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Recombinant human pigment epithelium-derived factor (PEDF): Characterization of PEDF overexpressed and secreted by eukaryotic cells

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Abstract

Pigment epithelium-derived factor (PEDF) is a serpin found in the interphotoreceptor matrix of the eye, which, although not a proteinase inhibitor, possesses a number of important biological properties, including promotion of neurite outgrowth and differential expression in quiescent versus senescent states of certain cell types. The low amounts present in the eye, together with the impracticality of using the eye as a source for isolation of the human protein, make it important to establish a system for overexpression of the recombinant protein for biochemical and biological studies. We describe here the expression and secretion of full-length glycosylated human recombinant PEDF at high levels (>20 μg/mL) into the growth medium of baby hamster kidney cells and characterization of the purified rPEDF by circular dichroism and fluorescence spectroscopies and neurite outgrowth assay. By these assays, the recombinant protein behaves as expected for a correctly folded full-length human PEDF. The availability of milligram amounts of PEDF has permitted quantitation of its heparin binding properties and of the effect of reactive center cleavage on the stability of PEDF towards thermal and guanidine hydrochloride denaturation.

Keywords: circular dichroism; fluorescence enhancement; heparin binding; mammalian overexpression; pigment epithelium-derived factor; serpin

Human pigment epithelium-derived factor (PEDF) is a secreted protein shown from its cDNA sequence to be a member of the serpin superfamily (Pignolo et al., 1993; Steele et al., 1993). The PEDF cDNA sequence predicts a precursor polypeptide of 418 residues, with a secretion signal peptide (residues 1–17) and one N-linked glycosylation site, Asn-Leu-Thr (residues 285–287). The protein was first identified as a product secreted by cultured pigment epithelium cells from fetal human retina (Tombran-Tink et al., 1991). It has also been identified as an extracellular component of the adult bovine eye in the retinal interphotoreceptor matrix (Wu et al., 1995) and in the vitreous and aqueous humors (Wu and Becerra, 1996). Several reports have demonstrated that PEDF is a neurotrophic factor that induces a neuronal phenotype in retinoblastoma cells (Tombran-Tink et al., 1991; Becerra et al., 1993; Steele et al., 1993; Wu et al., 1995) and promotes neuronal survival in cerebellar granule cell cultures (Taniwaki et al., 1995). PEDF gene expression has also been reported in a human fibroblast-like cell line from fetal lung (WI-38) (Pignolo et al., 1993), where the gene was shown to be differentially expressed in young versus senescent WI-38 cells, with 100-fold higher levels in the young cells. Low levels of PEDF protein were identified in the medium of WI-38 cells. However, the specific role of PEDF in this in vitro aging system is still unknown. These reports support a role for PEDF in development, survival, and maintenance of neuronal cells.

Given the important biological properties of PEDF, it is important to have an abundant source of PEDF for detailed biological, biochemical, and structural studies. Although the bovine protein can be isolated from the interphotoreceptor matrix and vitreous humor of the eye (Wu et al., 1995; Wu and Becerra, 1996), the yield is relatively low (1.3–7.2 μg per eye) and such a source is unlikely to be feasible for large-scale preparation of the human protein.

We describe here the high-level overexpression, purification, and characterization by biophysical, biochemical, and biological means of human rPEDF secreted by stably transfected baby ham-

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Abbreviations: BHK, baby hamster kidney; CD, circular dichroism; EPC-1, early PDL c-DNA-1; PDL, population doubling level; PEDF, pigment epithelium-derived factor; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonylfluoride.

ster kidney (BHK) cells. Yields of purified rPEDF of ~20 mg per liter of medium have been obtained. We found that the recombinant protein had properties consistent with correctly folded full-length PEDF, by all the criteria used, and that the reduction in its stability upon cleavage paralleled that of ovalbumin, another member of the serpin superfamily, treated similarly. Heparin binding was shown to give a 17% enhancement in tryptophan fluorescence. The affinity of rPEDF for heparin was also determined and shown to be comparable to that for other heparin–protein interactions in which the interaction is dominated by ionic interactions.

Results

Expression and purification of PEDF

Stable BHK cell transfectants were cycled between serum-containing medium and serum-free medium, as described in Materials and methods. Medium was changed every 24 h and only the conditioned serum-free medium collected for analysis. The presence of PEDF protein was analyzed in serum-free medium from pMA-PEDF and pBK-PEDF stable BHK cell transfectants by SDS-PAGE and Western analysis (not shown). The level of rPEDF production for both cell lines increased to an approximate plateau by cycle 3, but was greater than threefold higher in medium from BHK[pMA-PEDF] than from BHK[pBK-PEDF] cells. The amounts of rPEDF and other proteins were determined by densitometric scanning of Coomassie Blue stained SDS-PAGE gels. At cycle six the intensities for the 50 kDa PEDF band were 50 and 14% of total protein in the medium for pMA-PEDF and pBK-PEDF, respectively (not shown). After purification, the amount of rPEDF corresponded to 20% of total protein in the pMA-PEDF medium but only 2.5% of that in the pBK-PEDF medium. These estimates of percentage of PEDF indicate that the pMA-PEDF transfectants contained much higher levels of rPEDF at a higher percentage composition, and with resulting higher percentage of recovery. We therefore chose the BHK[pMA-PEDF] expression system for the large-scale production of rPEDF. Recombinant PEDF was purified from the pooled medium of the pMA-PEDF–transfected BHK cells by ammonium sulfate precipitation and cation exchange chromatography, as described in Materials and methods. A yield of 23 mg of highly purified rPEDF per liter of BHK[pMA-PEDF] medium was obtained.

