Pigment Epithelium-derived Factor Behaves Like a Noninhibitory Serpin

NEUROTROPHIC ACTIVITY DOES NOT REQUIRE THE SERPIN REACTIVE LOOP*

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S. Patricia Becerra‡§, Alessandra Sagasti‡, Patricia Spinella¶, and Vicente Notario

From the §Laboratory of Retinal Cell and Molecular Biology, National Eye Institute, National Institutes of Health, Bethesda, Maryland 20892, ¶Protein Expression Laboratory, Office of Intramural Research, Office of the Director, National Institutes of Health, Bethesda, Maryland 20892, and ©Department of Radiation Medicine, Georgetown University Medical Center, Washington D.C. 20007

Pigment epithelium-derived factor (PEDF), a neurite-promoting factor, has an amino acid primary structure that is related to members of the serine protease inhibitor (serpin) family. Controlled proteolysis of native PEDF (50 kDa) with either trypsin, chymotrypsin, elastase, or subtilisin yields in each case one major limited product of 46 kDa as analyzed by SDS-polyacrylamide gel electrophoresis. N-terminal sequence analysis of the isolated 46-kDa products indicates a favored cleavage region located toward the C-terminal end of PEDF. A proteolysed PEDF protein reaction mixture reveals two overlapping sequences: that of the N terminus of intact PEDF and that of an internal region, consistent with cleavage of PEDF about position 382. These data indicate that PEDF protein has a globular conformation with one protease-sensitive exposed loop that contains the homologous serpin-reactive site. Cleavage within the reactive-site loop of PEDF does not cause a conformational change in the molecules (the stressed (S) → relaxed (R) transition) and results in heat denaturation identical to its native counterpart. This lack of conformational change is also seen upon cleavage within the reactive-site loop of the noninhibitory serpin ovalbumin. Furthermore, the PEDF neurite-promoting function is not lost with cleavage of the exposed loop. Recombinant PEDF polypeptide fragments with larger truncations from the C-terminal end show neurotrophic activity. Our results clearly indicate that integrity of the PEDF homologous serpin reactive center is dispensable for neurotrophic activity. Thus, the PEDF induction of neurites must be mediated by a mechanism other than serine protease inhibition. Altogether our data indicate that PEDF belongs to the subgroup of noninhibitory serpins and that its N-terminal region confers a neurite-promoting activity to the protein. The neurotrophic active site of PEDF is separated from the serpin reactive-site loop, not only in the primary structure, but also in the folded protein structure.

PEDF1 was first described as a neurite-promoting factor that is released by human fetal retinal pigment epithelial (RPE) cells. It was reported that PEDF isolated from medium conditioned by human fetal RPE primary cultures promotes neurite outgrowth in cultured human retinoblastoma Y-79 cells (1). Information about the PEDF peptide sequence has permitted the isolation and cloning of a human PEDF cDNA (2). From cDNA clones, expression vectors were constructed (3, 4) and, in turn, specific antisera to PEDF were developed from the recombinant PEDF proteins (4, 5). Antiserum Ab-rPEDF has been instrumental in the identification of PEDF protein in physiological sources. PEDF is present in bovine eyes in the interphotoreceptor matrix (IPM), i.e. the extracellular matrix located between RPE and the neural retina, and is the sole IPM component responsible for the IPM neurite-promoting activity (5). In addition to the effect on retinoblastoma cells, PEDF has the capacity of promoting neuronal survival of primary cerebellar granule cell neurons (6). These observations support the idea that PEDF is secreted from the RPE and has a neurotrophic effect on retinal cells.

PEDF is a 50-kDa glycoprotein with a sequence homologous to members of the serpin family (2, 4, 5). Sequence analysis of the 418 amino acids in the human PEDF demonstrates a 27% identity to α1-antitrypsin, the serpin prototype. However, no inhibitory function of PEDF against serine proteases has yet been demonstrated. Serpins constitute a group of >40 proteins thought to share the same overall tertiary structure (7). Analysis of the serpin folded protein structure in solution indicates that the C-terminal region of all serpins has an exposed peptide loop that is highly susceptible to proteolysis. In the case of inhibitory serpins, the serpin-reactive site, P1, is located within the exposed loop, and the bond between residues at positions P1 and P1′ is cleaved by the target protease (where known). Cleavage of an inhibitory serpin by its target protease induces a change in conformation from stressed to relaxed (S → R) as revealed by an increase in stability to denaturation. The residue at position P1 binds at the primary specificity pocket of the target protease, and the protease and serpin form a complex that impairs further proteolytic activity. It is believed that the amino acid in position P1 helps define the specificity of inhibition for serpins. Thus, the serpin reactive center is a well defined structural-functional characteristic of serpin proteins. Not all serpins have a demonstrable inhibitory activity against a serine protease, e.g. chicken ovalbumin (8), rat angiotensinogen (9), and barley Z-protein (10) have no reported inhibitor

