Compartmentalization of PDGF on Extracellular Binding Sites Dependent on Exon-6-Encoded Sequences

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Abstract. The PDGFs are a family of molecules assembled as disulfide-bonded homo- and heterodimers from two distinct but highly homologous polypeptide chains (PDGF-A and PDGF-B). Two PDGF A-chain transcripts, which arise from alternative usage of the 69-bp exon 6 and exon 7, give rise to two forms of PDGF-A. In spite of the conservation of two PDGF A-chain forms over at least 350 million years, no differences in their biological activities have been identified. We have investigated the activity of the sequence encoded by the alternatively spliced exon 6 of the PDGF A-chain (peptide A_L). Addition of peptide A_L at 10^{-5}–10^{-9}M to cultured endothelium and smooth muscle induced a dose-dependent, 3–20-fold increase in PDGF in conditioned media within 30 min. Peptide A_L had no detectable effect on A- or B-chain transcript levels, and decrease in culture temperature did not prevent rapid release of PDGF. In human umbilical vein endothelial cells treated with peptide A_L, the PDGF released was principally PDGF-BB, while in smooth muscle cells it was primarily PDGF-AA. The capacity to induce release of PDGF is shared by the homologous peptide encoded by exon 6 of the B-chain of PDGF. Binding studies and cross-linking analysis are consistent with a charge-based association of exon 6 sequences with membrane- and matrix-associated heparan-sulfate proteoglycans. We hypothesize that translation of exon 6 of the A- or B-chain of PDGF results in compartmentalization of these forms of PDGF with HS-PG, whereas forms lacking this sequence would be soluble and diffuse.

CONSERVATION of the two highly homologous A- and B-chains of PDGF and the alternative usage of exon 6 of the A-chain over at least 350 million years of evolution (Mercola et al., 1988; Matoskova et al., 1989), suggest that each of the protein products may have different functional roles. The existence of the A- and B-chains of PDGF is partly explained by recent data establishing the existence of two distinct cell-surface PDGF receptors, one that binds either the A- or B-chain (termed the α-subunit) and the other that only binds the B-chain (the β-subunit) (Hart et al., 1988; Heldin et al., 1988, 1989; Seifert et al., 1989; Matsui et al., 1989*a*,*b*). The two receptor subunits are expressed in differing numbers and proportions on different cell types and are differentially regulated. As a consequence, the capacity of the different dimers of PDGF to induce chemotaxis and mitogenesis, as well as a number of other critical cell functions, depends on both the type of PDGF dimer present and the relative number and proportion of receptor subunits on the responding cell (Seifert et al., 1989; Ferns et al., 1990). However, no major differences have been demonstrated in receptor subunit binding or in biological activity for the two alternative forms of the PDGF A-chain (Beckmann et al., 1988; Ostman et al., 1989).

In the course of attempting to develop antibodies specific to each of the two distinct carboxy-termini (COOH-termini) of the long and short forms of the PDGF A-chain, we observed that immunization with a peptide representing the sequence unique to the alternatively spliced exon 6 (peptide A_L) resulted in antibodies that recognized the unrelated sequence of the COOH-terminus of the short form of the PDGF A-chain. These antibodies also recognized mature PDGF, although less well, that contained no sequences homologous with the exon 6 sequence. Although the basis of the response in these animals to peptide A_L is unclear, we pursued the hypothesis that peptide A_L may induce the release of endogenous PDGF. As demonstrated in this report, peptide A_L induces a temperature-independent release of PDGF from cultured human umbilical vein endothelial cells (HUVECs)[1] and smooth muscle cells (SMCs). On the basis of our observations, we propose a model in which the presence of exon-6–encoded sequences results in compartmentalization of these specific forms of PDGF on cell- and matrix-associated heparan-sulfate proteoglycan (HS-PG) while the alternatively spliced form of PDGF A-chain, lacking exon-6–encoded sequences, is secreted. Such differential compartmentalization could determine whether PDGF acts principally as a growth factor involved in cell–cell interactions, or whether it acts in a broader context as a paracrine growth factor.

1. *Abbreviations used in this paper*: HS-PG, heparan-sulfate proteoglycan; HUVEC, human umbilical vein endothelial cell; RRA, radioreceptor assay; SMC, smooth muscle cell.

Materials and Methods

Peptides

The peptides used in our experiments were synthesized by the Chemical Synthesis Facility of the Howard Hughes Medical Institute at the University of Washington. They were synthesized using standard automated solid phase synthesis chemistry which is discussed in detail in Stewart and Young (1984). After synthesis, the purity of the synthesized peptides was assessed by HPLC using a C18-reverse phase column.

Immunization of Rabbits and Evaluation of Antisera

The peptides were coupled to keyhole limpet hemocyanin (Calbiochem, San Diego, CA) through a cysteine added at the amino-terminal (NH_2-terminal) end of each peptide (Liu et al., 1979) using m-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Calbiochem, San Diego, CA). Rabbits were immunized with 200 μg of coupled peptide per injection, administered subcutaneously in RIBI adjuvant (RIBI Immunochem Research, Hamilton, MT) 2, 3, and 8 wk after the initial injection. Plasma was collected from the animals by collection into sodium citrate and centrifugation to remove the cells. Plasma samples were evaluated in an ELISA in which peptides (250 μg/ml, 100 μl/well) or dimeric forms of PDGF (25 ng/ml, 100 μl/well) were coated on 96-well multiwell immunoplate (Nunc, Denmark) overnight at 4°C. Different dilutions of plasma were incubated with the coated trays for 1 h at 37°C after blocking the plate with 10 mg/ml BSA. This was followed by incubation with biotin-conjugated goat anti-rabbit IgG (Vector Labs, Burlingame, CA), avidin-HRP (Vector Labs, Burlingame, CA), and developed with *o*-phenylenediamine (Sigma Chemical Co., St. Louis, MO) as the color reagent.

Cell Culture

Cultures of human arterial SMC were grown from explants of aortic media and maintained in Dulbecco-Vogt medium (Gibco Laboratories, Lawrence, MA) supplemented with 10% FBS (Hyclone, Logan, UT). HUVECs were obtained by collagenase digestion of umbilical cords as previously described (Wall et al., 1978) and maintained in RPMI-1640 (Whittaker Bioproducts, Walkersville, MD) containing 18% adult BSA (Hyclone, Logan, UT), 2.4% HUVEC growth factor (Gospodarowicz et al., 1983), and 60 μg/ml heparin (Sigma Chemical Co.). Dishes were coated with 1% gelatin (Sigma Chemical Co.) before plating the HUVEC. 24 h before initiation of experiments, the media were changed to 1% plasma-derived serum or 5% Carboxymethyl Sephadex fraction I (Raines and Ross, 1985) for the SMC and HUVEC, respectively. At the time of initiation of experiments, fresh medium was added to the cells along with increasing concentrations of the peptides.

Assays for PDGF

The levels of PDGF in media collected from cell cultures were determined by PDGF radioreceptor assay (RRA) on human skin fibroblasts using ^{125}I-PDGF-AB (Bowen-Pope and Ross, 1985) or using PDGF ELISA specific for different dimeric forms of PDGF. The chain-specific ELISAs use a mAb made to recombinant PDGF-AA, 127.2.2.2 (Hart et al., 1990), or a mAb specific for a 25-amino-acid peptide located near the COOH-terminus of the PDGF B-chain, PGF-007 (Shiraishi et al., 1989; Ross et al., 1990), in combination with rabbit anti-PDGF antisera from animals immunized with either recombinant PDGF-AA (αA) or PDGF-BB (αB) (Hart et al., 1990).