Characterization of recombinant PEDF

The purified rPEDF migrated as a protein of 50 kDa by SDS-PAGE under reducing conditions (Fig. 1a). The presence of carbohydrate in the recombinant protein was confirmed by an increase in gel mobility subsequent to N-glycosidase F treatment (Fig. 1b). N-terminal sequence determination of the recombinant PEDF resulted in a single sequence of NPASPPEEGSPD. This exactly matches the cDNA-derived pre-PEDF sequence starting at residue 21. It thus appears that the secreted protein is the full-length mature glycoprotein with ~5% carbohydrate and lacking only the first 20 residues, which encompass the hydrophobic leader. Alignment and comparison of the sequence of bovine PEDF with that of human PEDF, using the location of the N-terminus determined here, indicate that the mature human protein has two extra residues at the amino terminal end (Becerra et al., 1995; Perez-Mediavilla et al., 1996).

The circular dichroism spectrum of recombinant PEDF was as expected for a globular protein and was indistinguishable from that of PEDF isolated from bovine eyes (not shown), indicating that the recombinant protein is correctly folded. In addition, the CD spectrum was similar, though not identical, to that of another non-inhibitory serpin of similar size, ovalbumin (not shown). Deconvolution of the spectrum indicated that the differences between the ovalbumin and PEDF CD spectra are consistent with a smaller percentage of α-helix ($\Delta \sim 7\%$) and an increase in antiparallel β-sheet ($\Delta \sim 8\%$) in PEDF.

The tryptophan fluorescence emission spectrum was also consistent with a folded globular protein, having an emission maxi-

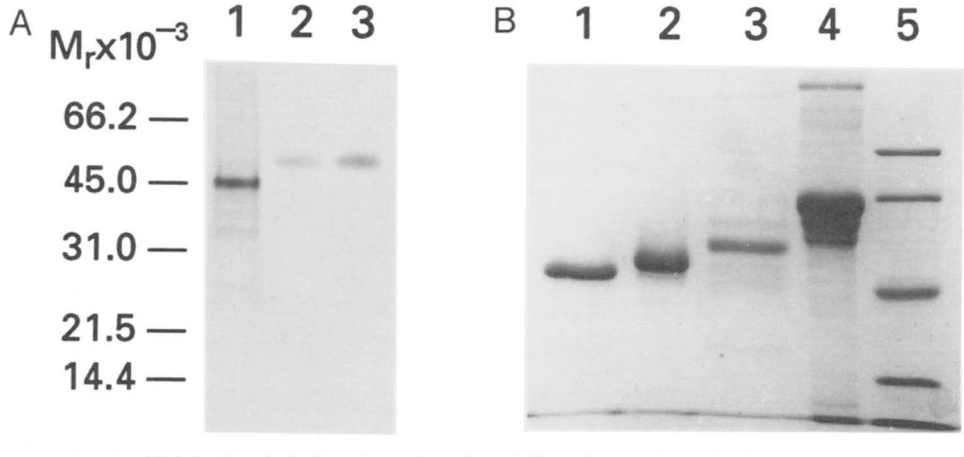

Fig. 1. Demonstration by SDS-PAGE of similar electrophoretic mobility of recombinant human and native bovine PEDF and of glycosylation of the recombinant human protein. (**A**) Lane 1, N-terminally truncated and non-glycosylated bacterially derived human PEDF (2.1 μg), isolated from inclusion bodies and corresponding to residues 44–418 (Becerra et al., 1993); lane 2, BHK cell-derived recombinant human PEDF (0.6 μg); lane 3, native bovine PEDF (1 μg) purified from the interphotoreceptor matrix of bovine eyes (Wu et al., 1995). (**B**) Increase in mobility of BHK-derived recombinant human PEDF following treatment with N-glycosidase F. Lane 1, N-glycosidase F-treated recombinant human PEDF; lane 2, untreated recombinant human PEDF; lane 3, N-glycosidase F-treated fetuin (a control reaction for the effect of removal of N-linked carbohydrate); lane 4, untreated fetuin; lane 5, molecular weight markers, phosphorylase (97.4 kDa), serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa).

