into pGEX-5X-1 (GE Healthcare, Piscataway, NJ, USA). The sequences of all PCR products were verified by sequencing with ABI3100 (Applied Biosystems, Foster City, CA, USA).

Cell culture and transfection

HEK293A cells (Invitrogen) were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. CHO cells stably expressing an active form of H-Ras (CHO-Ras cells) were kindly provided by S. Shirahata and Y. Katakura (Kyushu University, Fukuoka, Japan) and were cultured in minimum essential medium α (Sigma) supplemented with 2 mM L-glutamine, 10 mM HEPES-NaOH (pH 7.4), and 10% FBS. Cultured cells were transfected with expression vectors by the use of Lipofectamine2000 (Invitrogen) in accordance with the manufacturer's procedure. Pervanadate was prepared immediately before the use by mixing 50 mM sodium orthovanadate with 50 mM hydrogen peroxide for 15 min.

Immunoprecipitation and immunoblot

Cells were washed with ice-cold phosphate-buffered saline (PBS) and then lysed on ice in 1 mL of Buffer A [20 mM Tris–HCl (pH7.5), 150 mM NaCl, 1 mM EDTA, 1% TritonX-100] containing 1 mM phenylmethylsulfonyl fluoride, aprotinin (10 μg/mL) and 1 mM sodium vanadate. The lysates were centrifuged at 17 500 *g* for 20 min at 4 °C, and the resulting supernatants were subjected to immunoprecipitation and immunoblot analysis as previously described (Miyake *et al.* 2008).

Pull-down assay

GST proteins and GST fusion proteins were expressed in *E. coli*, and purified with glutathione-Sepharose beads (GE Healthcare) as previously described (Ito *et al.* 2003). Pervanadate-treated cells were lysed with Buffer A and centrifuged at 17 500 *g* for 20 min at 4 °C. The supernatants were incubated with GST or each GST fusion protein immobilized to glutathione-Sepharose beads in buffer A for 1 h at 4 °C. The beads were then washed extensively with buffer A and the proteins bound to the beads were subjected to SDS–PAGE, followed by immunoblot analysis.

Isolation of mouse IECs

The isolation of mouse IECs was carried out with a slight modification as previously described (Lee *et al.* 2006). Briefly, the small intestine was removed from adult mouse (C57BL/6), extensively rinsed with ice-cold PBS and cut open longitudinally. Tissues were then incubated in Hanks' balanced salt solutions containing 20 mM HEPES (pH 7.5) and 5 mM EDTA with rotation for 30 min at room temperature to detach IECs. The tissue debris was removed and IECs were collected by centrifuge at 150 *g* for 10 min at 4 °C. Collected IECs were washed with ice-cold PBS and used for further analysis.

Immunostaining

Cells were fixed with 4% paraformaldehyde for 30 min, incubated for 1 h with Buffer G (5% goat serum and 0.1% Triton X-100 in PBS) and then subjected to immunostaining with primary antibodies in the same buffer. The cells were then washed with PBS and exposed to secondary antibodies and rhodamine-conjugated phalloidin in Buffer G, followed by observation with a laser-scanning confocal laser-scanning microscope, LSM 5 Pascal (Zeiss, Oberkochen, Germany).

Analysis of lamellipodium formation

For analysis of lamellipodium formation, the morphology of cells stained with rhodamine-phalloidin was observed with the LSM 5 Pascal microscope. The number of lamellipodia formed at the cell periphery or at the tip of filopodium-like protrusions extending from the cell body was counted.

Statistical analysis

Data were presented as means ± SE and were analyzed by Student's *t* test or by analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test. A P value of <0.05 was considered statistically significant.

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Promotion of Cell Spreading and Migration by Vascular Endothelial-Protein Tyrosine Phosphatase (VE-PTP) in Cooperation With Integrins

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Vascular endothelial-protein tyrosine phosphatase (VE-PTP) is a receptor-type protein tyrosine phosphatase with a single catalytic domain in its cytoplasmic region and multiple fibronectin type III-like domains in its extracellular region. VE-PTP is expressed specifically in endothelial cells and is implicated in regulation of angiogenesis. The molecular basis for such regulation by VE-PTP has remained largely unknown, however. We now show that forced expression of VE-PTP promoted cell spreading as well as formation of lamellipodia and filopodia in cultured fibroblasts plated on fibronectin. These effects of VE-PTP on cell morphology required its catalytic activity as well as activation of integrins and Ras. In addition, VE-PTP-induced cell spreading and lamellipodium formation were prevented by inhibition of Src family kinases or of Rac or Cdc42. Indeed, forced expression of VE-PTP increased the level of c-Src phosphorylation at tyrosine-416. Moreover, the VE-PTP-induced changes in cell morphology were suppressed by expression of dominant negative forms of FRG or Vav2, both of which are guanine nucleotide exchange factors for Rho family proteins and are activated by tyrosine phosphorylation. Forced expression of VE-PTP also enhanced fibronectin-dependent migration of cultured fibroblasts. Conversely, depletion of VE-PTP by RNA interference in human umbilical vein endothelial cells or mouse endothelioma cells inhibited cell spreading on fibronectin. These results suggest that VE-PTP, in cooperation with integrins, regulates the spreading and migration of endothelial cells during angiogenesis.

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During mammalian embryonic development, aggregation of endothelial cell precursors, known as angioblasts, results in the formation of early blood vessels and development of a primitive vascular plexus, a process termed vasculogenesis (Jain, 2003; Coultas et al., 2005). The primitive vasculature subsequently undergoes a complex remodeling process, termed angiogenesis, that includes endothelial cell growth and migration as well as vessel sprouting and pruning, resulting in the development of a functional vascular system (Jain, 2003; Coultas et al., 2005). Development of the vascular system requires highly coordinated actions of a variety of regulators including cell surface receptors as well as adhesion molecules (Jain, 2003; Coultas et al., 2005). Vascular endothelial growth factor (VEGF) and its receptors, in particular VEGF receptor-2 (VEGFR-2), are important for early vasculogenesis as well as later angiogenesis (Coultas et al., 2005; Olsson et al., 2006; Holderfield and Hughes, 2008), whereas Tie-2, a receptor for angiopoietin, is thought to play a key role in angiogenesis (Jain, 2003; Coultas et al., 2005; Eklund and Olsen, 2006). Moreover, integrins, through their interaction with extracellular matrix (ECM), are thought to be important for various processes, including maintenance of the structural stability of blood vessels as well as the proliferation, migration, morphogenesis, and survival of endothelial cells, during both vasculogenesis and angiogenesis (Davis and Senger, 2005). In addition, VE-cadherin, an endothelial cell-specific cadherin, is essential for the formation of stable contacts between endothelial cells that underlie development and maintenance of blood vessels (Vestweber, 2008). Given that the most important receptors in vasculogenesis and angiogenesis, including VEGFR-2 and Tie-2, are receptor-type protein tyrosine kinases (PTKs; Jain, 2003; Eklund and Olsen, 2006; Olsson et al., 2006), protein tyrosine phosphatases (PTPs), which counterregulate protein tyrosine phosphorylation by PTKs, are also likely to play key roles in these processes.

VE-PTP (also known as PTPRB or PTPβ) is a receptor-type PTP (RPTP) with a single catalytic domain in its cytoplasmic region and multiple fibronectin (FN) type III-like domains in its extracellular region (Fachinger et al., 1999; Andersen et al., 2001; Alonso et al., 2004), being classified as an RPTP of the R3 subtype (Andersen et al., 2001). The expression of VE-PTP is restricted to endothelial cells and is especially prominent in those of arteries and arterioles (Fachinger et al., 1999; Bäumer

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et al., 2006; Dominguez et al., 2007). It becomes apparent along the primitive aorta at embryonic days 9 and 10 in mice, and it gradually increases in the entire vascular system in accordance with embryonic development (Dominguez et al., 2007). Ablation of VE-PTP in mice resulted in embryonic death at 10 days of gestation as a result of a variety of angiogenesis defects in both the embryo and yolk sac. Defects included failure of remodeling of the vascular plexus into large veins and branched vascular networks, suggesting that VE-PTP is important for angiogenic processes such as remodeling and maintenance of blood vessels rather than for vasculogenesis (Bäumer et al., 2006; Dominguez et al., 2007). However, the molecular basis for such functions of VE-PTP in development of the vascular system remains largely unknown.