Western Blot Analysis of PDGF Released by Peptide A_L

Concentrates of serum-free medium collections from cells treated with peptide A_L were concentrated in Centriprep microconcentrators (Amicon, W.R. Grace and Co., Beverly, MA) and then separated on 15% SDS polyacrylamide gels (Laemmli, 1970). Samples were analyzed under nonreduced and reduced conditions and transferred electrophoretically to nitrocellulose (Schleicher & Schuell, Inc., Keene, NH) in 25 mM tris, 192 mM glycine, 20% methanol, pH 8.3, for 16 h. For Western blot analysis, the nonspecific binding was blocked by incubation with 1% BSA in TBS for 1 h at room temperature with shaking, followed by incubation with affinity-purified goat anti-PDGF for 90 min at room temperature. After four washes with TBS, biotinylated second antibody specific for the primary antibody (Vector Laboratories, Burlingame, CA) was added and incubated for 60 min followed by an additional four washes. The peroxidase conjugate was developed with 0.03% H_2O_2, 0.074% DAB tetrachloride (Sigma Chemical Co.) in 0.05 M Tris-HCl, pH 7.25. The affinity-purified goat anti-PDGF, which recognizes all chains of PDGF, was prepared by repeated passage of DEAE-purified IgG over a column of Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ) to which 400 μg of purified PDGF had been coupled after cyanogen-bromide activation (March et al., 1974).

Metabolic Labeling Studies

Cultures of HUVEC were grown to confluence and six flasks (2×10^6 cells per flask) were changed to cysteine-free media containing 5% carboxymethyl sephadex fraction I (Raines and Ross, 1985) for 3 h before addition of ^{35}S-cysteine. The cultures were labeled for 16 h with 100 μCi/ml ^{35}S-cysteine (Amersham, 1,300 C/mmol). After 16 h, the culture media were replaced with complete medium for 2 additional h and subsequently were changed to Hepes-buffered media and incubated for 4 h at 4°. Three flasks were treated with diluent and three flasks received 200 μg/ml peptide B_{24}. At the end of the incubation, the media was collected and the cells were solubilized in 1% triton, 0.05% Tween-20, 0.5% deoxycholate in PBS. Media and cell pellets were subsequently incubated for 1 h at room temperature with anti-PDGF-BB (mouse monoclonal 120.1.2.1; Hart et al., 1990) coupled to Sepharose. In all cases, one half of the sample was incubated with anti-PDGF-BB Sepharose preincubated with 2 μg PDGF-BB. After removal of unbound supernatant, the gels were washed three times with PBS containing 0.5% deoxycholate and 2.5 mg/ml BSA followed by two washes with PBS containing 2.5 mg/ml BSA. Bound proteins were eluted with 1 N acetic acid and lyophilized for analysis by SDS-PAGE.

Peptide Binding Studies

Peptides were synthesized which contained a tyrosine residue added at the NH_2 terminus of peptide A_L and B-chain residues 133–144 (assuming residue 1 is the NH_2-terminal serine in the mature B-chain) to allow iodination of the peptides using iodobeads (Pierce Chemical Co., Rockford, IL). Cells were plated in 24-well cluster dishes and allowed to grow to confluence. Binding studies were performed essentially as previously described for PDGF (Bowen-Pope and Ross, 1985). Briefly, binding studies were performed with increasing concentrations of ^{125}I-peptides on cells plated in 24-well trays (Costar, Cambridge, MA). Cultures were rinsed once with cold PBS, incubated with 1 ml test solution in binding medium for 3 h at 4°C with continuous oscillatory mixing, rinsed three times with cold binding rinse, and the cell-bound ^{125}I-peptides were solubilized with 1 ml 1% Triton X-100 in distilled water for 4 min at room temperature. The wells were subsequently washed with 1 ml 4 M urea in PBS to extract matrix-associated peptides. Glycosaminoglycans used for competition studies were heparin from porcine intestinal mucosa, heparan sulfate from bovine kidney, chondroitin sulfate, type A from porcine cartilage, type B from porcine skin, and type C from shark cartilage (Sigma Chemical Co.).

Affinity Cross-linking Studies

^{125}I-peptides at 1 μg/ml were incubated with cells as described above in peptide binding studies for 3 h at 4°C. After washing three times, the cells were incubated with shaking for 10 min at 4°C with 1 mM Bis (sulfosuccinimidyl) suberate (Pierce Chemical Co.) in PBS (250 μl/well) made immediately before use. Wells were subsequently incubated with 20 mM Tris, 0.15 M NaCl, pH 7.5, for 2 min at 4°C, rinsed twice with binding rinse, drained, and then solubilized with SDS sample buffer (Laemmli, 1970) for 4 min with shaking at room temperature (200 μl/well). Before separation on SDS-PAGE, aliquots of cross-linked samples were incubated without further addition or in the presence of 1 U/ml heparinase (Sigma Chemical Co.) and 5 mM $CaCl_2$ for 2 h at 37°C. At the end of the incubation, mercaptoethanol was added to all samples (final concentration of 5%), and the samples were boiled and loaded on 5% SDS-PAGE gels with a 3% stacking gel. After SDS-PAGE, the gels were dried and exposed to Kodak X-Omat film at −70°C.

Results

Rabbits Immunized with the Peptide Encoding Exon 6 of the PDGF A-Chain Recognize the Distinct COOH-terminal Sequence of the Short Form of PDGF A-Chain

To further investigate the long (PDGF-A_L) and short (PDGF-A_S) forms of the PDGF A-chain, we attempted to

make antibodies that would specifically recognize PDGF-A_L and PDGF-A_S. Peptides containing the predicted sequence of the alternative COOH-termini of the long and short forms of the A-chain (Fig. 1 *a*) were synthesized and injected into rabbits. Each of three rabbits (two shown in Fig. 1 *b*, numbers 1 and 2) injected with the peptide encoded by exon 6 of the A-chain (peptide A_L) developed antibodies to that peptide, but also antibodies that recognized the distinct peptide sequence of the short form of the A-chain (peptide A_S) (Fig. 1 *b*). Rabbits injected with peptide A_L recognized peptide A_S better than rabbits injected with peptide A_S (numbers 3 and 4). At very high serum concentrations, rabbits injected with peptide A_L also recognized PDGF-AA and PDGF-AB (which contain only the peptide A_S COOH-terminal sequence) and mature PDGF-BB (that contains none of the homologous exon-6 "pro" sequence). Since peptide A_L and peptide A_S are not homologous (Fig. 1 *a*), it is unclear why rabbits injected with peptide A_L recognized the unrelated peptide as well as native human PDGF. However, one possible explanation for these observations is that injection of peptide A_L induced release of endogenous PDGF to which the rabbits developed antibodies. We, therefore, investigated the effects of the addition of peptide A_L to cultured HUVEC and SMC.