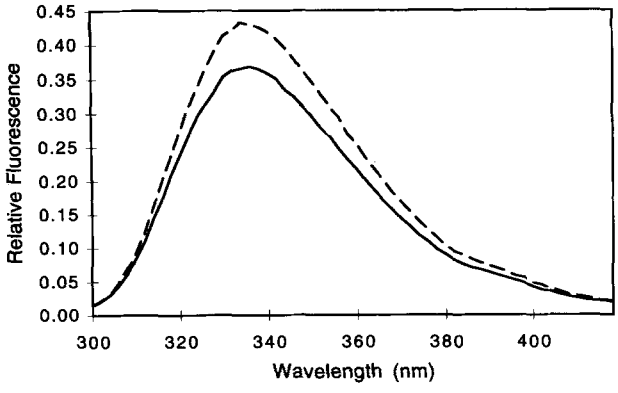

Fig. 2. Tryptophan fluorescence emission spectra of recombinant PEDF in the absence (solid line) and presence (dashed line) of heparin. PEDF, 0.7 μM, was in 20 mM sodium phosphate, pH 7.4. Upon addition of an excess of heparin, sufficient to saturate the heparin binding site (15 μM) an approximately 17% enhancement in fluorescence resulted, but without change in shape of the curve or the position of the maximum.

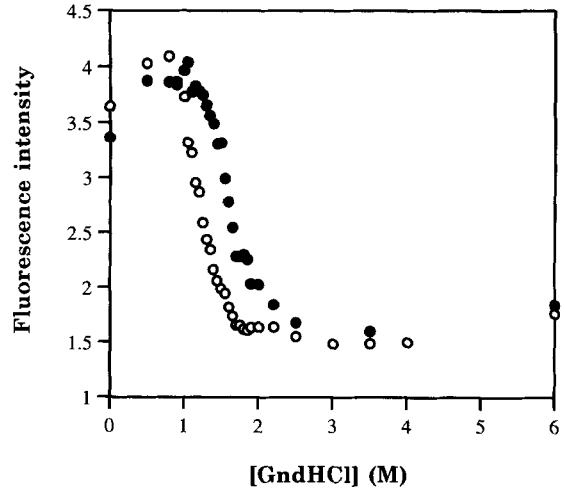

Fig. 3. Comparison of the stabilities of native and reactive center cleaved (by chymotrypsin) PEDF towards denaturation by guanidine hydrochloride. Filled circles, uncleaved human rPEDF; open circles, reactive center-cleaved human rPEDF. Separate samples were prepared, as described, at each guanidine hydrochloride concentration. The endogenous tryptophan fluorescence emission was monitored at 340 nm for each sample.

mum at 335 nm, compared to 348 nm for tryptophan in water (Lakowicz, 1983) (Fig. 2), which indicates at least partial burial of one or more of the three tryptophans present.

Effect of reactive center cleavage

It has been shown for PEDF isolated from bovine eyes that treatment with catalytic amounts of trypsin or chymotrypsin results in cleavage only within the reactive center region. This cleavage brings about neither an increase in stability nor a major change in secondary structure (Becerra et al., 1995), such as is seen for inhibitory serpins (Bruch et al., 1988; Gettins and Harten, 1988). rPEDF was also found to have similar limited and reactive center loop-specific susceptibility to proteolytic cleavage. Gradient SDS-PAGE confirmed that the recombinant PEDF treated with chymotrypsin had been specifically cleaved in the reactive center, by the release of a peptide of ~5 kDa and by a small increase in mobility for the residual PEDF band, consistent with cleavage close to the C-terminus (not shown). Reactive center loop cleavage gave a small but reproducible reduction in negative ellipticity at 220 nm in the CD spectrum of recombinant PEDF (not shown), consistent with a small loss of α-helix. The same type of change is seen for ovalbumin (not shown), where it is known from X-ray structures of the native and cleaved protein (Wright et al., 1990; Stein et al., 1991) that cleavage results in loss of the α-helix in the reactive center region, but without the other secondary structural changes that occur in inhibitory serpins and which involve insertion of the cleaved reactive center into β-sheet A as an additional strand.

Changes in the stability of cleaved and uncleaved PEDF towards unfolding by guanidine hydrochloride and towards heat denaturation were determined by fluorescence and CD spectroscopies, respectively. Both methods showed a small decrease in stability for cleaved rPEDF. The guanidine hydrochloride concentration for 50% unfolding was reduced from ~1.6 M for uncleaved rPEDF to ~1.2 M for cleaved PEDF (Fig 3). The T_m for thermal denaturation, determined from the midpoint of the change in CD ellipticity, was reduced by ~6.5 °C upon cleavage (Fig. 4), from 58 °C for uncleaved rPEDF to 51.5 °C for cleaved PEDF. These findings again parallel those for the non-inhibitory serpin ovalbumin (Shitamori and Nakamura, 1983). SDS-PAGE was used to confirm that no additional cleavage of chymotrypsin-treated rPEDF had occurred during the unfolding by any incompletely inactivated chymotrypsin present in the sample (not shown).