VE-PTP is thought to bind to Tie-2 and inactivate the receptor by mediating its dephosphorylation (Fachinger et al., 1999; Winderlich et al., 2009), resulting in inhibition of the proliferation of endothelial cells (Winderlich et al., 2009). VE-PTP is also thought to form a complex with VE-cadherin and thereby to promote its adhesive function (Nawroth et al., 2002; Nottebaum et al., 2008). Given that proper regulation of vascular endothelial cell morphology and migration by integrins is important for angiogenesis, we have now investigated whether VE-PTP also participates in such regulation. We found that VE-PTP promotes cell spreading and lamellipodium formation as well as cell migration in cooperation with integrins.

Materials and Methods

Antibodies and reagents

Mouse monoclonal antibodies (mAbs) to the Flag epitope tag (M2) and to β-Tubulin were obtained from Sigma (St. Louis, MO). Mouse mAbs to Cdc42 or to Rac were from Santa Cruz Biotechnology (Santa Cruz, CA). A mouse mAb to v-Src (Ab-1) was from Oncogene Science (Manhasset, NY), and rabbit pAbs to c-Src phosphorylated on Tyr^{416} or Tyr^{527} were from Cell Signaling Technology (Beverly, MA). A mouse mAb to the Myc epitope tag (9E10) was purified from the culture supernatant of hybridoma cells. Horseradish peroxidase-conjugated goat pAbs to rabbit immunoglobulin G (IgG) or to mouse IgG were from Jackson ImmunoResearch (West Grove, PA). Alexa Fluor 488-conjugated goat pAbs to rat IgG, to mouse IgG or to rabbit IgG as well as rhodamine-conjugated phalloidin were from Invitrogen (Carlsbad, CA). Cy3-conjugated goat pAbs to rat IgG were from Jackson ImmunoResearch. PP2, PP3, PD98059, and Y-27632 were obtained from EMD (Darmstadt, Germany). Wortmannin, FN, and poly-L-lysine were from Sigma, and laminin was from Invitrogen. Collagen type I-coated dishes were from Iwaki (Chiba, Japan). Dimethyl sulfoxide (DMSO) was from Wako (Tokyo, Japan), and May-Giemsa solution was from Merck (Darmstadt, Germany).

Plasmids

A full-length cDNA for mouse VE-PTP was obtained from Riken (Tsukuba, Japan). To construct an expression vector for wild-type (WT) VE-PTP (VE-PTP-WT), we subcloned the full-length cDNA into the pTracer-CMV vector (Invitrogen). For construction of an expression vector encoding a catalytically inactive form of VE-PTP (VE-PTP-C/S), in which a cysteine residue (Cys^{1905}) that is essential for PTP activity is replaced by serine, the mutated cDNA was generated by polymerase chain reaction (PCR)-ligation mutagenesis (Ali and Steinkasserer, 1995) with pTracer-CMV-VE-PTP-WT as the template. The PCR products were verified by sequencing with an ABI3100 instrument (Applied Biosystems, Foster City, CA). Plasmids encoding enhanced green fluorescent protein (EGFP)-RacT17N or EGFP-NWASP-CRIB fusion proteins were kindly provided by Y. Takai (Kobe University, Japan). A plasmid encoding Myr-Csk (rat Csk with a myristylation signal at its NH_2-terminus) was kindly provided by M. Okada (Osaka University, Japan). Expression vectors for a Myc epitope-tagged dominant negative mutant of mouse Vav2, a Flag epitope-tagged dominant negative mutant of human FRG, or a Myc epitope-tagged dominant negative mutant of human Tiam1 were described previously (Murata et al., 2006). For expression of EGFP, the pEGFP-N3 vector was obtained from Clontech (Palo Alto, CA) and a pCAGGS vector containing a full-length cDNA for EGFP was kindly provided by J. Miyazaki (Osaka University). A pGEX vector for a glutathione S-transferase (GST) fusion protein containing the Cdc42/Rac interactive binding (CRIB) domain of p21PAKα was kindly provided by E. Manser (Institute of Molecular and Cell Biology, Singapore).

Cell culture and generation of cell lines stably expressing VE-PTP

All cultured cells were maintained at 37°C under a humidified atmosphere of 5% CO_2 in air. Chinese hamster ovary (CHO) cells stably expressing an active form of H-Ras (CHO-Ras cells) were kindly provided by S. Shirahata and Y. Katakura (Kyushu University, Fukuoka, Japan) and were cultured in minimum essential medium α (Sigma) supplemented with 2 mM L-glutamine, 10 mM HEPES–NaOH (pH 7.4), and 10% fetal bovine serum (FBS; Sigma). CHO cells were cultured in Ham's nutrient mixture F-12 medium supplemented with 10% FBS. Human umbilical vein endothelial cells (HUVECs) (Kurabo, Osaka, Japan) were cultured in basal medium (HuMedia-EB2, Kurabo) supplemented with 2% FBS, recombinant human epidermal growth factor (10 ng/ml), recombinant human basic fibroblast growth factor (5 ng/ml), hydrocortisone (1 μg/ml), heparin (10 μg/ml), gentamicin (50 μg/ml), and amphotericin B (50 ng/ml) (Kurabo). Mouse endothelioma bEnd.3 cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. For generation of CHO-Ras cells stably expressing VE-PTP-WT or VE-PTP-C/S, CHO-Ras cells (5×10^5) were transfected with 2 μg of pTracer-CMV-VE-PTP-WT or pTracer-CMV-VE-PTP-C/S with the use of 3 μl of Lipofectamine 2000 (Invitrogen). The transfected cells were cultured in medium containing Zeocin (200 μg/ml) (Invitrogen), and colonies were isolated 14–21 days after transfection. Several cell lines expressing VE-PTP-WT or VE-PTP-C/S were then identified by immunostaining with mAbs to VE-PTP and by immunoblot analysis with pAbs to VE-PTP.

Generation of antibodies to VE-PTP

For generation of pAbs to mouse VE-PTP, an expression vector for a GST fusion protein containing the cytoplasmic region of VE-PTP (GST-VE-PTP-cyto) was constructed. A cDNA fragment encoding the cytoplasmic region of VE-PTP (amino acids 1645–1998) was amplified by PCR with the primers 5′-AATGTCGACAGAAAG-CTAGCCACAGCA-3′ (forward) and 5′-AGCGCGGCCGCTT-AATGTCTCGAGTAGAT-3′ (reverse), and the resulting PCR products were subcloned into pGEX-5X-1 (GE Healthcare, Piscataway, NJ). GST and GST-VE-PTP-cyto proteins were then prepared as described previously (Sadakata et al., 2009). Rabbits were injected with GST-VE-PTP-cyto, and the resulting pAbs to VE-PTP were purified from serum with the use of columns containing GST or GST-VE-PTP-cyto immobilized on CNBr-Sepharose (GE Healthcare) as described previously (Sadakata et al., 2009).

For generation of rat mAbs to mouse VE-PTP, an expression vector [pTracer-CMV-VE-PTP(FN1-3)-Fc] for the extracellular region of VE-PTP fused with human Fc was generated. A cDNA fragment for VE-PTP (amino acids 1–278) was thus amplified by PCR with the primers 5′-GGAATTCAGCGAGATGCTGAGG-CATGG-3′ (forward) and 5′-CTTTTCTAGAAGCGGTCCT-CACCAGTTTC-3′ (reverse), and the PCR products were subcloned into pTracer-Fc (Motegi et al., 2008; Sadakata et al.,

2009). CHO-Ras cells were transfected with pTracer-CMV-VE-PTP(FN1-3)-Fc and selected as described above, and several cell lines stably producing VE-PTP(FN1-3)-Fc were identified by immunoblot analysis of culture supernatants with horseradish peroxidase-conjugated goat pAbs to the Fc fragment of human IgG (Jackson ImmunoResearch). The VE-PTP(FN1-3)-Fc fusion protein produced by cells cultured in serum-free DMEM–F-12 (1:1, v/v) was purified from culture supernatants with the use of protein G-Sepharose 4 Fast Flow (GE Healthcare). Hybridoma cells producing specific antibodies were then generated as described (Motegi et al., 2008; Sadakata et al., 2009). In brief, purified VE-PTP(FN1-3)-Fc was injected into the hind foot pads of three Wistar rats three times at weekly intervals, after which lymphocytes were isolated from the draining lymph nodes and fused with P3U1 myeloma cells. Hybridoma clones producing mAbs that react with VE-PTP(FN1-3)-Fc but not with human IgG were identified by enzyme-linked immunosorbent assay. The mAbs were purified from serum-free culture supernatants of the hybridoma cells by column chromatography with protein G-Sepharose 4 Fast Flow.

