium binding (>4 h), approximately half of the radiolabeled PDGF was bound to α_2M under these conditions. *Panel B*, dissociation of ^{125}I·PDGF-BB from ^{125}I·PDGF-BB·α_2M complex was determined by incubating ^{125}I-PDGF-BB with α_2M for 24 h as shown in *panel A*, then incubating the isolated ^{125}I·PDGF-BB·α_2M complex for increasing time prior to FPLC separation. Again, the 20–28 min elution zone was pooled and radioactivity quantitated by γ-counting. An equilibrium of bound to free ^{125}I-PDGF-BB was reached by 6 h; *i.e.* approximately half of the PDGF dissociated by this time point.

BB dissociation at pH 7.4. Under these conditions, 50–60% of the bound ^{125}I-PDGF-BB dissociated from α_2M ($t_{1/2}$ = ~2.5 h) and an equilibrium was reached by ~6 h (Fig. 6*B*).

PDGF Remains Bound to α_2M following Proteolytic Conversion to the Fast Form—In order to determine whether or not PDGF would be released from slow α_2M during the proteolytic conversion of the α_2M from slow to fast by trypsin, recombinant human ^{125}I-PDGF-BB complexed to bovine slow α_2M at pH 7.5 was treated with a 4:1 molar excess (trypsin/α_2M) for 20 min prior to nondenaturing gel electrophoresis. Autoradiography of the ^{125}I·PDGF-BB·α_2M complex before and after trypsin exposure showed that this proteolytic treatment converted the α_2M to the electrophoretically fast form, but the ^{125}I-PDGF-BB remained bound to the trypsin-activated α_2M (Fig. 7).

Competition of PDGF Isoforms and Other Cytokines for ^{125}I-PDGF-BB Binding to α_2M—The three different isoforms of PDGF were tested for competitive binding to α_2M to evaluate the capacity of this binding protein to potentially modulate the activity of all of the PDGF dimers. Human recombinant ^{125}I-PDGF-BB (2 ng/ml) and bovine α_2M (200 μg/ml) were incubated in the absence or presence of an excess of nonradioactive PDGF-AA, -AB, or -BB (8 μg/ml) for 24 h at 37 °C. All three nonradioactive isoforms inhibited the majority of ^{125}I-PDGF-BB binding as determined by a decrease in the radioactivity associated with the slow bovine α_2M peak on the Superose 6 FPLC column (Fig. 8). The same experiment was performed using fast, methylamine-modified, α_2M. Importantly, several other cytokines that have been reported to bind α_2M (*i.e.* bFGF, IL-1β, IL-6, TNF-α, TGF-β1, and TGF-β2) were tested for their ability to compete with the ^{125}I-PDGF-BB for binding to both slow and fast α_2M. The column fractions containing the PDGF·α_2M complex (10–30 min elution time) were pooled and counted for radioactivity. Nonradioactive PDGF-AA, -AB, and -BB all inhibited complex formation between ^{125}I-PDGF-BB and α_2M or ^{125}I-PDGF-BB and α_2M-methylamine by 65–80% compared to control treatments that received no excess isoform (Fig. 9). IL-1β, IL-6, bFGF, TNF-α, TGF-β1, and TGF-β2 each at an excess concentration (8 μg/ml) did not inhibit the binding of ^{125}I-PDGF-BB to slow α_2M. TGF-β1 inhibited complex formation between ^{125}I-PDGF-BB and α_2M-methylamine by as much as 50%, while TGF-β2 inhibited this interaction by only 10%. IL-6 and bFGF inhibited ^{125}I-PDGF-BB binding to α_2M-methylamine 30–35%, while IL-1β and TNF-α had negligible inhibitory effects on this complex formation. Bovine α_2M and

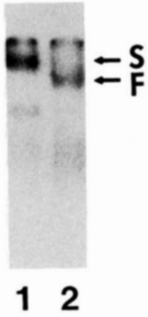

FIG. 7. **Autoradiography of ^{125}I-PDGF-BB bound to α_2M before and after conversion of slow bovine α_2M to the fast form by trypsin treatment.** Slow α_2M (100 μg) was incubated with ^{125}I-PDGF-BB (1 ng) in 500 μl of binding buffer for 24 h at 37 °C. Slow α_2M was converted to the fast form by treatment with trypsin for 20 min at 25 °C as described under "Materials and Methods." α_2M-trypsin complexes were purified by Superose 6 FPLC. ^{125}I·PDGF-BB·α_2M complexes were loaded onto 5% Tris-borate (nondenaturing) gels and electrophoresed as described under "Materials and Methods."