RPE, retinal pigment epithelium; Ab-rPEDF, rabbit polyclonal antiserum to human recombinant PEDF; PN-1/GDN, protease nexin-1/gliadervied nexin; HClI, heparin cofactor II; PAGE, polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography; HPLC, high pressure liquid chromatography; kb, kilobase pair(s).
activities and sequence comparison reveals that they are also members of the serpin family. As opposed to the inhibitory serpins, ovalbumin and angiotensinogen do not undergo serpin conformational change. The human PEDF sequence has amino acid Leu in position P₁, known to be specific for inhibition of chymotrypsin and chymotrypsin-like activities; however, the recombinant PEDF protein (positions 44–418) does not demonstrate inhibition against these activities (3). As in ovalbumin, angiotensinogen, and some dysfunctional serpins, PEDF has residues on the N-terminal side of the P₁ residue that are considered unfavorable for the insertion of the serpin loop into the A- sheet of the folded serpin protein. Incorporation of the serpin-exposed loop as an additional strand into the A- sheet explains the serpin conformational change S → R that establishes the inhibitory status of a serpin.

In this study we have investigated the overall conformation of PEDF protein and its inhibitory/substrate status. We have used native PEDF protein purified from bovine eyes (5) and recombinant PEDF polypeptides derived from a human PEDF expression vector, pRC-BH (3). Limited proteolysis of native PEDF with several classes of proteases showed that it is consistently vulnerable to cleavage at sites around the homologous P₁ site (position 382) leaving most of the molecule resistant to proteolysis. We show that, unlike inhibitory serpins, the thermal stability of PEDF did not increase upon cleavage at its exposed loop. PEDF without its exposed loop and even without larger segments from its C-terminal end retained its neurite outgrowth-inducing activity. From our results we conclude that PEDF indeed has a folded protein structure in solution typical of serpins and belongs to the subgroup of noninhibitory serpins. The N-terminal region of PEDF contains a neurotrophic active site which is distal from the serpin reactive loop. The structure-function relationships of the PEDF protein are discussed.

EXPERIMENTAL PROCEDURES

Materials—Bovine pancreas trypsin (i.e., 1-tosylamido-2-phenylthyl chloromethyl ketone-treated), bovine pancreas α-chymotrypsin (1-chloro-3-tosylamido-7-aminoo-2-heptanone-treated) were from Worthington; human neutrophil elastase was from Calbiochem; human plasma thrombin was from Sigma; Badillus subtilis subtilisin, Staphylococcus aureus V8 endoproteinase Glu-C, Lysobacter enzymogenes endoproteinase Lys-C, and restriction enzymes Asp718 and XhoI were from Boehringer Mannheim.

Preparation of PEDF Polypeptides—Native PEDF protein was purified from the IPM of fresh bovine eyes as described below (5). Briefly, IPM protein extract was subjected to 45–80% ammonium sulfate fractionation. The precipitated proteins were resuspended and dialyzed against 100 mM NaCl, 50 mM sodium phosphate, pH 6.5, 1 mM dithiothreitol, 10% glycerol, and layered onto a Mono-S column (HR 5/5) on an FPLC system. PEDF was eluted with a linear gradient of 0–100 mM NaCl in the above buffer with a flow rate of 0.8 ml/min. Finally, the PEDF-containing fractions were pooled and subjected to gel filtration chromatography attached to an HPLC system (Gold Beckmann) using a 7.5 mm × 60-cm TSK-Gel G3000SW HPLC column (Toyo Soda, Inc.), equilibrated with phosphate-buffered saline, pH 7.4, at a flow rate of 1 ml/min. PEDF protein eluted as a single peak with a retention time immediately behind that for ovalbumin (M, 43,000). The final PEDF sample was stored at −80°C. Sequence analysis on the purified protein indicated that the N terminus of the mature bovine PEDF protein started at position 23 of the human PEDF precursor sequence.

