PTP-ε, a tyrosine phosphatase expressed in endothelium, negatively regulates endothelial cell proliferation

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Am J Physiol Heart Circ Physiol 281: H396–H403, 2001.—The vascular endothelium is a dynamic interface between the blood vessel and circulating factors and, as such, plays a critical role in vascular events like inflammation, angiogenesis, and hemostasis. Whereas specific protein tyrosine kinases have been identified in these processes, less is known about their protein tyrosine phosphatase (PTP) counterparts. Because there are endothelium-specific PTKs like KDR/Flk-1, we hypothesized that endothelial cell-specific PTPs should also exist. We utilized a RT-PCR/differential hybridization strategy to identify a growth-regulated PTP, PTP-ε, that is expressed preferentially in endothelium. Overexpression of PTP-ε decreases proliferation by 60% in human umbilical vein endothelial cells (HUVEC) but not in smooth muscle cells or fibroblasts. In contrast, overexpression of PTP-ε (D284A), a catalytically inactive mutant, has no significant effect on HUVEC proliferation. These data provide the first functional characterization of PTP-ε in endothelial cells and identify a novel pathway that negatively regulates endothelial cell growth. Such a pathway may have important implications in vascular development and angiogenesis.

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TYROSINE PHOSPHORYLATION is a critical event in signal transduction pathways that regulate fundamental cellular processes such as cell proliferation, differentiation, and cytoskeletal function (36). The phosphotyrosine content of target substrates in these processes is governed by a balance between the actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) (8). Like the well-characterized PTKs, PTPs constitute a large and diverse family of enzymes. In fact, more than 80 PTPs have been identified, with an additional 300+ members estimated to exist (55). Whereas great progress has been made in illustrating the structural diversity within this large enzyme family, relatively little is known of the physiological functions of individual PTPs.

All of the structurally diverse PTPs contain at least one highly conserved 240-amino acid catalytic domain. These catalytic domains are defined by the presence of a PTP signature motif, (I/V)HCXAGXXR(S/T)G, which forms the “catalytic pocket” of the enzyme (35, 36). The signature motif functions as a phosphate-binding cleft in which a cysteine residue is sterically positioned for a nucleophilic attack on the phosphorous atom of the phosphotyrosyl residue of a substrate. For catalysis to proceed efficiently, the phenolic oxygen of the tyrosine residue of the substrate must be protonated by a nearby aspartic acid residue, serving as a general acid. Mutation of either of these two essential residues results in a catalytically inactive “substrate-trapping” mutant (15, 20).

Tyrosine phosphorylation events are critical for many endothelial cell (EC) processes. PTK inhibitors decrease EC migration (41), block induction of adhesion molecules (50), and attenuate growth factor-mediated proliferation (17). The repertoire of PTKs involved in the earliest events of EC proliferation is relatively well characterized. They include, but are not limited to, the receptor PTKs for basic fibroblast growth factor and vascular endothelial growth factor (VEGF) (53). EC proliferation also requires the activities of cytosolic PTKs like Jak-1 (10) and focal adhesion kinase (28). Of all EC PTKs, perhaps the most intriguing are the receptor PTKs for VEGF, Flt-1 and KDR/Flk-1, because they are expressed almost exclusively in the endothelium (43) and are essential for EC development (16, 44).

Whereas critical roles for PTKs in the endothelium have been delineated, little is known about their PTP counterparts. Because there are endothelium-specific PTKs like KDR/Flk-1, we hypothesized that endothelium-specific PTPs should also exist. We utilized a PCR-based differential hybridization strategy to identify a growth-regulated PTP, PTP-ε, that is expressed preferentially in endothelium compared with other vascular cell types. Overexpression of PTP-ε in human umbilical vein EC (HUVEC) using a retroviral strategy...
results in a phenotype that grows more slowly than control cells, indicating a negative role for PTP-ε in EC proliferation.

