Mapping the Type I Collagen-binding Site on Pigment Epithelium-derived Factor

IMPLICATIONS FOR ITS ANTIANGIOGENIC ACTIVITY

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Pigment epithelium-derived factor (PEDF), a neurotrophic and antiangiogenic serpin, is identified in tissues rich in collagen, e.g. cornea, vitreous, bone, and cartilage. We show that recombinant human PEDF formed complexes with collagens from the bovine cornea and vitreous. We have examined the direct binding of PEDF to collagen I and found that interactions were ionic in nature and occurred when PEDF and collagen I were both in solution, when either one was immobilized, or even when collagen I was denatured under reducing conditions. 125I-PEDF bound to immobilized collagen I in a saturable fashion (K_D = 123 nM). Compared with neurotrophic PEDF-derived peptides, ovalbumin and angiogenic inhibitors, only full-length PEDF competed efficiently with 125I-PEDF for the binding to immobilized collagen I (EC_50 = 3 µg/ml). The collagen-binding region was analyzed using controlled proteolysis and chemically modified PEDF. Cleavage of the serpin exposed loop did not prevent binding to collagen I. Conjugation of lysines with fluorescein increased the collagen binding affinity. However, treatment of PEDF with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide abolish ed it, implicating the PEDF aspartic and/or glutamic acid residues in its interaction with collagen I. A negatively charged region on the surface of the PEDF molecule is rich in acidic residues (Glu^41, Glu^42, Glu^43, Asp^44, Asp^64, Asp^256, Asp^258, Glu^290, Glu^291, Glu^296, Asp^299, Glu^304) available to interact directly with positively charged areas of collagen. This represents the first collagen-binding site described for a serpin, which in PEDF, is distinct from its heparin-binding region, neurotrophic active site, and its serpin exposed loop. The collagen-binding property of PEDF may play a role in surface localization and modulation of its antiangiogenic effects in the eye and bone.

Pigment epithelium-derived factor (PEDF) is an extracellular protein that has neurotrophic and antiangiogenic activities expressed mainly in compartments of the eye. It acts in neuronal survival and differentiation on photoreceptor cells and on neuronal cells of the retina and central nervous system (1–5). Several reports show that PEDF is a major inhibitor of neovascularization and is responsible for excluding vessels from invading the cornea, vitreous, and retina (6–8). These biological activities are of great importance for the development, morphology, and vision process in the eye.

PEDF is highly secreted by a variety of cells and associates intimately with extracellular matrix (5, 9–12). In particular, cells of the retinal pigment epithelium secrete PEDF protein, which associates with the interphotoreceptor matrix consistent with its affinity for binding glycosaminoglycans, e.g. heparin and heparan sulfate (13, 14). Other areas in the eye that contain PEDF include the vitreous gel and the cornea. In the bovine vitreous, PEDF accumulates at 20 nM accounting for <1% of the total protein of cell-free extracts (15). In the human cornea, PEDF immunolabeling has been detected by pathways independent of its serine protease inhibition potential (16). Outside of the eye, PEDF has been detected in teeth, bone, and cartilage matrix (10). It is worth noting that in addition to containing PEDF and being rich in collagen, the adult vitreous, cornea, and bone and cartilage matrixes are vessel-free.

Collagens comprise a family of 19 proteins with a broad range of structural and physiological functions, which are strictly located in the extracellular space. They are composed of three chains that fold to form at least one triple helical domain. All collagens are present in tissues as homotrimeric and/or heterotrimeric assemblies and have specific tissue distributions and functions, e.g. they are involved in hemostasis, wound healing, cell adhesion, and migration. In the cornea and vitreous they are known to give transparency, strength, and elasticity. Collagen type I is the major protein of the cornea (94% of the total collagen in the bovine cornea) and coassembles in heterotypic fibrils with collagen type V. Collagen type II is the main protein of the vitreous (70–80%) followed by collagen types V and IX (17).

