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Pigment epithelium-derived factor: Neurotrophic activity and identification as a member of the serine protease inhibitor gene family

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ABSTRACT **Cultured pigment epithelial cells of the fetal human retina secrete a protein, pigment epithelium-derived factor (PEDF), that induces a neuronal phenotype in cultured human retinoblastoma cells. Morphological changes include the induction of an extensive neurite meshwork and the establishment of corona-like cellular aggregates surrounding a central lumen. The differentiated cells also show increases in the expression of neuron-specific enolase and the 200-kDa neurofilament subunit. Amino acid and DNA sequence data demonstrate that PEDF belongs to the serine protease inhibitor (serpin) family. The PEDF gene contains a typical signal-peptide sequence, initiator methionine codon, and polyadenylylation signal and matches the size of other members of the serpin superfamily (e.g., α_1-antitrypsin). It lacks homology, however, at the putative serpin reactive center. Thus, PEDF could exert a paracrine effect in the embryonic retina, influencing neuronal differentiation by a mechanism that does not involve classic inhibition of serine protease activity.**

The retinal pigment epithelium (RPE) develops in advance of and lies adjacent to the neural retina. A closed compartment between the two cell layers contains the interphotoreceptor matrix and many soluble secretory products of RPE and neural retinal cells. In the human, the interphotoreceptor matrix is present by 20 weeks of gestation and contains at least one specific marker, the interphotoreceptor retinoid-binding protein (1). Nutrients, metabolites, or trophic factors exchanged between the RPE and neural retina must pass through the interphotoreceptor matrix. RPE cells, for example, are thought to synthesize and secrete a photoreceptor survival-promoting factor, PSPA (2). Cultured RPE cells also synthesize a number of other, well-known trophic factors including PDGF (3), FGF (4), TGF-α (5), and TGF-β (6). It is thus possible that these or other, unknown factors derived from the RPE could influence neural retina development.

We have used human Y79 retinoblastoma cells as a test system to study the effects of possible differentiating factors in conditioned medium from cultured human fetal RPE cells. Y79 cells are of neural retina origin and thought to be the tumorous counterpart of early, undifferentiated retinoblasts (7). Conditioned medium from human fetal RPE cells induces a high level (>90%) of neuronal differentiation in Y79 cells—e.g., an elaborate meshwork of neurite-like processes (8). Treated cells also exhibit enhanced levels of neuron-specific enolase (NSE) and neurofilament 200-kDa subunit protein (NF-200), molecular indices of neuronal maturation (8). Recently, we have identified a 50-kDa protein, pigment epithelium-derived factor (PEDF), as the neurotrophic agent (9) in RPE conditioned medium. We now report that this extracellular neurotrophic protein is a member of the serpin gene family of serine protease inhibitors.§

MATERIALS AND METHODS

Amino Acid Sequencing. PEDF, purified from conditioned medium of cultured human fetal RPE cells by HPLC (9), was reduced and alkylated as follows. The sample was dried and dissolved in 50 μl of 8 M urea/0.4 M ammonium bicarbonate, pH 8.0, and 5 μl of 45 mM dithiothreitol (Calbiochem, San Diego, CA) was added. After heating at 50°C for 15 min, the solution was cooled to 4°C and 5 μl of 100 mM iodoacetic acid (Sigma) was added. After 15 min, the solution was diluted to a concentration of 2 M urea and subjected to digestion with trypsin [Boehringer Mannheim; enzyme/substrate ratio of 1:25 (wt/wt)] for 22 hr at 37°C. Tryptic peptides were separated by narrow-bore reverse-phase HPLC on a Hewlett–Packard 1090 instrument equipped with a 1040 diode-array detector, using a Vydac C_{18} column (2.1 mm × 150 mm). The gradient was modified from that described by Stone *et al.* (10). Solvent A was 0.06% trifluoroacetic acid/water and solvent B was 0.055% trifluoroacetic acid/acetonitrile; a gradient of 5% B at 0 min, 33% B at 63 min, 60% B at 95 min, and 80% B at 105 min with a flow rate of 150 μl/min was used. The column effluent was monitored at 210 and 277 nm and the UV spectrum (209–321 nm) of each peak was obtained. Samples for amino-terminal sequence analysis were applied to a Polybrene precycled glass-fiber filter and subjected to automated Edman degradation (Harvard Microchemical Facility, Boston) on an ABI model 477A gas-phase protein sequencer (program NORMAL 1). The resulting phenylthiohydantoin amino acid fractions were manually identified with an on-line ABI model 120A HPLC instrument and Shimadzu CR4A integrator.

