Extracellular Phosphorylation Converts Pigment Epithelium-Derived Factor From a Neurotrophic to an Antiangiogenic Factor

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ABSTRACT

The pigment epithelium derived factor (PEDF) belongs to the superfamily of serine protease inhibitors (serpin). Two distinct functions have been attributed to this factor, which can act either as a neurotrophic or as an antiangiogenic factor. Beside its localization in the eye, PEDF was recently reported to be present also in human plasma. We found that PEDF purified from plasma is a phosphoprotein, which is extracellularly phosphorylated by protein kinase CK2 (CK2) and to a lesser degree, intracellularly, by protein kinase A (PKA). CK2 phosphorylates PEDF on two main residues, Ser24 and Ser114, and PKA phosphorylates PEDF on one residue only, Ser227. The physiological relevance of these phosphorylations was determined by using phosphorylation site mutants. We found that both CK2 and PKA phosphorylations of PEDF markedly affect its physiological function. The fully CK2 phosphorylation site mutant S24,114E abolished PEDF neurotrophic activity but enhanced its antiangiogenic activity, while the PKA phosphorylation site mutant S227E reduced PEDF antiangiogenic activity. This is a novel role of extracellular phosphorylation that is shown here to completely change the nature of PEDF from a neurotrophic to an antiangiogenic factor.
INTRODUCTION

The pigment epithelium derived factor (PEDF) was originally identified in conditioned medium of fetal human retinal pigment epithelium cell cultures\textsuperscript{1,2}. It shares sequence and structure homology to members of the superfamily of serine protease inhibitors (serpin)\textsuperscript{3}, however, it does not serve as an inhibitor of any protease activity\textsuperscript{4}. PEDF was first described as a neurotrophic factor that induces a specific neuronal phenotype in retinoblastoma cells\textsuperscript{3}. The neurotrophic activity of PEDF was also demonstrated by its ability to support neuronal survival\textsuperscript{5}, and its ability to protect neurons against neurotoxic effects\textsuperscript{6}. Structure-function studies have shown that this neurotrophic activity is exerted by the amino terminal edge (44-mer, amino acid residues 78-121) of the human PEDF, and that it is mediated through a ~80kDa membranal receptor, which is abundant in retinoblastoma cells\textsuperscript{7}, and in neural retinal cells\textsuperscript{8}.

Beside its neurotrophic activity, PEDF was recently demonstrated as one of the most potent natural inhibitors of angiogenesis\textsuperscript{9}. Thus, it was found that PEDF inhibits not only bFGF-induced migration of endothelial cells under \textit{in vitro} conditions, but also bFGF-induced neovascularization in an avascular rat cornea. Furthermore, addition of anti-PEDF antibodies (Abs) to rat corneas was found to stimulate the invasion of new vessels into these corneas\textsuperscript{9}, suggesting that PEDF plays a physiological regulatory role in retinal angiogenesis. Additionally, PEDF was shown to be a very potent inhibitor of neovascularization in a murine model of ischemia-induced retinopathy\textsuperscript{10}. The antiangiogenic activity of PEDF was associated with endothelial cell apoptosis\textsuperscript{10}, probably by increasing Fas ligand (FasL) mRNA and surface FasL in these cells\textsuperscript{11}.

It was recently reported that beside its expression in multiple sites in the eye, PEDF is also present in human plasma at a physiologically relevant concentration\textsuperscript{12}. In the last decade several reports have described the possibility that protein kinases might function as a regulatory device not only intracellularly but also in the cell exterior (for review, see\textsuperscript{13} and\textsuperscript{14}).
These reports described the presence of membrane-bound ectoprotein kinases (on the outer cell surface) and soluble secreted exoprotein kinases (detached from the cell). Additionally it was shown that these ecto or exoprotein kinases do have several substrates in the circulating blood including the coagulation cofactors Va and VIII\textsuperscript{15,16} as well as vitronectin\textsuperscript{14}. The main protein kinases that seem to exert exokinase activity are protein kinase A (PKA\textsuperscript{14,17}) and protein kinase CK2 (CK2\textsuperscript{13}). For example it was shown that vitronectin is phosphorylated by PKA\textsuperscript{14} and this phosphorylation modulate its interaction with PAI-1\textsuperscript{18}. In addition, phosphorylation by CK2 changes intracellular signaling by vitronectin\textsuperscript{19}, indicating that both PKA and CK2 play an important regulatory role in the circulating blood.

Since PEDF was found to be present in human plasma, we aimed to determine whether PEDF in circulating blood serves as a substrate for exokinases. We report here that PEDF purified from human plasma is a phosphoprotein. It is phosphorylated in the serum mainly by CK2 on two main residues, Ser24 and Ser114, but also by PKA on Ser227. We found that PEDF is functionally modulated by extracellular phosphorylation. The CK2 phosphorylated PEDF had a reduced neurotrophic activity, while its antiangiogenic activity was significantly increased. On the other hand, PKA phosphorylation reduced the PEDF antiangiogenic activity but had only slight effect on its neurotrophic activity. This is a novel role of extracellular phosphorylation that is shown here to completely change the nature of the physiological activity of a circulating protein.
METHODS

**Reagents and antibodies** – Recombinant human CK2 was from Calbiochem (Darmstadt, Germany), the catalytic subunit of PKA was purified as described\textsuperscript{20}. Active ERK was purified as described\textsuperscript{21}. Full-length human PEDF cDNA was provided by Dr. N. Bouck (Northwestern University, Chicago, IL, USA). Phosphothreonine Ab was from Zymed Laboratories, Inc (San Francisco, CA). Phosphotyrosine Ab (PY99) was from Santa Cruz Biotechnology (Santa Cruz, CA). pERK, gERK phosphoserine Abs, bFGF, α-casein and dephosphorylated casein were from Sigma (Rehovot, Israel). Polyclonal Ab against PEDF was developed by the Ab Unit of the Weizmann Institute of Science.