Recombinant PEDF polypeptides were produced in Esherichia coli from expression vectors containing human PEDF cDNA fragments. Preparation of DNA fragments, ligation reactions, and bacterial transformations were performed as described before (11). Deletion mutant plasmids were derived from pRC-BH, an expression vector with PEDF coding sequences generated by shuttle plasmid cloning (3). Plasmid pRC-BH (5.8 kb) was digested with PvuII and the ends of the 3.2-kb DNA fragment self-ligated to obtain pRC-BP. Plasmid pRC-BH was digested with XhoI and HindIII, their ends filled in with Klenow fragment for a blunt-end ligation to produce pRC-BX (5.2 kb). Plasmid pRC-BH was digested with Asp718 and HindIII, their ends filled in with dNTPs and Klenow fragment followed by blunt-end ligation to obtain pRC-BA (4.8 kb) DNA. PEDF amino acid positions in the expression plasmids were as follows: BH, 44–418; BP, 44–267; BX, 44–228; BA, 44–121. Expression of PEDF genes in bacteria, cell lysis, and protein isolation from inclusion bodies were performed as described before for pRC-BH (3). Polyacrylamide gel bands BP and BX were further purified by S-Sepharose chromatography in 4 mM urea in 50 mM phosphate buffer, pH 6.5, and eluted with a NaCl linear gradient. Fractions containing BP and BX were pooled and stored at −80°C.

Proteolytic Reactions of PEDF—Trypsin and a-chymotrypsin reaction mixtures contained 20 mM Tris-Cl, pH 7.5, 150 mM NaCl, and 1 mM EDTA. Subtilisin, endoproteinase Glu-C, endoproteinase Lys-C, and elastase reaction mixtures contained 20 mM Tris-Cl, pH 8.8, and 10% glycerol. Protein amounts, incubation times, and temperatures were indicated. The reactions were stopped by freezing in dry ice and the addition of SDS-PAGE sample buffer. Before use, the activity of each protease was confirmed by assaying cleavage of a polypeptide substrate of 39 kDa used previously in limited proteolysis (12), followed by SDS-PAGE analysis of products.

Thrombin Inhibition Solution Assays—Thrombin activity was assayed against tosyl-glycyl-prolyl-arginine-4-nitroanilide acetate (Chromozym TH; Boehringer Mannheim) following the manufacturer’s protocol. Reactions were conducted at 25°C in 50 mM Tris-Cl, pH 8.3, and 200 mM NaCl, with 35 ng/ml thrombin and a substrate concentration of 0.12 μM. Reaction mixtures were in a volume of 60 μl. Incubation was monitored on a Beckman DU-30 spectrophotometer at 405 nm. The rates of hydrolysis of substrate were found to be linear, at about 0.044 OD₅₄₀ rng/min. For inhibition assays, thrombin was preincubated with PEDF in PBS (20 μl) at 37°C for 1 h, in the absence or presence of heparin. Preincubation periods longer than 3 h inactivated thrombin by more than 50%. The final concentration of heparin in the reaction mixture was 7 units/ml, and that of PEDF varied between 17.5 and 70 ng/ml.

Preparation of Cleaved PEDF—PEDF was treated with subtilisin at 1:60 (w/w) for 30 min at 25°C. PEDF cleavage was confirmed by SDS-PAGE. The reaction was stopped by addition of 9 mM phenylmethylsulfonyl fluoride (final concentration) when used in heat stability assays. PEDF was used by resuspending it 1:50 (w/w) at 25°C for 20 min when used in neurite outgrowth assays. Trypsin treatment of PEDF was at a ratio of 1:100 (w/w) for 120 min at 25°C. PEDF cleavage was confirmed by SDS-PAGE analysis and N-terminal sequencing of the products. Cleaved PEDF was separated from trypsin and other products by gel filtration on a TSK-3000 column attached to an HPLC system. Cleaved PEDF was then concentrated by ultrafiltration using Centricon-30 (Amicon) concentrators.

Heat Stability Assays—Aliquots of PEDF and cleaved-PEDF protein were each incubated at 15 μg/ml for the subtilisin-treated and at 150 μg/ml for the trypsin-treated samples, in 75 mM Tris, 75 mM glycine, 75 mM sodium phosphate, pH 7.6, at increasing temperatures between 30 and 100°C for 2 h as described before (13). Proteins were fractionated by centrifugation at 14,000 × g for 30 min at 4°C. Residual soluble PEDF protein was quantified by densitometry of immunoblot analysis with Ab-I-PEDF using 4-chloro-1-naphthol as the color development reagent (5).