Cell Culture. The Y79 retinoblastoma cell line (American Type Culture Collection) was maintained in suspension culture (7). Cells were harvested by centrifugation for 5 min at 600 × *g*, washed in prewarmed serum-free medium, centrifuged again, and suspended at 10^6 cells per ml in serum-free medium with or without HPLC-purified PEDF (50–500 ng/ml) (9) for a 7-day incubation period prior to attachment to poly(D-lysine)-coated flasks (8). A number of growth factors were tested under similar conditions: NGF at 10–300 ng/ml, PDGF at 1–5 ng/ml, TGF-α at 5 ng/ml, TGF-β at 5 ng/ml, FGF at 25–220 ng/ml, and EGF at 25–100 ng/ml (11).

Cell Analyses. Morphology of attached cells was monitored daily by phase-contrast microscopy (Nikon inverted Diaphot TMD microscope) of living cells or by differential interference contrast microscopy (Olympus BHS-BH2 microscope)

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Abbreviations: RPE, retinal pigment epithelium; PEDF, pigment epithelium-derived factor; NSE, neuron-specific enolase; NF-200, neurofilament 200-kDa subunit protein.

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§The sequence reported in this paper has been submitted to the GenBank/EMBL data base (accession no. M76979).

of cells fixed with 4% paraformaldehyde in 0.1 M sodium cacodylate buffer. Expression of NSE and NF-200 detected by monoclonal antibodies (Sigma) was monitored as described (9) by either epifluorescence microscopy (Olympus BHS-BH2 microscope) or microspectrofluorometry (Farrand microscope spectrum analyzer). Microspectrofluorometry readings were $\mu A \times 100$ with a time constant of 0.3 sec; the specimen size was ≈2 mm (about 100 cells per aggregate) with a target size of 15 μm (approximately that of one cell).

Cloning. From PEDF peptide sequences (JT-3, TSLEDFYLDEERTVRVPMM; JT-8, ALYYDLISSPDIHGTYKELLDTVTAPQXN) and codon usage data, oligodeoxynucleotides were constructed (oFS5665, 5′-AGYAAYTTYTAYGAYCTSTA-3′; oFS5667, 5′-CTYTCYTCRTCSAGRTARAA-3′) on an ABI 392 DNA/RNA synthesizer and used as primers in a polymerase chain reaction (PCR) experiment. A human fetal eye Charon BS cDNA library (a gift of A. Swaroop, University of Michigan) was amplified once (12) and screened by PCR (13) using a Techne thermal cycler and standard reagents (GeneAMP, Perkin–Elmer/Cetus) except that $MgSO_4$ was used at 3 mM. The amplified fragment was isolated by electrophoresis in a 3% NuSieve 3:1 gel (FMC Biochemicals, Rockland, ME), recovered by use of NA-45 DEAE-cellulose paper (Schleicher & Schuell) (12), and labeled with [α-^{32}P]dCTP (Amersham) by random priming (kit from Boehringer Mannheim). This probe was used to screen 200,000 plaque-forming units of the same library; positive clones were isolated (12) and the DNA was purified with Qiagen maxi preparation protocols (Qiagen, Chatsworth, CA). The inserts were cut out with *Not* I (BRL), circularized with T4 DNA ligase (New England Biolabs), transformed into competent *Escherichia coli* Sure cells (Stratagene), and plated out on ampicillin/5-bromo-4-chloro-3-indolyl β-D-galactopyranoside plates (12). White colonies were selected for preparation of plasmid DNA (Qiagen plasmid miniprep protocol). Purified plasmids were digested with *Eco*RI/*Hin*dIII (BRL) and electrophoresed in a 0.7% agarose gel to determine the size of the inserts. One of these, πFS17, was selected for mapping and subsequent sequencing (Sequenase 2.0 protocol and reagents, United States Biochemical). Sequence analysis was performed with the MacVector (IBI–Kodak) software package and the GenBank sequence data bank (IntelliGenetics).