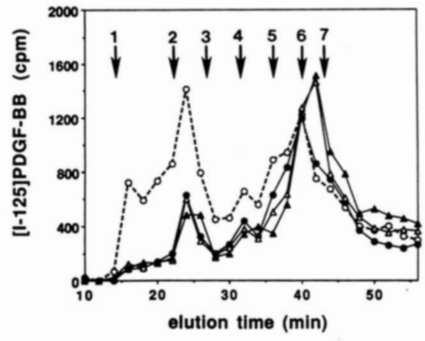

FIG. 8. **Superose 6 FPLC chromatography demonstrating competition of human recombinant PDGF-AA, -AB, and -BB dimers for ^{125}I-PDGF-BB binding to slow bovine α_2M.** ^{125}I-PDGF-BB (1 ng) was mixed with 100 μg of α_2M in 500 μl of Ham's F-12 binding buffer with HEPES, $CaCl_2$, and 0.25% BSA, pH 7.4, in the absence (*open circles*) or presence of 5 μg of nonradioactive human recombinant PDGF-AA (*open triangles*), -AB (*closed triangles*), or -BB (*closed circles*) for 24 h at 37 °C prior to loading chromatography in phosphate-buffered saline, pH 7.4. α_2M eluted between 20–28 min (see Fig. 1 for immunoreactive profile). All three nonradioactive PDGF isoforms inhibited the formation of the ^{125}I-PDGF-BB·α_2M complex by ~70% (see Fig. 5). *Arrows* indicate molecular mass markers: *1*, blue dextran (V_0); *2*, thyroglobulin (669 kDa); *3*, apoferritin (440 kDa); *4*, β-amylase (200 kDa); *5*, BSA (66 kDa); *6*, carbonic anhydrase (29 kDa); and *7*, cytochrome *c* (12.4 kDa).

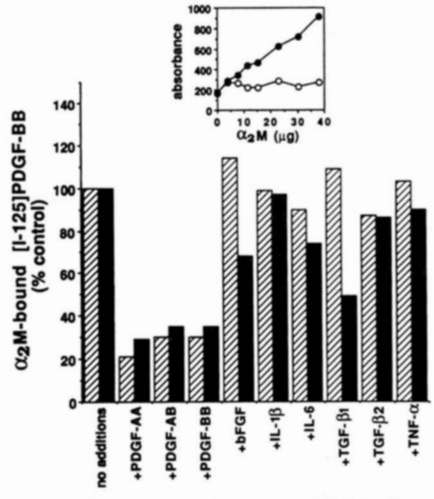

FIG. 9. **Competition of the known α_2M-binding cytokines for recombinant ^{125}I-PDGF-BB binding to slow bovine α_2M and fast α_2M-methylamine.** ^{125}I-PDGF-BB (1 ng) was mixed with 100 μg of either slow bovine α_2M (*hatched bars*) or α_2M-methylamine (*hatched bars*) in binding buffer in the absence or presence of 5 μg of nonradioactive cytokine. The amount of radioactivity bound to α_2M in the 10–30-min elution zone of the gel filtration column (see Fig. 4) was pooled for each column run. None of the cytokines tested, other than the PDGF isoforms, competed for slow α_2M, while bFGF, IL-6, and TGF-β1 prevented complex formation between PDGF-BB/α_2M 30–50%. Inactivation of the proteinase binding capacity of the slow α_2M by methylamine was measured by the trypsin binding assay described under "Materials and Methods" (*inset*); *closed circles* show that slow α_2M possesses trypsin binding activity that is proportional to an increase in absorbance, while methylamine-activated α_2M (*open circles*) does not bind trypsin.

α_2M-methylamine bound approximately equivalent amounts of ^{125}I-PDGF-BB on the gel filtration column, a result similar to that obtained with human ^{125}I-PDGF-AB binding to human α_2M and α_2M-methylamine on a nondenaturing gel (Fig. 2). Bovine α_2M and α_2M-methylamine were not run on a nondenaturing gel to test for differences in electrophoretic migration because, unlike human α_2M-methylamine, bovine α_2M-methylamine does not migrate as a fast form in a nondena-

turing gel (27). For this reason, the complete conversion of bovine α_2M to α_2M-methylamine was determined by the inability of the methylamine-treated α_2M to bind and thus inactivate trypsin (Fig. 9). Trypsin that is trapped within the α_2M molecule is still able to react with small molecules such as the BAPNA reagent (50), producing a yellow product that increases in proportion to the amount of slow α_2M in the reaction mixture (Fig. 9). These data demonstrated that methylamine treatment inhibited the trypsin binding capacity of bovine slow α_2M.