Studies on structure-function relationships have revealed that PEDF is a glycoprotein of 50 kDa that folds like members of the superfamily of serine protease inhibitors (serpins) (13, 18, 19), but its neurotrophic and antiangiogenic activities are mediated by pathways independent of its serine protease inhibition potential (1, 4, 7, 19). The molecular mechanisms of action are better understood for its neurotrophic activities than for its antiangiogenic properties. Upon interactions with receptors on the surface of cells, PEDF can activate the necessary signal transduction events for neurotrophic activities (20–22). The binding sites for receptors are distinct and non-overlapping from those for glycosaminoglycans, with affinities for PEDF >1000-fold different (K_D ~3 nM for the receptor; K_D ~4 µM for heparin) and locations in the folded PEDF protein molecule separated about 180° (12, 14, 20, 21). The receptors interact with a neurotrophic region span-

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‡ The abbreviations used are: PEDF, pigment epithelium-derived factor; Fl, fluorescein; PBS, phosphate-buffered saline; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl; MES, 4-morpholineethanesulfonic acid; BSA, bovine serum albumin; NHS, N-hydroxysuccinimide; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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ning amino acid positions 75–121 in the human PEDF (termed 44-mer), while heparin binds to a region rich in basic amino acids (lysines and arginines) located opposite to the 44-mer. Both of these binding sites are distinct and away from the homologous serpin reactive loop.

Given the antiangiogenic activities of PEDF and colocalization with collagen in extracellular matrix, it was of interest to study the binding between these two proteins for structure-function studies. We have examined the interactions of PEDF with collagen present in bovine eyes and developed assays for PEDF-collagen binding. Using recombinant human PEDF polypeptides, synthetic PEDF peptides, and three different chemically modified PEDF versions, we have analyzed the binding site of PEDF to collagen I. We discuss how these data may aid in further understanding the antiangiogenic functions of PEDF.

EXPERIMENTAL PROCEDURES

Materials and Reagents—PEDF was purified from the cultivating media of baby hamster kidney cells containing an expression vector for human PEDF as described (12). 125I-PEDF and Fl-PEDF were prepared as described previously (14, 21). Collagen type I purified from rat tail, collagen type III from human placenta, and 24-well plates coated with collagen I were all purchased from BD Biosciences. Collagen type II purified from chicken sternal cartilage, monoclonal antibodies to bovine skin collagen type I (mouse IgG1 isotype, clone COL-1), pig collagen type II (mouse IgG1 isotype, COL-2), and human collagen type III (mouse IgG1 isotype, FH-7A), and heparin immobilized on acrylamide beads were obtained from Sigma. The monoclonal antibody to PEDF, anti-PEDF, was obtained from Molecular Probes. Protease inhibitor tablets and Lumi-LightPLUS Western blotting kit were from Roche Molecular Biochemicals. Vectastain ABC elite kit for immunoreactions was obtained from Vector Laboratories. Centricon-100 and centricon-30 devices were obtained from Millipore. PBS was prepared from 10× phosphate-buffered saline, pH 7.4, from Invitrogen (catalog number 70011-044). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (EDC) was obtained from Vector Laboratories. Centricon-100 and centricon-30 were from Pierce. Ethylenediamine was from Acros, and ethanolamine was from J. T. Baker Inc.

Preparation of Cornea and Vitreous Extracts—Bovine eyes were purchased from J. W. Truth & Sons, Baltimore, MD. After dissection of the eye, the corneas were removed, suspended in a solution of cold 20 mM HEPES, pH 7, 100 mM KCl, 1 mM EDTA containing protease inhibitors at 10 ml per cornea, and homogenized with a Polytron (Brinkmann PT 3000) set at 10,000 rpm for 20 s. The homogenized material was separated from tissue and cellular debris by centrifugation at 100,000 exclusion limit, while free PEDF molecules of 50 kDa were isolated. A protein concentration of 0.5–0.8 mg/ml.

EDC Treatment of PEDF—Pure PEDF (2.25 mg/ml; 45 μm) was mixed with 6 mM ethylenediamine or 12 mM ethanolamine and increasing concentrations of EDC (as indicated) in 0.1 MES, 0.9%/NaCl, pH 4.7. The mixtures were incubated at room temperature for 2 h. To purify the conjugates, 2 ml of PBS were added to the mixtures and then subjected to ultrafiltration through centricon-30 (M, cut-off ~ 30,000). This was repeated four times. The protein concentration in the final samples ranged between 0.5–0.8 mg/ml.