Biological activity of recombinant PEDF

PEDF secreted by human fetal retinal pigment epithelial cells in culture (Steele et al., 1993) or isolated from bovine eyes (Wu et al., 1995; Wu and Becerra, 1996) induces neuronal differentiation in retinoblastoma Y79 cells. Nanomolar concentrations of the native

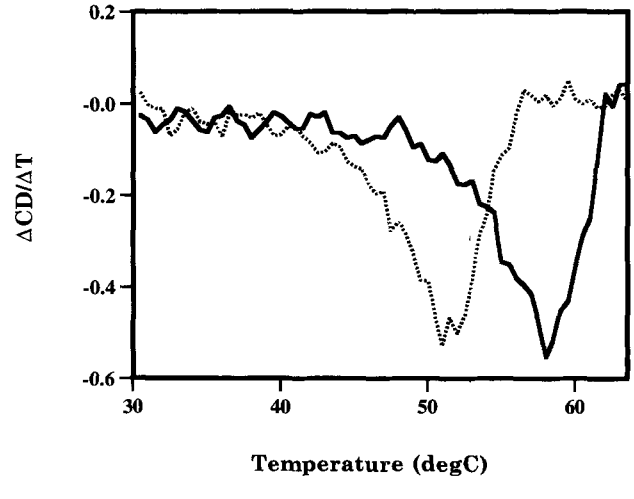

Fig. 4. Comparison of the thermal stabilities of native and chymotrypsin-cleaved PEDF followed by change in ellipticity at 222 nm. Solid line, uncleaved human rPEDF; dashed line, reactive center-cleaved human rPEDF. First derivatives of the temperature dependencies are plotted to more accurately show the mid-point of the unfolding transition, which correspond to the mimina.

protein are sufficient to induce neurite processes that are visible by light microscopy. Detection of processes starts at 3 days post-attachment and increases with time. Between days 7 and 11 post-attachment the processes are present in more than 50% of the treated cells. The levels of PEDF used in these assays (typically 1–4 nM) are even less than those found in the interphotoreceptor matrix (~250 nM) (Wu et al., 1995) or the vitreous humor (~32 nM) (Wu and Becerra, 1996) of the eye. Recombinant BHK-derived human PEDF was tested for its ability to promote such neurite outgrowth. Both recombinant PEDF and bovine PEDF isolated from interphotoreceptor matrix showed the same effect when added at the same concentration (Fig. 5). Processes were detected as early as 3 days post-attachment and extended with time. Processes were observed in 50% of cells by 7 days post-attachment. It has also been shown that bacterially derived rPEDF promotes neuronal survival in cerebellar granule cell cultures (Taniwaki et al., 1995) and also protects these neurons against glutamate-induced neurotoxicity (Taniwaki et al., 1996). The BHK-derived rPEDF afforded protection when added at 6.9 nM to the culture medium of cerebellar granule cells 30 min prior to addition of glutamate (J.P. Schwartz, pers. comm.), and is thus neurotrophically active. These properties collectively demonstrate that recombinant BHK-derived human PEDF has the same neurotrophic activity as natural PEDF.

Heparin–PEDF interactions

Recent experiments have shown qualitatively that PEDF binds to heparin and other glycosaminglycans (Alberdi and Becerra, 1996). Such binding may be of importance in vivo for surface localization of PEDF, as is the case for another specific heparin-binding serpin antithrombin (de Agostini et al., 1990). We therefore examined the strength of the heparin–PEDF complex using two different approaches.

The first approach used changes in PEDF tryptophan fluorescence upon complex formation with heparin. Addition of an excess of a defined length heparin (~30 sugars) to PEDF at I 0.05 gave an ~17% enhancement of fluorescence, but without a change in position of the emission maximum (Fig. 2). When the same heparin was titrated into rPEDF, a saturable increase in fluorescence was seen, with a maximum enhancement of ~17% (data not shown). Attempts to fit the data to a simple binding equation were complicated by a superimposed small time-dependent reduction in fluorescence intensity that may have been due to surface absorption at the low ionic strength used or to photodecomposition. Although fits could be obtained to the titration data, the confidence in the accuracy of the resulting K_d was not high.

To obtain more reliable binding data, an affinity partitioning method (Materials and methods), as modified by Olson et al. (Olson et al., 1991), was used to determine the K_d for the heparin–rPEDF complex. From measurements of the displacement of rPEDF from heparin–agarose by increasing concentrations of the same defined molecular weight high-affinity heparin a K_d of 4.1 $\pm$ 0.3 μM was determined by non-linear least-squares fitting of the data (Fig. 6). This K_d is three to four orders of magnitude weaker than for the same heparin to antithrombin under comparable conditions, but is very similar to that of low affinity heparin to antithrombin. For the latter, most of the binding energy is thought to come from relatively non-specific ionic interactions (Streusand et al., 1995), which is likely to also be the case for PEDF–heparin interactions.