DISCUSSION

Earlier studies on plasma-derived PDGF introduced the concept that this growth factor forms covalent bonds with its binding protein, α_2M (17, 18). In the process of isolating and purifying PDGF from plasma and macrophage supernatants, we and others observed that it was necessary to acidify these biological fluids in order to separate the PDGF from its higher molecular weight-binding proteins before the growth factor could be detected by immunoassay or receptor assay (16, 19). The principal PDGF-binding proteins in these fluids were identified as α-macroglobulins and they were found to inhibit the binding of PDGF to either its cell-surface receptor or anti-PDGF antibodies, presumably by masking the receptor and antibody recognition site on the growth factor (19). The observation that PDGF could be detected after acidification suggested that at least a portion of the PDGF complexed to α_2M was bound noncovalently. The issue of covalent *versus* noncovalent binding of PDGF to α_2M is key to understanding the biological role(s) that this binding protein could serve in affecting the growth promoting activity of this cytokine. For example, α_2M has been proposed as a clearance protein for PDGF released into the circulation following platelet degranulation and the PDGF·α_2M complex could be cleared in the liver via α_2M receptors on hepatocytes (52). In extravascular tissues, macrophages, among other cell types, produce PDGF-like molecules and α_2M (19, 53, 54), and the proliferative response of fibroblasts to PDGF may be inhibited or enhanced by α_2M, depending on whether it is in the slow form or the fast receptor-recognized conformation (15). Thus, α_2M could serve as a clearance pathway for PDGF in the circulation and in extravascular tissues, but also as a positive or negative regulator of growth factor activity. We postulated that the control of PDGF-stimulated growth by α_2M likely involves the release of the growth factor from this binding protein, allowing PDGF to bind to its own receptor and trigger a mitogenic response (15). For this reason, the observation that the majority of the PDGF is bound to α_2M noncovalently (Figs. 3 and 4) and the demonstration that approximately 50% this growth factor can be released from its binding protein by lowering the pH of the incubation medium from 7.5 and 4.0 (Fig. 5) or in a time-dependent manner at pH 7.4 (Fig. 6) are consistent with our hypothesis that PDGF binding to α_2M is reversible. Such information is basic to our understanding of the mechanism(s) by which the binding protein influences cytokine activity.

Human ^{125}I-PDGF-AB and recombinant human ^{125}I-PDGF-BB both bound human or bovine slow α_2M, as well as fast α_2M that was prepared by reaction with either methylamine, plasmin, or trypsin. All three recombinant PDGF isoforms were observed to compete for ^{125}I-PDGF-BB binding to either α_2M or α_2M-methylamine, suggesting that these isoforms all bind to a similar site on the α_2M molecule and that conversion from the slow to fast conformation by methylamine does not alter PDGF isoform binding. Indeed, α_2M and α_2M-methylamine that had been incubated with radiolabeled PDGF and then isolated by nondenaturing gel electrophoresis were found to contain approximately equivalent amounts of human ^{125}I-PDGF-AB or human recombinant ^{125}I-PDGF-BB. It will be of interest to determine whether or not the biological activities of PDGF-AA, -AB, and -BB are modulated by α_2M in a similar manner. Interestingly, the ^{125}I-PDGF-BB remained bound to bovine α_2M after the ^{125}I-PDGF-BB·α_2M complex was treated with an excess of trypsin to convert the α_2M to fast form (Fig. 7). Thus, proteinases apparently do not displace PDGF bound to α_2M, and these data suggest that α_2M could serve to protect PDGF from proteolytic degradation. Such a role for α_2M has been suggested for IL-6, which is inactivated by trypsin, but retains IL-6-like activity in the presence of trypsin when complexed to α_2M (46). These observations also suggest that PDGF and IL-6 interact with α_2M by a mechanism different from that of proteinases and primary amines.

The PDGF·α_2M complex could provide a readily obtainable source of PDGF *in vivo* since the growth factor can be removed under physiological conditions *in vitro*. Kinetic studies showed that under conditions which are similar to those found in plasma, *i.e.* high [α_2M] and relatively low [PDGF], half of the PDGF bound α_2M in a time-dependent manner reaching equilibrium after 4 h at 37 °C and subsequently half of this α_2M-bound PDGF dissociated from isolated PDGF/α_2M complex more slowly and reached a new equilibrium after 6 h at 37 °C (Fig. 6). A calculation of the rate constants for association (k_1) and dissociation (k_2), which must assume an excess of radioligand over receptor (or binding protein), were not performed on these kinetic data due to the low molarity of ^{125}I-PDGF-BB (6.7×10^{-11} M) relative to α_2M (3×10^{-7} M); *i.e.* there was likely an excess of binding sites for PDGF. Further studies using saturation binding and kinetic studies are in progress to address the relative affinities of PDGF for α_2M *versus* the PDGF cell-surface receptor. The time course data shown in Fig. 6 for association of ^{125}I-PDGF-BB with α_2M are closely similar to the time course of ^{125}I-bFGF binding α_2M (44); *i.e.* low concentrations of both bFGF and PDGF binding to 200 μg/ml α_2M reached an equilibrium state at about 4 h. ^{125}I-PDGF-BB was released rapidly from the ^{125}I-PDGF-BB·α_2M complex by lowering the pH over a physiological range of 7.4–4.0 (Fig. 5). Such low pH could be encountered within lysozomes following internalization of the PDGF·α_2M complex via the α_2M receptor. In this case, it would be of interest to learn whether or not released PDGF (which is acid stable) could then be recycled to the cell-surface and remain bioactive. Thus, it is conceivable that PDGF could be dissociated from the PDGF·α_2M complex by either depletion of unbound PDGF via internalization by PDGF cell-surface receptors (which would favor the release of α_2M-bound PDGF to establish a new extracellular equilibrium) or PDGF could be released under conditions where pH is reduced.