Sequence Analysis—N-terminal sequence determination of peptides was performed using Applied Biosystems model 477 sequencer following the manufacturer’s protocols. Amino acid sequencing of PEDF proteins was carried out on 7–10 μg of protein in solution. Sequence determination of PEDF proteolytic fragments was performed on protein that had been resolved by SDS-PAGE, transferred to Immobilon poly(vinylidene difluoride) membrane (Millipore), and stained briefly with Coomassie Blue (14). The membrane pieces containing stained bands were subjected to amino acid sequencing.

Neurite Outgrowth Analysis—Human Y-79 retinoblastoma cells were grown and treated for neurite outgrowth as described in Becerra et al. (3). Addition of effectors to 2-m1 cultures was as indicated and at a final PEDF concentration as indicated. The differentiation state of the cultures was monitored by light microscopy at intervals after attachment.

Other Methods—Protein concentration was determined by the Bradford assay. Protein concentration was also determined by the Bio-Rad protein assay kit. SDS-PAGE of proteins was performed according to Laemmli (16). All protein markers were from Bio-Rad.
from bovine eyes. By incubation with trypsin or α-chymotrypsin at a protease:substrate ratio of 1:100 (w/w), native PEDF of 50 kDa was digested in a one-step fashion into a product of 46 kDa, i.e., a decrease of 4 kDa. Complete substrate utilization was achieved by 120 min (Fig. 1, lanes 4 and 7). A band corresponding to the released low molecular weight peptide was not readily identified by Coomassie Blue staining of the gel.

To determine the position(s) of the cleavage sites, isolated tryptic and chymotryptic 46-kDa fragments were subjected to automated amino acid sequencing from their N termini. Both fragments shared the same sequence with the undigested PEDF protein (Table I). Sequence analysis on the intact protein indicated that the N terminus of bovine PEDF starts at position 23 of the human PEDF precursor sequence. Note that there is a basic amino acid, Arg, at position 48 that is a potential site for trypsin cleavage, and two aromatic amino acids, Phe-Phe, at positions 46 and 47 that are potential sites for α-chymotrypsin cleavage. To determine the sequence of the low molecular weight fragment, analysis was performed on total reaction mixtures of PEDF treated with trypsin or α-chymotrypsin (at a ratio of 1:100 for 120 min). Two overlapping sequences were obtained, one corresponding to the N terminus of intact PEDF starting at Asp$^{23}$ and the other corresponding to the internal C-terminal region starting at positions 382 and 383 for the tryptic and chymotryptic fragment, respectively (Table I). Note that the internal sequence (20 amino acid residues) shares 95% identity with the human PEDF (see Fig. 7).

This sequence has potential sites for chymotrypsin cleavage, e.g., amino acids Phe (positions 384, 395, and 397), Leu (positions 386 and 390), and for trypsin cleavage, e.g., Arg (position 399). These results clearly indicate that the 46-kDa fragments represent the N-terminal domain of the protein and that limited tryptic and chymotryptic cleavage sites are located between positions 381/382 and 382/383 of the PEDF molecule. Position 382 maps to the homologous P$_1$ site and is occupied by amino acid Leu.

Proteolytic digestion of PEDF with subtilisin at a protease:substrate ratio of 1:120 generated a 46-kDa fragment, and within 60 min of proteolysis, most of the PEDF substrate molecules were converted to product (Fig. 2, lanes 2 and 3). Sequence analysis of the isolated 46-kDa fragment indicated that it started at Thr$^{32}$ (Table I). Treatment with elastase, at a protease:PEDF ratio of 1:1, also generated a 46-kDa polypeptide fragment (data not shown), and sequence determination of the 46-kDa elastase fragment revealed that two-thirds of the molecules began at Asp$^{23}$ and one-third at Ser$^{30}$ (Table I). The loss of nine or seven amino acid residues cannot account for an overall size reduction of ~4 kDa. This implies that PEDF has limited cleavage sites for subtilisin and elastase located about position 380, i.e., around the P$_1$ position. However, treatment of PEDF with endoproteinase Glu-C or endoproteinase Lys-C, at a higher protease:substrate ratio (1:24 and 1:57, respectively) than with subtilisin, did not generate a size reduction of the 50-kDa PEDF fragment (Fig. 2, lanes 4–7). The N-terminal sequence of the 50-kDa endoproteinase Glu-C and 50-kDa endoproteinase Lys-C fragments started at Ala$^{28}$ and at Asp$^{23}$, respectively (Table I). Note that there are several acidic amino acids, Asp and Glu, at more internal positions, 34, 41, 42, 43,