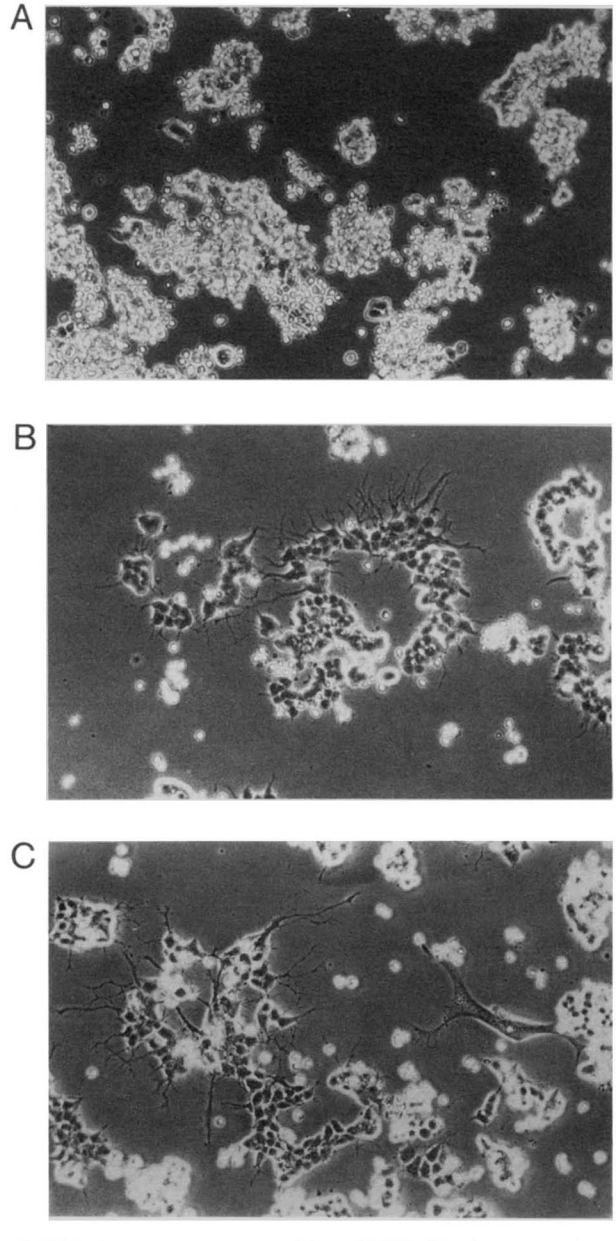

Fig. 5. Biological activity of recombinant PEDF. Neurite-outgrowth assays of purified recombinant human PEDF were performed on human retinoblastoma Y-79 cells. Two milliliters of Y-79 cell cultures (1.25×10^5 cells/mL) growing in serum-free medium were treated without PEDF (**A**); 200 ng recombinant human PEDF (**B**); or 200 ng native bovine PEDF purified from interphotoreceptor matrix (Wu et al., 1995) (**C**). After 7 days of treatment cells were attached to poly-D-lysine–coated plates. Morphological differentiation was monitored daily. Photographs of cells at nine days post-attachment are shown.

Discussion

We have shown here that the BHK cell system we have developed for stable expression of human PEDF results in secretion into the growth medium of correctly folded, glycosylated, and biologically active rPEDF at high levels and at high percentage of total protein. This has permitted facile purification of many milligram amounts of human rPEDF. Although both pMAStop and the commercial pBK plasmids are satisfactory expression vectors, the SV40