Immunoprecipitation and immunoblot analysis

Cells were washed with ice-cold phosphate-buffered saline (PBS) and then lysed on ice with RIPA buffer [20 mM Tris–HCl (pH 7.6), 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS] containing 1 mM phenylmethylsulfonyl fluoride (PMSF), aprotinin (10 μg/ml), and 1 mM sodium vanadate. The lysates were centrifuged at 21,000*g* for 15 min at 4°C, and the resulting supernatants were subjected to immunoprecipitation or immunoblot analysis as described previously (Murata et al., 2006; Miyake et al., 2008).

Immunostaining

Cells were fixed with 4% paraformaldehyde for 30 min, incubated for 1 h with buffer G (5% goat serum and 0.1% Triton X-100 in PBS), and then subjected to immunostaining with primary antibodies in the same buffer. The cells were washed with PBS, exposed to secondary antibodies or rhodamine-conjugated phalloidin in buffer G, and observed with a laser-scanning confocal microscope (LSM 5 Pascal; Zeiss, Oberkochen, Germany) or with a fluorescence microscope (BX51; Olympus, Tokyo, Japan).

Analysis of lamellipodium formation and cell spreading

For analysis of lamellipodium formation, the morphology of cells stained with rhodamine–phalloidin was observed with the LSM 5 Pascal microscope. The number of lamellipodia formed at the cell periphery or at the tip of filopodium-like protrusions extending from the cell body was counted. For evaluation of cell spreading, cells stained with rhodamine–phalloidin were examined with the BX51 microscope, and the captured images were analyzed for cell area with the use of ImageJ software (NIH, Bethesda, MD).

Assay of activated Rac and Cdc42

Activated Rac and Cdc42 were assayed as described previously (Miyashita et al., 2004). In brief, cells were lysed in a solution containing either 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 10 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, and aprotinin (10 μg/ml) for the Rac assay or 50 mM Tris–HCl (pH 7.4), 100 mM NaCl, 1% Triton X-100, 10 mM $MgCl_2$, 0.2% sodium deoxycholate, 1 mM dithiothreitol, 1 mM PMSF, and aprotinin (10 μg/ml) for the Cdc42 assay. The cell lysates were incubated for 60 min at 4°C with a GST fusion protein that contained the CRIB domain (amino acids 70–106) of rat p21PAKα and was bound to glutathione-Sepharose beads (GE Healthcare). Lysate proteins that bound to the beads were subjected to immunoblot analysis with mAbs to Rac or to Cdc42. The total abundance of each small GTP binding protein was also determined by immunoblot analysis of cell lysates.

Assay of cell migration

Cell migration was assayed with a Transwell apparatus (Corning, New York, NY) as described previously (Motegi et al., 2003). In brief, cells were deprived of serum for 8 h, harvested, and resuspended in serum-free culture medium. The cell suspension (3×10^4 cells in 100 μl) was then transferred to a polycarbonate filter (pore size, 8 μm; Corning) in the upper compartment of a Transwell apparatus, and 600 μl of serum-free medium containing FN (20 μg/ml) was added to the lower compartment. Cells that had migrated to the lower chamber during incubation for 2 h were fixed with 4% paraformaldehyde and then stained with May-Giemsa solution. The stained cells in the upper chamber were removed with a cotton swab. The migrated cells were then observed with a light microscope (model DM IRB; Leica, Wetzlar, Germany), and the number of cells in five independent fields (20×) was counted. The images were also captured with a charge-coupled device camera (Penguin 600CL; Pixera, San Jose, CA).

Adenoviral vector construction and infection

For construction of adenoviral vectors encoding VE-PTP-WT or VE-PTP-C/S, the corresponding full-length cDNAs were separately cloned into pENTR11-CMV, which was generated by subcloning the cytomegalovirus (CMV) promoter/BGH poly(A) expression cassette of pTracer-CMV into pENTR11 (Invitrogen). The resulting shuttle vectors, pENTR11-CMV/VE-PTP-WT and pENTR11-CMV/VE-PTP-C/S, were then subjected to recombination with the adenoviral vector pAd-PL-DEST (Invitrogen) to yield pAd-VE-PTP-WT and pAd-VE-PTP-C/S, respectively. To construct an adenoviral vector encoding EGFP, we generated pENTR11-CAG/EGFP by subcloning a DNA fragment containing the CMV enhancer, chicken β-actin gene promoter, EGFP cDNA, and poly(A) signal of the rabbit β-globin gene from pCAGGS-EGFP into pENTR11. The resulting vector was subjected to recombination with pAd-PL-DEST to generate pAd-EGFP. Adenoviruses encoding VE-PTP-WT, VE-PTP-C/S, or EGFP were then generated from pAd-VE-PTP-WT, pAd-VE-PTP-C/S, or pAd-EGFP with the use of a ViraPower Adenoviral Expression System (Invitrogen). Adenoviruses were purified with the use of Vivapure adenoPACK 20 (Vivascience, Hannover, Germany), and their titers were determined by a modified version of the median tissue culture infectious dose ($TCID_{50}$) method with the use of HEK293 cells (Kanegae et al., 1994).

HUVECs were incubated for 1 h with adenoviruses at a multiplicity of infection of 30 in HuMedia-EB2. The cells were then washed and maintained in fresh culture medium for 42 or 72 h before experiments.

RNA interference (RNAi)

Adenoviral RNAi vectors pAd-shRNA-hVE-PTP #1 and #2, each of which encodes a human VE-PTP short hairpin RNA (shRNA), were constructed. Two sequences, 5′-TCGGATCCTACTCTTC-AATGATT-3′ and 5′-AGGTACCTGGTGTCCATCAAAGT-3′, corresponding to nucleotides 1922–1944 or 4956–4978, respectively, of human VE-PTP mRNA (GenBank accession no. NM001109754.1), were selected for construction of the pAd-shRNA-hVE-PTP vectors, which direct the synthesis of the corresponding 23-bp double-stranded target sequence. Two pairs of 67-nucleotide sequences, each of which contains a targeting sequence and its reverse complementary sequence (pair 1,5′-GATCGCCTCGGATCCTACTCTTCAATGATTTT-CAAGAGAAATCATTGAAGAGTAGGATCCGATTTTA-3′ and 5′-AGCTTAAAATCGGATCCTACTCTTCAATGATTTCTC-TTGAAAATCATTGAAGAGTAGGAT CCGAGGG-3′; pair 2, 5′-GATCCCCAGGTACCTGGTGTCCATCAAAGTTTCAAG-AGAACTTTGATGGACACCAGGTACCTTTTTA-3′ and 5′-AGCTTAAAAAGGTACCTGGTGTCCATCAAAGTTCTC-TTGAAACTTTGATGGACACCAGGTACCTGGG-3′), were synthesized. After annealing, each pair of oligonucleotides was

inserted into pENTR11-H1, which was generated by subcloning a DNA fragment containing the H1 RNA polymerase III promoter and a termination signal of pSUPER (OligoEngine, Seattle, WA) into pENTR11. The resulting vectors, pENTR11-H1-shRNA-hVE-PTP #1 and #2, were subjected to recombination with pAd-PL-DEST to produce pAd-shRNA-hVE-PTP #1 and #2, respectively. Generation of adenoviruses encoding the shRNAs specific for human VE-PTP mRNA and infection of HUVECs with the adenoviruses was performed as described above.

To construct the expression vectors shRNA-mVE-PTP-EGFP #1 and #2, each of which encodes both a mouse VE-PTP shRNA and EGFP, we selected two sequences, 5′-TCCTATTCGGATAGACAACTTTA-3′ and 5′-CCTCACTGAGGGTAACAGT-3′, corresponding to nucleotides 459–481 or 771–789, respectively, of mouse VE-PTP mRNA (GenBank accession no. NM029928.2). Two pairs of 67- or 59-nucleotide sequences, each of which contains a targeting sequence and its reverse complementary sequence (pair 1, 5′-GATCCCCTCCTATTCGGATAGACAACTTTATTCAAGAGATAAAGTTGTCTATCCGAATAGGATTTTA-3′ and 5′-AGCTTAAAATCCTATTCGGATAGACAACTTTATCTCTTGAATAAAGTTGTCTATCCGAATAGGAGGG-3′; pair 2, 5′-GATCCCCCCTCACTGAGGGTAACAGTTTCAAGAGAACTGTTACCCTCAGTGAGGTTTTA-3′ and 5′-AGCTTAAAACCTCACTGAGGGTAACAGTTCTCTTGAAACTGTTACCCTCAGTGAGGGGG-3′), were synthesized, and each pair of oligonucleotides was inserted into pSUPER after annealing. The resulting vectors, pSUPER-mVE-PTP #1 and #2, as well as pCAGGS-EGFP were used to produce shRNA-mVE-PTP-EGFP #1 and #2 as previously described (Murata et al., 2006; Kusakari et al., 2008). A vector encoding both an shRNA for human SHP-2 and EGFP (shRNA-hSHP-2-EGFP) was constructed from an shRNA vector for human SHP-2 (pSUPER-hSHP-2; kindly provided by M. Hatakeyama, University of Tokyo, Japan; Higashi et al., 2004) and pCAGGS-EGFP as described above.