![Fig. 1. Controlled proteolysis of PEDF with trypsin or chymotrypsin.](image)

**TABLE I**

| Protease       | Size of peptide$^a$ | N-terminal sequence$^b$ |
|----------------|---------------------|-------------------------|
| Undigested     | 50                  | $^{23}$DAQGEAGSLTPES?GAPVEEDPFFRV |
| Trypsin$^c$    | 46                  | $^{23}$DAQGEAG |
| (1/3)          |                     | $^{36}$LTFFLDYHLNQPFIFVL?DT |
| (2/3)          | <5                  | $^{23}$DAQGEAG |
| Chymotrypsin$^c$| 46                  | $^{36}$TFFLDYHLNQPFIFVL?DT |
| (1/3)          |                     | $^{32}$TPES |
| (2/3)          | <5                  | $^{24}$AGSLTPES |
| Elastase$^c$   | 46                  | $^{23}$DAQGE |
| (2/3)          |                     | $^{34}$SLTPES |
| (1/3)          | 46                  | $^{23}$DAQGEAG |
| Subtilisin     | 46                  | $^{36}$LTFFLDYHLNQPFIFVL?DT |
| Endoproteinase Glu-C | 50                  | $^{23}$DAQGE |
| Endoproteinase Lys-C | 50                  | $^{34}$SLTPES |

$^a$ Estimated from SDS-PAGE.

$^b$ Amino acids are given in single letter code. Numbering corresponds to the derived sequence from a human PEDF cDNA where residue 1 is the first position derived for the precursor PEDF polypeptide (2) and position 23 corresponds to the first residue of the mature bovine protein (5).

$^c$ Two N-terminal sequences were obtained, parentheses indicate the molar ratio of their first amino acid.
and 44, that are potential sites for endoproteinase Glu-C cleavage. Thus, sequence and size analysis of these fragments revealed that the protease-sensitive region of bovine PEDF does not contain Asp, Glu, or Lys. The limited tryptic site and the lack of an endoproteinase Lys-C site suggests that bovine PEDF has Arg around its P1 position. In contrast, the known human sequence has Asp and lacks Arg around P1.

PEDF Is Not a Substrate or an Inhibitor of Thrombin—The PEDF and heparin cofactor II (HCII) sequences have Leu at position P1. HCII forms a complex with thrombin and the rate of inhibition of thrombin by HCII increases 1000-fold when heparin or dermatan sulfate is present (17). At the same time, thrombin is the physiological target for the neurotrophic serpin protease nexin-1/glia-derived nexin (PN-1/GDN) that has Arg at position P1 (18). Therefore, it was of interest to examine whether PEDF would form an inhibitory complex with thrombin. Incubation of PEDF with thrombin in the absence or presence of heparin did not generate a complex or a proteolytic product (Fig. 3). In solution assays of thrombin inhibition, preincubation of thrombin with PEDF did not decrease the rate of hydrolysis of the thrombin substrate, not even when heparin was present (data not shown). Thus, PEDF did not behave as an inhibitor of thrombin.

S → R Transition Assay—From the information above, the preferred cleavage site on PEDF for several proteases falls within the serpin-exposed loop and specifically for trypsin next to the homologous P1 site. Inhibitory and noninhibitory serpins can be classified a priori by the thermal stability properties of their unmodified and cleaved species. The stability to heat of intact PEDF protein and trypsin-PEDF was assayed and is shown in Fig. 4. In solution assays of thrombin inhibition, preincubation of thrombin with PEDF did not decrease the rate of hydrolysis of the thrombin substrate, not even when heparin was present (data not shown). Thus, PEDF did not behave as an inhibitor of thrombin.