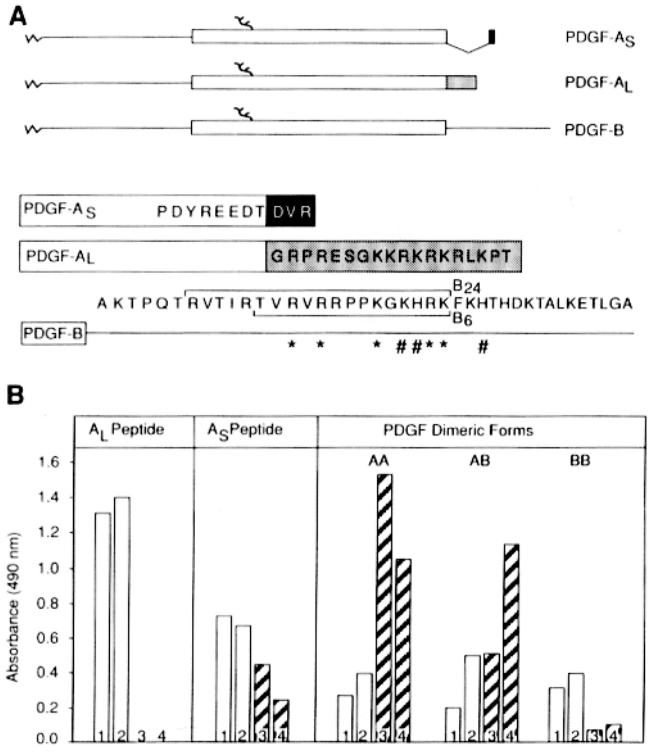

Figure 1. Relative recognition of peptides and PDGF by rabbits immunized with peptides encoding distinct COOH-termini of the long and short forms of the PDGF A-chain. (*A*) The indicated peptides encoding the distinct COOH-termini of the long and short forms of the PDGF A-chain were synthesized and coupled to keyhole limpet hemocyanin for immunization of rabbits as described in experimental procedures. The two forms of the mature PDGF A-chain (*boxed area*) arise from alternative usage of the 69-bp exon 6 (*gray box*). Peptide A_L is the sequence encoded by exon 6 (*gray box*), while peptide A_s contains sequence shared by the long and short forms of the A-chain encoded by exon 5 and three unique residues encoded by exon 7 (*black box*). The B-chain contains C-terminal "pro" sequences (*underlined sequence, no box*), encoded by the B-chain exon 6, that share significant homologies with A-chain exon 6 (indicated by *asterisks*). A number of conservative substitutions that would maintain a similar charge distribution are also apparent (indicated by *crosses*). B-chain peptides synthesized for binding and competition studies are indicated by brackets. (*B*) ELISA data obtained with four rabbits (numbers indicated inside bars) immunized with peptide A_L (rabbits 1 and 2, *open bars*) and peptide A_s (rabbits 3 and 4, *hatched bars*). The indicated peptides (25 ng/well) or different dimeric forms of PDGF (2.5 ng/well) were used to coat 96-well microtiter plates (Nunc), and the ELISA performed as described in Materials and Methods. Plasma obtained from the immunized rabbits 7 d after the fourth injection of coupled peptide were evaluated at dilutions of 1:800 for peptide A_L, 1:200 for peptide A_s, and 1:10 for the PDGF dimeric forms. The absorbance observed with preimmune plasma for each of the animals has been subtracted from the absorbance observed at the same dilution of immune plasma.

The Peptide Encoding Exon 6 of the PDGF A-Chain Induces Release of PDGF from Cultured HUVEC and SMC

In both HUVEC and SMC, addition of the peptide encoded by exon 6 at 10^{-9}M to 10^{-5}M induces a dose-dependent increase in the PDGF in the conditioned medium 2 h after addition of peptide A_L (Fig. 2). Although each of these cells constitutively produces PDGF in culture, the amount present in the medium following a 2-h incubation with the peptide is the equivalent of 69 or 104 h of constitutive synthesis by HUVEC and SMC, respectively. The kinetics of the 3–20-fold increase in PDGF detected in the conditioned medium were rapid in both cell types (Fig. 3). A similar effect is not induced by the addition of an 11-amino acid peptide that codes for the COOH-terminal sequence of the spliced pep-

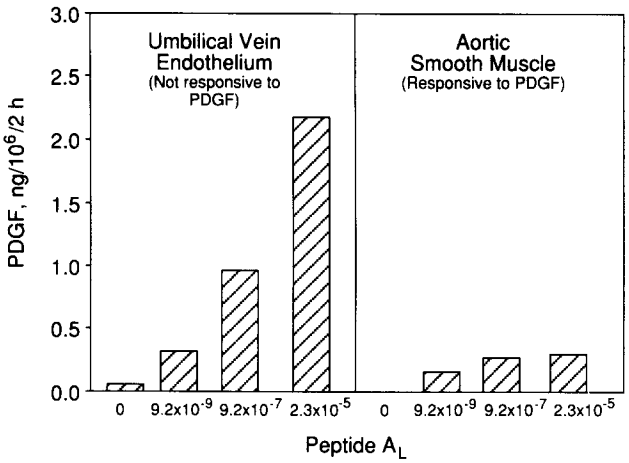

Figure 2. The peptide encoding exon 6 of PDGF A-chain induces a dose-dependent release of PDGF activity from HUVEC and SMC. The peptide encoding exon 6 of PDGF A-chain (peptide A_L) was added to cultured HUVEC and SMC at the indicated concentrations and the media were collected after 2 h for the determination of the level of PDGF. PDGF levels were determined by PDGF RRA (Bowen-Pope and Ross, 1985) and were normalized to cell number ($\times 10^6$). The dose response data is representative of three replicative experiments and two separate cell isolates for each cell type. Media from the untreated cells were collected after 24 h due to the lower levels of PDGF secretion, and represent the mean of four separate collections which were normalized to the PDGF production over 2 h.

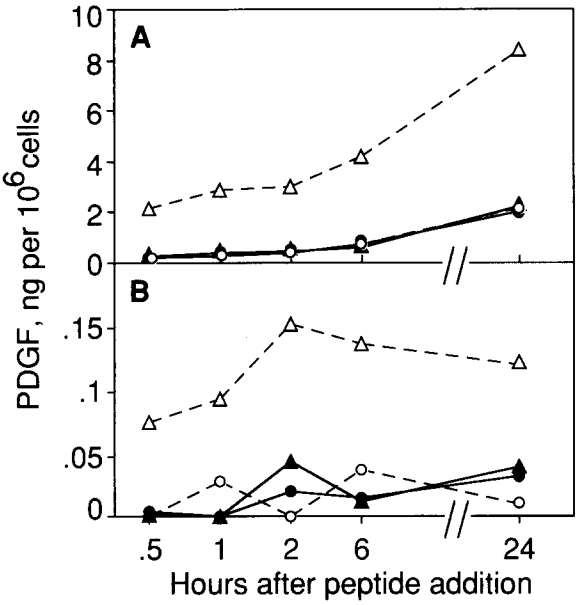

Figure 3. Peptide A_L induces rapid and specific release of PDGF from HUVEC and human arterial SMC. HUVEC (*A*) or human arterial SMC (*B*) were cultured for varying periods of time in the presence of 2×10^{-5}M peptide A_L (△), peptide A_s (▲), an 11-amino acid peptide coding for the COOH-terminal sequence of the short form of the A-chain, a combination of two basic peptides (●; CVRKKPIFKKATVTLEDHLACK and HHRS; matched for molar concentrations of arginine and lysine in peptide A_L), or diluent (○; 10 mM acetic acid). At the indicated time period, after addition of the peptides, the media was collected and cell number was determined. The PDGF content of the collected media was determined by PDGF RRA. No significant differences in cell numbers were observed with the different treatments. The time course plotted is representative of three replicative experiments and three separate cell isolates for each cell type.

tide A_S, or by a peptide mixture containing equivalent molar concentrations of lysine and arginine residues. Treatment with peptide A_L does not affect the secretion of another constitutively produced growth modulator, transforming growth factor-β (TGF-β; data not shown).