**Cell cultures** - Human Y-79 retinoblastoma cells (ATCC, Manassa, VA) were grown in MEM supplemented with 2mM L-Glutamine and 15% fetal calf serum (FCS). HEK-293T cells were cultured in DMEM F-12 supplemented with 10% FCS. HUVEC were grown in M-199 supplemented with 20% FCS, 25µg/ml ECGS mitogen (BT-203, Biomedical Technologies Inc, Stoughton, MA), and 5U/ml heparin.

**Construction of rPEDF mutants** – Full-length PEDF cDNA was used as a template for oligonucleotide-site directed mutagenesis kit (Clontech, Palo Alto, CA). Pure PCR products digested by Hind III and EcoRI were ligated into the multicloning site of pcDNA3. DNA sequencing analysis confirmed the nucleotide sequence of the PEDF mutants.

**Transient expression of mutants in HEK-293T cells** – pcDNA3 carrying mutants were introduced into HEK-293T cells using the LipofectAMINE reagent (Life Technologies Inc, Grand Island, NY) according to the manufacturer’s instructions. The transfected cells were serum starved (3 days, serum-free) after which the PEDF mutants were purified on a Ni\textsuperscript{2+} column (Amersham, UK) according to the manufacturer’s instructions.

**Purification of PEDF from human plasma** – plPEDF was purified from human citrated plasma (1L) by a 9-20% PEG cut followed by DEAE-Sephacel column (2.9 x 40 cm) and
heparin agarose column that was developed stepwise. The fractions were pooled (~20ml), dialyzed against buffer D (20mM Tris-HCl, pH 7.4), and applied onto a Mono Q-FPLC column (1ml, Pharmacia, Sweden), which was developed with a linear NaCl gradient in buffer D. PEDF was eluted at 0.2M NaCl and usually yielded 1mg pure PEDF (4°C all steps).

**Alkaline phosphatase treatment of PEDF** – Recombinant PEDF (50µg/ml) or plPEDF (50µg/ml) were incubated with alkaline phosphatase conjugated to acrylic beads (50U/ml) or with sepharose CL-4B beads as control (45min, 30°C). Beads were pre-equilibrated with BSA (1mg/ml), Tris-HCL (50mM pH 8.0), and EDTA (0.1mM). Reaction was arrested by centrifugation. The supernatant was further subjected to *in vitro* phosphorylation.

**In vitro phosphorylation of PEDF** – The phosphorylation assay (40µl) contained either rPEDF, plPEDF or rPEDF mutants (50µg/ml). For CK2: the constituents were CK2 (4µg/ml), glycerol (2%), NaCl (20mM), β-mercaptoethanol (0.1mM), MgCl₂ (20mM), [γ³²P]-ATP (10µM), poly-L-lysine (200nM), and Tris-HCL (50mM pH 7.4). For PKA: pure catalytic subunit of PKA (2.5µg/ml), MgCl₂ (10mM), heparin (50µg/ml), [γ³²P]-ATP (10µM), and Tris-HCL (50mM pH 6.5). For human plasma: phosphatase treated PEDF (30µg/ml), fresh human plasma, MgCl₂ (20mM), [γ³²P]-ATP (20µM), Tris (50mM pH 7.4) with or without PKA inhibitor (PKI, 1µg/ml) or heparin (100µg/ml). Reactions were for 45min at 30°C. Then, boiled sample buffer was added, and the samples were subjected to 10% SDS-PAGE.

**Determination of ERK phosphorylation** – Serum starved cells were treated with rPEDF, plPEDF or the various rPEDF mutants (10nM unless otherwise specified) for the indicated times. Following stimulation pERK and gERK were detected using the appropriate Abs.

**Neurite outgrowth assay** - Human Y-79 retinoblastoma cells were assayed for neurite outgrowth as previously described. Briefly, one ml of a Y-79 cell suspension (2.5 x 10⁵ cells/ml) were incubated with rPEDF, plPEDF or the various rPEDF mutants (20nM) in the
cell’s medium. After 7 days the cells were transferred to poly-D-lysine coated plates, and their neurite outgrowth was monitored by light microscopy at various periods of time.

**Aortic ring assay** – The aortic ring assay was modified from \(^{23}\). Briefly, thoracic aortas were dissected from 10-12 weeks old BALB/C mice and transferred to Petri dish containing BIO-MPM-1. After removing excess perivascular tissue, transverse cuts of 1mm long were made. The rings were embedded in collagen (prepared as in \(^{24}\)) mix (7 parts collagen, 1 part 10 x MEM, and 2 parts 0.15M NaHCO\(_3\), 800\(\mu\)l) in 24-well plates. Medium (500\(\mu\)l BIO-MPM-1 containing penicillin-streptomycin and the examined reagent) was added to the embedded rings, and the plates were incubated at 37\(^\circ\)C in a humidified incubator. Medium containing reagents was replaced 3 times a week. After 10-12 days, the rings were fixed with 4% formaldehyde and stained with crystal violet (0.02%). The effect of each factor was examined in 2 wells (4 rings) per assay, and was repeated at least 3 times.