Statistical analysis

Data are presented as means ± SE and were analyzed by Student's *t*-test or by analysis of variance (ANOVA) followed by Bonferroni's post hoc test. A *P*-value of <0.05 was considered statistically significant.

Results

Forced expression of VE-PTP promotes cell spreading and lamellipodium formation in CHO cells in a manner dependent on integrins and Ras

To investigate the role of VE-PTP in regulation of cell morphology, we used a CHO cell line (CHO-Ras) that was originally designed to express high levels of exogenous protein as a result of transformation with an active form of human H-Ras in which Glu^{61} is mutated to Leu (Katakura et al., 1999; Kusakari et al., 2008). We generated CHO-Ras cell lines that stably express WT (VE-PTP-WT) or catalytically inactive mutant (VE-PTP-C/S) forms of mouse VE-PTP. From the several cell lines obtained, we selected two VE-PTP-WT lines (CHO-Ras-VE-PTP-WT1 and -WT2) and one VE-PTP-C/S line (CHO-Ras-VE-PTP-C/S) for further analysis. Immunoblot analysis revealed the increased levels of WT or mutant VE-PTP proteins (~230 kDa) in these cell lines compared with the virtually undetectable amount of endogenous VE-PTP in mock-transfected CHO-Ras cells (Fig. 1A). The levels of VE-PTP-WT expressed in CHO-Ras cells (WT1 and WT2) were markedly higher than that of VE-PTP endogenously expressed in cultured endothelial cells, such as HUVECs or bEnd.3 cells (Supplementary Fig. S1). For evaluation of the effects of forced expression of VE-PTP on cell morphology, serum-deprived cells were detached from culture dishes, replated on cover glasses coated with either FN or poly-L-lysine, and cultured for up to 120 min before staining of cellular F-actin with rhodamine-conjugated phalloidin. CHO-Ras-VE-PTP-WT1 or -WT2 cells plated on FN exhibited marked cell spreading and formation of both lamellipodia and filopodia 120 min after plating (Fig. 1B). These morphological characteristics were apparent as early as 30 min after replating but were most pronounced at 120 min (Supplementary Fig. S2). Such effects were also observed in the presence of serum (data not shown). Formation of lamellipodia was frequently observed at the distal end of filopodium-like protrusions extended by CHO-Ras-VE-PTP-WT cells (Fig. 1B). VE-PTP immunoreactivity was detected as dotlike structures

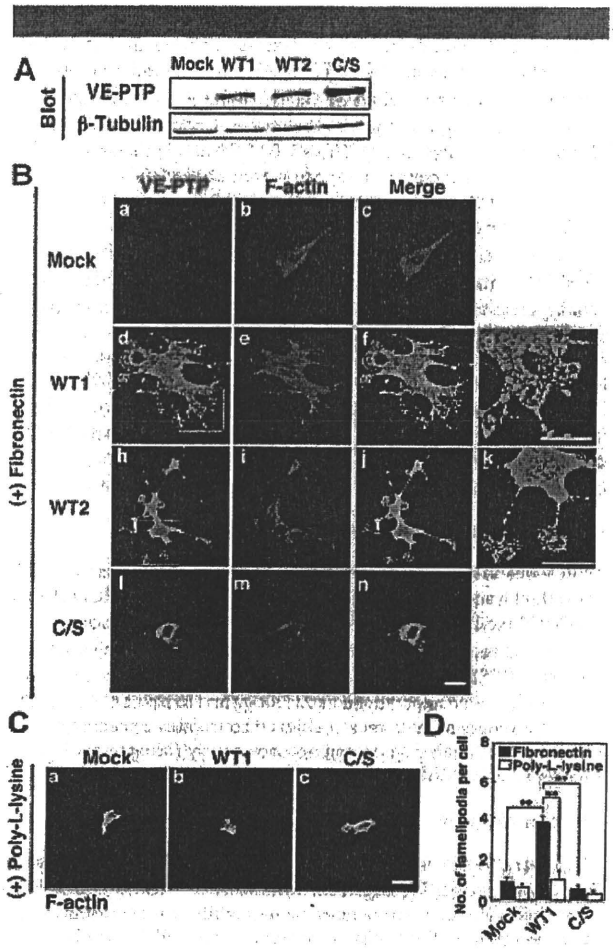

Fig. 1. Effects of forced stable expression of VE-PTP on cell spreading and lamellipodium formation in CHO-Ras cells plated on fibronectin or poly-L-lysine. A: Lysates of mock-transfected CHO-Ras cells (Mock), of CHO-Ras-VE-PTP-WT cells (WT1 and WT2), or of CHO-Ras-VE-PTP-C/S cells (C/S) were subjected to immunoblot analysis with pAbs to VE-PTP or mAbs to β-Tubulin (loading control). Results are representative of three independent experiments. B: Cells as in A were deprived of serum for 8 h, replated on cover glasses coated with fibronectin (20 μg/ml), and cultured in serum-free medium for 2 h. The cells were then fixed and subjected to immunostaining with mAbs to VE-PTP (d,g,h,k,l) and to staining of F-actin with rhodamine–phalloidin (b,e,i,m). Enlarged images of the boxed regions in d and h are shown in g and k, respectively. Bars, 20 μm. C: Cells as in A were deprived of serum for 8 h, replated on cover glasses coated with poly-L-lysine (50 μg/ml), and cultured in serum-free medium for 2 h. They were then fixed and stained with rhodamine–phalloidin (a–c). Bar, 20 μm. D: Cells treated as in B or C were evaluated for the number of lamellipodia as described in the Materials and Methods Section. Data are means ± SE from 20 cells for each condition and are representative of three independent experiments. **$P<0.01$ (ANOVA and Bonferroni's test) for the indicated comparisons.

both at the periphery and inside of CHO-Ras-VE-PTP-WT cells (Fig. 1B), consistent with previous observations of cultured cells expressing exogenous VE-PTP (Saharinen et al., 2008). These morphological characteristics of CHO-Ras-VE-PTP-WT cells were not observed in either mock-transfected CHO-Ras cells or CHO-Ras-VE-PTP-C/S cells (Fig. 1B). Quantitative analysis confirmed that lamellipodium formation by CHO-Ras-VE-PTP-WT cells plated on FN was indeed greater than that apparent in mock-transfected CHO-Ras cells or CHO-Ras-VE-PTP-C/S cells (Fig. 1D). These results thus suggested that the PTP activity of VE-PTP is required for the morphological phenotype of CHO-Ras-VE-PTP-WT cells. Cell spreading in the various cell lines was markedly reduced in extent when the cells were cultured on cover glasses coated with poly-L-lysine (Fig. 1C), compared with that apparent with the corresponding cells plated on FN (Fig. 1B). In addition, lamellipodium formation by CHO-Ras-VE-PTP-WT cells on poly-L-lysine was markedly inhibited compared with that apparent with the corresponding cells plated on FN (Fig. 1D). Moreover, plating of CHO-Ras-VE-PTP-WT cells on collagen type I resulted in marked enhancement of both cell spreading and lamellipodium formation, whereas plating of the cells on laminin increased cell spreading (Supplementary Fig. S3). These results suggested that activation of integrins by ECM is indispensable for induction of marked cell spreading and lamellipodium formation in CHO-Ras-VE-PTP-WT cells.

Transient expression of VE-PTP-WT, but not that of VE-PTP-C/S or of EGFP, in CHO-Ras cells plated on FN also induced changes in cell morphology similar to those apparent in the stably transfected CHO-Ras-VE-PTP-WT cells (Fig. 2A,B), suggesting that these changes in CHO-Ras-VE-PTP-WT cells were not attributable simply to a clonal effect of stable transfection. To examine whether activated Ras is also important for the pronounced cell spreading and lamellipodium formation in CHO-Ras-VE-PTP-WT cells, we induced transient expression of VE-PTP-WT in parental CHO cells, which do not express the activated form of H-Ras. Forced expression of VE-PTP-WT in parental CHO cells also tended to promote formation of lamellipodia (Fig. 2B), but this effect was not statistically significant and was markedly smaller than that in CHO-Ras cells. In contrast, when cell spreading was evaluated by measurement of the area occupied by CHO cells plated on FN, this parameter was increased significantly by forced expression of VE-PTP-WT compared with that apparent in cells expressing VE-PTP-C/S or EGFP (Fig. 2C). These results thus suggested that forced expression of VE-PTP in cooperation with integrins and Ras enhances cell spreading and lamellipodium formation in CHO cells. The VE-PTP-induced formation of lamellipodia appeared to be especially dependent on activation of Ras.