Peptide A_L (200 μg/ml) has no detectable effect on HUVEC or SMC PDGF A- or B-chain transcript levels 15 and 30 min, 1, 2, 4, 6, and 24 h after addition of peptide A_L (data not shown). Neither addition of the peptide to cultures at 4°C nor 3-h pretreatment of the cells with cycloheximide blocks the early release of PDGF in response to the addition of peptide A_L (Table I). Transport from the RER and stimulated exocytosis of secretory proteins are severely inhibited below 20°C (Saraste et al., 1986) or by blocking protein synthesis. Thus, these observations are consistent with the possibility that peptide A_L may regulate the release of PDGF from preformed storage pools. Interestingly, peptide A_L-induced release of PDGF does not require the PDGF receptor to be active since PDGF is released from HUVEC (Figs. 2 and 3), which do not express PDGF receptors (Heldin et al., 1981; Bowen-Pope and Ross, 1982; Smits et al., 1989). Peptide A_L also does not compete for binding to the PDGF receptor on fibroblasts or SMC (data not shown).

Table I. Cycloheximide and Decreased Temperature Do Not Prevent Peptide-induced Release of PDGF

	PDGF, ng/10^6 cells/2 h	
	HUVEC	SMC
Experiment 1		
untreated	1.15 ± 0.23	0.15 ± 0.01
3 h cycloheximide	1.28 ± 0.36	0.13 ± 0.03
Experiment 2		
37°C	0.64 ± 0.01	0.10 ± 0.06
4°C	0.64 ± 0.08	0.09 ± 0.01

HUVEC or SMC were cultured as described in Fig. 2. In experiment 1, the cultures were treated with 5 μg/ml cycloheximide for 3 h before addition of peptide A_L. Protein synthesis, as determined by ^{3}H-leucine incorporation into TCA-precipitable protein, was inhibited by 80 and 93% in the HUVEC and SMC cultures, respectively. In both experiments, the HUVEC cultures were treated with 2.3×10^{-5} M peptide A_L, and the SMC cultures with 9.2×10^{-5} M peptide A_L. The PDGF levels were determined by RRA and are expressed as the mean ± SEM from three doses determined in triplicate. These two experiments are representative of duplicate experiments.

Characterization of the Form of PDGF Released by the Peptide Encoded by Exon 6 of the PDGF A-Chain

To determine which dimeric forms of PDGF are released after treatment with peptide A_L, chain-specific antibodies (Hart et al., 1990; Ross et al., 1990) were used to evaluate HUVEC- and SMC-conditioned media (Table II). After

Table II. Dimeric Form of PDGF Released after Treatment with the Peptide Encoding Exon 6 of the PDGF A-Chain (A_L)

		PDGF assays			
		Chain-specific ELISA			RRA with ^{125}I-AB
		Antisera			
		127.2.2.2 (αA)	007 (αA)	007 (βB)	
Cell treatment	Time	PDGF			
	h	*ng/10^6*			
HUVEC					
none	24	0.32	0.28	7.8	0.82
diluent	4	0.04	0.02	0.12	0.17
1×10^{-8} M A_L	4	0.04	0.02	0.63	0.32
1×10^{-6} M A_L	4	0.04	0.02	3.28	0.75
2.3×10^{-5} M A_L	4	0.08	0.26	12.9	1.70
SMC					
none	96	0.18	NS	NS	0.4
diluent	4	NS	NS	NS	NS
1×10^{-8} M A_L	4	0.06	NS	NS	0.14

PDGF ELISA, using chain-specific antibodies or a PDGF RRA on human fibroblasts, were utilized to evaluate the PDGF content of media collected from cells treated with different concentrations of peptide A_L. The chain-specific ELISAs use a mAb made to recombinant PDGF-AA, 127.2.2.2 (Hart et al., 1990) or a mAb to a 25-amino acid peptide located near the COOH-terminus of the PDGF B-chain, PGF-007 (Shiraishi et al., 1989; Ross et al., 1990) in combination with rabbit anti-PDGF antisera from animals immunized with either recombinant PDGF-AA (αA) or PDGF-BB (αB) (Hart et al., 1990). If the relative recognition of PDGF-AA and PDGF-BB is normalized to PDGF-AB (1.0), the signals for PDGF-AA and PDGF-BB detected with the antibody 127.2.2.2 (αA) are 50 and NS; with antibody 007 (αA) NS and 0.06; and with antibody 007 (αB) NS and 3.0. ^{125}I-PDGF-AB was the radioligand for the PDGF RRA on human dermal fibroblasts as previously described (Bowen-Pope and Ross, 1985). NS indicates that no significant signal was detected.

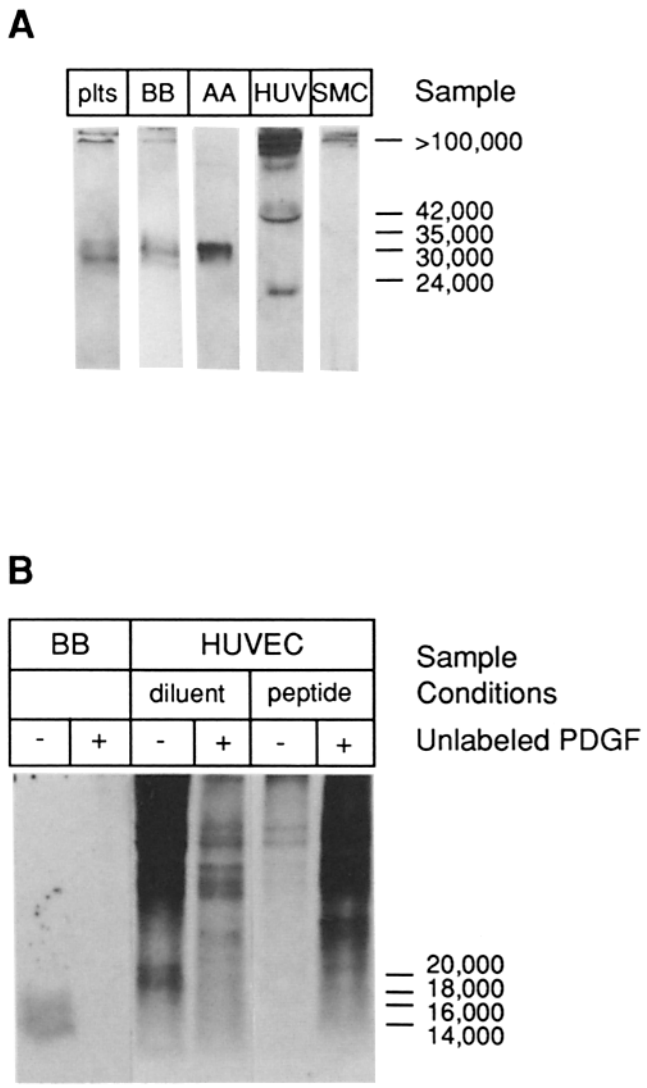

Figure 4. Exon 6 peptides induce release of a larger form of PDGF-AA from smooth muscle cells and a larger form of PDGF-BB from HUVEC. (*A*) Cultures of human SMC or HUVEC were treated as described in Fig. 1, except at the time of initiation of the experiment, the cultures were changed to serum-free medium (Hepes buffered) and incubated with 2.3×10^{-5} M peptide A_L for 4 h at room temperature. The media were concentrated ~200× (Centriprep concentrators, Amicon) and 5 μl analyzed per lane by SDS Western blot analysis after separation on 15% SDS-PAGE as described in Materials and Methods. The samples analyzed included a thrombin release of human platelets, PDGF-BB isolated from human platelets (*BB*), recombinant PDGF-AA (*AA*), and the SMC and HUVEC (*HUV*) medium concentrates from peptide A_L-treated cells. Samples were analyzed under nonreducing conditions. The blots were incubated with the affinity-purified anti-PDGF, which recognizes all chains of PDGF. The molecular weights of the principal bands are shown on the right. (*B*) Cultures of HUVEC were metabolically labeled with ^{35}S-Cysteine for 16 h as described in Materials and Methods. After a 2-h chase, replicate cultures were treated for 4 h at 4° with either diluent or 8.3×10^{-5} M peptide B_{24}. The cell layers were collected, incubated with anti-PDGF-BB coupled to Sepharose in the presence (+) or absence (−) of unlabeled PDGF-BB, and the immunoprecipitated species were analyzed by SDS-PAGE under reducing conditions followed by autoradiography. Immunoprecipitation of mature ^{125}I-PDGF-BB is shown for comparison.