RESULTS

PEDF Effects. If not treated with PEDF, most Y79 cells remain morphologically undistinguished (Fig. 1*A*). Within 24 hr of attachment, however, >50% of cellular aggregates previously treated in suspension with PEDF (50 ng/ml) for 7 days begin to extend neurite-like processes (Fig. 1*B*). The differentiated phenotype is neuron-like, with numerous processes projecting from cells growing in aggregates and complex interactions of processes between aggregates. Some aggregates are compact (Fig. 1*B*) whereas others are corona-like in appearance (Fig. 1*C*). Cells in the corona-like structures arrange themselves in a ring, creating a central, lumen-like area. Numerous processes radiate outward from the corona-like aggregates, with few processes projecting into the luminal space. Occasional single differentiated cells are seen; these generally appear unipolar (Fig. 1*D*) or bipolar (Fig. 1*E*) in nature. A number of well-characterized growth factors and natural agents (NGF, PDGF, FGF, TGF-α, TGF-β, and EGF), used at concentrations known to elicit physiological responses in other cell systems, do not demonstrate similar neurotrophic effects on the Y79 cells (11).

By day 7 in treated cultures, an elaborate meshwork of processes can be seen radiating from virtually all aggregates (Fig. 1*F*), with polarity similar to that of the outgrowths observed emanating from the corona-like structures (see Fig. 1*C*). Spinous projections and numerous varicosities are present along the length of most of the processes (Fig. 1*G*). Flattened expansions are seen at the distal tips of many processes; these are usually spiked (Fig. 1*G*), fan-shaped (Fig. 1*H*), or club-shaped (Fig. 1*I*). Thus, the PEDF-induced phenotype appears to be similar in architecture to that of mature neuronal cells of the nervous system.

Low expression of both NSE and NF-200 is observed in attached, undifferentiated cells. In PEDF-treated cells, however, immunocytochemical staining for NSE (Fig. 1 *C–E*) and NF-200 (Fig. 1 *F–I*) is dramatically increased, as assessed by quantitative microspectrofluorometry: for untreated cells, NSE signal = 117, NF-200 signal = 30; for treated cells, NSE signal = 485, NF-200 signal = 590; background = 0. PEDF therefore markedly affects the expression of neuronal marker molecules as well as morphology of the cultured retinoblastoma cells.

Amino Acid Sequencing and cDNA Cloning. Trypsin digestion of purified PEDF and amino acid analysis of the fragments yielded a series of peptides as underlined in Fig. 2. Based on three of these sequences (JT-3 and JT-8), oligonucleotides were constructed that allowed cloning of candidate cDNAs. Two of these, oFS5666 and oFS5667, yielded a PCR amplification product of expected size (about 350 base pairs), which was used to isolate full-length clones from the human fetal eye library. Of the eight positive clones isolated and analyzed, the largest (1.5-kilobases, πFS17PEDF) was selected for sequence analysis. Analysis revealed a 1503-base sequence with a long open reading frame encoding 418 amino acids, a typical ATG start codon, and a polyadenylylation signal (Fig. 2). All previously determined PEDF peptide sequences align perfectly with the translated product of the clone. The deduced sequence also contains a stretch of hydrophobic amino acids that could serve as a signal peptide. The predicted molecular mass of the protein is 46.3 kDa but the actual mass could be higher due to possible glycosylation at an N-linked consensus sequence (NLT in Fig. 2) as well as numerous possible phosphorylation sites (not shown). Comparison of the peptide sequences and the translated product with the GenBank data bank (September 24, 1991) indicates that PEDF is a unique protein but one with significant homology to the serpin (serine antiprotease) gene family as exemplified by human α_1-antitrypsin (HUMA1AT, Fig. 3). The peptide sequence thought to be the active site involved in the antiprotease activity of α_1-antitrypsin is underlined in Fig. 3. Interestingly, there is little similarity in this region between PEDF and other members of the serpin family as represented by α_1-antitrypsin, though the absolutely conserved glycine residue at P_{15} is present.