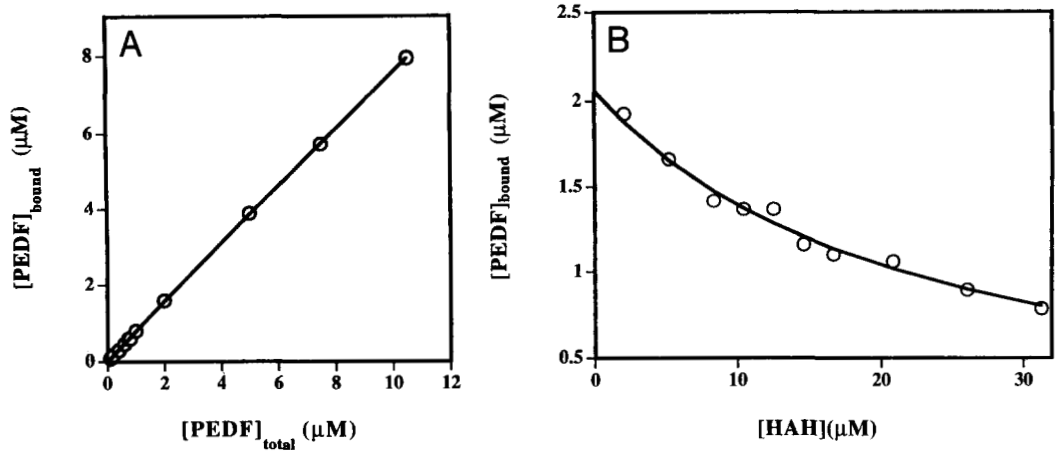

Fig. 6. Competitive displacement of PEDF from heparin–agarose as a function of added heparin (M_r ~ 9000) under conditions of low ionic strength (I 0.05). (**A**) Binding of rPEDF to heparin-Sepharose in the absence of competing heparin. (**B**) Displacement of matrix-bound rPEDF by added high-affinity heparin. Human rPEDF–heparin–agarose complex was incubated with the indicated concentration of heparin and the consequent partitioning of the rPEDF between matrix-bound and solution phases determined from the concentration of rPEDF remaining in solution. The solid line is the non-linear least-squares fit of the experimental data to the polynomial binding equation (Olson et al., 1991) and corresponds to a K_d of 4.1 ± 0.3 μM, with one heparin binding site per rPEDF.

promoter-driven pMAStop appears to give several-fold higher expression levels than does the RSV promoter-driven pBK plasmid. pMAStop will therefore be the vector of choice for future expression of wild-type and variant PEDF species. The levels of expression by pMA-PEDF transfected BHK cells (~20 mg L^{-1}) are similar to those we have found for expression of other serpins (Fan et al., 1993; Hood et al., 1994) and of human α_2-macroglobulin (Gettins et al., 1994) using the same expression system. Although the bovine protein can be isolated from the interphotoreceptor matrix of bovine eyes (Wu et al., 1995; Wu and Becerra, 1996), the yield is relatively low (~1.3 μg per eye) and such a source is unlikely to be favorable for large-scale isolation of the human protein. The advantages of this mammalian expression system over the earlier described expression in *E. coli* (Becerra et al., 1993) are twofold. First, the expressed protein is a full-length glycosylated mature protein rather than a non-glycosylated N-terminally truncated species. Most importantly, however, the PEDF is correctly folded as a globular protein and is consequently soluble in physiological buffers, rather than requiring >2 M urea to remain in solution, as was found for the truncated, resolubilized *E. coli* expression product.

The ability to produce high levels of folded rPEDF has allowed more sample-intensive biophysical methods to be used to characterize PEDF than was possible previously. Using CD spectroscopy to examine the secondary structure of native and proteolytically cleaved rPEDF, we have shown that PEDF behaves very similarly to the non-inhibitory, non-loop–insertable serpin ovalbumin. Thus, cleavage at the P1–P1′ bond does not result in the major structural rearrangements seen with inhibitory serpins in which the whole of the reactive center from P14 to P1 inserts into β-sheet A as a central strand. Instead, the modest changes in the CD spectrum are consistent with changes being restricted only to the reactive center region itself and with the reactive center region probably being helical in the native structure and unstructured when cleaved, such as occurs for ovalbumin (Wright et al., 1990; Stein et al., 1991). This conforms to the growing consensus that both inhibitory and non-inhibitory serpins, with the exception of the heparin-regulatable antithrombin, have exposed helical reactive center regions (Stein et al., 1991; Wei et al., 1994; Song et al., 1995). The reduction in thermal stability of ~6.5 °C also parallels the behavior of ovalbumin, for which a more modest 4.4 °C reduction in temperature of unfolding has been seen (Shitamori and Nakamura, 1983). This contrasts with the very large *increases* in stability seen for inhibitory serpins upon cleavage and insertion of the cleaved reactive center loop into β-sheet A (Bruch et al., 1988; Gettins and Harten, 1988). Unfolding by guanidine hydrochloride also occurs at a lower concentration in the reactive center cleaved form of PEDF, whereas loop-insertable serpins show a large increase in stability to unfolding by guanidine hydrochloride upon cleavage (Bruch et al., 1988).

The interaction of rPEDF with heparin (K_d ~ 4 μM), although not as strong as that of antithrombin with the same high affinity heparin (K_d ~ 10 nM), is similar to that of antithrombin with heparin chains that lack the high affinity sequence (~5 μM) (Gettins et al., 1993). Further work will be needed to determine how specific the interaction is for heparin compared with other glycosaminoglycans and what the nature of the interactions are.

If the fluorescence enhancement of ~17% upon heparin binding is due to direct contact with one or more of the three tryptophans, rather than as a result of a heparin-induced conformational change, it may help to localize the heparin binding site, since, from comparison of the sequences of PEDF with other serpins of known structure, it is likely that tryptophans 197 (W194 in α_1-proteinase inhibitor) and 344 (I340 in α_1-proteinase inhibitor) are buried and that only tryptophan 160 (Y160), located on helix F, is accessible to solvent. Such a position for a heparin binding site in PEDF would be on the opposite side of β-sheet A than the heparin binding site in antithrombin. Consistent with differently located heparin binding sites in PEDF and antithrombin is the absence of the high concentration of lysine and arginine residues in PEDF on the A and D helices, which are thought to form a major part of the heparin binding site in antithrombin.