treating HUVEC with peptide A_L, most of the increase in PDGF is detected with the B chain–specific antibody PGF-007 (Shiraishi et al., 1989; Ross et al., 1990) and with a second antibody that is also specific for the B-chain (Hart et al., 1990). In contrast, SMC appear to release principally PDGF-AA after treatment with peptide A_L.

The nature of the dimeric forms of PDGF released by peptide A_L was further analyzed by Western blot (Fig. 4 *a*). Using an affinity-purified goat polyclonal anti-PDGF IgG that recognizes all three dimeric forms of PDGF (Raines et al., 1989), species with ~24,000, 35–42,000, and greater than 100,000 M_r were detected under nonreducing conditions in the HUVEC media after treatment with peptide A_L. The species >100,000 and 35–42,000 M_r are larger than the mature protein (~27–31,000) (Fig. 4 *a*). The 35–42,000-D species is consistent with the estimated size of the precursor B-chain molecule, which contains sequence encoded by exon 6 but lacks the NH_2 terminal "pro" sequence (Igarashi et al., 1987). After reduction, the principal species present in the HUVEC media are also larger than the mature B-chain protein (data not shown). Analysis of the PDGF species released into the medium by SMC treated with peptide A_L also demonstrates principally M_r species greater than mature PDGF-AA: a band at ~24,000 D, a doublet at 35,000 D, and multiple bands larger than 100,000 D under nonreducing conditions (Fig. 4 *a*). While the 35–42,000-D species from endothelial cells and 35,000-D species from SMC are consistent with the expected size of forms containing exon 6, the identity of the larger and smaller forms will require analysis with sequence-specific antisera.

The nature of the cell-associated forms of PDGF was also analyzed in HUVEC after labeling with ^{35}S-Cysteine (Fig. 4 *b*). Immunoprecipitates of diluent-treated cells contained 18000- and 20,000-D species after reduction that were not detected in cells treated with peptide B_{24}, a peptide homologous with peptide A_L encoded by exon 6 of the B-chain (Fig. 1 *a*), for 4 h at 4°. These species are again consistent with the estimated size of the precursor B-chain molecules which contain sequence encoded by exon 6 but lack the NH_2-terminal "pro" sequence (Igarashi et al., 1987). They are also larger than the 14,000- and 16,000-D chains observed in mature forms of PDGF-BB (Fig. 4 *b*). Furthermore, these studies establish that the PDGF released by addition of the peptide encoded by exon 6 is synthesized by the cells.

Peptides Encoded by Exon 6 of Either the A- or B-Chain of PDGF Induce Release of PDGF and Bind to Extracellular Binding Sites

Since peptide A_L induced the release of principally higher molecular weight species of both PDGF-AA and PDGF-BB, depending on the cell type (Fig. 4), we hypothesized that peptide A_L was competing for a cell binding site for the sequence encoded by exon 6 that is shared by the COOH terminus of the long form of the A-chain and by precursors of the B-chain that contain the COOH-terminal "pro" sequences encoded by exon 6 of the B-chain (Fig. 1 *a*). If this were true, the homologous sequence encoded by exon 6 of the B-chain should also be able to induce release of PDGF. As shown in Table III, two peptides containing different portions of the B-chain exon 6 sequence also induce release of PDGF from

Table III. Peptides Encoded by Exon 6 of the PDGF B-Chain Also Induce Release of PDGF from Human SMC and HUVEC

Treatment	PDGF, ng/10^6 cells	
	HUVEC	SMC
M		
2.3×10^{-5} peptide A_L	6.61	0.52
2.1×10^{-5} peptide B_{24}	10.0	0.89
2.7×10^{-5} peptide B_6	12.9	ND

Cultures of human SMC and HUVEC were prepared as described in Fig. 2 and incubated for 4 h in the presence of 2×10^{-5} M of the indicated peptides. Peptides A_L, B_{24}, and B_6 are shown in Fig. 1. The levels of PDGF were determined by PDGF RRA.

endothelium and smooth muscle. Each of the B-chain peptides contains a number of basic amino acids and contains sequences homologous to the peptide encoded by exon 6 of the A-chain (Fig. 1 *a*). Addition of randomized peptides containing the same amino acids as the exon 6 peptides, but with the basic amino acids alternating with nonbasic amino acids (YRGKPRVKTRSKGRPKVRTK and YRGKPRVKTRSRGRP), did not induce release of PDGF (data not shown).

To further determine whether the peptide encoded by exon 6 is involved in the binding of larger forms of PDGF to cells, we synthesized these peptides with an added NH_2-terminal tyrosine residue to facilitate ^{125}I binding studies. The peptide sequences encoded by exon 6 of the A- and B-chain bind specifically to both HUVEC (Fig. 5 *a*) and SMC (Fig. 5 *b*). The estimated K_D of binding for each peptide is $2–4 \times 10^{-6}$, and the estimated number of binding sites is $4–8 \times 10^9$ for endothelial cells and 1×10^8 for SMC. Randomized peptides containing the same number of basic residues also bind to HUVEC and SMC with a comparable number of binding sites and binding affinity (data not shown). However, exon 6 B-chain peptides or randomized peptides were 10–30-fold less effective than peptide A_L in competing for ^{125}I-peptide A_L binding (Fig. 6 *a*).

The large number of binding sites and the basic nature of the peptides are consistent with the possibility that the exon 6 sequences might bind to HS-PG. This possibility is supported by the ability of heparin and heparan sulfate, but not chondroitin sulfate A, B, or C, to compete for binding of peptide A_L to HUVEC (Fig. 6 *b*). The exon 6 peptides also bind to extracellular matrix as shown by extraction with 4 M urea (Fig. 6, *c* and *d*). Similar relative competition can be observed with the B-chain peptides and the randomized peptide in the cell-associated and extracellular matrix competition studies (Fig. 6, *a* and *c*). Involvement of HS-PG in both the cell-associated and extracellular matrix binding is suggested by the observation that heparin and heparan sulfate compete for binding, while chondroitin sulfate A, B, and C do not compete at the concentrations tested (Fig. 6, *b* and *d*). Similar relative competition with peptides and glycosaminoglycans was observed with human SMC (data not shown).