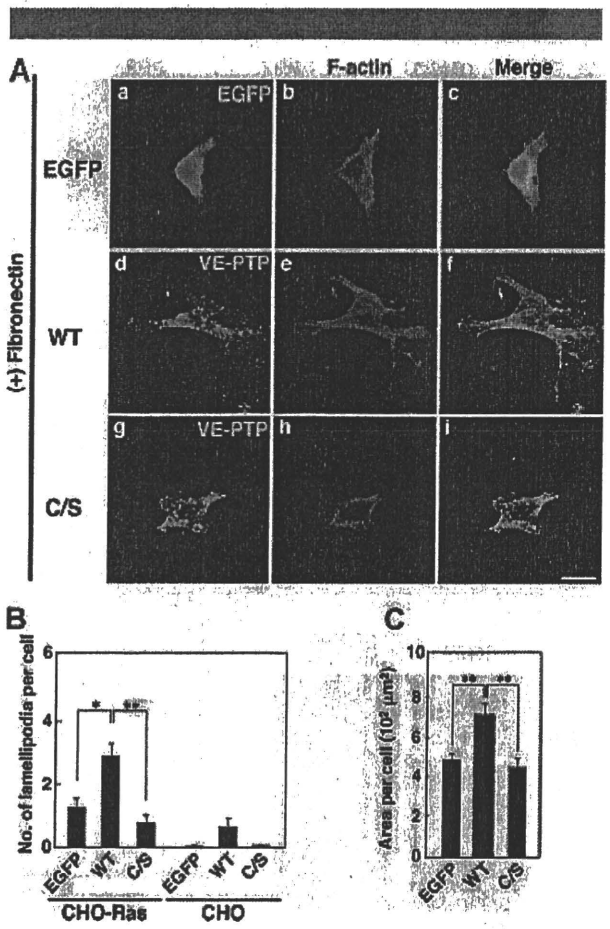

Fig. 2. Effects of transient expression of VE-PTP on cell spreading and lamellipodium formation in CHO-Ras cells or parental CHO cells plated on fibronectin. A: CHO-Ras cells transiently transfected with an expression vector for EGFP (a–c), VE-PTP-WT (WT; d–f), or VE-PTP-C/S (C/S; g–i) were deprived of serum for 8 h, replated on cover glasses coated with fibronectin, and cultured in serum-free medium for 2 h. The cells were then fixed, stained with rhodamine–phalloidin (b,e,h), and either immunostained with mAbs to VE-PTP (d,g) or monitored for EGFP fluorescence (a). The merged images are shown in c, f, and i. Bar, 20 μm. B: CHO-Ras or CHO cells transiently transfected and treated as in A were evaluated for the number of lamellipodia per transfected cell (identified by VE-PTP immunofluorescence or EGFP fluorescence). C: CHO cells were transiently transfected and treated as in A. The area occupied by each transfected cell was then determined as described in the Materials and Methods Section. Data in B and C are means ± SE from 20 cells for each condition and are representative of three independent experiments. *$P < 0.05$ and **$P < 0.01$ (ANOVA and Bonferroni's test).

Role of Src family kinases (SFKs) in VE-PTP-induced cell spreading and lamellipodium formation

We next investigated the intracellular signaling molecules that mediate the effects of VE-PTP on cell spreading and lamellipodium formation. We first examined the effects of inhibitors specific for SFKs, mitogen-activated protein or extracellular signal-regulated kinase kinase (MEK), phosphoinositide 3-kinase (PI3K), or Rho-kinase, all of which are implicated in the regulation of cell morphology. We found that PP2, an inhibitor of SFKs (Hanke et al., 1996; Murata et al., 2006), markedly inhibited cell spreading and lamellipodium formation in CHO-Ras-VE-PTP-WT cells plated on FN (Fig. 3A). In contrast, neither DMSO (vehicle) nor PP3, an inactive analog of PP2 (Hanke et al., 1996; Murata et al., 2006), affected the morphology of these cells (Fig. 3A). Quantitative analysis of lamellipodium formation confirmed that PP2, but not PP3, prevented lamellipodium formation in CHO-Ras-VE-PTP-WT cells as well as in mock-transfected CHO-Ras cells, whereas neither the MEK inhibitor PD98059, the PI3K inhibitor wortmannin, nor the Rho-kinase inhibitor Y-27632 affected this parameter in CHO-Ras-VE-PTP-WT cells (Fig. 3B).

The PTK Csk is thought to inhibit the activity of SFKs by phosphorylating their COOH-terminal tyrosine residue (Honda et al., 1997; Murata et al., 2006), and a membrane-targeted form of Csk (Myr-Csk), which contains a myristylation signal at the NH_2-terminus, has been shown to inhibit the activity of SFKs more effectively than does WT Csk (Honda et al., 1997; Murata et al., 2006). Forced expression of Myr-Csk prevented cell spreading and lamellipodium formation in CHO-Ras-VE-PTP-WT cells as well as in mock-transfected CHO-Ras cells (Fig. 3C,D). Together, these results suggested

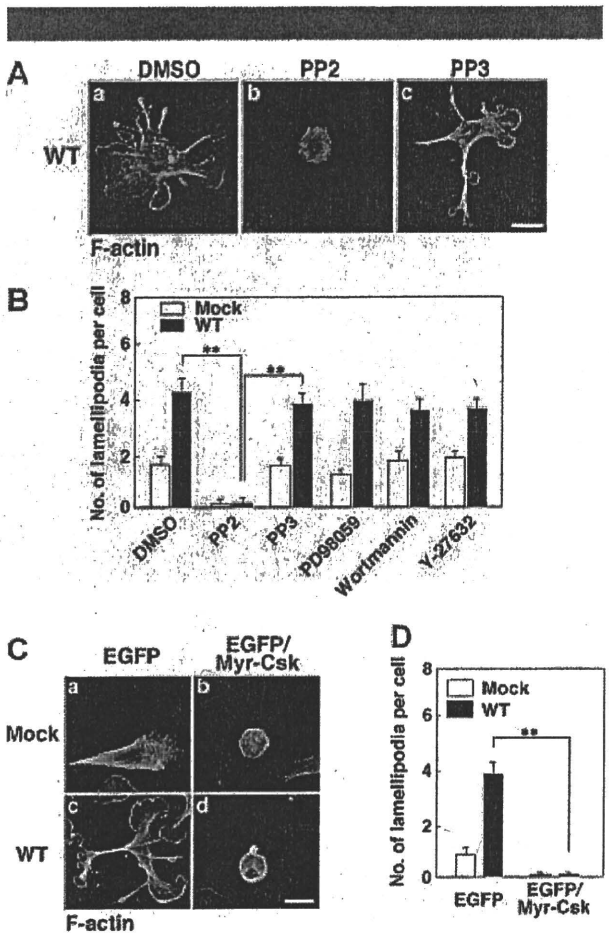

Fig. 3. Participation of SFKs in VE-PTP-induced cell spreading and lamellipodium formation. A: CHO-Ras-VE-PTP-WT cells (WT) were deprived of serum for 8 h, replated on fibronectin-coated cover glasses, and cultured for 2 h in serum-free medium containing 0.05% DMSO (a), 5 μM PP2 (b), or 5 μM PP3 (c). The cells were then fixed and stained with rhodamine–phalloidin (a–c). Bar, 20 μm. (B) Mock-transfected CHO-Ras cells (Mock) or CHO-Ras-VE-PTP-WT cells were deprived of serum for 8 h, replated on fibronectin-coated cover glasses, and cultured for 2 h in serum-free medium containing 0.05% DMSO, 5 μM PP2, 5 μM PP3, 50 μM PD98059, 50 μM wortmannin, or 5 μM Y-27632. The cells were then fixed and stained with rhodamine–phalloidin for evaluation of the number of lamellipodia. C: Cells as in B were transiently transfected with an expression vector for EGFP (a–d) and either an expression vector for Myr-Csk (b,d) or the corresponding empty vector (a,c), after which they were replated on fibronectin-coated dishes and cultured for 24 h in medium containing 10% FBS. The cells were then fixed and stained with rhodamine–phalloidin (a–d). EGFP fluorescence was monitored to identify transiently transfected cells. Bar, 20 μm. D: The number of lamellipodia per transiently transfected cell for cells treated as in C. Data in B and D are means ± SE from 20 cells for each condition and are representative of three independent experiments. $^{**}P < 0.01$ (ANOVA and Bonferroni's test).

that SFKs are important for VE-PTP-induced cell spreading and lamellipodium formation in CHO-Ras cells plated on FN.