**Matrigel Plug Angiogenesis Assay** – Matrigel (BD Biosciences, MA; 0.5ml/mouse) containing bFGF (300ng/ml), with or without PEDF (20nM) was injected subcutaneously into the flank of 8 weeks old nude mice as described \(^{25}\). On day 7, mice were sacrificed, plugs were removed, fixed (4% formaldehyde), paraffin embedded and sectioned. Sections were stained using Hematoxilin-Eosin (H&E). Endothelial cells/microvessels infiltrating the Matrigel were confirmed by Masson’s Trichrome staining.

**RESULTS**

PEDF in plasma is a phosphoprotein. PEDF, which was identified as a neurotrophic and antiangiogenic factor in the eye, was recently found to be present also in circulating blood \(^{12}\). Since it was demonstrated that exokinases are able to phosphorylate plasma proteins, we studied whether PEDF can be a target for phosphorylation by these kinases. Two forms of PEDF used in the study were PEDF purified from human plasma (plPEDF) and recombinant PEDF (rPEDF), which was expressed in HEK-293T cells and purified from the serum free
medium of these cells. To examine whether plPEDF is indeed a phosphoprotein, we first immunobloted samples of plPEDF and rPEDF with various anti-phospho amino acid Abs. Both proteins were specifically recognized by anti-phospho-Ser Ab but not by anti-phospho-Thr, or by anti-phospho-Tyr Abs (Fig.1A). As positive controls, we used active phosphorylated ERK (pERK), which was recognized both by anti-phospho-Tyr and anti-phospho-Thr, and α casein, which was recognized only by anti-phospho-Ser Ab. We therefore concluded that plPEDF and rPEDF are phosphorylated on Ser residue(s).

The existence of an extracellular PKA as well as CK2 activities is well documented\textsuperscript{14,16}. Analysis of the primary amino acid sequence of PEDF revealed the existence of several putative phosphorylation sites for CK2, as well as for PKA. In order to examine whether PEDF can be phosphorylated by one of these protein kinases, we have pretreated rPEDF and plPEDF with immobilized alkaline phosphatase prior to in vitro phosphorylation reaction by CK2 and PKA. Phosphorylated products were subjected to SDS-PAGE followed by Western blot, and the membrane used were first exposed to autoradiography and then immunobloted with anti-PEDF Ab. Pretreatment of plPEDF with alkaline phosphatase (Fig. 1B) significantly increased CK2, and to a lesser extent PKA phosphorylation of the protein. The PKA and CK2 phosphorylation of rPEDF following phosphatase treatment were also increased, but not as significantly as plPEDF (Fig. 1C).

To further verify that CK2 phosphorylation of PEDF can occur in plasma, we pretreated plPEDF with alkaline phosphatase following its phosphorylation by fresh human plasma. A phosphorylated product that corresponds exactly to PEDF was detected by the autoradiogram (Fig. 1D left panel). Heparin, which is an inhibitor of CK2\textsuperscript{26}, and PKI\textsuperscript{27} which inhibits PKA inhibited this reaction (Fig. 1D, right panel). Taken together, our results indicate that PEDF is phosphorylated in the circulating blood on the CK2 sites. The small amount of phosphorylation in the secreted rPEDF may be a result of cellular phosphorylation.
Fig. 1

A. 

B. 

C. 

D. 

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Fig. 1: PEDF in plasma is a phosphoprotein. A, Aliquots of rPEDF, plPEDF active (phosphorylated) ERK, phosphorylated α-casein (phos cas) and dephosphorylated α-casein (dephos cas) were subjected to 10% SDS-PAGE (1 µg/lane) and immunobloted with anti-phospho Ser, Thr, or Tyr Abs in the presence or absence of the appropriate phosphorylated amino acid (2.5mM). As a control, the samples were blotted with anti-PEDF, anti-phosphorylated ERK (αpERK) and anti-general ERK (αgERK) Abs, or stained with gel code for the α-casein. B, rPEDF (2 µg) or plPEDF (2 µg) were incubated with alkaline phosphatase (APase) conjugated to acrylic beads (2U) or with protein A sepharose CL-4B (1.6mg) for 45min at 30°C. Following incubation, samples were centrifuged in order to remove the phosphatase, and the supernatants were subjected to in vitro CK2 or PKA phosphorylation as described under Material and Methods. Phosphorylated products were analyzed by 10% SDS-PAGE and blotting which was followed by exposure to autoradiography (Auto, upper panel) and immunoblot with anti-PEDF Ab (lower panel). C, Quantitative analysis of the experiment depicted in Fig. 1B (n=4). D, PEDF purified from human plasma was subjected to alkaline phosphatase treatment as described above. Thereafter, aliquots (1.5 µg) were incubated with fresh human plasma (8 µl) and [γ^32P]-ATP (20 µM, 6Ci/mmol) in the presence or absence of PKA inhibitor (PKI, 1 µg/ml) or heparin (100 µg/ml). Control samples were subjected to in vitro CK2 or PKA phosphorylation as described in Fig. 1B. Phosphorylated products were analyzed as described in Fig 1B. Vn – plasma vitronectin

CK2 and PKA phosphorylate PEDF in vitro. After showing that plPEDF is a phosphoprotein that can be phosphorylated by CK2 and PKA, we undertook to further analyze these phosphorylations. Thus, rPEDF and plPEDF were incubated with CK2 and [γ^32P]-ATP, with an increasing concentration of poly-L-lysine, which activates CK2 in vitro. Both rPEDF and plPEDF were phosphorylated by CK2 (Fig. 2A), and as reported for calmodulin, the phosphorylation of PEDF was dependent on the presence of poly-L-lysine. Additionally, CK2 phosphorylation of rPEDF was stronger than the phosphorylation of identical amount of plPEDF (Fig. 2A), indicating that some of the plPEDF sites are already phosphorylated. Heparin was found to inhibit CK2 phosphorylation of PEDF (Fig. 2B).

