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Pigment Epithelium-derived Factor Receptor (PEDF-R): A Plasma Membrane-linked Phospholipase with PEDF Binding Affinity

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Abstract

Pigment epithelium-derived factor (PEDF), a multifunctional protein, acts in retinal differentiation, survival and maintenance by interacting with high affinity receptors on the surface of target cells. We have recently identified PEDF-R, a new member of the patatin-like phospholipase domain-containing 2 (PNPLA2) family with characteristics of a PEDF receptor. The PEDF-R sequence reveals a patatin-like phospholipase domain toward its amino-end, and four transmembrane domains interrupted by two extracellular loops and three intracellular regions along its polypeptide sequence. This newly identified protein is present on the surface of retina and RPE cells, and has the expected transmembrane topology. It has specific and high binding affinity for PEDF, and exhibits a potent phospholipase A_2 activity that liberates fatty acids. Most importantly, PEDF binding stimulates the enzymatic phospholipase A_2 activity of PEDF-R. In summary, PEDF-R is a novel component of the retina that is a phospholipase-Iinked membrane protein with high affinity for PEDF. The results suggest a molecular pathway by which PEDF ligand/receptor interactions on the cell surface could generate a cellular signal. These conclusions enhance our understanding of the role of PEDF as a neurotrophic survival factor.

4.1 Introduction

Pigment epithelium-derived factor (PEDF), a non-inhibitory member of the serine protease inhibitor superfamily (SERPIN), is a multifunctional protein involved in neuronal survival and differentiation (Barnstable and Tombran-Tink 2004; Becerra 2006; Bouck 2002). It was discovered as a 50-kDa protein released by cultured pigment epithelial cells from fetal human retina (Tombran-Tink et al. 1991). PEDF is ubiquitously expressed and distributed in the human body (Singh et al. 1998). The retinal pigment epithelium (RPE) expresses the highest levels of *PEDF* transcripts am ong ocular tissues (Becerra et al. 2004; Perez-Mediavilla et al. 1998) and secretes the protein product into the interphotoreceptor matrix (Tombran-Tink et al. 1995; Wu et al. 1995). PEDF acts to promote photoreceptor and retinal neuron cell survival (Cayouette et al. 1999; Takita et al. 2003), and prevents the pathological invasion of neovessels (Dawson et al. 1999). Decreased levels of PEDF have been linked to several retinal diseases, such as age-related macular degeneration (AMD), diabetic retinopathy, and neuroretinal dystrophies (Duh et al. 2004; Holekamp et al. 2002; Ogata et al. 2004). The importance of PEDF in the development, maintenance, and function of the retina and CNS is evident in animal models for inherited and light-induced retinal degeneration, elevated intraocular pressure, retinopathy of prematurity, as well as for degeneration of spinal cord motor neurons (Bilak et al. 1999; Cao et al. 2001; Cayouette et

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al. 1999; Dawson et al. 1999; Duh et al. 2002). The above observations have prompted development of clinical trials on the efficacy of PEDF in the context of AMD (Chader 2005). Although the mechanisms of neuroprotection and angiogenesis inhibition remain unknown, it has been implied that they are associated with receptor interactions at cell-surface interfaces.

4.2 Identification of a PEDF Receptor

The surface of normal and tumor retina cells exhibit high affinity binding for PEDF ligands (K_D = 2–8 nM) (Alberdi et al. 1999; Aymerich et al. 2001). Plasma membranes of retina cells from different species contain specific PEDF-binding com ponents that migrate as 80–85-kD a proteins by SDS-PAGE (Alberdi et al. 1999; Aymerich et al. 2001). Yeast 2-hybrid experiments performed using a com m ercial fetal human liver cDNA library as target and human PEDF plasmids as bait, reveal about 50 clones with potential PEDF interacting genes, but only a few of the sequences are of interest (Notari et al. 2006). One of them, clone 12c, contains a cDNA with 100% identity to a fragment of a new mRNA transcript isolated from the RPE of a human eye, which we have termed *PEDF-R*.