We therefore next investigated whether forced expression of VE-PTP indeed affected the activity of SFKs in CHO-Ras cells plated on FN. Autophosphorylation of SFKs (at Tyr^{416} for avian c-Src) results in an increase in PTK activity (Roskoski, 2004), with the extent of autophosphorylation being thought to reflect that of PTK activity. In contrast, phosphorylation of the COOH-terminus of SFKs (at Tyr^{527} for avian c-Src) by Csk results in inhibition of PTK activity (Roskoski, 2004).

Immunoprecipitation and immunoblot analysis with pAbs to the Tyr^{416}-phosphorylated form of c-Src revealed that the level of autophosphorylation of c-Src was markedly increased in CHO-Ras-VE-PTP-WT cells compared with that in mock-transfected CHO-Ras cells at 120 min after replating on FN-coated dishes (Fig. 4A,B). This increase in phosphorylation of c-Src at Tyr^{416} was also apparent at 30–60 min after replating of CHO-Ras-VE-PTP-WT cells (data not shown). In contrast, immunoprecipitation and immunoblot analysis with pAbs to the Tyr^{527}-phosphorylated form of c-Src showed that the level of c-Src phosphorylation at this residue was slightly but not significantly reduced in CHO-Ras-VE-PTP-WT cells compared with that in mock-transfected CHO-Ras cells (Fig. 4A,B).

Immunostaining with pAbs to the Tyr^{416}-phosphorylated form of c-Src revealed a substantial increase in the level of immunoreactivity, especially at the periphery of lamellipodia, in CHO-Ras-VE-PTP-WT cells, compared with that apparent in mock-transfected CHO-Ras cells (Fig. 4C), indicative of SFK activation in this region of CHO-Ras-VE-PTP-WT cells. In

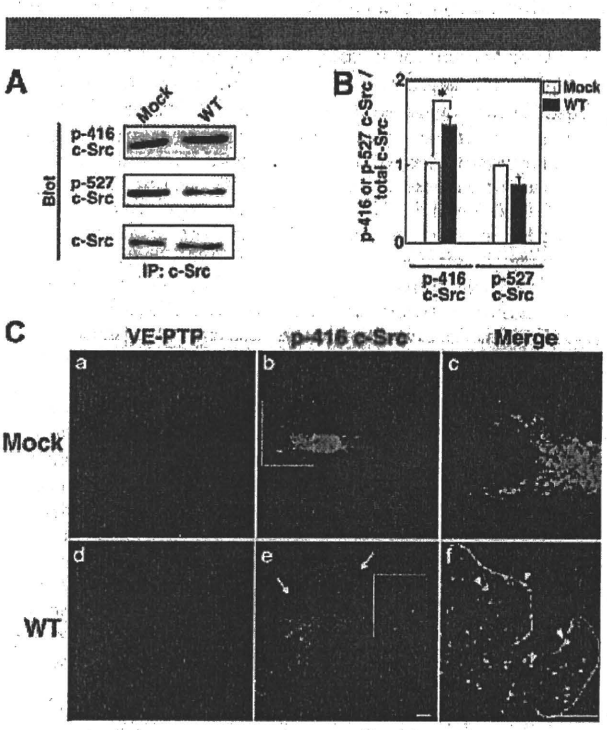

Fig. 4. Effect of forced expression of VE-PTP on SFK activation in CHO-Ras cells plated on fibronectin. A: Mock-transfected CHO-Ras cells (Mock) or CHO-Ras-VE-PTP-WT cells (WT) were deprived of serum for 8 h, replated on fibronectin-coated dishes, and cultured in serum-free medium for 2 h. The cells were then lysed and subjected to immunoprecipitation with mAbs to v-Src, and the resulting precipitates were subjected to immunoblot analysis with mAbs to v-Src as well as with pAbs to phospho-Tyr^{416} (p-416) or phospho-Tyr^{527} (p-527) forms of c-Src. B: Immunoblots similar to those in A were subjected to densitometric analysis, and the ratio of the band intensity for p-416 or p-527 c-Src to that for total c-Src was calculated. Data are expressed relative to the corresponding value for mock-transfected CHO-Ras cells and are means ± SE from four separate experiments. $^{*}P < 0.05$ (Student's t-test). C: Cells as in A were deprived of serum for 8 h, replated on cover glasses coated with fibronectin, and cultured in serum-free medium for 2 h. They were then fixed and subjected to immunostaining with mAbs to VE-PTP (a,d) and pAbs to p-416 c-Src (b,e). Enlarged images corresponding to the boxed regions in b and e are shown in c and f, respectively. Arrows in e indicate signals for p-416 c-Src along the periphery of lamellipodia. Arrowheads in f indicate colocalization of immunoreactivity for VE-PTP and p-416 c-Src. Results are representative of three independent experiments. Bars, 10 μm.

addition, immunoreactivity for the Tyr^{416}-phosphorylated form of c-Src overlapped in part with that of VE-PTP both within and at the periphery of lamellipodia (Fig. 4C).

Roles of Rac and Cdc42 as well as of Vav2 and FRG in the VE-PTP-induced morphological changes in CHO-Ras cells plated on fibronectin

Formation of lamellipodia and filopodia is promoted by activation of the small GTPases Rac and Cdc42, respectively (Takai et al., 2001; Etienne-Manneville and Hall, 2002). Indeed, forced expression of a dominant negative mutant of Rac1 (RacT17N) fused with EGFP markedly inhibited cell spreading and lamellipodium formation in both CHO-Ras-VE-PTP-WT cells and mock-transfected CHO-Ras cells plated on FN (Fig. 5A,B). NWASP-CRIB specifically binds the GTP-bound (active) form of Cdc42 and thereby inhibits its activity (Miyashita et al., 2004). Expression of an EGFP fusion protein of NWASP-CRIB also inhibited cell spreading and formation of lamellipodia in CHO-Ras-VE-PTP-WT cells (Fig. 5A,B). We therefore next determined whether the activity of Rac or Cdc42 was increased in CHO-Ras-VE-PTP-WT cells with the use of a GST-PAK pull-down assay. We found that the activity of Cdc42 in CHO-Ras-VE-PTP-WT cells plated on FN-coated dishes was indeed increased compared with that apparent in mock-transfected CHO-Ras cells (Fig. 5C,D). The activity of Rac in CHO-Ras-VE-PTP-WT cells plated on FN also tended to be increased compared with that in mock-transfected CHO-Ras cells, although this difference was not statistically significant (Fig. 5C,D). Together, these results suggested that activation of Rac or Cdc42 participates in the morphological changes apparent in CHO-Ras-VE-PTP-WT cells plated on FN.

Vav2, a guanine nucleotide exchange factor (GEF) for Rho family proteins, is tyrosine-phosphorylated and thereby activated by c-Src (Marignani and Carpenter, 2001; Servitja et al., 2003). FRG, another GEF specific for Cdc42 and Rac, is also tyrosine-phosphorylated and activated by c-Src (Fukuhara et al., 2003; Miyamoto et al., 2003). We therefore next examined whether Vav2 or FRG might participate in VE-PTP-induced cell spreading and lamellipodium formation. We first determined the effects of forced expression of a dominant negative mutant of Vav2 (Vav2-DN) in which Leu^{212} is replaced with Gln. By analogy with Vav1, this substitution would be expected to abolish the catalytic activity of Vav2, and the mutant thus acts in a dominant negative manner (Kodama et al., 2000; Kawakatsu et al., 2005). Forced expression of the Vav2 mutant resulted in marked inhibition of the enhanced cell spreading and lamellipodium formation apparent in CHO-Ras-VE-PTP-WT cells plated on FN (Fig. 6). Similarly, forced expression of a dominant negative mutant of FRG (FRG-DN), which lacks both DH and PH domains of FRG (Fukuhara et al., 2003; Miyamoto et al., 2003; Murata et al., 2006), also inhibited the enhanced cell spreading and formation of lamellipodia in CHO-Ras-VE-PTP-WT cells (Fig. 6). In contrast, expression of a dominant negative mutant of the Rac GEF Tiam1 (Tiam1-DN), which lacks the DH domain, had no effect on the morphological changes in CHO-Ras-VE-PTP-WT cells (Fig. 6). These results thus suggested that Vav2 and FRG are important for the specific morphological characteristics of CHO-Ras-VE-PTP-WT cells plated on FN.