We then analyzed whether PEDF is an in vitro substrate of PKA as well. Therefore, rPEDF and plPEDF were incubated with the pure catalytic subunit of PKA and [γ^32P]-ATP in the presence of heparin, which stimulates PKA phosphorylation of several substrates. Both rPEDF and plPEDF were equally phosphorylated by PKA in the presence of heparin (Fig. 2C) in a PKI-inhibited manner (not shown), indicating that both proteins contain only a small amount of phosphate incorporated to the PKA site.
Fig. 2

A. Autoradiogram

B. Coom

C. Autoradiogram

D. Autoradiogram

E. Tryptic peptides of the 20kDa fragment
Trytic peptides of the 30kDa fragment
N-terminal sequence of the 30kDa fragment
Putative CK2 phosphorylation site
Glycosylation site

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Fig. 2: CK2 and PKA phosphorylate PEDF in vitro. A, rPEDF and pPEDF were incubated with CK2 holoenzyme, \([\gamma^{32}\text{P}]-\text{ATP}\) and increasing concentrations of poly-L-lysine (PLL) as described under Material and Methods. As a control, rPEDF and pPEDF were incubated in the same mix in the absence of CK2. After 45 min at 30°C, the reaction was arrested by boiling for 5 min in sample buffer, and the samples were subjected to 10% SDS-PAGE. The gel was stained with Coomassie blue (Coom, lower panel), dried, and subjected to autoradiography (Auto, upper panel). B, rPEDF and pPEDF (50 µg/ml) were incubated with CK2 holoenzyme (4 µg/ml), \([\gamma^{32}\text{P}]-\text{ATP}\) (10 µM, 6 Ci/mmol), poly-L-lysine (200 nM) and increasing concentrations of heparin (Hep). Phosphorylation and analysis were followed as of panel A. C, rPEDF and pPEDF (50 µg/ml) were incubated with the pure catalytic subunit of PKA (2.5 µg/ml), heparin (50 µg/ml) and \([\gamma^{32}\text{P}]-\text{ATP}\) (10 µM, 6 Ci/mmol). Phosphorylation and analysis were as of panel A. D, rPEDF was digested with trypsin as described under Material and Methods. At the indicated times, aliquots were removed from the reaction mixture, centrifuged, and sample buffer was added to the supernatant. Samples were boiled and subjected to 12.5% SDS-PAGE followed by silver stain. Right panel, rPEDF was phosphorylated by CK2 and loaded on a G25 Sephadex column to remove the excess of \([\gamma^{32}\text{P}]-\text{ATP}\). The eluted fraction was then subjected to trypsin digestion and subjected to 12.5% SDS-PAGE followed by autoradiography. E, Alignment of the tryptic peptides revealed by mass spectrometry and N-terminus sequence analysis that were obtained from the trypsin digested fragments of rPEDF within a schematic representation of PEDF.

Localization of the CK2 phosphorylation site(s) in PEDF. CK2 phosphorylates Ser or Thr immersed in acidic sequence within proteins and peptides\(^{30}\). The minimum requirement for CK2 phosphorylation is depicted by the sequence S/T-X-X-D/E. The presence of additional Asp or Glu residues at positions −3, +1, +2, +4, +5, or +7 improves the phosphorylation efficacy. By examining the primary sequence of PEDF for potential phosphorylation sites, 11 putative sites that meet the minimal consensus requirements were found. These are S24, S114, T121, S195, T219, T226, S227, T287, S328, S336, and T354. Of these, S24, S114, S195, T226, S227 and T287 were considered as preferred targets since they contain additional acidic residues in the preferred positions.

In an attempt to identify the actual CK2 phosphorylation site(s) in PEDF, rPEDF was digested with trypsin. This partial digestion yielded two major fragments with an apparent molecular weight of 20kDa and 30kDa (Fig. 2D). We then phosphorylated rPEDF by CK2 and digested the phosphorylated protein with trypsin. Only the 20kDa fragment was phosphorylated by CK2 (Fig. 2D), indicating that the CK2 phosphorylation site is located
within the 20kDa fragment. We found that the fragment could not be sequenced by Edman degradation since it was blocked, indicating that it is the N-terminal fragment of PEDF. The 30kDa fragment was sequenced by Edman degradation and was found to start at amino acid Glu198. Mass spectrometry revealed more peptides in the 30kDa fragment (Fig. 2E) confirming its C-terminal position. Since the CK2 phosphorylation sites are located within the 20kDa fragment, we concluded that Ser24 and/or Ser114 are the sites of CK2 phosphorylation. However, because the combined mass of the fragments is smaller than that of full-length rPEDF, it is possible that an additional CK2 phosphorylated fragment, which run out of the gel, was also formed.