Since several other cytokines bind to α_2M we sought to establish if these growth factors compete for PDGF binding to α_2M. Excess concentrations of TGF-β1, TGF-β2, TNF-α, bFGF, IL-1β, and IL-6 were tested for their inhibitory potency in preventing complex formation between ^{125}I-PDGF-BB and α_2M or α_2M-methylamine. None of these cytokines inhibited the binding of ^{125}I-PDGF-BB to the slow form of α_2M (Fig. 9). These data suggest that PDGF isoforms could bind to slow α_2M unhindered in the presence of cytokine mixtures *in vivo*. In contrast, some of these cytokines competed for PDGF binding to fast α_2M. TGF-β1, but not TGF-β2, inhibited complex formation between ^{125}I-PDGF-BB and α_2M-methylamine by ~50%. While both TGF-β1 and TGF-β2 bind to fast α_2M (42), it is conceivable that these two factors have

differing affinities for α_2M or bind to different sites on the α_2M molecule. Thus, the TGF-β1-binding site on the fast α_2M could overlap or allosterically modulate the PDGF-binding site. Similarly, IL-6 and bFGF inhibited complex formation between ^{125}I-PDGF-BB and α_2M-methylamine by ~20–30%, which could suggest some overlap or allosteric hinderance by PDGF and these cytokines binding simultaneously to fast α_2M. TGF-β1 has been reported to inhibit the binding of ^{125}I-bFGF binding to α_2M, while PDGF does not compete for this interaction (44). It is conceivable that certain α_2M-binding cytokines, such as TGF-β1 and bFGF, could mediate the release of PDGF from the fast form of α_2M, but not the slow form. Other cytokines apparently do not interfere with PDGF binding to α_2M. For example, TNF-α binds fast α_2M-methylamine (43) but did not inhibit the formation of the PDGF-BB-α_2M-methylamine complex (Fig. 9). Such interactions between these cytokines and the two forms of α_2M are likely to be complex and require further study.

A variety of growth-promoting cytokines, including PDGF, are likely to be involved in the processes of normal tissue maintenance and repair, and their abberant expression may well be linked to pathogenic disorders such as pulmonary fibrosis and atherogenesis that are characterized by an increase in cell proliferation and extracellular matrix production (7). A growing number of studies suggest that α_2M could play a role in modulating the biological activity, clearance, and degradation of these cytokines in either the circulation or in extravascular tissues (36–48). Because α_2M also serves as a proteinase inhibitor and since proteinases irreversibly convert α_2M to a fast or receptor-recognized form, this adds another layer of complexity to the problem of understanding the mechanisms by which a number of cytokines interact with a common binding protein. Proteinases apparently are capable of "turning on" the modulatory effects that α_2M has for some cytokines in at least two different ways: 1) cytokines such as TNF-α (43) and IL-1β (45) preferentially bind fast α_2M (42) and thus the activity of these cytokines would not be expected to be directly regulated by slow form α_2M, and 2) PDGF binds both native and proteinase-reacted forms and α_2M converted to the fast form by methylamine synergistically enhances the growth promoting activity of PDGF, while the slow form inhibits PDGF-stimulated growth (15). It is presently unclear whether or not α_2M-proteinase complexes potentiate or inhibit the growth promoting activity of the different PDGF isoforms, and this is the subject of ongoing studies. Understanding the interactions between the network of proteinases, α_2M, and growth factors which bind α_2M will be fundamental to our knowledge of cytokine function *in vivo*.

In summary, the majority of the PDGF-α_2M association is noncovalent and all three PDGF isoforms (-AA, -AB, and -BB) bind similarly to α_2M. PDGF binds to slow and fast α_2M and the slow to fast conversion by trypsin does not dissociate bound PDGF. PDGF-BB dissociates slowly from isolated PDGF·α_2M complex ($t_{1/2}$ = 2–3 h) at pH 7.4 and reaches an equilibrium state after 6 h. Furthermore, a decrease in pH from 7.4 to 4.0 causes a rapid, progressive release of PDGF from α_2M. Because PDGF binding to α_2M is reversible, α_2M could release PDGF near the cell surface in close proximity to its own receptor.

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