**EXPERIMENTAL PROCEDURES**

**Cell cultures.** Saos-2 osteosarcoma cells, HeLa epidermoid carcinoma cells, HepG2 hepatoma cells, RD rhabdomyosarcoma cells, IM-9 B lymphoblastoid cells, human microvascular EC (HMEC-1), COS7 kidney cells, HCN glioblastoma cells, CEMCT T-cell leukemia cells, HEK293 embryonic kidney cells, and primary culture HUVEC, human fibroblasts, human aortic smooth muscle cells (HASMC), human skeletal muscle cells (HSKM), bovine aortic EC (BAEC), and rat aortic smooth muscle cells (RASMC) were grown as described (52).

**RT-PCR, construction of PTP PCR libraries, and differential hybridization.** PCR amplification was performed on randomly primed cDNA with degenerate primers corresponding to the conserved sequences in the catalytic domains of known PTPs. The sense primer was 5'-AC/GT/CTTGGA(A/G)GI-GATGGA(A/G)TTGGG-3', corresponding to the amino acid sequence (H/D)FWRM(I/V)W, and the antisense primer was 5'-GGGAC(G/A)/TA/AA(G/A)AT/TCIGGGCA-3', corresponding to the amino acid sequence WPD(F/H)GVP. PCR products were gel purified and ligated into the vector pCR II (Invitrogen).

After transformation, 100 recombinant clones were selected, and differential hybridization was performed as described (24). Duplicate filters were hybridized with a 32P-labeled RT-PCR-generated PTP probe from either HUVEC or HASMC. Signals were detected by autoradiography. To identify the PTP probes in the library, recombinant plasmids were sequenced and compared with GenBank entries.

**In situ hybridization.** cDNA probes were labeled with [35S]UTP to generate sense and antisense riboprobes. Sections were prepared by fixation in 4% paraformaldehyde and embedded in paraffin. Hybridization was performed at 55°C for 12 h. Slides were washed and coated with emulsion, exposed in the dark for 4 wk, and counterstained with hematoxylin and eosin. All sections were examined by bright-field microscopy. Magnification was ×200.

**Generation of PTP-ε and PTP-α constructs.** The full-length cDNAs for PTP-ε and PTP-α were cloned into the BamHI and XhoI sites, respectively, of the pLXIN retroviral vector (Clontech) in the sense orientation using a PCR-based strategy. A catalytically inactive mutant, PTP-ε (D284A), was generated by mutation of aspartic acid 284 to alanine using oligo 5'-CAGCTGGCCCGCCTTCGGAGTGC-3', corresponding to the amino acid sequence (29).

**Retroviral-mediated gene delivery.** pLXIN-based constructs containing either green fluorescent protein (GFP), PTP-ε, PTP-α (D284A), or PTP-ε alone were stably transfected into PT67 packaging cells using G-418 selection to generate a homogenous population of cells producing retrovirus. Primary culture HUVEC, HASMC, or human fibroblasts were infected with retroviruses derived from plasmids pLXIN, pLEIN-GFP, pLXIN-PTPε, pLXIN-PTPε (D284A), or pLXIN-PTPα as previously described (30) to produce a population of cells expressing the gene of interest. Overexpression of PTP-ε and PTP-ε (D284A) was confirmed by Northern blot analysis, and cells were used at equivalent passages (usually 2–3 passages after viral transduction) in experiments.

**Proliferation assays.** For growth curves, virally transduced cells were plated at a density of 1 × 10⁴ cells/well in six-well plates. At each indicated time point, cells were harvested and counted. Each experiment utilized a different set of virally transfected cells. Data are the means ± SE of three experiments done in triplicate. For thymidine-uptake studies, cells were plated at a density of 4.2 × 10³ cells/well in 24-well plates. Cells were incubated in the presence of 2 μCi/ml [3H]thymidine for 3 h and harvested as described (37). Data are the means ± SE of four experiments done in triplicate. For growth curve and thymidine-uptake analysis, a t-test was applied using SigmaStat software. Statistical significance was accepted at P < 0.05.