DISCUSSION

The present study, along with previous work (8, 9), identifies a protein, PEDF, secreted by human fetal RPE cell cultures and shows that it is a potent neurite-promoting factor for human Y79 retinoblastoma cells. This induction of neuronal differentiation and maturation in the neoplastic counterpart of the human neural retina indicates the possibility of an important parallel role for PEDF in normal embryological development of the retina. Y79 cells induced to differentiate with PEDF not only assume the architectural phenotype of neurons but also demonstrate increases in biochemical markers (NSE and NF-200) indicative of relative neuronal maturity. In addition, the formation of striking corona-like aggregates by PEDF-treated cells mimics the orderly arrangement of neurons in the developing nervous system (including retina) as the cells begin to functionally organize. These structures also are reminiscent of the "rosettes" found in human retinoblastoma tissue that are thought to be attempts at photoreceptor neuron formation (16). PEDF might therefore not only influence

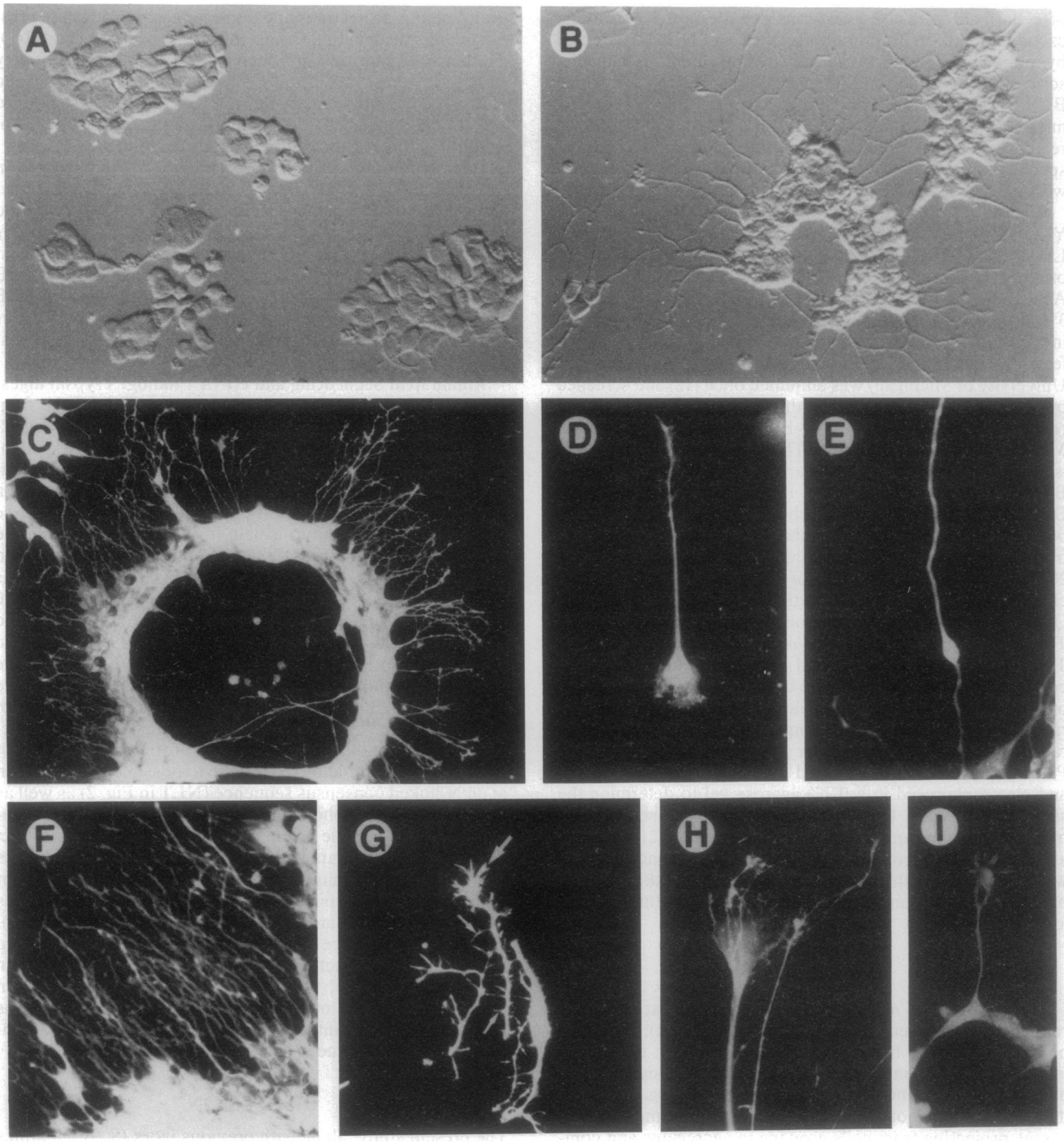

FIG. 1. Effects of PEDF on Y79 cell morphology. Cells were treated in suspension culture for 7 days with or without PEDF (50 ng/ml) and then placed in attachment culture in the absence of PEDF for another 1–7 days. *A* and *B* show differential interference contrast microscopic images; C–E are anti-NSE fluorescence micrographs; F–I are anti-NF-200 fluorescence