4.3 In Silico Information

The newly identified *PEDF-R* transcript has a coding capacity of 504 amino acids and four *N*-glycosylation consensus sites. The sequence shares strong homology with members of the Ca^{2+}-independent PLA_2 ($iPLA_2$)/desnutrin/patatin-like phospholipase domain-containing protein 2 (PNPLA2) family (Jenkins et al. 2004; Notari et al. 2006). M embers of the PNPLA2 family have demonstrable triglyceride lipase, triacylglycerol transacylase and phospholipase activities. The *PEDF-R* nucleotide and its derived amino acid sequences match those of human TTS-2.2 and iPLA2ζ, and share high identity to mouse desnutrin and ATGL, all of which are synonyms of PEDF-R. The *PEDF-R* gene contains 10 exons with the initiating methionine codon in the second exon (Fig. 4.1, top panel). The structure of the exon/intron junctions reflects to a certain degree the proposed domain structure of the protein (Fig. 4.1, bottom panel). Hydrophobicity plots predict a transmembrane nature for the PEDF-R polypeptide product with 4 transmembrane (TM) domains interrupted by 2 extracellular loops and 3 intracellular regions. The region of the first two TM domains plus the smallest extracellular loop located between them is flanked by introns. Each of the other TM domains and the smallest intracellular loop are also flanked by introns; while the longest extracellular loop and the last intracellular region are com posed entirely of exons.

4.4 Expression and Distribution in the Retina

Most tissues of several species express *PEDF-R* transcripts (Notari et al. 2006). Although adipose tissues express the highest levels among tissues, *PEDF-R* mRNA is clearly expressed in ocular tissues, especially in the neural retina and the RPE (Table 4.1). The RPE cell lines ARPE-19 and H-Tert, and the retina precursor cell lines R28 and RGC-5 also express significant levels of *PEDF-R* transcripts. In the native retina PEDF-R protein is distributed in the RPE and in the inner segments of the photoreceptors, and at lower levels, in the inner nuclear and retinal ganglion cell layers (Notari et al. 2006). It partitions with plasma membrane proteins by biochemical cell fractionation. Western blots with a specific antiserum to PEDF-R clearly demonstrate the presence of a single immunoreacting band that migrates as an 83-kDa protein in RPE, retina, ARPE-19, RGC-5 and R28 cell membrane fractions (Fig. 4.2).

4.5 Transmembrane Topology

Specific biotinylation of cell-surface proteins labels PEDF-R in ARPE-19 cells, demonstrating that PEDF-R is a plasma membrane protein in these cells (Notari et al. 2006). Immunocytochemistry of ARPE-19 cells using antibodies to specific peptides designed from presumptive extracellular (R^A) and intracellular (R^C) regions of the PEDF-R sequence (Fig. 4.1, bottom panel) provides information of the PEDF-R transmembrane topology. Only the anti-R^A antibody stains non-permeabilized cells, while both antibodies detect PEDF-R in permeabilized ARPE-19 cells (Notari et al. 2006). These results demonstrate that the R^A and R^C regions of PEDF-R are extracellular and intracellular, respectively, as predicted from the PEDF-R sequence.

4.6 Binding to PEDF Ligands

Epitope-tagged PEDF-R can be overexpressed using a cell-free system and the recombinant PEDF-R polypeptide can be purified by affinity column chromatography. Binding assays unequivocally show that the recombinant protein binds to PEDF when either one is immobilized or in solution. PEDF-R can rapidly and reversibly interact with PEDF sensor chips in a specific fashion by Surface Plasmon Resonance (Notari et al. 2006). The kinetic parameters reveal high binding affinity for the PEDF:PEDF-R interactions with a dissociation constant (K_D = ~3 nM) that is similar to the that of PEDF to intact cells. Furthermore, fluorescein-labeled human recombinant PEDF (Fl-PEDF) binds specifically to cell surfaces with a pattern similar to that observed with anti-R^A antibody (Notari et al. 2006). These results demonstrate that PEDF binds to PEDF-R at the plasma membrane interface.