A role for the HS-PG in exon 6 binding is further supported by affinity cross-linking studies (Fig. 7). Peptide A_L and peptide B_6 were bound and cross-linked as high molecular weight complexes on human SMC. Even with a 5% separating gel, the high molecular weight complexes did not enter the separating gel (suggesting a molecular weight

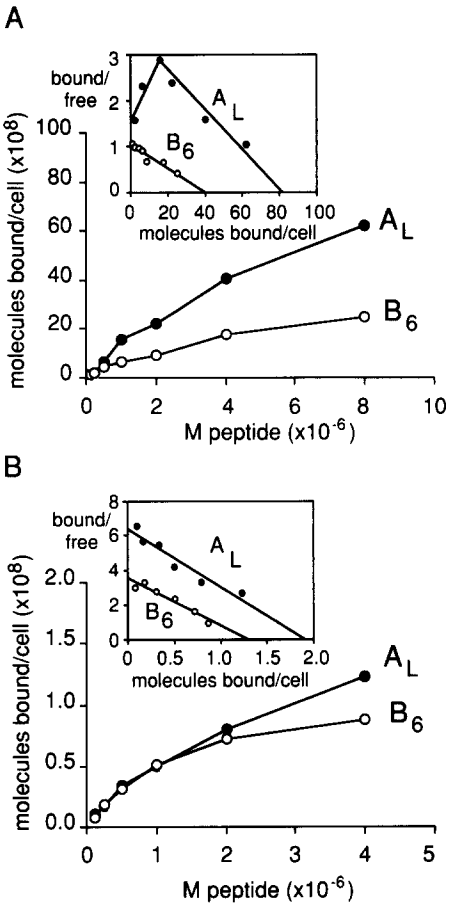

Figure 5. Peptides representing exon 6 of the PDGF A- and PDGF B-chain bind specifically to human SMC and HUVEC. Saturation binding analysis of ^{125}I-peptide A_L (●) and ^{125}I-peptide B_6 (○) to HUVEC (*A*) or human carotid SMC (*B*). The results are plotted as the number of specifically bound ^{125}I-labeled ligand molecules/cell at each concentration tested, and represent the average of duplicate determinations. Nonspecific binding, determined at 5×10^{-8} and 5×10^{-7}M, averaged 11.5 and 16% of total bound counts on SMC, and 6.2 and 7.5% of total bound counts on HUVEC for peptide A_L and peptide B_6, respectively. The inset shows the binding data plotted according to the method of Scatchard (1949). In the Scatchard plot of the data, bound/free is $\times 10^{15}$ for HUVEC and $\times 10^{13}$ for SMC.

greater than 300,000 D). Treatment of the membrane-associated material with heparinase before separation on SDS-PAGE significantly reduced the radioactivity associated with the complex. The high molecular weight complex that did not enter the 5% separating gel was not observed in the presence of excess unlabeled peptide. Further characterization of this high molecular weight complex on agarose gels and 3–12% gradient gels suggests that the exon 6 peptide is bound to hydrophobic HS-PG (Raines, E. W., E. Schönherr, M. Kinsella, T. N. Wight, and R. Ross, unpublished observations).

Discussion

Model of Proposed Compartmentalization of PDGF Containing Exon-6-Specific Sequences

A model depicting the proposed interactions between the

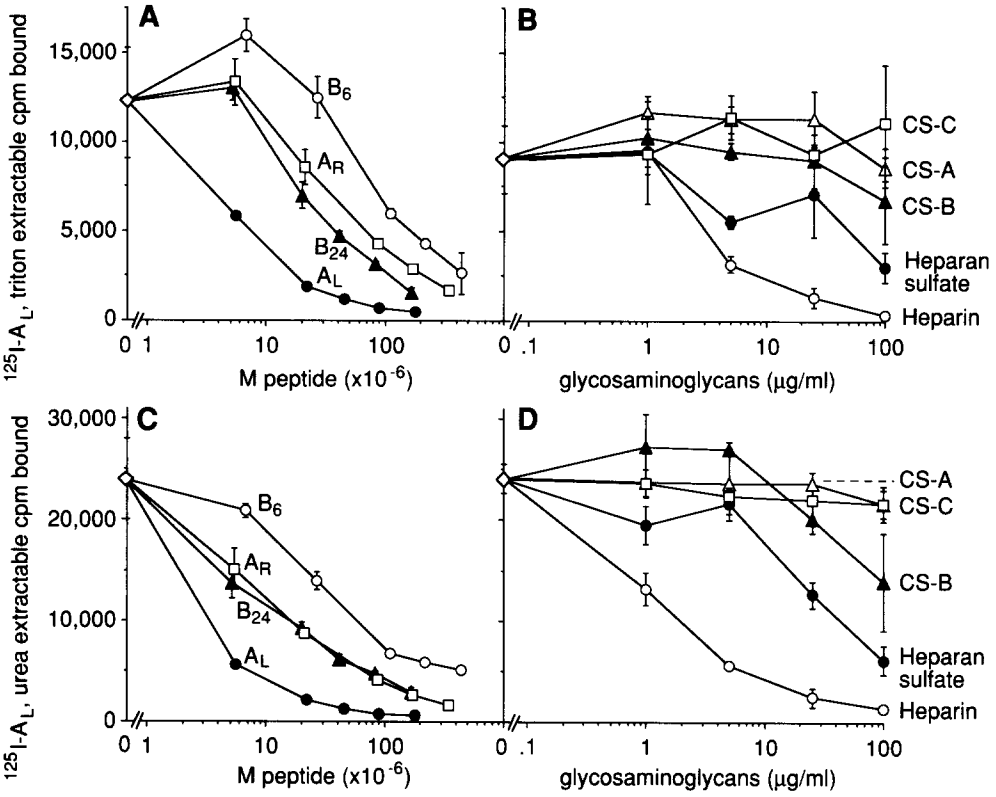

Figure 6. Competition binding studies with peptides related to peptide-A_L and glycosaminoglycans on HUVEC. Competition studies were performed with peptides related to peptide-A_L (*A* and *C*) or glycosaminoglycans (*B* and *D*). The indicated molar concentrations of peptide-A_L (●), peptide B_{24} (▲) and B_6 (○), and a randomized peptide, A_R (□), containing the same number of lysine and arginine residues as A_L, were incubated with HUVEC for 3 h at 4°C with shaking (*A* and *C*). Alternatively, HUVEC were incubated with purified glycosaminoglycans heparin (○), heparan sulfate (●), chondroitin sulfates, type A (△), type B (▲), and type C (□) for 3 h at 4°C (*B* and *D*). After 3 h, ^{125}I-peptide-A_L was added and incubated for an additional 1 h at 4°C. Cells were washed and first membrane-associated peptide was solubilized with 1% Triton X-100 in H_2O (*A* and *B*) and then matrix-associated peptide (*C* and *D*) was extracted with 4M urea in PBS. The sequence of the randomized peptide A_R is YRGKPRVKTRSKGRPKVRTK.

exon-6-coded peptides and HUVEC or SMC is shown in Fig. 8. This report demonstrates that, when added to cultured HUVEC or SMC, the peptide A_L induces a rapid, dose-dependent, temperature-independent release of PDGF into the culture medium. PDGF release is specific to the sequences encoded by exon 6 of the A- and B-chains. Depending on the cell type, most of the PDGF-AA or PDGF-BB released by exposure to peptide A_L exhibited apparent molecular weight larger than the mature proteins, consistent with the expected size of the long form of the A-chain, or precursor form of the B-chain containing exon-6–encoded sequences. The peptides encoded by exon 6 of PDGF A- and B-chains bind specifically to a relatively large number of low-affinity binding sites present on cultured HUVEC and SMC. Characterization of these binding sites by competition studies, and analysis of cross-linked species, are consistent with the association of the exon-6–encoded basic residues with HS-PG on the cell surface and in the extracellular matrix. Thus, we hypothesize that transcription and translation of exon 6 of the A- or B-chain of PDGF can result in these forms of PDGF remaining cell and/or matrix associated. Association of PDGF with the HS-PG would depend on the presence of the exon-6–encoded sequence in the secreted PDGF as well as expression of the HS-PG. Localization of pro-PDGF-BB (the form that contains the exon-6–encoded sequence) on such extracellular binding sites could also be altered by the presence of enzymes capable of cleaving the COOH-terminal "pro" sequence containing the exon 6 binding sequence to release mature PDGF-BB (Fig. 8). Enzyme(s) may cleave the long form of the A-chain at multiple mono-, di-, and polybasic cleavage sites (Fisher and Scheller, 1988) within the exon-6–encoded sequence and therefore release a cleaved form of PDGF-A_LA_L from the cell surface (Fig. 8). It is also possible that release of heparanases from platelets and neutrophils (Vlodavsky et al., 1991) and heparin from mast cells (Fransson, 1985), may also regulate soluble and diffusible forms of exon 6 containing PDGF molecules.