Enhancement of fibronectin-dependent cell migration by VE-PTP

Given that VE-PTP markedly enhanced cell spreading and lamellipodium formation on FN, we next examined the effect of forced expression of VE-PTP on FN-dependent cell migration. Cell migration was determined with the use of a Transwell assay, in which serum-deprived cells were plated in the upper chamber and the number of cells that had migrated into the lower chamber containing serum-free medium with or without

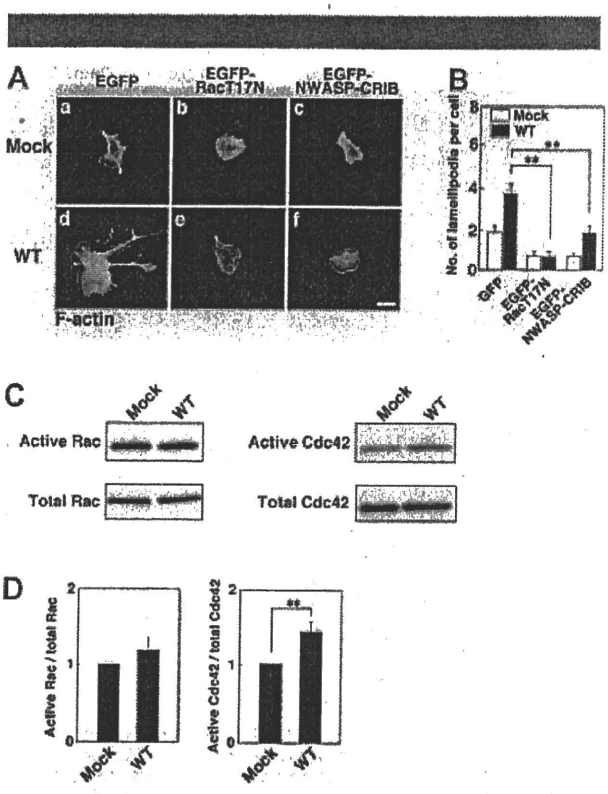

Fig. 5. Importance of Rac and Cdc42 for VE-PTP-induced morphological changes in CHO-Ras cells plated on fibronectin. A: Mock-transfected CHO-Ras cells (Mock; a–c) or CHO-Ras-VE-PTP-WT cells (WT; d–f) were transiently transfected with expression vectors for EGFP (a,d) or for EGFP fusion proteins containing either a dominant negative mutant of Rac1 (RacT17N; b,e) or NWASP-CRIB (c,f). The cells were deprived of serum for 8 h, replated on cover glasses coated with fibronectin, and cultured in serum-free medium for 2 h. They were then fixed and stained with rhodamine–phalloidin (a–f). EGFP fluorescence was monitored to identify transiently transfected cells. Bar, 20 μm. B: The number of lamellipodia per transiently transfected cell in experiments similar to that shown in A was determined. Data are means ± SE from 20 cells for each condition and are representative of three independent experiments. **$P < 0.01$ (ANOVA and Bonferroni's test). C: Mock-transfected CHO-Ras cells or CHO-Ras-VE-PTP-WT cells were deprived of serum for 8 h, replated on fibronectin-coated dishes, and cultured in serum-free medium for 2 h. The cells were then lysed, and the GTP-bound (active) forms of Rac or Cdc42 were precipitated with a GST fusion protein containing the CRIB domain of p21PAKα. The resulting precipitates were subjected to immunoblot analysis with mAbs to Rac (left upper part) or to Cdc42 (right upper part). Whole cell lysates were also subjected directly to immunoblot analysis with the same mAbs to determine the total amounts of Rac (left lower part) or Cdc42 (right lower part). D: Immunoblots similar to those shown in C were subjected to densitometric analysis, and the ratio of the band intensity for GST-PAK-bound Rac to total Rac or for GST-PAK-bound Cdc42 to total Cdc42 was calculated. Data are expressed relative to the corresponding value for mock-transfected CHO-Ras cells and are means ± SE from four independent experiments. **$P < 0.01$ (Student's t-test).

FN was counted after 2 h. The number of migrating cells was significantly greater for CHO-Ras-VE-PTP-WT cells than for either mock-transfected CHO-Ras cells or CHO-Ras-VE-PTP-C/S cells (Fig. 7). Cell migration was minimal for all cell lines in the absence of FN.

Importance of VE-PTP for cell spreading in HUVECs and bEnd.3 cells

We investigated whether VE-PTP is important for integrin-mediated regulation of cell morphology in HUVECs, which

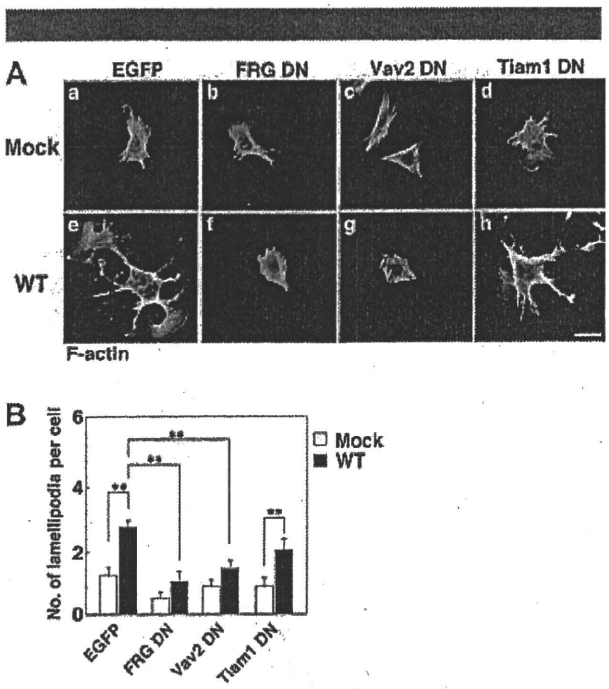

Fig. 6. Importance of Vav2 and FRG for VE-PTP-induced morphological changes in CHO-Ras cells plated on fibronectin. A: Mock-transfected CHO-Ras cells (Mock; a–d) or CHO-Ras-VE-PTP-WT cells (WT; e–h) were transiently transfected with expression vectors for EGFP (a,e), a Flag epitope-tagged dominant negative form of FRG (FRG DN; b,f), or Myc epitope-tagged dominant negative forms of Vav2 (Vav2 DN; c,g) or Tiam1 (Tiam1 DN; d,h). The cells were deprived of serum for 8 h, replated on cover glasses coated with fibronectin, and cultured in serum-free medium for 2 h. They were then fixed and stained with rhodamine–phalloidin (a–h). Cells expressing Flag epitope-tagged FRG DN or Myc epitope-tagged Vav2 DN or Tiam1 DN were identified by immunostaining with mAbs to the Flag or Myc epitopes; those expressing EGFP were identified on the basis of EGFP fluorescence. Bar, 20 μm. B: The number of lamellipodia per transiently transfected cell was determined for cells treated as in A. Data are means ± SE of values from 20 cells for each condition and are representative of three independent experiments. **$P < 0.01$ (ANOVA and Bonferroni's test).

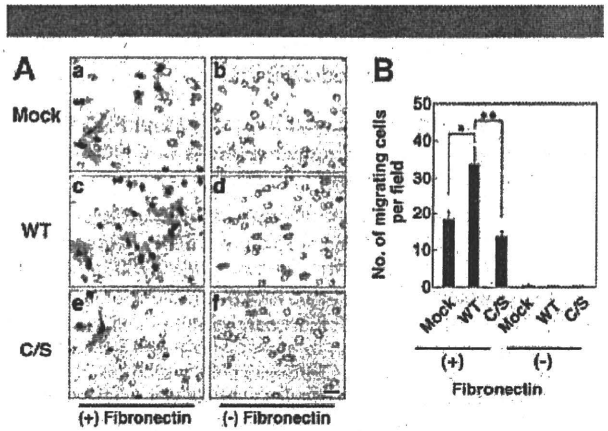

Fig. 7. Enhancement of fibronectin-dependent cell migration by VE-PTP. A: Mock-transfected CHO-Ras cells (Mock; a,b), CHO-Ras-VE-PTP-WT cells (WT; c,d), or CHO-Ras-VE-PTP-C/S cells (C/S; e,f) were deprived of serum for 8 h, resuspended in serum-free medium, and seeded into the upper chamber of a Transwell apparatus. Serum-free medium with or without fibronectin (20 μg/ml) was then added to the lower chamber and the seeded cells were cultured for 2 h, after which cells that had migrated through the filter were fixed and stained with May-Giemsa solution. Bar, 20 μm. B: The number of cells that had migrated into the lower chamber in experiments similar to that in A was determined. Data are means ± SE for five independent microscopic fields and are representative of three independent experiments. *$P < 0.05$ and **$P < 0.01$ (ANOVA and Bonferroni's test).