4.7 Phospholipase Activity

The patatin-like phospholipase domain of PEDF-R has sequence homology to the catalytic domain of two well-known PLA enzymes, Patatin B2 and cytosolic PLA_2. The PLA active site of these enzymes is formed by a Ser-Asp catalytic dyad within the conserved motifs GXSXG and DXG/A (Hirschberg et al. 2001; Rydel et al. 2003). The sequence of human PEDF-R reveals structural homologies to these motifs, having a serine residue in position 47 (S^{47}) and aspartic acid residue in position 166 (D^{166}) (Fig. 4.1, bottom panel). Functional studies have demonstrated that the amino acids S^{47} and D^{166} are crucial for lipase activity of PEDF-R (Smirnova et al. 2006). These residues are located in the membrane interface by the second and third TM2 and TM3 domain of the transmembrane PEDF-R topology. The location of these critical residues suggests that the Ser-Asp catalytic dyad in PEDF-R is in the vicinity of potential phospholipid substrates.

PLA assays demonstrate that the recombinant PEDF-R exhibits PLA activity that liberates fatty acids from phospholipid substrates. The concentration response curve shows that the PLA specific activity of the recombinant PEDF-R is higher (>4-fold) than that of the commercial hog pancreas PLA_2 (Notari et al. 2006). Bromoenol lactone, a known inhibitor of triolein lipase activity for $iPLA_2S$, inhibits the PLA activity of PEDF-R. A pH curve reveals optimum enzymatic activity at pH 7.5 for recombinant PEDF-R protein. More interestingly, PEDF increased the PLA activity of PEDF-R, and, in contrast, PEDF did not affect commercial hog PLA_2 activity (Notari et al. 2006). These results demonstrate that PEDF stimulates the release of fatty acids and lysophosphatidic acid from phospholipids catalyzed by the PLA activity of PEDF-R.

4.8 PEDF-R Activity in Retinal Cells

Recent studies have demonstrated PEDF as a survival factor for R28 cells in response to serum starvation (Murakami et al.2008). R28 cells, rat retinal cells expressing neuronal genes, contain functional cell-surface PEDF-R, as detected by immunoblotting of R28 plasma membrane fractions (Fig. 4.2). Moreover, these fractions exhibit PLA activity (Fig. 4.3a) similar to human recombinant PEDF-R protein (Notari et al. 2006). Interestingly, the PLA activity of the R28 fractions further increases upon preincubation with PEDF ligand (Fig. 4.3b), similar to the activities of human recombinant PEDF-R and ARPE-19 plasma membrane fractions (Notari et al.2006).

The membrane PEDF-R protein is labile in solution upon extraction with detergents from the lipid environment of membranes. Extracted PEDF-R can readily lose activity upon storage. However the enzyme can be stabilized by adding 0.8 μM PEDF to the solubilization buffer used to extract proteins from membrane pellets obtained by high speed centrifugation. This approach increases protein solubility and the enzymatic activity of PEDF-R (Fig. 4.3c), which is further stimulated by preincubation of the fraction with PEDF ligand (Fig. 4.3c). These results suggest that binding of the membrane-embedded PEDF-R to PEDF ligands does not saturate its PLA activity and can provide structural stability to enhance activity. Thus, PEDF-R has a potential role to mediate the survival effect of PEDF on R28 cells.

4.9 Conclusions

PEDF-R is a newly-identified membrane-linked receptor with lipase activity and high binding affinity for PEDF present in the retina. The protein contains a functional phospholipase domain and a transmembrane topology for PEDF-R with extracellular loops available to interact with the PEDF ligand. The PEDF-mediated stimulation of the PLA activity o f PEDF-R supports the idea that PEDF signaling is mediated by the released fatty acids and lysophospholipids from phospholipids at the cell-surface interface. In the retina, DHA is one of the most abundant fatty acids in the membrane phospholipids, which in turn is the precursor of neuroprotectin Dl, a neuroprotectant and antioxidant agent in the retina, RPE and CNS (Bazan 2005). The PEDF:PEDF-R interactions could participate in the generation of neuroprotectin Dl via its PLA activity to act as a bioactive signal mediator for the PEDF neurotrophic activity (Bazan et al. 2005). In summary, these findings suggest a molecular pathway by which PEDF ligand/receptor interactions on the cell-surface could generate a cellular signal.