It is unclear why subcutaneous injection of the exon 6 sequence in rabbits induced antibodies to different forms of PDGF and the unrelated A_S sequence. However, we hypothesize that the peptide may induce release of PDGF from epidermal cells in the skin which stain strongly immunohistochemically with PDGF-B–specific antibodies (Sasahara, M., E. W. Raines, and R. Ross, unpublished observations). Examination of the mechanism of action of the peptide in vivo will be necessary to determine whether the peptides may possibly be useful clinically to alter the delivery of PDGF; to promote release of endogenous PDGF in a diffusible form; or even to develop antibodies to PDGF in certain patient populations.

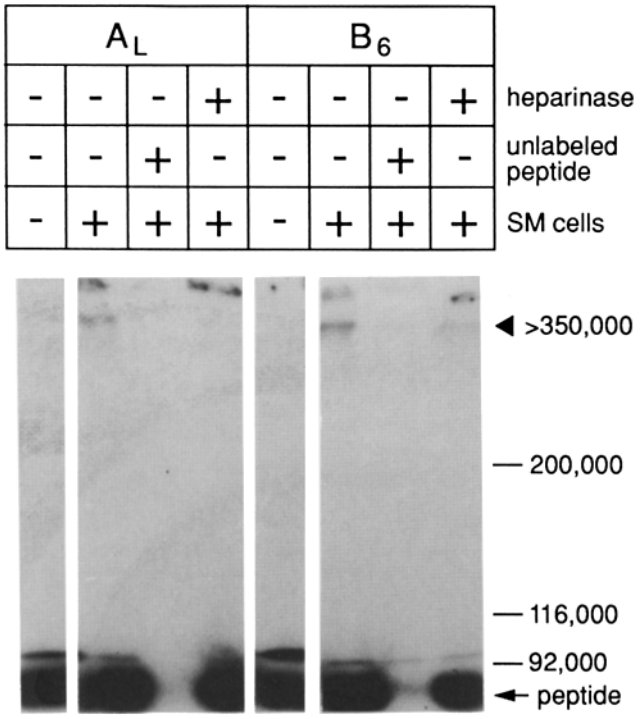

Figure 7. Characterization of the exon-6 binding site. Affinity-labeled and cross-linked ^{125}I-peptide-A_L complexes and ^{125}I-peptide-B_6 complexes isolated from human SMC were analyzed by SDS-PAGE. Human SMCs were incubated with 5×10^{-7}M ^{125}I-peptide A_L or ^{125}I-peptide B_6 for 3 h at 4°C in the presence or absence of unlabeled peptide A_L or B_6. After washing to remove unbound peptide, peptide bound to the cells was cross linked with 1 mM Bis (sulfosuccinimidyl) suberate (Pierce Chemical Co.) for 5 min at 4°C, washed, and then cross-linked complexes were solubilized in SDS sample buffer. Prior to separation on 5% SDS-PAGE, some samples were incubated with heparinase for 2 h at 37°C. ^{125}I-peptides not incubated with cells were also separated by SDS-PAGE. The arrowhead indicates the separating gel interface and the arrow, the migration position of free peptide. Nonspecific binding for peptides A_L and B_6 was 2.1 and 6.3% of total bound and cross-linked counts. All samples were run in the presence of 5% mercaptoethanol added prior to boiling and loading on the gels. Molecular weight standards are indicated on the right.

The Sequence Encoded by Exon 6 of PDGF A- and B-Chain Is Extremely Basic

The sequences encoded by exon 6 of the PDGF A- and B-chains share only 28–50% homology, depending on the alignment and the number of residues included. However, they each contain a large number of basic amino acids. A number of unique properties have been ascribed to such high local concentrations of basic amino acids. Polylysine-containing peptides, including the COOH-terminal segment of the human c-Ki-ras 2 protein, affect membrane protein kinases, phosphatidylinositol kinases, and adenylate cyclase (Gatica et al., 1987; Fujita-Yamaguchi et al., 1989), and the Lys-Arg-Lys-Arg sequence found in exon 6 of PDGF-B but not PDGF-A is found in melittin and can inhibit adenylate cyclase (Cook and Wolff, 1977). Specific sequences within exon 6 of PDGF-A and PDGF-B have been demonstrated to encode nuclear targeting signals (Lee et al., 1987; Maher et al., 1989). Basic sequences, including those of FGF and platelet factor-4, have been associated with increased cell adhesion, protein-glycosaminoglycan interactions, and inhibition of angiogenesis (Rideout et al., 1985; Baird et al., 1988; Cardin and Weintraub, 1989; Maione et al., 1990). Another family of molecules, the ras proteins, also contains a polybasic domain. Such a domain, together with palmitoylation and farnesylation has been demonstrated to be essential for plasma membrane targeting (Hancock et al., 1990). Investigation of such possible interactions may reveal localizations and biological activities of PDGF "precursors" containing exon 6 as well as possible activities of released free peptide.

It is interesting to note that the basic sequence encoded by exon 6 and alternative splicing of exon 6 is conserved in the PDGF-related protein, vascular endothelial growth factor, or vascular permeability factor (Keck et al., 1989; Leung et al., 1989; Tischer et al., 1989; Betsholtz et al., 1990). Exon 6 of vascular endothelial growth factor/vascular permeability factor codes for 6 of the 7 consecutive basic residues encoded by exon 6 of the A-chain (KKRKRKR) with the sequence reversed (KRKRKK). We hypothesize that the long form of vascular endothelial growth factor/vascular permeability factor may also be localized extracellularly, depending on peptide binding sites and processing enzymes.

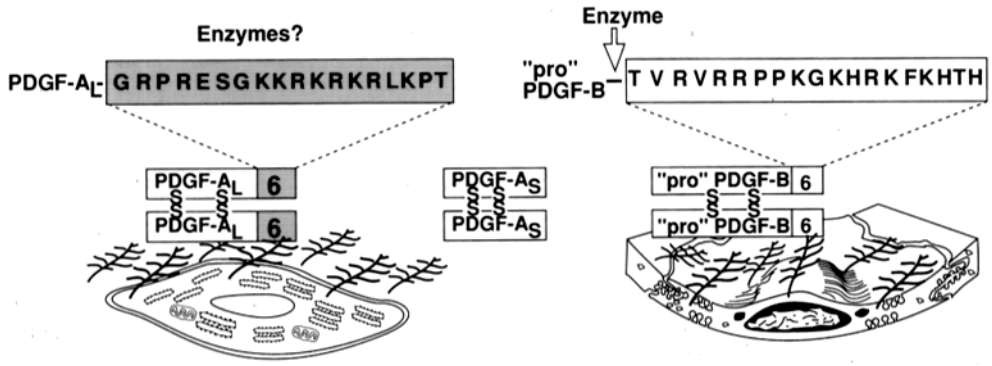

Figure 8. Model of proposed compartmentalization of PDGF containing exon-6-specific sequences. We hypothesize that translation of exon 6 of the A- or B-chain of PDGF results in selective compartmentalization of these forms of PDGF (PDGF-A_LA_L and "pro-PDGF-BB") on cell- and matrix-associated HS-PG. However, the alternatively spliced form of PDGF A-chain (PDGF-A_SA_S), lacking exon-6-encoded sequences would not bind to HS-PG and would therefore be secreted and diffusable. Addition of competing peptide (as shown in this report) or enzymes capable of cleaving the COOH-terminal exon 6 binding region could also result in increased levels of soluble PDGF. Such differential compartmentalization may provide a means whereby PDGF can act as a growth factor involved in either cell–cell interactions or targeting PDGF-producing cells to tissue sites rich in PDGF receptors, or in a broader context as a paracrine growth factor.