micrographs. (*A*) Control cells not treated with PEDF at 5 days in attachment culture. (×300.) (*B*) Treated cells at 1 day in attachment culture. (×300.) (*C*) Corona-like aggregate at 5 days. (×145.) (*D*) Single unipolar cell at 5 days. (×300.) (*E*) Single bipolar cell at 5 days. (×300.) (*F*) Complex meshwork of neurite-like processes at day 7 in attachment culture. (×190.) (*G*) Spinous projections (medium arrows) and varicosities (tiny arrows) on processes with spike-shaped tip (large arrow) at day 7. (×190.) (*H*) Fan-shaped tip of process at day 7. (×190.) (*I*) Club-shaped tip of process at day 7. (×190.)

morphological and biochemical development of individual retinal cells but also provide organizational cues important to attaining a final, mature tissue architecture.

cDNA sequence analysis indicates a molecular mass of about 46.3 kDa for the PEDF protein moiety. This is somewhat lower than the apparent size of 50 kDa observed on SDS/PAGE (9). The difference may be due to posttranslational modification, since a glycosylation consensus sequence is present in the predicted protein (Fig. 2). As would be expected in a secreted protein, the translated product exhibits an amino-terminal hydrophobic stretch of amino acids that agrees well with known signal-peptide sequences (17). The putative initiator methionine appears to be correctly designated when the sequence of PEDF is compared with those of other serpin superfamily members (see below). A typical polyadenylylation signal is also present.