Heparin Affinity Chromatography—EDC-treated PEDF was subjected to heparin affinity column chromatography (14). EDC-treated PEDF (20 μg) was diluted in a 0.6-mL total volume of buffer S (20 mM Tris-HCl pH 7.5, 150 mM NaCl) and loaded onto a 1-mL amount of heparin immobilized on acrylamide beads (0.5 ml bed volume). The flow-through was collected and the column washed with 10 column volumes of buffer S. The bound protein was eluted with a step gradient of NaCl in buffer S (0.6 ml per fraction). The flow-through and eluted fractions were concentrated using Centricon-30 before SDS-PAGE. SDS-PAGE—Proteins of the sample were loaded onto a 10% acrylamide gel and electrophoresed using a 5% stacking gel. The proteins were transferred to nitrocellulose membranes by wet transfer using a Trans-Blot transfers system (Bio-Rad). The membranes were blocked in 5% nonfat dry milk in Tris-buffered saline (TBS; 20 mM Tris/HCl, pH 7.5, 150 mM NaCl) for 20 min at room temperature with gentle rocking and washed four times with TBS for 1 min. The membranes were blocked with blocking solution (Lumi-LightPLUS kit) for 1 h at room temperature with gentle rocking before incubation with 2 ml Fl-PEDF in blocking solution for 1 h at room temperature with gentle rocking. The membranes were rinsed and washed four times for 3 min each with TBST (TBS with 0.05% Tween 20). Bound Fl-PEDF was detected by incubating in a 1:1000 dilution of anti-fluorescein (IgG fraction), anti-Fl, was from Molecular Probes. Protease inhibitor tablets and Lumi-LightPLUS Western blotting kit were from Roche Molecular Biochemicals. Vectastain ABC elite kit for immunoreactions was obtained from Vector Laboratories. Centricon-100 and centricon-30 were from Pierce. Ethylenediamine was from Acros, and ethanolamine was from J. T. Baker Inc.

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instructions. Briefly, the membranes were sequentially incubated in a 1:100,000 dilution of mouse anti-PEDF or 1:200,000 dilution of rabbit anti-Fl in Blocking Solution, then in secondary antibody (anti-mouse IgG-POD (1:1,000) or anti-rabbit IgG-POD (1:20,000)) and finally in Lumi-LightPLUS substrate solution and then exposed to Kodak Biomax ML film. For colorimetric immunostaining, the membranes were sequentially incubated in a 1:750 dilution of anti-collagen I or anti-collagen III, then in biotinylated anti-mouse IgG (H\(_2\)L) (1:1000) and followed by ABC solution (Vectastain ABC elite kit). Color was developed with horseradish peroxidase (HRP) color development reagent (Bio-Rad).

### RESULTS

**PEDF Binds to Collagens of Cornea and Vitreous**—Because PEDF is a natural component of cornea and vitreous, we evaluated its ability to interact with proteins from these ocular components. Binding reactions were performed at 4°C to allow interactions to occur with minimal proteolytic degradation. Using a method based on size-exclusion ultrafiltration, a PEDF of 50 kDa was retained by the membrane (exclusion limit of \(M_r 100,000\)) only when incubated with the ocular extracts (Fig. 1A). The amount of bound PEDF increased with increasing amounts of corneal and vitreal protein in the reactions. Next, we used ligand affinity chromatography to identify PEDF-binding proteins in the cornea and vitreous. Several proteins of the cornea and vitreous had binding affinity for immobilized PEDF, including proteins that comigrated with collagen I and immunoreacted with antibodies to collagens (Fig. 1B). Note that the commercial collagen I also bound to the PEDF affinity resin. Ligand blotting of collagen I showed that PEDF bound to immobilized denatured collagen I and comigrating proteins from vitreous and cornea after SDS-PAGE under reducing conditions (Fig. 1C). These data demonstrated that PEDF can bind to bovine corneal and vitreal collagens in solution and when immobilized.