Biological Activity of Cleaved PEDF—To investigate the role for the PEDF serpin-exposed loop in the neurite-promoting function, we tested PEDF cleaved at its exposed loop for induction of neurite outgrowth in human retinoblastoma Y-79 cells in culture. Samples of cleaved PEDF were prepared by limited proteolysis with subtilisin or trypsin as above. The subtilisin reaction mixture containing the cleaved PEDF 46-kDa fragment (positions 32–380) was incubated at 75 °C for 20 min to heat-inactivate the protease and used for analysis. The results showed that cleaved PEDF induced morphological differentiation in Y-79 cell cultures, as did the unmodified PEDF protein (Fig. 5). Induction of neurite outgrowth from the Y-79 cell cultures was also observed with the 46-kDa tryptic fragment.
Incubation at 75°C did not inactivate the PEDF protein from differentiating the retinoblastoma cells. These observations showed that integrity of the serpin-exposed loop is dispensable for neurotrophic activity. Thus, action as a serpin inhibitor is not what mediates the PEDF-induced neurotrophic activity.

Truncation of the C-terminal Region of PEDF Does Not Affect Biological Activity—From the above observations the core of the PEDF protein (positions 32–381) is sufficient for inducing neurite outgrowth. Also, it has been shown that recombinant PEDF (amino acid positions 44–418) showed neurotrophic activity (3). To map the location involved in neurite outgrowth we created expression constructs of human PEDF sequence with further truncations from the C-terminal end: BP (positions 44–267), BX (positions 44–229), and BA (positions 44–121).

The resulting expression vectors produce polypeptides of 223, 185, and 77 PEDF residues, respectively. Purification of BP, BX, and BA was carried out from the inclusion bodies of their hosts, followed by S-Sepharose column chromatography for BP and BX in 4 M urea to maintain the peptides in solution. BP and BX were eluted with a linear gradient, and peak fractions containing pure BP and BX, as well as BA in inclusion body extracts, were used for analysis. The results showed that BP, BX, and BA exhibit neurite outgrowth-inducing activity (Fig. 6). Thus, even when large segments from the C-terminal end of PEDF were deleted, the neurotrophic activity is retained.

**DISCUSSION**

Analyses of the primary structure of the human PEDF sequence predict that the PEDF protein shares the common
serpin tertiary structure. By automated assembly of protein blocks for data base searching (19) the human PEDF protein sequence shows five high scoring peptide blocks that align with more than 50 serpin sequences (data not shown). This represents a strong relationship for PEDF to the serpin family. In addition, its linear sequence (418 residues) contains small insertions and deletions that are compatible with the three-dimensional serpin model, i.e. α₁-antitrypsin, and the highly conserved residues considered critical for maintaining the serpin spatial structure (7). However, the actual folded protein conformation of native PEDF has not yet been investigated. In the studies presented here, we have used native bovine PEDF protein, which based on its strong immunoreactivity with antiserum against human PEDF is presumed to share sequence homology with the human protein (5). Our data confirm that the bovine and human sequences are highly homologous within an internal region (Fig. 7). Controlled proteolysis has proven successful in analyzing the structural conformation of proteins in solution and, in particular, the common overall structure of serpins (20). Analysis of the PEDF products from several proteases indicates that the overall native conformation of PEDF protein includes a highly protease-resistant core consisting of most of the residues from the N-terminal region, and a protease-sensitive area located around the homologous serpin-reactive site P₁ (Table I). P₁ is occupied by Leu at position 382 in the bovine and human sequences. Our limited proteolysis data demonstrate that native PEDF has the common serpin structure, i.e. a globular conformation with an exposed loop located at the C-terminal end of the molecule. A circular dichroism spectrum confirms that native bovine PEDF protein...
contains 35% β structures as shown for the folded structure of serpins (data not shown). Thus, not only the linear but also the folded protein conformation of PEDF in solution is homologous to the serpin family of proteins.

There are now two serpins that are known to be neurotrophic factors, PN-1/GDN and PEDF. PN-1/GDN promotes neurite outgrowth from different neuronal cell types, including neuroblastoma as well as primary hippocampal and sympathetic neurons (21, 22), and rescues motor neurons from naturally occurring and axotomy-induced cell death (23). The physiological target for PN-1/GDN has been identified as thrombin (18, 24). Sequence comparison between PEDF (2) and PN-1/GDN (25) reveals 23% identity and 48% homology (3). Human and rat PN-1/GDN have Arg in position P1, that is specific for thrombin inhibition; while both human and bovine PEDF have Leu in P1 (Fig. 7), specific for chymotrypsin. However, PEDF (native or recombinant) is not an inhibitor of α-chymotrypsin3 (3). The effect of PEDF on thrombin was investigated since HCII, a serpin with Leu in P1, changes its target specificity to thrombin when in the presence of sulfated polysaccharides (17). In contrast to PN-1/GDN, we have found that PEDF does not decrease the hydrolysis of thrombin substrate or form serpin: proteinase complexes with thrombin, even in the presence of sulfated polysaccharides (Fig. 3). Thus, PEDF is not an inhibitor of thrombin and is structurally and biochemically distinct from the well-established neurotrophic serpin PN-1/GDN.