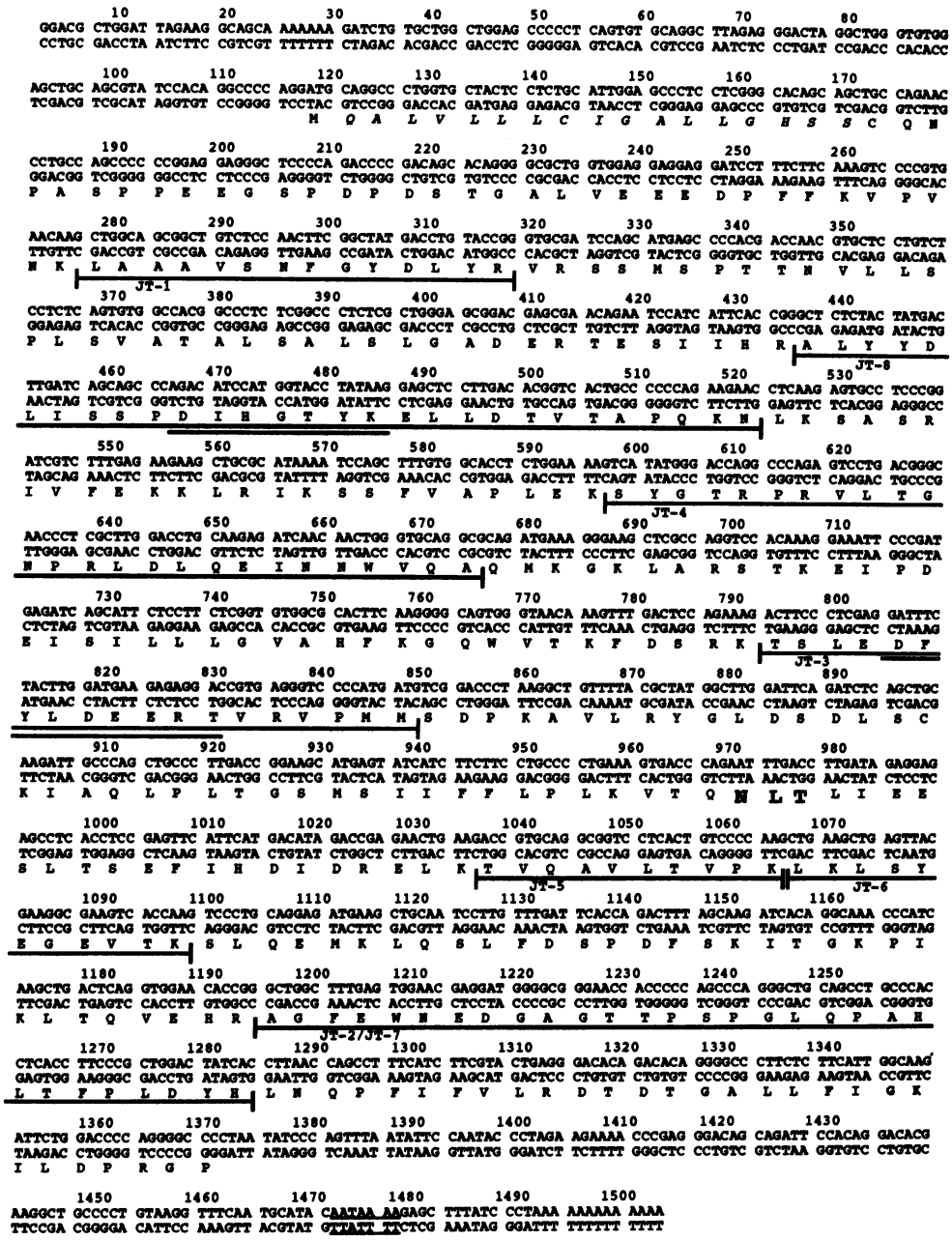

FIG. 2. Complete nucleotide and amino acid sequence of πFS17PEDF. The underlined amino acid sequences are those obtained from microsequencing of purified PEDF that exactly match the nucleotide-derived sequence. The doubly underlined sequences show regions used for construction of oligonucleotide primers for PCR. The 5′ italicized amino acid sequence indicates the putative signal peptide. The large, bold lettering (NLT) is a consensus sequence for putative N-linked glycosylation. The 3′ underlined nucleotides (1471–1477) represent the polyadenylylation signal.

Amino acid and cDNA analyses indicate that PEDF shares considerable sequence homology with members of the serpin superfamily of serine protease inhibitors, including the archetypical human α_1-antitrypsin (18). PEDF exhibits 27% amino acid identity with human α_1-antitrypsin and 42% homology when conservative substitutions are taken into account (Fig. 3). Similarly, it shares approximately 26% and 27% identity with human α_2-plasmin inhibitor and human α_1-antichymotrypsin, with 43% and 44% conserved homology, respectively. It should be noted that this is within the range of homology between other members of the serpin superfamily [e.g., 23–26% identity between α_2-plasmin inhibitor and other serpins (14)]. Serpins seem to have evolved from a common ancestral gene at least 500 million years ago and share a similar tertiary structure with a reactive center located on an exposed ("strained") loop near the carboxyl terminus (15). More than 40 members of the serpin family have now been identified, most retaining the inhibitory function of the ancestral protein (19). While PEDF is generally similar to previously reported serpin family proteins, it is interesting that it lacks significant homology to a proposed consensus sequence for the serpin reactive-center region (20) and is thus unlikely to demonstrate antiprotease activity. In this context, it should be noted that some members of the serpin family have developed specialized roles such as carriers of lipophilic molecules (thyroxine- and cortisol-binding globulins) or peptide hormone precursors (angiotensinogen).