**Binding of PEDF to Collagens in Solution**—Then we assayed the binding of PEDF to purified collagen types I and II in solution. Fig. 2 shows that PEDF bound to each type of collagen, unlike the non-inhibitory serpin ovalbumin. The binding was collagen concentration-dependent with an apparent effi-
assays with PEDF had higher affinity for collagen type I compared with the other types, we analyzed these interactions in more detail. PEDF had higher affinity for collagen type I compared with the other types (Fig. 2B).

Because PEDF radiolabeled with $^{125}$I was used as a ligand for collagen I binding. Solution assays showed that 43% of the 200 nM $^{125}$I-PEDF was retained by ultrafiltration in reactions with collagen I (20 g/ml), in contrast to 5% without collagen I, which decreased with an excess of unlabeled PEDF over the radioligand (data not shown). Thus, as with unmodified PEDF, the $^{125}$I-PEDF modified on tyrosines by iodination formed specific complexes with collagen I in solution.

To determine the binding parameters of the PEDF-collagen I interactions, solid-phase binding assays were performed with soluble radioligand and immobilized collagen I on plastic. The binding reactions were under the same conditions as the solution assays above; however, one advantage was that they could be performed with lower concentrations of radioligand. $^{125}$I-PEDF at 2 nM bound to the immobilized collagen I and the unlabeled ligand competed efficiently for the binding with a half-maximal competition concentration of EC$_{50}$ = 1.69 pg/ml (34 nM PEDF) (Fig. 3A). A binding saturation curve was calculated (Fig. 3B), and the binding parameters revealed an affinity of K$_D$ = 124 nM for the PEDF-collagen I interactions.

Radioligand competition assays were performed with PEDF fragments. BH is a bacterially derived recombinant human PEDF sequence between positions 44–418, and the 34-mer and 44-mer peptides are synthetic peptides designed from the human PEDF sequence between positions 44–77 and 78–121, respectively. The 44-mer peptide contains the receptor-binding region of PEDF and has neurotrophic activity on retinoblastoma cells (20). Fig. 3C shows that additions of an excess of BH inhibited the $^{125}$I-PEDF binding, comparable with additions of unlabeled PEDF. The presence of 75 mM urea in the binding reactions decreased the $^{125}$I-PEDF binding to 60%, probably due to partial unfolding of the ligand. However the 34-mer and 44-mer peptides did not have a major effect on the binding of PEDF to collagen I. Similarly, increasing concentrations of angiostatin or endostatin, established antiangiogenic inhibitors, did not compete with $^{125}$I-PEDF for binding to collagen I.

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Fig. 3. Binding of $^{125}$I-labeled PEDF to collagen I. Recombinant human PEDF labeled with $^{125}$I was used as ligand for collagen I in solid-phase assays. A, binding to immobilized collagen I was with 100 ng/ml (2 nM) $^{125}$I-PEDF and increasing concentrations of unlabeled PEDF. The bound-to-total ratio without unlabeled PEDF was 3.3%. Amounts of radioactivity of $^{125}$I-PEDF bound per well ± S.D. are shown. Each point was the average of triplicate assays. B, binding isotherm of PEDF to immobilized collagen I. Binding data of A was transformed and subjected to nonlinear regression using GraphPad Prism™. Equations for one- and two-site binding (hyperbola) were fitted and compared with an F test. The best fitted equation was for one-site binding with best-fit values of B$_{max}$ = 0.59 ± 0.02 pmol per well and K$_D$ = 123.5 ± 18.12 nM. Specific binding ± S.E. is shown. The inset displays the Scatchard transformation of the binding data. C, radioligand competition. Bar graph of $^{125}$I-PEDF per well with PEDF fragments in molar excess over the radioligand, as indicated. Binding was with 2.8 nM radioligand, and each point was the average of triplicate assays ± S.D. Human PEDF fragments were BH (amino acid positions 44–418), 33-mer (positions 44–77), and 44-mer (78–121, containing the neurotrophic active site of PEDF). Reactions for BH (0 and 50×) contained 75 mM urea. D, inhibition of $^{125}$I-PEDF binding by collagen I in solution. Plot of $^{125}$I-PEDF bound per well versus increasing concentrations of collagen I. Each point was the average of duplicate assays ± S.D.