Fig. 3

- Diagram A shows the auto and Coomassie blue staining of rPEDF with mutations S24A, S24E, S114A, S114E, S24,114A, and S24,114E. The relative phosphorylation (% of rPEDF) is measured from 0 to 275.

- Diagram B shows the auto and Coomassie blue staining of rPEDF with mutations S227A and S227E.
Fig. 3: Identification of CK2 and PKA phosphorylation sites of PEDF by site directed mutagenesis. A, rPEDF and rPEDF mutants (indicated) were phosphorylated by CK2 as described under Material and Methods. Reaction was arrested by sample buffer and samples were subjected to 10% SDS-PAGE. The gel was stained with Coomassie blue (Coom, lower panel), dried, and subjected to autoradiography (Auto, upper panel). B, rPEDF and rPEDF mutants (indicated) were phosphorylated by PKA. Samples were subjected to 10% SDS-PAGE, stained with Coomassie blue (Coom, lower panel), dried, and subjected to autoradiography (Auto, upper panel). C, Quantitative analysis of the autoradiogram depicted in Fig. 3A and B (n=6).

Identification of the CK2 phosphorylation site(s) by site directed mutagenesis. To further study the CK2 phosphorylation sites in PEDF, we constructed single or double site mutants by replacing Ser at position 24, 114 with Ala (S24A, S114A and S24,114A) or with Glu (S24E, S114E and S24,114E). rPEDF and its mutants were purified from the medium of the transfected HEK-293T cells and subjected to phosphorylation by CK2. Mutation of S24A significantly reduced CK2 phosphorylation (Fig. 3), while the S24E mutation reduced phosphorylation only to a moderate extent. The S114A mutant significantly reduced CK2 phosphorylation, while the double mutant S24,114A almost completely abolished this phosphorylation. We concluded that both Ser24 and Ser114 are the main sites for CK2 phosphorylation of PEDF. Surprisingly, both S114E and S24,114E mutations significantly increased phosphorylation compared with CK2 phosphorylation of rPEDF (Fig. 3). This unexpected result implies that probably mutation of this residue to Glu leads to the exposure of additional potential phosphorylation sites that were normally covered. Analysis of the three dimensional structure of PEDF\textsuperscript{31} revealed that Thr121 is spatially close to Ser114 and may serve as the additional site. However, since these sites may be covered upon phosphate incorporation to Ser24 and Ser114, it is possible that Thr354 is the other phosphorylated site. This site might have been phosphorylated by CK2 but was not detected in the tryptic digest because it was included in a small fragment that was not present on the gels. Nonetheless, our results indicate that PEDF is phosphorylated by CK2 mainly on residues Ser24, and Ser114.
Identification of the PKA phosphorylation site by site directed mutagenesis. PKA phosphorylates Ser or Thr residues adjacent to at least two consecutive basic residues, depicted by the consensus sequence of R/K-R/K-X-S/T. By examining the primary sequence of PEDF for potential PKA phosphorylation sites, we found one such putative site at Ser227. In order to confirm this PKA phosphorylation site in PEDF, we constructed a single site mutant by replacing Ser227 either with Ala (S227A) or with Glu (S227E). The rPEDF and the mutants were purified as described above and subjected to phosphorylation by PKA. Mutation of Ser227 to Ala or Glu completely abolished PKA phosphorylation of both rPEDF and pPEDF (Fig. 3), indicating that this residue is indeed the PKA site in PEDF.

A three dimensional structure analysis of the CK2 and PKA phosphorylation sites in PEDF revealed that Ser114 and Ser227 residues are exposed and can be accessible to interact with potential kinases. Ser24 is not included in the crystal structure however the location of the N-terminus is spatially converging to Ser114. Therefore, from the structural point of view, these residues may well serve as substrate candidates for phosphorylation.

Activation of ERK by PEDF in endothelial cells. It was previously shown that CK2 phosphorylation of vitronectin significantly enhances endothelial cell adhesion. We undertook to study the effect of PEDF and its phosphorylated forms on the signaling and physiological responses of endothelial cells. Therefore, serum-starved endothelial cells were incubated with rPEDF or with pPEDF, and cell lysates were analyzed for MAPKs and PKB activity using anti-phospho Abs. PKB as well as JNK, p38MAPK or ERK5 were not significantly affected in any of the conditions used (not shown). On the other hand, rPEDF caused a small (x5) but reproducible activation of ERK phosphorylation in endothelial cells, whether obtained from a human source (e.g., HUVEC; Fig. 4A) or from a bovine source (e.g., BAEC; not shown). The maximal activation of ERK1/2 was obtained after 15 min with 10nM PEDF. Interestingly, the activation obtained with pPEDF was higher than that with rPEDF in HUVEC (Fig. 4B) as well as in BAEC (not shown).
Fig. 4: The effect of rPEDF, plPEDF and the various rPEDF mutants on ERK/MAPK activation in HUVEC. A, HUVEC were serum-starved for 16hrs and then stimulated with different concentrations of rPEDF for the indicated times. Cytosolic extracts (30 µg) were subjected to immunoblotting with anti-pERK (αpERK, upper panel) or anti-gERK (αgERK, lower panel) Abs. The positions of ERK2 and ERK1 are indicated. B, HUVEC were serum-starved for 16hrs and then stimulated with rPEDF (10nM) or with plPEDF (10nM) for the indicated times. Cytosolic extracts (30 µg) were subjected to immunoblotting as described above. C. HUVEC were serum-starved for 16 hrs and then stimulated with rPEDF (10nM), plPEDF (10nM), or with the various rPEDF mutants (10nM) for 15min. Cytosolic extracts (30µg) were subjected to immunoblotting as described above. D, Quantitative analysis of immunoblots depicted in panel C and D (n=5).
The effect of rPEDF mutants on ERK activation. Because of the differences in ERK activation between plPEDF and rPEDF, we used this system to examine whether the phosphorylation mutants indeed mimic the effect of phosphorylation on PEDF activity. When used to stimulate HUVEC, the CK2 phosphorylation site mutants S24A and S24E did not have a significant effect, while S114A and S114E mutants demonstrated slightly reduced ability to stimulate ERK phosphorylation (Fig. 4C). However, significant effects were found with the double mutants, as S24,114A had a reduced effect, while S24,114E enhanced ERK phosphorylation. These effects were even stronger than the effects of rPEDF or plPEDF respectively. The higher activity of S24,114E suggests that the two Glu residues indeed mimic the activity of phosphorylated PEDF. However, plPEDF is incompletely phosphorylated in contrast to the existence of negatively charged residues in positions 24 and 114 of all molecules of the S24,114E. Similarly, the activity of S24,114A was lower than that of rPEDF suggesting that a small fraction of the rPEDF molecules is phosphorylated on Ser 24 and 114. Thus, the mutants S24,114E and S24,114A further extent the phosphorylation-dependent differences between plPEDF and rPEDF.