Most pertinent to the present study is the neurotrophic activity exhibited by some of the serpins and their role(s) in development (21, 22). Rapid neurite extension, for example, is observed after treatment of neuroblastoma cells with leupeptin or hirudin but not other serine protease inhibitors,

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HUMA1AT      MPSSVSWGILLAGLCCLVPVSLAEDPQGDAAQKTDTSHHDQD--HP
PEDF       MQALVLLLCIGALLGHSSCQNPASPPEEGSPDPDSTGALVEEEDPFFKV

HUMA1AT    TFNKITPNLAEFAFSLYRQLAHQSNSTNIFFSPVSIATAFAMLSLGTKA
PEDF       PVNKLAAAVSNFGYDLYRVRSSMSPTTNVLLSPLSVATALSALSLGADE

HUMA1AT    DTHDEILEGLNFNLTEIPEAQIHEGFQELLRTLNQPDSQLQLTTGNGLF
PEDF       RTESIIHRALYYDL--ISSPDIHGTYKELLDTVTAPQKNLK-SASRIVF

HUMA1AT    LSEGLKLVDKFLEDVKKLYHSEAFTVNFGDHEEAKKQINDYVEKGTQGK
PEDF       -EKKLRIKSSFVAPLEKSYGTRPRVLT-GNPRLDLQEINNWVQAQMKGK

HUMA1AT    IVDLVKELDRDTVFALVNYIFFKGKWERPFEVKDTEDEDFHVDQVTTVK
PEDF       LARSTKEIPDEISILLLGVAHFKGQWVTKFDSRKTSLEDFYLDEERTVR

HUMA1AT    VPMMKRLG-MFNIQHCKKLSSWVLLMKYLGNATAIFFLPDE--GKLQHL
PEDF       VPMMSDPKAVLRYGLDSDLSCKIAQLPLTGSMSIIFFLPLKVTQNLTLI

HUMA1AT    ENELTHDIITKFLENEDRRSASLHLPKLSITGTYDLKSVLGQLGITKVF
PEDF       EESLTSEFIHDIDRELKTVQAVLTVPKLKLSYEGEVTKSLQEMKLQSLF

                                          P15                P1P1'
HUMA1AT    SNGADLSGVTEEAPLKLSKAVHKAVLTIDEKGTEAAGAMFLEAIPMSIP
PEDF       -DSPDFSKITGK-PIKLTQVEHRAGFEWNEDGAGTTPSPGLQPAHLTFP

                P8'
HUMA1AT    PEVKFNKPFVFLMIEQNTKSPLFMGKVVNPTQK
PEDF       LDYHLNQPFIFVLRDTDTGALLFIGKILDPRGP
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FIG. 3. Amino acid alignment of PEDF with human α_1-antitrypsin (HUMA1AT). Two dots indicate a conserved amino acid; a single dot indicates a conservative change. The reactive center and neighboring residues (P_{15} to $P_{8'}$) are underlined in HUMA1AT (14, 15).

indicating specificity of the effect (21). Glia-derived nexin (GDN), a serpin from cultured glioma and glial cells, promotes neurite extension and also potentiates the neurite-promoting activity of nerve growth factor (NGF) (22). It has been proposed that a balance between protease and protease-inhibitor activities regulates neurite outgrowth (22, 23). In the developing retina, an extracellular "gelatinase–collagenase" activity, as well as other possible protease activities, has recently been shown to be associated with growing processes from cultured neurons (23). Unfortunately, little is known about the actual mechanism of action of the proteases and their inhibitors (e.g., serpins) in inducing cellular differentiation and the overall physiological importance of these factors in central nervous system development. Our system, however, may provide a convenient *in vitro* model for addressing these problems.

Since a subretinal space is present by 5 months in the human fetal eye (1), the embryonic RPE could secrete PEDF into the interphotoreceptor matrix, where it would modulate retinal neurite outgrowth and maturation when retinoblast migration had ceased. PEDF also could act to directly inhibit cell proliferation and/or retinoblast movement and thus signal for final differentiation. It will be interesting to determine whether this effect is through a classic serpin activity or an as yet unknown mechanism. In any event, the present work demonstrates the potential for a novel paracrine effect within the central nervous system, where a fetal neuroepithelial cell secretes a specific protein into an extracellular matrix compartment that could direct the development of adjacent neurons.

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