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$^a$ C. Meyer, personal observations.

$^3$ S. P. Becerra, unpublished observations.
test for displacement of bound $^{125}$I-PEDF from the immobilized collagen I, additions of increasing concentrations of collagen I resulted in a gradual loss in radioligand binding, reaching ~40% inhibition with 100 µg/ml collagen I (Fig. 3D).

Surface Plasmon Resonance Assays—Biacore kinetic and affinity analyses were performed on the interaction between PEDF and collagen I (Table I), and little or no nonspecific binding was observed with this method. Binding was reproducible and yielded data suitable for analysis. When the assay was performed in an orientation where the PEDF monomer protein was in solution (following a simple 1:1 interaction mechanism), the resultant affinities from both steady-state analysis and kinetically derived $K_d$ matched those obtained from solid-phase assays with radioisotopes in solution. When the orientation was reversed, and the trimeric collagen was in solution as the analyte, the kinetics became somewhat complicated probably due to avidity effects of multivalency (e.g. more than one PEDF-binding site on a collagen molecule). This observation was consistent with the cooperative binding found above with collagen in solution (see Fig. 2). Even though the measured affinities fell within a 10-fold range (i.e. 100–800 nM) for the bivalent steady-state model proposed for collagen binding to immobilized PEDF, and were roughly consistent with affinities measured in the opposite orientation (i.e. collagen I surface), the results from the latter orientation (i.e. collagen I surface) and the simple 1:1 model are to be preferred.

Effect of NaCl on the PEDF-Collagen I Interactions—To elucidate the type of interactions between PEDF and collagen I, we examined the effect of increasing the ionic strength in the reactions using NaCl. Fig. 4 shows that the binding of both unmodified PEDF and $^{125}$I-PEDF to collagen I was lost with an increase in NaCl concentration. The affinity of both PEDF versions for collagen I decreased significantly with 400 mM NaCl and 600 mM NaCl and was completely lost with ≥1 M NaCl. These data showed that the binding of PEDF to collagen was sensitive to increasing ionic strength, implying an ionic nature for their interactions, and that the modification on tyrosines by conjugation with $^{125}$I did not affect this binding characteristic of PEDF.

Modifications of PEDF at Lysines with Fluorescein Increase Its Binding Affinity for Collagen I—Given the ionic nature of the PEDF-collagen I interactions, we investigated the role that charges of the protein play on collagen I binding. The three-dimensional structure of PEDF has revealed two distinct areas with opposite ionic potentials on its surface (14, 24). In previous studies, we demonstrated that modification at the positively charged lysine residues of PEDF abolished its binding affinity for polyanions, such as glycosaminoglycans (14, 21). To determine whether the basic region of PEDF is required for collagen binding, we used PEDF chemically modified with an activated N-hydroxysuccinimide ester form of fluorescein, which reacts with primary amines such as those on basic lysine residues. Binding of Fl-PEDF to increasing concentrations of collagen I was tested in solution assays. Fig. 5 shows that about 50 ng of Fl-PEDF were retained in assays with 2.5 µg/ml collagen I, which increased more than 5-fold with 10 µg/ml collagen I. In contrast, the bound unmodified PEDF was not detected with 2.5 µg/ml collagen I and barely reached 50 ng with 10 µg/ml collagen I. Fig. 5B shows a comparison of the affinities for collagen I of unmodified PEDF and Fl-PEDF in the presence of NaCl. The modified Fl-PEDF required a higher ionic strength (1350 mM NaCl) to release from collagen I than the unmodified PEDF (300 mM NaCl). These results showed that the modifications to PEDF did not prevent binding to collagen I, but rather increased it. In addition to excluding the basic region of the PEDF protein as site for interaction with collagen I, these data show that neutralization of its positive charged lysines increased its binding affinity for collagen I. Furthermore, the homologous serpin reactive loop of PEDF has a chymotryptic site (19). PEDF and Fl-PEDF were cleaved by controlled proteolysis with chymotrypsin and tested for collagen I binding. Fig. 5C shows that the binding ability was not abolished upon cleavage of the homologous serpin reactive loop of either PEDF or Fl-PEDF, indicating that this region is also dispensable for the collagen I interactions.