Although the neurite-promoting biological activity of PEDF has been shown to be present in the *E. coli*-derived recombinant protein, and even to be expressed by fully denatured PEDF (Becerra et al., 1993) and by C-terminally truncated PEDF (Becerra et al., 1995), it is still essential to use correctly folded rPEDF for bio-

logical studies if the full physiological role of PEDF is to be elucidated. This is particularly true if binding to heparin or other surface glycosaminoglycans is a necessary part of localizing PEDF activity in vivo, which would probably require the intact PEDF protein. Such a requirement for correctly folded protein is well illustrated by another non-inhibitory serpin, angiotensinogen, which acts as a substrate for renin to produce the biologically active peptide angiotensin I and subsequently angiotensin II, by cleavage from the N-terminus. The affinity of angiotensinogen for renin are modified (Oxvig et al., 1995) by formation of a specific high molecular weight ternary complex of angiotensinogen with other proteins (Oxvig et al., 1993). Such complexes are present particularly during pregnancy (Tewksbury and Tryon, 1989), and may confer an advantage during a period in which complications involving hypertension can occur.

In conclusion, we have established an excellent expression system for human rPEDF that has already allowed quantitation of some of the biochemical and biophysical properties of this biologically active serpin and shown that in its structural properties it behaves like the prototypic non-inhibitory serpin ovalbumin. The large amounts now available and the ability to carry out site-directed mutagenesis should in the future permit a fuller characterization of the structural requirements for, and role of, the biological activities of PEDF.

Materials and methods

Construction of plasmids for expression of PEDF

Two PEDF cDNA-containing expression plasmids were constructed; pMA-PEDF and pBK-PEDF. The plasmid πFS17, containing the human PEDF cDNA in pBluescript II KS phagemid (Steele et al., 1993), was used for construction of the PEDF expression vectors. PEDF cDNA was excised by digestion of plasmid πFS17 with *EcoR1* and *HindIII* and ligated into the multiple cloning region of the expression vector pBK-RSV (Stratagene), cut with the same restriction endonucleases. This gave the PEDF expression vector pBK-PEDF. The pBK-PEDF construct was digested with *Sal I* and *XbaI* and the released PEDF cDNA ligated into the expression vector pMAStop (Fan et al., 1993) (gift from Dr. Gerd Zettlmeiβl) cut with the same restriction endonucleases. This gave the expression vector pMA-PEDF. The resulting PEDF expression vectors pBK-PEDF and pMA-PEDF contained the PEDF coding sequences under the control of the RSV or SV40 promoters, respectively.

Expression of PEDF in BHK cells

BHK cells (BHK-21, ATCC CCL10) were co-transfected with the plasmids pSV2dhfr, pRMH140, and either pBK-PEDF or pMA-PEDF, as described previously for expression of antithrombin (Fan et al., 1993). Stably transfected cells were selected by resistance to neomycin and to gradually increased levels of methotrexate. Stable transfectants were grown to confluence in 1.4 L roller bottles at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and containing 10 μM methotrexate and 400 μg mL^{-1} neomycin to maintain selection for stable transfectants. The cells were cycled between this medium and serum-free medium, with a cycle time of two days. Only medium from serum-free cycles was harvested and used for PEDF isolation.

Protein purification

Serum-free cycles of medium from BHK cells transfected with pBK-PEDF or pMA-PEDF were collected and pooled for eight cycles. The pooled medium (725 mL) was precipitated with 80% ammonium sulfate. The precipitate was resuspended in 30 mL 20 mM Na-phosphate buffer pH 7.4, containing 150 mM NaCl. The resuspended material was dialyzed against 50 mM Na-phosphate buffer pH 6.2, containing 50 mM NaCl, 1 mM dithiothreitol, and 10% glycerol. The particulate matter was removed by centrifugation and filtration through an 0.45 μM membrane. PEDF in the filtrate was further purified by cation-exchange chromatography on S-Sepharose (200 mL bed volume), pre-equilibrated in the pH 6.2 buffer, as previously described (Wu and Becerra, 1996). Briefly, the sample was loaded, washed with 20-column volumes of the equilibrating buffer, and PEDF eluted with a 50–500 mM NaCl gradient. PEDF-containing fractions were pooled and concentrated by vacuum ultrafiltration, using a 10 kDa cutoff membrane. Purified PEDF was stored at −80 °C until needed.

Neurite outgrowth analyses

Human retinoblastoma Y-79 cells were grown and treated for neurite outgrowth as described (Becerra et al., 1995). Two milliliters of Y79 cell cultures (1.25×10^5 cells/mL) growing in serum-free medium were treated with a solution (25 μL) containing different amounts of PEDF in 1% BSA in phosphate-buffered saline. After seven days of treatment cells were attached to poly-D-lysine–coated plates. The differentiation state of the cultures was monitored by light microscopy at intervals after attachment.