**RESULTS**

**Identification of PTPs expressed in human EC.** We employed a strategy of degenerate PCR amplification and differential hybridization to identify novel and differentially expressed PTP genes in vascular EC (Fig. 1A, left). This procedure relies on the fact that all known PTPs contain highly conserved amino acid sequences within their catalytic domains and has been used successfully to evaluate PTP expression in other cell types (54). Of the 100 clones sequenced, 92 encoded known PTPs (Fig. 1A, right) and 8 encoded genomic sequences and/or non-PTP-related cDNAs.

![Fig. 1. Identification of protein tyrosine phosphatase (PTP)-ε as a differentially expressed PTP. A, left: diagram of the approach undertaken to identify differentially expressed PTP mRNAs in endothelial cells (EC). RT-PCR was performed on total RNA from subconfluent human umbilical vein EC (HUVEC) using degenerate primers to highly conserved amino acid sequences within PTP catalytic domains. These amplifiers were identified by sequence analysis. Right: PTPs identified in vascular EC by this approach. B: differential hybridization analysis of PTP clones to rapidly identify PTPs that are expressed in an endothelial-restricted fashion. cDNAs from PTP clones were immobilized onto duplicate membranes, and each was hybridized with radiolabeled RT-PCR-generated PTP probes from either HUVEC (left) or human aortic smooth muscle cells (HASMC; right). Only PTP-ε clones exhibited a differential pattern of expression (C).](http://ajpheart.physiology.org/Downloaded from http://ajpheart.physiology.org/ by 10.220.33.6 on November 9, 2017)
To screen for EC PTPs that are expressed in a tissue-specific fashion, we utilized a differential hybridization procedure that allowed us to rapidly examine the relative expression of multiple clones in a semiquantitative fashion. cDNAs from the 100 clones described above were immobilized onto duplicate filters, and each filter was hybridized with an RT-PCR-generated PTP probe from either HUVEC or HASMC (Fig. 1B). All six clones that were highly expressed in HUVEC, but hybridized negligibly with the HASMC probe, encoded PTP-ε, a previously identified PTP of unknown tissue distribution and function. In contrast to clones encoding PTP-ε, other PTP clones did not exhibit differential expression. The results of our differential hybridization experiments suggested that PTP-ε might be an EC-specific or restricted PTP. PTP-ε is a member of the receptor-like family of PTPs and consists of a small extracellular region, a single transmembrane region, and two phosphatase domains (27).

**Preferential expression of PTP-ε in vascular EC.** To explore the possibility that PTP-ε is preferentially expressed in EC, Northern blot analysis was used to probe for the presence of PTP-ε mRNA in EC and non-EC in culture (Fig. 2A). A single major band (5.5 kb) denoting PTP-ε mRNA was observed, and this mRNA was easily detected in HMEC-1, BAEC, and HUVEC. In contrast, PTP-ε mRNA was not detected in primary culture HASMC and RASMC or in the cell lines HeLa, RD, Saos-2, IM-9, HepG2, COS7, HCN, HSKM, CEMC7, and HEK293. For comparison, we examined the expression of the related phosphatase PTP-α, which was expressed broadly in all cell lines examined. In HUVEC, we estimated expression of PTP-ε mRNA to be approximately twofold that of PTP-α. These experiments confirm the results of our differential hybridization screen (Fig. 1B) and indicate that PTP-ε is expressed in a highly restricted pattern in vascular EC in culture. Consistent with these results, PCR-based screens have failed to identify PTP-ε mRNA in HASMC or human fibroblasts (data not shown).

To examine the vascular expression of PTP-ε mRNA in vivo, we performed in situ hybridization analysis of baboon brachial arteries using sense and antisense PTP-ε riboprobes. Specific hybridization of an antisense PTP-ε probe was localized to the continuous layer of EC lining the artery, whereas no signal was observed in adjacent cells (Fig. 2B, top). The specificity of hybridization was demonstrated by a lack of signal using the sense PTP-ε riboprobe (Fig. 2B, bottom). A similar endothelial-restricted expression pattern was observed in venous and arteriolar sections (data not shown). These experiments confirm the results of our differential hybridization screen and indicate that PTP-ε expression is highly restricted to EC in vivo.