Differences in ERK activation were observed also with the PKA mutants. Thus S227A completely inhibited the ability of rPEDF to induce ERK1/2 phosphorylation, whereas the S227E mutant had only a slight inhibitory effect (Fig. 4D). Similar results were obtained with BAEC (not shown). These results further indicate that rPEDF is secreted as a phosphorylated protein on residue 227, in agreement with the phosphatase study above. Removal of the phosphate abolishes the PEDF-induced ERK phosphorylation, while Glu at this position elevated the PEDF effect. Taken together, our results indicate that the Glu or Ala mutants indeed mimic either the phosphorylated or non-phosphorylated forms of PEDF.
Fig. 5: The effect of rPEDF, pPEDF and the various rPEDF mutants on PEDF neurotrophic activity. The cells (2.5 x 10^5 cells/ml) were incubated with rPEDF, pPEDF, or the various rPEDF mutants (all at 20nM) in MEM supplemented with 2 mM L-glutamine, antibiotics, and 0.1% ITS. After 7 days in culture, the cells were transferred onto poly-D-lysine coated plates and their morphology and differentiation state was monitored by light microscopy at various periods of time. The Y-79 morphology at 10 days post-attachment is shown. dia= diameter.

B. Quantitative analysis of the results presented in panel A. Student t-test was used to analyze statistical significance of the differences between cells treated with rPEDF and cells treated with the various PEDF forms (* P < 0.01, ** P < 0.05; n=6).
The effect of rPEDF mutants on its neurotrophic activity. We examined whether CK2 as well as PKA phosphorylation of PEDF can modulate its neurotrophic activity. Thus, we tested rPEDF, pPEDF and the mutants for their ability to induce differentiation in human retinoblastoma Y-79 cells in culture. Indeed rPEDF and pPEDF induced neuronal differentiation (cell aggregation and neurite outgrowth) in Y-79 cells, where the effect of rPEDF was more pronounced compared to pPEDF (Fig. 5). The CK2 phosphorylation site mutants S24E/S24A and S114E/S114A had only small effects, as they all induced neuronal differentiation of the Y-79 cells. However, much less neurite-like processes and cell aggregates were observed when cells were treated with the S24,114E mutant. With this mutant, the cells formed small corona-like structures but were very compact without any sprouts projecting from the cells, and this inhibitory effect was stronger than that of pPEDF. On the other hand, cells treated with the S24,114A mutant exhibit neurite outgrowth and big aggregates similar to rPEDF. Mutation of the PKA phosphorylation site S227E revealed a different phenotype, where colonies were smaller, fewer and randomly spread, although their processes were clearly observed. Therefore, PKA phosphorylation has a limited influence on the neurotrophic effect of PEDF while CK2 phosphorylation significantly reduces this neurotrophic effect.