| Method                      | Orientation          | Affinity | Kinetics             |
|-----------------------------|----------------------|----------|----------------------|
| Radioisotope                | Collagen surface     | 123      | NA                   |
| Biacore kinetics            | Collagen surface     | 135      | $k_a = 2.62 \times 10^5$ M$^{-1}$ s$^{-1}$ |
| Biacore steady-state        | Collagen surface     | 135      | $k_a = 2.33 \times 10^5$ M$^{-1}$ s$^{-1}$ |
| Biacore kinetics (bivalent) | PEDF surface         | 351      | $k_a = 1.65 \times 10^5$ M$^{-1}$ s$^{-1}$ |
| Biacore steady-state        | PEDF surface         | 115      | NA                   |
| Biacore bivalent steady-state| PEDF surface         | 826      | NA                   |

* NA, not available.
Modified PEDF at Aspartic Acid and Glutamic Acid Loses Its Binding to Collagen I—To determine whether the negatively charged area of PEDF is directly involved in binding to collagen I, we treated PEDF with EDC. EDC modifies carboxyl groups, such as the ones in aspartic acid and glutamic acid residues. Treatments were with increasing concentrations of EDC in the presence of a molar excess of amine containing reagents such as ethylenediamine or ethanolamine to prevent protein-protein cross-linking. The resulting PEDF proteins migrated as 50-kDa monomers, with minimal formation of polymers detected only with the highest concentration of EDC (Fig. 6A). To test whether the treatments had affected the basic region of PEDF, the EDC-treated PEDF proteins were subjected to heparin affinity column chromatography. Fig. 6B shows that the treated proteins had retained their heparin-binding site with a higher heparin affinity as the EDC concentration increased, implying that their folded conformation had been maintained. Note that those treated in the presence of excess of ethylenediamine had slightly higher heparin affinity than ethanolamine (compare samples 3 and 6), probably due to differences in charge-to-protein ratio. The modified PEDF proteins were then tested for collagen I binding in solution. Treatments with increasing concentrations of EDC resulted in a gradual loss of affinity for binding collagen I (Fig. 6C). These results demonstrated that the aspartic and glutamic acid residues of PEDF were required for its binding to collagen I and implied that they are directly involved in interactions with collagen I.

**Effect of Polyanions on the PEDF-Collagen I Interactions**—The requirement of negative charges/acidic region of PEDF for the binding to collagen I suggests interactions at positively charged regions in collagen I. Heparin was used as a polyanionic competitor for the binding of the acidic region of PEDF to collagen I. Solution binding assays were under ionic strength conditions in which the PEDF-heparin interactions do not occur (see Ref. 14). Fig. 8 shows that increasing concentrations of heparin competed with unmodified PEDF molecules for the binding to soluble trimeric collagen I. These results imply that PEDF shares a positively charged binding region in collagen I.

**Fig. 5.** Binding of fluoresceininated PEDF to collagen I. Solution binding assays with 25 μg/ml PEDF or Fl-PEDF to collagen I. A, binding reactions were with increasing concentrations of collagen I, as indicated. Lanes on the right were with PEDF or Fl-PEDF not subjected to ultrafiltration. B, binding reactions were with 10 μg/ml collagen I and increasing concentrations of NaCl, as indicated. Western analysis using anti-PEDF is shown for A and B. C, PEDF and Fl-PEDF were cleaved with chymotrypsin at the serpin exposed loop and assayed for collagen I binding in solution. A Coomassie Blue-stained gel of bound ligands is shown. **Fig. 6.** Binding of EDC-treated PEDF to collagen I. PEDF protein samples were treated with increasing concentrations of EDC in the presence of ethylenediamine (samples 1–5) or ethanolamine (samples 4–6). A, SDS-PAGE of each purified EDC-treated PEDF protein (2 μg protein/lane). Reactions were with 0 mM EDC (lanes 1 and 4), 1.3 mM EDC (lanes 2 and 5), and 13 mM EDC (lanes 3 and 6). B, EDC-treated PEDF samples were subjected to heparin affinity column chromatography. Flow-through and eluted fractions with a step gradient of NaCl were resolved by SDS-PAGE. Numbers to the left correspond to protein loaded to each column and were as lanes in A. C, the EDC-PEDF was assayed for binding to collagen I in solution. Bound PEDF was resolved by SDS-PAGE. Numbers to the top correspond to protein ligand in each reaction and were as lanes in A. All gels shown were stained with Coomassie Blue. *, monomers; **, dimmers; ***, trimers.