**PTP-ε expression is serum responsive.** The catalytic domains of PTP-ε share 70–80% sequence identity with PTP-α, a phosphatase known to mediate cell proliferation and transformation by dephosphorylating, and hence activating, c-Src (56). On the basis of this finding, we hypothesized that PTP-ε might also regulate cell proliferation, and we examined its expression under conditions of high and low EC growth rates. When HMEC-1 were cultured in serum-free medium, PTP-ε mRNA was downregulated by 60% after 6 h (Fig. 3A), whereas PTP-α mRNA levels remained essentially unchanged. Addition of 10% fetal bovine serum to HMEC-1 after 3 h of starvation rescued PTP-ε mRNA expression. Similarly, addition of VEGF (20 ng/ml) to these cells after 3 h of starvation resulted in partial rescue of PTP-ε expression. Interestingly, the pattern of expression of PTP-ε mRNA was identical to that for mitogen-activated protein kinase (MAPK) phosphatase (MKP-1) in this cell type (Fig. 3A). Similar results were obtained when these experiments were performed with HUVEC (80% decrease in PTP-ε mRNA after 6 h; Fig. 3B).
PTP-ε negatively regulates EC proliferation. Because PTP-ε is growth factor responsive, we hypothesized that PTP-ε modulates EC growth. Since traditional mechanisms of transfection provided less-than-optimal results in primary cell cultures like HUVEC, we implemented a highly efficient retroviral-mediated approach for gene delivery to allow for stable gene expression in primary culture HUVEC. HUVEC were infected with retrovirus containing the cDNAs for wild-type PTP-ε, PTP-α, GFP, or empty vector. We also generated a catalytically inactive mutant of PTP-ε [PTP-ε (D284A)], in which aspartic acid 284 was replaced by alanine. This mutation resides in the membrane-proximal catalytic domain of PTP-ε, which contains essentially all of the phosphatase activity of the enzyme (29). Inactivation of PTP-ε was confirmed by measuring the phosphatase activities of wild-type and D284A-mutant glutathione-S-transferase fusion proteins (data not shown). When HUVEC were virally transduced with GFP, 100% of cells expressed GFP (Fig. 4A). Overexpression (15- to 20-fold) of both wild-type and mutant PTP-ε cDNA in HUVEC populations was observed in Northern blot analyses (Fig. 4B). We performed phosphotyrosine immunoblotting in these cells to exclude the possibility that overexpression of PTP-ε exerted nonspecific effects on tyrosine phosphorylation. These experiments indicated that total protein phosphorylation events were not grossly affected by overexpression of PTP-ε (data not shown).

Growth curve analysis revealed that overexpression of PTP-ε in HUVEC results in a 46% reduction in proliferation (Fig. 5A). This difference was statistically significant by the second day of the growth curve (P < 0.05). Similarly, DNA synthesis in HUVEC overexpressing PTP-ε was 60% of control values (Fig. 5B; P < 0.05). To discriminate between effects on cell proliferation and apoptosis, the nuclei of transduced HUVEC were stained with 4,6-diamidino-2-phenylindole and visualized by fluorescence microscopy. We saw no difference in apoptotic indexes between HUVEC overexpressing PTP-ε and those transduced with vector controls (data not shown). This growth-inhibitory activity seemed specific for PTP-ε insofar as PTP-α overexpression had no significant effect on growth in HUVEC (although a weak effect of PTP-α on growth of HUVEC that escaped our ability to detect a statistically significant difference cannot be excluded) (Fig. 5A). The catalytic activity of PTP-ε is required for its proliferative effects, because PTP-ε (D284A) did not attenuate EC proliferation (Fig. 5, A and B). It should be noted that PTP-ε (D284A) behaved as a loss-of-function mutant in these studies, although the strong growth conditions used may have masked any dominant negative effects exerted by this protein. In any event, these studies indicate that PTP-ε is a negative regulator of EC proliferation.