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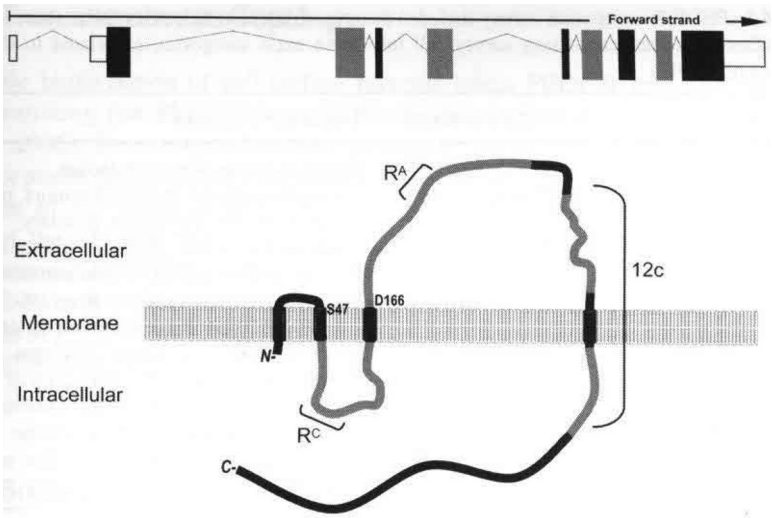

Fig. 4.1.

In silico information of gene, transcript and protein structure of PEDF-R. (**Top**) Transcript summary information for human PEDF-R obtained from http://www.ensembl.org for ENST00000336615 (Hubbard et al. 2005). PEDF-R gene has 10 exons (*top line*), the transcript length is 2,071 bps (exons are illustrated by *boxes*; coding regions are *black* and *grey*; introns are *lines flanking boxes*); and the translation length is 504 amino acid residues. (**Bottom**) Computer programs predict a transmembrane topology for PEDF-R. Exons are mapped on the illustration and are coded as in **Top Panel**. Features illustrated are: the coding region corresponding to the yeast-2 hybrid clone p12c; the locations of peptide antigens for antibodies Ab-R^A and Ab-R^C, and the Ser^{47}-Asp^{166} catalytic dyad

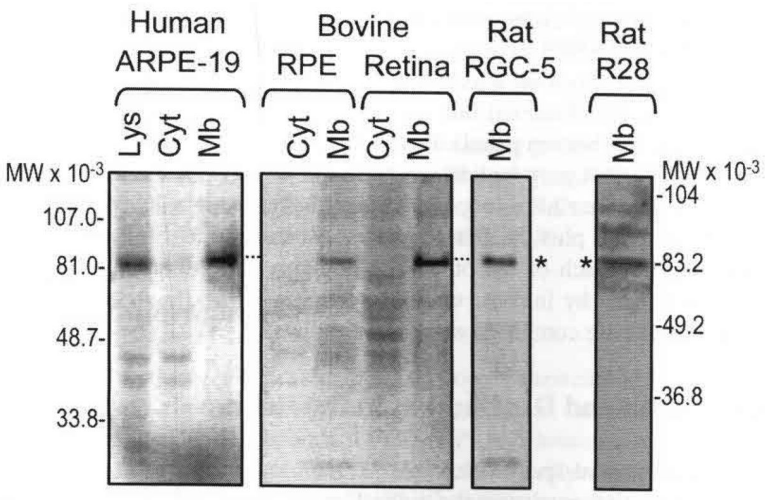

Fig. 4.2.