**Fig. 7.** Effect of heparin on the binding of PEDF to collagen I. Solution assays of PEDF-collagen I binding were in the presence of increasing concentrations of heparin, as indicated. A Coomassie Blue-stained gel of bound PEDF is shown. Lane to the left corresponds to 2 μg of PEDF as standard protein.
with heparin and further verify that the binding site for collagen I in PEDF is formed by a negatively charged region on the surface of the protein.

DISCUSSION

In this study we have characterized and analyzed the interactions between PEDF and collagen and provided evidence for a collagen I-binding site on PEDF. Our results show that the extracellular neurotrophic and antiangiogenic serpin PEDF binds to sites on collagens I–III under physiologic conditions. Interactions can occur when PEDF and collagen I are both in solution, when one is immobilized, or even when collagen I is denatured under reducing conditions. We present evidence for ionic interactions between a negatively charged area in the intact and correctly folded PEDF protein rich in glutamic acid and aspartic acid residues and positively charged area(s) in collagen I molecules. These interactions may occur in vivo and may play important roles in regulating the local availability of PEDF and/or in modulating its biological activities, e.g. antiangiogenic activities in the cornea and vitreous.

PEDF has the highest affinity for collagen I among collagens I–IV immobilized (10) and in solution, as shown here. When collagens are in solution, the PEDF-collagen interactions have a cooperative binding nature as demonstrated by the preferred binding equation for sigmoidal dose response. When collagen is immobilized, both the radioligand binding and Biacore analyses resulted in the same affinity measurement for the PEDF-collagen I interactions (Table I), confirming and validating each other as a means for measuring affinity. In addition, real-time surface plasmon resonance analysis revealed that this interaction has rapid kinetics and is transient. In other words, when the local concentration of reactants falls below the $K_p$ of the interaction, the complex is likely to rapidly dissociate. This is likely to provide additional insight into the binding mechanism in vivo. For example, transient interactions in the range of $10^{-7}$ to $10^{-5}$ M are typical with molecules involved in cell adhesion (25–29).

Our data also provide evidence for a collagen I-binding site on PEDF. The affinity of PEDF for immobilized collagen I ($K_D = -130$ nM) is distinct from its affinity for neurotrophic receptors ($K_D = -3$ nM) and glycosaminoglycans ($K_D = -4$ μM for PEDF-heparin) (12, 14, 20), implying a distinct and separate binding site for collagen I on the PEDF protein molecule. In this regard, the lack of competition by the 44-mer peptide containing the PEDF receptor-binding site (20, 21) is in agreement with separate and non-overlapping binding sites for collagen I and the neurotrophic receptor. The fact that modifications of lysines of PEDF, e.g. Fl-PEDF, abolishes the binding affinity for heparin (14, 21) but not for collagen I, also points to distinct and non-overlapping binding sites for glycosaminoglycans and collagens. Moreover, these three binding sites are independent of the homologous serpin reacting loop in PEDF.