The specificity of the effects of PTP-ε on EC proliferation was tested by comparing overexpression of PTP-ε in HUVEC, HASMC, and human fibroblasts. Retroviral-mediated overexpression produced PTP-ε levels that were comparable in the three cell types (data not shown).
Although the basal proliferative rates varied among cell types, PTP-ε only caused a significant reduction in DNA synthesis when overexpressed in HUVEC (Fig. 6). In fact, a slight, but nonsignificant, increase in proliferative rates was noted in fibroblasts. As in previous experiments, PTP-ε(D284A) had no effect on proliferative rates in any cell type. Similar cell type-specific effects were obtained in cell count experiments (data not shown). These results indicate that the effects of PTP-ε are, at least to some extent, cell type dependent.

DISCUSSION

Degenerate RT-PCR techniques have been used successfully to identify EC-specific receptor tyrosine kinases, most notably the VEGF receptor KDR/Flk-1 (32). KDR/Flk-1 is expressed almost exclusively in the endothelium (32, 40), and mice deficient in this receptor PTK die in utero due to lack of EC growth, indicating an essential role for this receptor in angiogenesis and vasculogenesis (44). The importance of tyrosine phosphorylation in EC signaling events is demonstrated by the fact that the PTK inhibitor genistein prevents VEGF-induced proliferation of EC in vitro (17).

The existence of EC-specific PTKs prompted us to seek EC PTPs using an analogous approach. Although our screen identified several PTPs in HUVEC, we did not detect PTP-μ and PTP-β/vascular endothelial PTP, which are also known to be expressed in the endothelium (1, 14, 51). PTP-μ differs from PTP-ε in that it contains four fibronectin type III repeats and one immunoglobulin domain in its extracellular region (35). Likewise, PTP-β contains a large extracellular region containing 16 fibronectin type III repeats (27). These large ectodomains can interact with the extracellular matrix (22, 38), promote cell adhesion (57), and transduce signals generated by cell-cell contact in vivo (2, 3). Expression of both PTP-μ and PTP-β is upregulated in response to increasing cell density (18, 21), which could explain why they were undetected in our analysis of subconfluent proliferating HUVEC. In contrast to these two endothelial-restricted PTPs, PTP-ε has an exceptionally small extracellular region lacking any known ligand-binding motifs (27), and our data suggest that PTP-ε is more likely to play a role in modulating proliferative events rather than cell-cell signaling. That distinct functions for the different EC PTPs exist is reminiscent of the different roles played by EC-specific PTKs in endothelial function (45).
Although the existence of PTP-ε is known, its tissue distribution and function have, until now, not been fully described. PTP-ε is expressed in cells derived from a subset of murine mammary tumors (13) and may play an accessory role to modulate the development of mammary hyperplasia in vivo (11). PTP-ε expression is also seen during neuronal cell differentiation of rat pheochromocytoma PC12D cells (34) and is induced in human U373-MG astrocytoma cells in response to interleukin-1 (42). Likewise, PTP-ε expression is upregulated in murine leukemia M1 cells (47) and in human promyelocytic leukemia HL60 cells (12) during differentiation. Expression of PTP-ε in cells derived from neural tissue may be relevant to neural development, insofar as mice deficient in PTP-ε show impaired myelination (39). In transformed cell types, PTP-ε appears to be associated with cell differentiation, perhaps by inhibiting proliferation during the differentiation process. On the basis of our data, EC are unique among vascular cells in their constitutive expression of PTP-ε.

On the basis of our present understanding of the functions of PTPs, we sought to define the function of PTP-ε in EC. For these studies, we developed a highly efficient retroviral gene transfer system for constitutive overexpression of wild-type and mutant PTP-ε to analyze the function of PTP-ε in EC cultures. HUVEC that overexpressed PTP-ε proliferated at a significantly slower rate than did cells virally transduced with a control retroviral vector. The catalytic activity of PTP-ε was clearly required for this decrease in proliferation, because the catalytically inactive PTP-ε (D284A) mutant had no significant effect on EC proliferation. Taken together, these data indicate that PTP-ε mediates an inhibitory pathway that regulates EC proliferation.