Subcellular localization of PEDF-R in retinal cells. Cell lysates (Lys) were fractionated by high speed centrifugation (80,000×*g*) into cytosolic (cyt) and membrane fractions (Mb). Western blots of fractions were immunostained with anti-PEDF-R^A antibody. Total protein loaded in lanes were as follows: (ARPE-19, Lys) 19.8 μg; (ARPE-19, Cyt) 12 μg; (ARPE-19, Mb) 6.5 μg; (RPE, Cyt) 67 μg; (RPE, Mb) 10 μg; (Retina, Cyt) 71 μg; (Retina, Mb) 10 μg; (RGC-5, Mb) 9.5 μg; and (R29, Mb) 5.7 μg respectively. The *asterisks* point to the migration position of PEDF-R

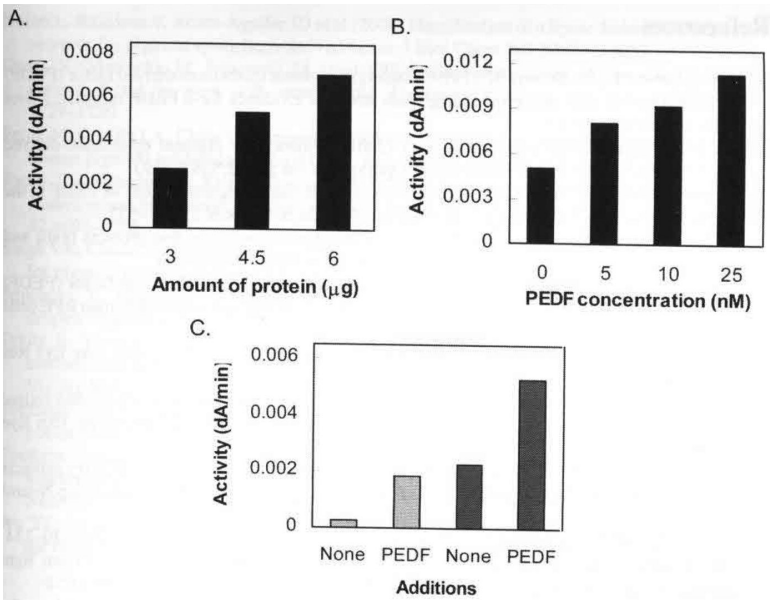

Fig. 4.3.

PLA activity of PEDF-R. Detergent-soluble membrane fractions from R28 cells were assayed for PLA activity using (1,2-dilinoleoyl)-phosphatidylcholine as substrate and lipoxygenase as the coupling enzyme in reaction buffer (50 mM Tris-HCl, pH 7.5, 3 mM deoxycholate) as described (Jimenez-Atienzar et al. 2003). Formation of the product was measured spectrophotometrically by increase in the absorbance at 234 nm per min (dA/min; *y*-axis). (**a**) Dose response of PLA activity of R28-derived PEDF-R. Proteins from R28 cell membranes were solubilized with phosphate buffered saline pH 6.5 containing 0.1% NP-40, and the PLA activity was determined for increasing amounts of detergent-soluble protein fractions, (**b**) Effects of PEDF on the R28-derived PEDF-R activity. Extracts were preincubated with PEDF for 10 min and then assayed for PLA activity. Concentrations of PEDF used in each assay are indicated in the *x*-axis. (**c**) PEDF-R proteins were solubilized from ARPE-19 membrane protein precipitate with phosphate buffered saline pH 6.5 containing 0.5% CHAPS in the absence (*grey boxes*) or presence of 0.8 μM PEDF (*black boxes*). PLA activity was measured as in **Panel B** in the absence (*none*) or presence of 10 nM PEDF

Table 4.1

PEDF-R expression survey includes sources of interest. Selected cDNA sources from the UniGene reported expression survey fall into three main categories, as related to the key functions of PEDF

Ocular sources	Neuronal sources	Tumorigenic sources
Retinal pigment epithelium (RPE) Choroid Optic nerve Fetal eye Lens Retina Eye anterior segment Lacrimal gland	White matter Hypothalamus Brain Hippocampus Medulla	Retinoblastoma, adenocarcinoma, chondrosarcoma, germ cell tumors, pooled, squamous cell carcinoma, pituitary adenomas, serous papillary carcinoma, choriocarcinoma, epithelioid carcinoma, leiomyosarcoma cell line, anaplastic oligodendroglioma, epithelioid carcinoma cell line, renal carcinoma, myeloma, astrocytoma grade IV, hepatocellular carcinoma, ductal carcinoma, duodenal adenocarcinoma, chronic lymphotic leukemia, meningioma, melanoma, adrenal adenoma