The effect of PEDF mutants on its antiangiogenic activity ex-vivo. To examine the effect of phosphorylation on the antiangiogenic activity of PEDF, we used the ex-vivo aortic ring assay in the presence of bFGF as an angiogenic model. In the presence of bFGF (50ng/ml), aortic rings from BALB/C mice developed numerous vessels-like sprouts as compared to the rings that were treated with serum free medium (Fig. 6). As expected, pPEDF significantly inhibited the bFGF-induced vessel formation. However, the inhibitory effect of rPEDF was less pronounced than that of pPEDF, as rearrangement towards vessel formation and small number of vessels structure were observed when rPEDF and bFGF were added together.
Fig. 6: The antiangiogenic activity of the various rPEDF forms on bFGF-induced vessel sprouting in the ex-vivo aortic ring assay. A. Aortic rings from BALB/C mice embedded in collagen matrix were exposed to rPEDF, plPEDF, S24,114A mutant, S24,114E mutant, S227A mutant or S227E mutant (10nM) in the presence or absence of bFGF (50ng/ml) in the serum free BIO-MPM-1 medium. Control rings were treated with serum free BIO-MPM-1 medium, or with bFGF (50ng/ml). Following 10 days of incubation, rings were fixed and stained with crystal violet (0.02%) to illustrate sprouting and vessels formation. Representative micrographs of ring of each arm of the experiment are shown. Micrographs were taken under X4 and X10 objective. B. Quantitative analysis of the assay described in panel A. Student t-test was used to analyze statistical significance of the differences between rings treated with bFGF and rings treated with the combination of bFGF and the various PEDF forms. (* P < 0.01; n=6).
We then examined the effects of the phosphorylation site mutants. When incubated together with bFGF, the CK2 non-phosphorylated double mutant, S24,114A, exhibited an antiangiogenic activity that was similar to or slightly less than that of rPEDF, where rearrangement towards vessels could be seen, but clear vessels did not form. On the other hand, the CK2 phosphorylated mutant, S24,114E, appeared to be a very significant antiangiogenic factor, even stronger than plPEDF, as it did not allow any vessel formation. The PKA non-phosphorylated mutant, S227A, inhibited the bFGF-induced vessel formation similarly to rPEDF, while the PKA phosphorylated mutant, S227E, had less antiangiogenic activity. S227E alone was not proangiogenic and its effect on the bFGF-induced angiogenesis was reduced as compared to rPEDF. We therefore conclude that phosphorylation of PEDF on its CK2 sites significantly enhanced the antiangiogenic activity of PEDF, while the phosphorylation on its PKA site may slightly reduce its antiangiogenic activity.

The effect of rPEDF mutants on its antiangiogenic activity in vivo. To further assess the effect of phosphorylation on PEDF antiangiogenic activity in vivo we have used the Matrigel plug assay in the presence of bFGF as an angiogenic model. Thus, liquid Matrigel supplemented with the various treatments was injected subcutaneously into CD-1 nude mice. The Matrigel polymerized to form a plug, which was removed after a week and analyzed for its angiogenic response. As expected, control plugs treated with PBS or PEDF alone showed very little angiogenic response (Fig. 7). bFGF-impregnated plugs elicited a robust angiogenic activity, as judged by the large number of blood vessels infiltrating into the plug. plPEDF significantly inhibited the bFGF-induced vessel infiltration, while the inhibitory effect of rPEDF was significantly less pronounced. As showed in the aortic ring assay, the S24,114E mutant had even stronger antiangiogenic activity relative to plPEDF, as plugs treated with this mutant had very little angiogenic response. In contrast, plugs treated with bFGF and S227E had much less antiangiogenic activity reflected in many infiltrating vessels. In
addition plgs treated with bFGF and S24,114A mutant or S227A mutant appeared similar to those treated with bFGF and rPEDF (not shown). These results further support that CK2-phosphorylated PEDF enhanced the antiangiogenic activity of PEDF, while the phosphorylation on its PKA site may reduce this activity.

Fig. 7

A. PBS  
   rPEDF  
   plPEDF  
   bFGF + S227E  
   bFGF + S24,114E  

B. Microvessels (number/field)

Fig. 7: The antiangiogenic activity of the various rPEDF forms on bFGF-induced neovascularization in the in vivo Matrigel plug assay. A, CD-1 nude mice were subcutaneously injected with 0.5ml Matrigel containing rPEDF, plPEDF, S24,114E mutant and S227E mutant (all at 20nM) in the presence or absence of bFGF (300ng/ml). Control plugs were combined with PEDF (20nM) or bFGF (300ng/ml) only. After 7 days, mice were sacrificed and Matrigel plugs were excised, fixed in 4% formaldehyde, embedded in paraffin, sectioned and stained. Representative fields of H&E staining of thin sections from Matrigel plugs of each arm of the experiments are shown (X40 magnification). B. Angiogenesis was measured by counting the number of blood vessels/field for three different cross sectional area of each Matrigel plug. Student t-test was used to analyze statistical significance of the differences between plugs treated with bFGF and plugs treated with the combination of bFGF and the various PEDF forms (* P < .01, ** P < .05; n=3).
DISCUSSION

In this report we demonstrated that PEDF is present in the human plasma as a phosphoprotein, with phosphates incorporated to two of its CK2 sites and to a lesser extent on one PKA site. rPEDF is also phosphorylated on the sites of these kinases but to a much lower extent. Indeed, rPEDF can be phosphorylated by CK2 and PKA in vitro (Fig. 1A-C), and these phosphorylations can be achieved also by the whole plasma (Fig. 1D). These results indicate that the phosphorylation may occur, at least in part, in the circulating blood and may serve as a regulatory mechanism of PEDF activity.

The two different sources of PEDF used in our study, rPEDF and pIPEDF exhibited different physiological activities in the assays used. We examined whether the physiological differences between the two proteins are induced by the differences in their phosphorylation. Thus, we constructed several phosphorylation site mutants that were supposed to mimic either the phospho (Glu) or non-phospho (Ala) forms of PEDF. First we determined whether the mutants indeed behave like their phosphorylated counterparts. Therefore, we compared the mutants to rPEDF and pIPEDF for their ability to induce ERK phosphorylation. We found that these constructs even amplify the differences in phosphorylation between pIPEDF and rPEDF (Fig. 4). This could be due to the fact that while rPEDF is slightly phosphorylated by CK2, S24,114A is not phosphorylated at all on these sites. On the other hand, part of the molecules of pIPEDF seem to be phosphorylated on the CK2 site, while the S24,114E represent a homogeneous PEDF population with a negative charge at positions 24 and 114. Unlike the significant differences in the CK2 sites, pIPEDF and rPEDF were found to be phosphorylated to a small extent on Ser227. However, S227A represent molecules that are not phosphorylated at all, while S227E represents fully phosphorylated Ser227. We conclude that the amount of negative charges on residues 24 and 114 is in the following order...
S24,114E > plPEDF >> rPEDF > S24,114A, while the phosphorylation on Ser227 is in the order S227E >> plPEDF = rPEDF > S227A.