At first glance, increased PTP-ε expression in response to proliferative stimuli (Fig. 3) seems inconsistent with the antimitogenic effects of PTP-ε. However, a very similar growth regulatory mechanism has been reported to regulate MAPK signaling. In this pathway, the protein levels of the dual-specificity MKPs (MKP-1 and MKP-2) are also increased in response to proliferative stimuli and subsequent MAPK activity (4, 5). These phosphatases, in turn, dephosphorylate MAPK on critical tyrosine and threonine residues to inactivate the kinase and turn off the proliferative signal (7). The upregulation of MKP-1 and MKP-2 by growth factors, and their downregulation as cells exit the cell cycle, imply that these phosphatases are required for “fine-tuning” of the MAPK-dependent proliferative response. In addition, the data suggest that these growth-inhibitory phosphatases are not required, and are therefore downregulated, in conditions when their substrates are not themselves phosphorylated, which occurs under conditions when growth is already arrested by external signaling events. On the basis of this precedent and the identical pattern of regulation of PTP-ε and MKP-1 expression in HUVEC (Fig. 3A), we speculate that increased PTP-ε expression in response to mitogenic stimuli provides a means to modulate growth, in a manner similar to that of the MAPK phosphatases, by dephosphorylating as-yet-unknown substrates that are phosphorylated after mitogenic stimulation and required for EC proliferation. Our current efforts focus on the identification of PTP-ε substrates in vascular EC.

Because PTPs contain very highly conserved catalytic domains, it is believed that the diverse protein sequences flanking these domains must convey substrate specificity (31). These noncatalytic regions frequently serve a regulatory function, including ligand binding for receptor PTPs and targeting of cytoplasmic PTPs to defined subcellular locations (15). More recently, these regions have been found to contain phosphotyrosine residues and proline-rich sequences that can interact with Src homology (SH)-2 and SH-3 domains, respectively, to promote protein-protein interactions (9, 19, 26). These interactions can then couple a PTP directly to a specific substrate, as in the case of PTP-PEST binding to p130cas (19). Alternatively, these domains can also bind to an adapter protein like Grb-2, which can subsequently bind additional proteins, bringing the PTP and substrate(s) into close proximity. In fact, PTPs have been found in multimeric complexes containing Grb2 (9) and PTKs (6, 26) through both SH-2 and SH-3 interactions. Of particular interest in the context of the experiments presented here, PTP-ε was recently reported to associate with Grb-2 in vivo through interactions between the COOH-terminus of PTP-ε and the SH-2 domain of Grb-2 (48).

Unlike ubiquitous PTPs that can negatively regulate EC signaling events under some circumstances, such as human cellular protein tyrosine phosphatase A, Src homology region 2-containing phosphatase (SHP)-1, and SHP-2 (23, 25, 46, 49), PTP-ε is not broadly expressed and is therefore more likely to regulate cell type-specific proliferative events under endogenous conditions. Given the proximity of this membrane-associated PTP with growth factor receptors, one can envision that these receptors may be directly dephosphorylated and inactivated by PTP-ε. In support of this notion, ectopic expression of PTP-ε in hamster kidney cells can negatively regulate signaling via the insulin receptor, which is also a receptor PTK (33). Alternatively, PTP-ε could also regulate the proliferative response by modifying the phosphotyrosine content of downstream cytosolic signaling molecules. Because PTP-ε contains many potential SH-2- and SH-3-binding domains, as well as a functional Grb-2-binding domain (48), it is likely to exist in a multimeric complex with its substrates. Studies are currently under way in our laboratory to identify the physiological substrate(s) for PTP-ε to understand more clearly the function of this EC PTP in cell proliferation.

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