CD spectroscopy

CD spectra of intact and reactive center-cleaved PEDF were recorded on a Jasco 710 spectrophotometer. A path length of 1 mm, PEDF concentration of 0.6 mg mL^{-1} and temperature of 25 °C were used. Spectra were recorded from 200 to 260 nm, with a band width of 1 nm, response time of eight seconds, and scan rate of 5 nm min^{-1} and are the average of three scans. For rPEDF, the spectra are additionally the average of three separate experiments; for ovalbumin, the spectra are the average of two separate experiments. A buffer baseline was subtracted from all spectra. For thermal denaturation studies, ellipticity at 222 nm was monitored as a function of temperature. A 2 nm band width, response time of 16 s and temperature scan rate of 0.5 °C per minute were used. Deconvolution of CD spectra to estimate secondary structure used the program SELCON (self-consistent method of protein secondary structure estimation) (Sreerama and Woody, 1993) and the protein basis sets provided by the program authors, for which proteins secondary structure had been assigned by the method of Levitt and Greer (1977).

Fluorescence spectroscopy

Tryptophan fluorescence emission spectra were used both to monitor PEDF unfolding as a functional of guanidine hydrochloride concentration and to examine the effect of heparin binding on PEDF conformation. Measurements were made on an SLM8000 spectrofluorimeter. For guanidine hydrochloride denaturation studies emission was monitored at 340 nm, with excitation at 280 nm. Band widths of 4 nm for excitation and 16 nm for emission were used. Separate samples were used for each guanidine hydrochlo-

ride concentration, with pre-incubation for one hour prior to recording of the fluorescence intensity, to ensure that equilibrium had been attained. Complete emission spectra were recorded with excitation and emission band widths of 4 nm and with excitation at 280 nm.

Determination of heparin dissociation constant by affinity partitioning

The method of affinity partitioning, as modified by Olson et al. (Olson et al., 1991), was used to determine the dissociation constant for the PEDF–heparin complex. This method uses the competitive displacement of PEDF bound to an affinity matrix by the ligand of interest to enable determination of the dissocation constant. The analysis does not depend on the nature of the affinity matrix interaction, but does require an initial empirical determination of the interaction between PEDF and the matrix (heparin–agarose). This was then used in the subsequent analysis of the displacement of PEDF from the affinity matrix by added heparin to allow determination of K_{AB}, the solution dissociation constant for the PEDF–heparin complex (Olson et al., 1991). Since it was anticipated that the interaction might not be as tight as that for specific heparin–antithrombin interactions, the measurements were made at a relatively low ionic strength (I 0.05) to reduce unfavorable salt effects on the binding interactions.

For determination of the interaction between PEDF and the heparin–agarose matrix, a series of 1 mL solutions of PEDF in I 0.05 buffer and covering a [PEDF] range from 0.1 to 10.5 μM, were mixed with 200 μL of a 1:3 suspension of heparin–agarose. The mixtures were incubated for one hour at room temperature with gentle agitation. The heparin–agarose beads were spun down and the supernatants collected. The amount of unbound PEDF was determined from the concentration of PEDF in the supernatant, and was calculated from the intrinsic protein fluorescence, calibrated by a PEDF fluorescence standard curve.

For the competitive displacement measurements, a series of 200 μL samples of 2.6 μM PEDF and heparin ($M_r \sim 9000$) concentrations from 0 to 31.25 μM were mixed with 40 μL of a 1:3 suspension of heparin–agarose. The mixtures were incubated for one hour at room temperature with gentle agitation. Solution [PEDF] was determined from the intrinsic protein fluorescence corrected for the effect of heparin. PEDF remaining bound to the matrix was determined by difference from the total [PEDF].

Determination of the heparin–PEDF dissociation constant (K_{AB}) used the analysis developed by Olson et al. (Olson et al., 1991) and involved initial fitting of the PEDF–matrix interaction data to a second-degree polynomial and use of the resulting coefficients to solve the binding equation by a Newton-Raphson iteration.

Gel electrophoresis of native, cleaved, and deglycosylated PEDF

SDS-PAGE was run either on 10% linear or 10–20% gradient gels according to the procedure of Laemmli (Laemmli, 1970). Protein bands were visualized by staining with Coomassie brilliant blue. Cleaved PEDF was generated by treatment of PEDF with chymotrypsin at a w/w ratio of 100:1 for two hours at 25 °C in 50 mM sodium phosphate buffer at pH 7.4. The reaction was stopped by addition of PMSF to a final concentration of 1 mM. PEDF was deglycosylated by treating the denatured protein with N-glycosidase F.

Other methods

Protein analysis was carried out by SDS-polyacrylamide gel electrophoresis and by immunoreaction of western transfers with polyclonal anti-PEDF antiserum, as previously described (Wu and Becerra, 1996). Total protein concentration was determined by Bradford assay using the BioRad Protein Assay kit and with bovine serum albumin as standard. N-terminal sequence determination was preformed using an Applied Biosystems 477 sequencer, following the manufacturer's protocols.

Materials

TLCK-treated chymotrypsin, methotrexate, neomycin, and PMSF were from Sigma. Precast 10–20% gradient gels were from Bio Rad.

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