Previous reports demonstrated that the recombinant His-tagged PEDF is functionally identical to the PEDF isolated from bovine eyes\textsuperscript{9,10,33}. Here we show that although rPEDF may induce part of the activities of plPEDF, these two proteins still represent significant differences in their effects, which are dependent on their degree of phosphorylation. Thus, we observed a CK2-dependent differences in the ability of PEDF to induce neuronal differentiation in retinoblastoma cells where both rPEDF and S24,114A mutants induced neuronal differentiation, while the S24,114E had almost no neurotrophic effect. These results correlated with the extent of CK2 phosphorylation, and clearly indicate that lack of CK2 phosphorylation makes PEDF a superior neurotrophic factor. On the other hand the results with the PKA mutants indicated that although this phosphorylation may play a role in the proliferation of the cells, it probably does not affect the neurotrophic activity of PEDF.

Dependence on the extent of phosphorylation was also observed in the two angiogenic assays used in this study namely the aortic rings assay (Fig. 6), and the Matrigel plug assay (Fig. 7). In both this assays however, the phosphorylation increased, rather than decreased, the effect. Thus, S24,114E appeared to be a very significant antiangiogenic factor and its inhibitory effect was more pronounced when compared to plPEDF antiangiogenic activity. Treatment with S227E reduced the antiangiogenic effect of PEDF, and in both assays the effect of this mutant was even smaller than that of rPEDF. Therefore, it is possible that phosphorylation of Ser227 and possibly Ser24 and Ser114 induce a general anti-proliferative effect of PEDF.

The present data reveal different physiological functions of PEDF that are dependent on the phosphorylation state of the protein. Indeed the various functions of PEDF that are well described in the literature reveal a protein that can maintain dual activity. PEDF is a very
effective neuroprotective\textsuperscript{5,6}, as well as antiangiogenic\textsuperscript{9,10} factor. The mechanism involved in PEDF’s various functions has not yet been revealed. This is mainly due to lack of information on the nature of the receptor for PEDF. Although an \textasciitilde80kDa interacting protein has been reported for PEDF in retinoblastoma (Y-79) and neuronal cells\textsuperscript{7,8}, it is not clear whether this is indeed the receptor or another type of regulatory protein. Therefore, it is possible that PEDF is exerted by two distinct receptors or by one receptor that may be influenced differently by the phosphorylated or non-phosphorylated PEDF.

Additional support for a functional change of PEDF that is phosphorylation dependent was demonstrated by the degree of ERK activation. A significant effect was found with the double mutants, as S24,114A reduced ERK activation, while S24,114E enhanced ERK activation to a higher degree than the stimulation achieved by pPEDF. Apparently, none of the forms of PEDF that has been used were able to significantly inhibit the bFGF-induced ERK activation (data not shown). Therefore, there is no correlation between the activation of ERK and either the antiangiogenic or the neurotrophic activities of PEDF. The lack of correlation, together with the low extend of the ERK activation, indicate that PEDF signaling is not mediated by the ERK cascade.

Evidence for the existence of an extracellular PKA and CK2-like activity was documented in several laboratories. One of the substrates for these extracellular kinases is vitronectin that was found to be a major substrate for PKA in blood\textsuperscript{14}. This phosphorylation attenuates the vitronectin binding to PAI-1, and consequently modulates this important physiological function of vitronectin\textsuperscript{18}. Additionally it was shown that urokinase plasminogen activator (u-PA) is phosphorylated on Tyr residue\textsuperscript{34}, and the phosphorylated u-PA exhibits a lower affinity for PAI-1 and PAI-2 than the non-phosphorylated form\textsuperscript{35}. These studies together with our current findings support the involvement of extracellular protein phosphorylation as a mechanism of regulatory cellular processes. Thus, our findings indicate
that the extracellular phosphorylation of PEDF may well be a physiological regulatory mechanism that control PEDF multifaceted functions, and determines its specific outcome.

In summary we have shown that PEDF purified from human plasma (pPEDF) is a phosphoprotein, which is phosphorylated in the circulation by CK2 and to a lesser degree also by PKA. The physiological relevance of these phosphorylations was determined by comparing rPEDF to the phosphorylation site mutants in regard to their neurotrophic and antiangiogenic activities. We found that both CK2 and PKA phosphorylations of PEDF markedly affect its physiological function. The fully CK2 phosphorylation site mutant S24,114E abolished PEDF neurotrophic activity and enhanced its antiangiogenic activity, while the PKA phosphorylation site mutant S227E reduced PEDF antiangiogenic activity. Therefore, phosphorylation may determines the specific physiological outcome of PEDF, and as such is the first demonstration that extracellular phosphorylation causes a complete change of function of a circulating protein.

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REFERENCES


Extracellular phosphorylation converts pigment epithelium-derived factor from a neurotrophic to an antiangiogenic factor

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