To establish the substrate/inhibitor status of PEDF, our approach was based on the fact that cleavage of inhibitory serpins at or near the reactive site is followed by a dramatic transformation of structure from a stressed (S) to a relaxed (R) form (26). For example, cleavage of the reactive center loop of PN-1/GDN, α1-antitrypsin and antithrombin III gives the typical stressed to relaxed (S → R) change in thermal stability (27, 28). As opposed to inhibitory serpins, the noninhibitory serpins do not undergo the S → R conformational change, as detected by heat stability, transverse urea gels, and even more quantitative 1H and 31P NMR spectroscopic data on native and cleaved ovalbumin and angiotensinogen species (13, 29, 30). Comparison of x-ray structures of cleaved forms of α1-antitrypsin and ovalbumin supports the conformational change specific for inhibitory members. The two residues that constitute the reactive center, P1′ and P1, in the native α1-antitrypsin are located 67 Å apart in the cleaved species while they remain close in the cleaved ovalbumin (31). However, this lack of S → R transition for noninhibitory serpins has an exception. The hormone carriers, cortisol- and thyroxine-binding globulins, are reported to undergo serpin conformational change upon cleavage (32). It has been proposed that, in these two noninhibitory serpins, the S → R change is utilized to modulate a different property of the protein, such as transport in the hormone carrier globulins. Thermal stability curves for unmodified and cleaved PEDF reveal that PEDF lacks the S → R conformational change upon cleavage at its exposed loop (Fig. 4). Interestingly, PEDF becomes thermodynamically unstable at lower temperatures than ovalbumin with denaturation temperatures differing by 20 °C. The thermal stability curves for PEDF resemble more the ones for angiotensinogen than the ones for ovalbumin as reported by Stein et al. (13) and presume that PEDF and angiotensinogen (29) have a greater tendency to denature or unfold than ovalbumin. The lack of S → R transition for PEDF may be explained by the presence of unfavorable residues in its P12 region (3). Recently, Pemberton et al. (33) have reported that a newly identified serpin, maspin, is not a protease inhibitory serpin. Similar to the study presented here, the authors show that the tumor suppressor maspin does not undergo the S → R transition or inhibit trypsin-like serine proteases. In PEDF, as in angiotensinogen, ovalbumin, and maspin, the S → R transition may serve no useful purpose and therefore has been lost by evolutionary change. Thus, PEDF behaves like a typical noninhibitory serpin.

Our data demonstrate that while the C-terminal exposed loop is dispensable, the N-terminal region of PEDF confers a neurotrophic function to the protein (Figs. 5 and 6). Consequently, the neurotrophic induction must be mediated by other than PEDF serpin inhibition. Experiments with cerebellar granule cells have also shown that PEDF polypeptide fragments lacking the exposed loop have a neurotrophic survival effect (6, 34). From these observations two distinct regions are identified on the PEDF primary structure: 1) a proximal region (BA) with a neurotrophic domain located within residues 44–121 and 2) a distal region with an exposed loop around position P1 (Leu182) (Fig. 7). The sequence of the proximal region is apparently unique, with the highest degree of homology to members of the serpin family (20–30%). Spatial models for PEDF based on the three-dimensional structures of α1-anti-
trypsin and ovalbumin (7, 35) show that the proximal BA region, composed of putative α-helices A, B, C, and part of D, is located to the opposite pole from the exposed loop. This model is in agreement with the fact that the PEDF neurotrophic activity is not lost with cleavage of the exposed loop. Thus, in the folded protein structure of PEDF the neurotrophic domain is also separated from the exposed loop.

Altogether, the results presented here demonstrate that PEDF has the protein conformation of a serpin, but inhibition of serine proteases by PEDF cannot account for its neurotrophic activity. The initial biochemical step(s) for the complex biological effect of neurite outgrowth is still unknown. However, the relationships between structure of PEDF and its biological function suggest that the mode of action would include the interaction(s) between peptide residues from its proximal BA region and molecules associated to the membrane of target cells that would then trigger subsequent events of signal transduction.

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