The loss of the affinity of PEDF for collagen I with an increase in ionic strength, a lower pH, or modifications of carboxylic groups of PEDF, e.g. EDC-treated PEDF, is consistent with an acidic/negatively charged region of PEDF as a binding site for collagen I. The three-dimensional structure of PEDF has two distinct regions with opposite ionic potentials located almost 180° apart from each other (14, 24). The positively charged region is rich in basic amino acid residues, lysines and arginines, with side chains containing NH₃⁺ exposed to the surface of the protein, while the negatively charged region is rich in aspartic and glutamic acids, Glu¹⁴¹, Glu¹⁴², Glu¹⁴³, Asp¹⁴⁴, Asp¹⁴⁵, Asp¹⁴⁶, Glu¹⁴⁹, Glu¹⁵³, Glu¹⁵⁶, Glu¹⁵⁹, and Glu¹⁶⁴, with side chains containing COO⁻ groups exposed to the surface of the protein (Fig. 8). As the pH is lowered the carboxyl groups become protonated, and the attracting force between the acidic region of PEDF and collagen I decreases (Fig. 2D). At the same time, however, in regions that were electroneutral at pH 7, as the carboxyl groups become protonated, the number of positively charged amino groups that are no longer neutralized increases, and the attracting force for polyanions increases. When the basic region is blocked, e.g. Fl-PEDF, the number of positive charged amino groups decreases causing a reduction in attracting the negatively charged heparin (21) and an increase in attraction for collagen I (Fig. 6). Conversely, when the acidic region of PEDF is blocked, e.g. EDC-treated PEDF, the number of negatively charged carboxyl groups diminishes, causing a reduction in the attracting force for positively charged areas in collagen I and an increase in attraction for heparin (Fig. 7). Together our data...
demonstrate that all or part of the aspartic and glutamic acids forming the acidic region of the PEDF protein are available to interact directly with positively charged areas in collagen I. In the three-dimensional structure of PEDF, this acidic region, corresponding to the collagen I-binding site, is distinct and non-overlapping from both of the binding sites for the neurotrophic receptor and heparin and all away from the serpin exposed loop (see Fig. 8).

Our data also provide evidence for PEDF-binding sites in the collagen I molecule. The competition between heparin and PEDF for collagen I binding points to overlapping binding sites for these two ligands in collagen I. The binding affinity of heparin for collagen I ($K_D = 150$ nM) is similar to that of PEDF-collagen I, suggesting that heparin and PEDF can be interchangeable ligands for collagen I. Mapping of ligand-binding sites in collagen I has shown that collagen I has positively charged areas that interact with glycosaminoglycans (31). Several sites on collagen I have been proposed to mediate heparin/heparan sulfate binding to type I collagen. One of them at position 87–90 of the (1) overlaps with one of the integrin $\alpha$2$\beta$1-binding sites and is a near neighbor of the integrin $\alpha$2$\beta$1-binding sites. These observations suggest that the binding of heparin/heparan sulfate proteoglycans and/or PEDF to collagen I could modulate integrin-collagen interactions, which play a role in cell adhesion an important event in angiogenesis. In addition, triple helical peptides of the basic N-terminal sequence of this area were found to be inhibitory in the collagen I-mediated endothelial cell tube formation in vitro, an assay for angiogenesis (30). In the same fashion, in corneal neovascularization assays or other angiogenesis assays requiring the presence of collagen, PEDF could regulate endothelial cell adhesion and migration on collagen.

Comparison with serpins known to bind to collagen I such as HSP47 and maspin shows that the PEDF-collagen I interactions have binding parameters more similar to those for HSP47 but biological activities resembling those of maspin. For example, using surface plasmaphoresis with immobilized collagen I and maspin as analyte, Blacque and Worral (29) found a $K_D$ of 60 nM with an association rate constant ($k_a$) of $3.2 \times 10^8$ M$^{-1}$ s$^{-1}$, and a dissociation rate constant ($k_d$) of $0.002$ s$^{-1}$, whereas MacDonald and Balbininger (32) studying the binding of mouse and chicken HSP47 to native bovine collagen I by fluorescence quenching and cooperative binding, observed a collagen concentration at half-saturation ($K_{sat}$) of 110–140 nM and a Hill coefficient of 3.2–4.3. However, the collagen binding affinity of PEDF for extracellular matrix may be even higher for that of individual extracellular matrix molecules, as its affinity for collagen increases when the glycosaminoglycan-binding site of PEDF is neutralized and vice versa and could even change with the pH of the extracellular matrix. Therefore, qualitative and quantitative changes of extracellular matrix molecules, e.g. collagen, glycosaminoglycans, as well as pH changes of extracellular matrix, which occur through development, with aging and in certain pathologic conditions, such as corneal dys
Mapping the Type I Collagen-binding Site on Pigment Epithelium-derived Factor: IMPLICATIONS FOR ITS ANTIANGIOGENIC ACTIVITY
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