PDGF-BB is Cell Associated

Studies of cells transfected with the PDGF B-chain-homologues $p28^{sis}$ or with PDGF B-chain have suggested that most of the active PDGF-BB remains membrane associated, whereas cells transfected with PDGF-AA release most of the PDGF into the medium (Robbins et al., 1985; Johnsson et al., 1985; Igarashi et al., 1987; Stevens et al., 1988; Bywater et al., 1988; Beckmann et al., 1988; Lokeshwar et al., 1990). No obvious structural motifs, such as hydrophobic stretches that might cause retention of PDGF-BB, have been observed. Recent experiments with PDGF-A and -B chimeric molecules correlated the presence of the last 13 residues of exon 6 with membrane localization (LaRochelle et al., 1990). Smaller dimeric forms of PDGF-BB (30, 24, and 21 kD), lacking exon 6 sequences, have also been identified in membrane fractions after metabolic labeling of transfected cells (Robbins et al., 1985; Stevens et al., 1988; Bywater et al., 1988). It is, therefore, possible that other sequences in the mature protein, or protein modification, such as isoprenylation (Maltese, 1990) may also be involved in cell association by different forms of PDGF-BB.

The Long and Short Forms of PDGF-AA

Although PDGF-A_L and PDGF-A_S arise from alternative splicing of exon 6 and differ only in their COOH-terminal sequence (Fig. 1), both proteins are biologically active (Collins et al., 1987; Beckmann et al., 1988; Bywater et al., 1988; Ostman et al., 1989). They were originally observed in human tumor cells and HUVEC (Collins et al., 1987; Betsholtz et al., 1986; Tong et al., 1987; Rorsman et al., 1988). However, *Xenopus* oocytes also synthesize both forms of the A-chain mRNA (Mercola et al., 1988). Examination of normal tissues has demonstrated expression of both A-chain transcripts, with the short form being the predominant species (Matoskova et al., 1989; Young et al., 1990). Expression of PDGF-A_LA_L and PDGF-A_SA_S in *Saccharomyces cerevisiae* has allowed more detailed comparison of the binding and mitogenic properties of each form of the A-chain (Östman et al., 1989). No significant differences were detected in binding specificity, binding affinity, or mitogenic potency between PDGF-A_SA_S and PDGF-A_LA_L. Rather than differing in biological activity, our observations suggest that the exon-6–containing form of the A-chain remains cell associated, whereas the spliced form is secreted.

In previous metabolic labeling studies of NIH-3T3 cells transfected with the long or short forms of the PDGF A-chain, no differences in the cellular compartmentalization of PDGF-A_L and PDGF-A_S were detected, although the expression levels were at least 20-fold higher with the long form of the A-chain (Beckmann et al., 1988). In addition, when PDGF-A and -B chimeric molecules were expressed in the same cells, the presence of exon 6 sequences of either the A- or the B-chain demonstrated roughly comparable levels of PDGF immunoreactive protein in crude membrane preparations. Only chimeras containing exon 6 of the A-chain were efficiently secreted (LaRochelle et al., 1990). Although these observations appear to be contrary to the hypothesis proposed in this study, the possible role of enzymes capable of releasing the mature forms (Fig. 8) is supported by a recent study using inhibitors of proteolysis (LaRochelle et al., 1991). Monensin treatment of cells transfected with the long form of PDGF A-chain blocked secretion and proteolytic processing of PDGF A-chain. Cell-surface–retained PDGF A-chain could be released by treatment with suramin in a manner indistinguishable from that of cell-surface–retained PDGF B-chain. Our hypothesis is further supported by observations in the RAT-1 cell line in which transfectants expressing the short form of PDGF-AA secreted several fold more immunoreactive PDGF-like protein than the long form of PDGF-AA, containing the exon-6–encoded sequences, in spite of similar mRNA levels (Bywater et al., 1988). Coincident with our studies, analysis of transfectants of PDGF B-chain in COS cells identified sequences within exon 6 of the A- and B-chain that appear to mediate cell association (Östman et al., 1991). The regions identified in both the LaRochelle (1991) and Östman (1991) studies are the same as those identified in this study. It is most likely that differences between the different cell expression systems previously reported represent variations in expression levels of PDGF, HS-PG, processing enzymes, or combinations of these factors.

Cell and Matrix Association May Focus and Prolong the Activity of Growth Factors

Both cell and matrix association of growth factors suggests the existence of several possibly important regulatory mechanisms. A number of growth-regulatory molecules, including EGF (Gray et al., 1983; Scott et al., 1983), transforming growth factor-α (Brachmann et al., 1989; Wong et al., 1989; Anklesaria et al., 1990; Teixido et al., 1987; 1990), vaccinia growth factor (Stroobant et al., 1985), colony-stimulating factor (Rettenmier et al., 1987), and tumor necrosis factor-α (Kriegler et al., 1988) are produced as part of membrane-anchored precursors that may interact with receptors on the surface of adjacent cells. In each of these cases, proteolytic processing of these membrane precursors generates soluble factors. This is not always an efficient process and can lead to local accumulation. It has been hypothesized that such membrane-associated growth factors may not only be active, but may prolong the biological response as compared with soluble, diffusible factors. Differentiation inhibiting activity, a factor that specifically inhibits embryonic stem cell differentiation, has been shown to be produced through alternative transcripts as a diffusible protein and in an immobilized form associated with the matrix (Rathjen et al., 1990). Other growth factors, such as FGF (Baird et al., 1987; Vlodavsky et al., 1987; Folkman et al., 1988; Saksela et al., 1988; Saksela and Rifkin, 1990), TGF-β (Andres et al., 1989), and granulocyte-macrophage colony-stimulating factor (Gordon et al., 1987; Roberts et al., 1988) have also been shown to associate with specific matrix constituents, primarily HS-PG. These matrix-associated forms have been proposed to participate in functions such as localized retention, protection from proteolytic degradation, delivery, and clearance. Enzymes can release these "stored" growth regulatory molecules. Although it is not known whether cell-associated PDGF is active, a number of the same possibilities could be proposed for the exon-6–derived forms of PDGF. The short form of the A-chain, or exon 6 containing forms of PDGF in which the exon 6 sequence is removed by enzymes, would be secreted and able to diffuse and stimulate cells beyond the local environment. PDGF with exon 6 sequences, however,

would be immobilized and confined to specific sites containing HS-PG. Such interactions may be important in developmental and repair processes that depend on specific cell–cell interactions or targeting PDGF-producing cells to tissue sites rich in PDGF receptors.

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