express endogenous VE-PTP (Nawroth et al., 2002). Adenovirus-mediated overexpression of VE-PTP-WT, but not that of EGFP or VE-PTP-C/S, promoted the spreading of HUVECs plated on FN (Fig. 8A,B). Expression of VE-PTP-C/S actually resulted in slight inhibition of cell spreading in HUVECs plated on FN. We next examined the effect of RNAi-mediated depletion of endogenous VE-PTP on the morphology of HUVECs plated on FN. Infection of HUVECs with an adenovirus encoding the human VE-PTP shRNA shRNA-hVE-PTP #2 markedly reduced the amount of endogenous VE-PTP, whereas that with adenoviruses encoding either shRNA-hVE-PTP #1 or EGFP had no such effect (Fig. 8C). Consistent with these results, expression of shRNA-hVE-PTP #2, but not that of EGFP or shRNA-hVE-PTP #1, markedly inhibited spreading of HUVECs on FN (Fig. 8D,E). Finally, transfection of mouse endothelioma bEnd.3 cells, which also express endogenous VE-PTP (Nawroth et al., 2002), with shRNA vectors for mouse VE-PTP, but not with control vectors, markedly inhibited cell spreading on FN (Supplementary Fig. S4).

Discussion

PTPs are generally considered to function as negative regulators on the basis of their ability to oppose the effects of PTKs. Indeed, VE-PTP is thought to bind to Tie-2, a receptor-type PTK, and to inactivate it by dephosphorylation (Fachinger et al., 1999; Winderlich et al., 2009), thereby inhibiting proliferation of endothelial cells (Winderlich et al., 2009). In contrast, we have now shown that forced expression of VE-PTP promoted cell spreading and formation of both lamellipodia and filopodia in cultured fibroblasts plated on FN. It also enhanced FN-dependent cell migration. All of these effects of VE-PTP were found to be dependent on its PTP activity, suggesting that they are mediated by tyrosine dephosphorylation of a substrate (or substrates) for this enzyme. The effects of VE-PTP on cell morphology were also dependent on integrin activation. Engagement of integrins by ECM proteins such as FN results in activation of a variety of signaling molecules including SFKs, focal adhesion kinase (FAK), as well as PI3K, Ras-MEK, and Rho (Rüegg and Mariotti, 2003; Brunton et al., 2004). Inhibition of SFKs abolished the effects of VE-PTP on cell spreading and formation of lamellipodia in cells plated on FN, suggesting that these effects of VE-PTP are mostly attributable to enhancement of integrin-induced activation of SFKs.

The mechanism by which VE-PTP up-regulates the activity of SFKs is unclear. However, we found that the level of autophosphorylation of c-Src was increased by forced expression of VE-PTP. Given that the extent of autophosphorylation reflects the PTK activity of SFKs, this observation indicates that VE-PTP increases the activity of c-Src. Indeed, RPTPs of the R4 subtype, such as PTPα and PTPε, are also thought to contribute to the activation of c-Src (Gil-Henn and Elson, 2003; Pallen, 2003). Tyrosine phosphorylation of the COOH-terminal region of PTPα or PTPε promotes the binding of these enzymes to the SH2 domain of c-Src, resulting in disruption of the closed conformation formed by interaction between the SH2 domain and phosphorylated Tyr^{527} at the COOH-terminus of c-Src. The phosphorylated Tyr^{527} residue, which negatively regulates the activity of c-Src, is thereby exposed and rendered susceptible to dephosphorylation by PTPα or PTPε (Gil-Henn and Elson, 2003; Pallen, 2003). We have recently found that VE-PTP also contains a tyrosine

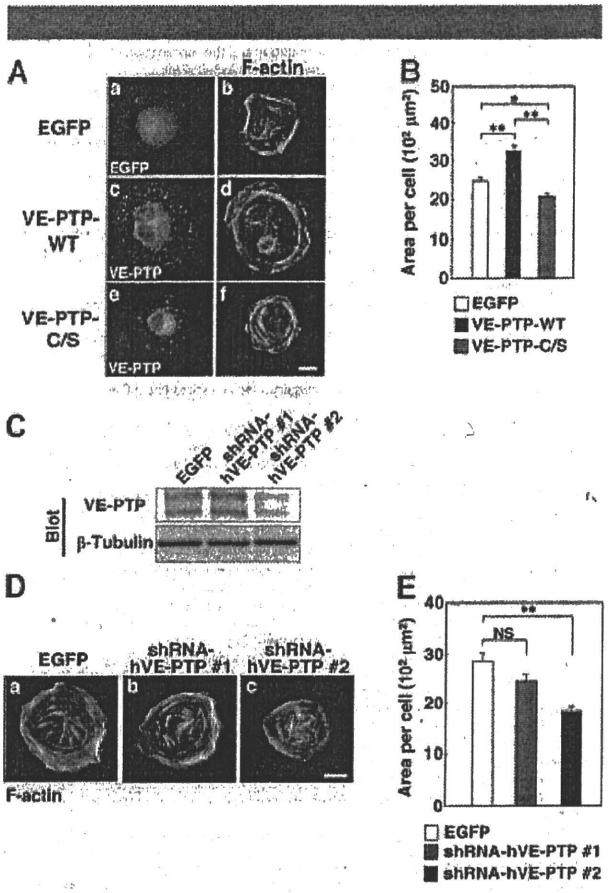

Fig. 8. Role of VE-PTP in cell spreading in HUVECs. A: HUVECs infected with adenoviruses encoding EGFP (a,b), VE-PTP-WT (c,d), or VE-PTP-C/S (e,f) were cultured in HuMedia-EB2 supplemented with 0.5% FBS for 8 h, replated on fibronectin-coated cover glasses, and cultured for an additional 1 h. The cells were then fixed and stained with rhodamine–phalloidin (b,d,f), and infected cells were identified by immunostaining with mAbs to VE-PTP (c,e) or by monitoring of EGFP fluorescence (a). Bar, 20 μm. B: The area occupied by infected HUVECs treated as in A was determined. Data are means ± SE from 174 cells for each condition in six separate experiments. *$P < 0.05$ and **$P < 0.01$ (ANOVA and Bonferroni's test). C: HUVECs were infected for 72 h with adenoviruses encoding EGFP or shRNAs specific for human VE-PTP mRNA (shRNA-hVE-PTP #1 or #2). Cell lysates were then prepared and subjected to immunoblot analysis with pAbs to VE-PTP or mAbs to β-Tubulin. Results are representative of three independent experiments. D: HUVECs infected with adenoviruses as in C were incubated in HuMedia-EB2 supplemented with 0.5% FBS for 8 h, replated on fibronectin, and cultured for 1 h. The cells were then fixed and stained with rhodamine–phalloidin (a–c). Bar, 20 μm. E: The area occupied by infected HUVECs treated as in D was determined. Data are means ± SE from 50 cells for each condition and are representative of three independent experiments. **$P < 0.01$ (ANOVA and Bonferroni's test); NS, not significant.

residue in the COOH-terminal region that undergoes phosphorylation (Yoji Murata and Takashi Matozaki, unpublished observation). VE-PTP may therefore up-regulate the activity of c-Src in a similar manner to PTPα or PTPε. Tyrosine phosphorylation of Cbp or paxillin promotes the recruitment of Csk to the plasma membrane, resulting in inhibition by Csk of the PTK activities of SFKs through phosphorylation of their COOH-terminal tyrosine residues (Ren et al., 2004; Zhang et al., 2004). Cbp or paxillin might thus also be a target for VE-PTP, with their dephosphorylation by VE-PTP preventing inhibition of SFK activity by Csk. In the present study, we found that forced expression of VE-PTP-WT in CHO-Ras cells plated on FN resulted in a marked increase in immunoreactivity for active forms of SFKs, with this increase being especially prominent at the leading edge of lamellipodia. This immunoreactivity overlapped in part with that for VE-PTP. These results thus suggest that integrins, activated SFKs, and VE-PTP may be colocalized, thereby their interaction contributing to proper regulation of cell spreading or cell morphology.

The VE-PTP-induced cell spreading and lamellipodium formation observed in cultured cells plated on FN was prevented by inhibition of Rac or Cdc42. In addition, we showed that forced expression of VE-PTP increased the activity of Cdc42 and tended to increase that of Rac. Engagement of integrins by ECM triggers activation of Rac and Cdc42 (DeMali et al., 2003; Brunton et al., 2004; Huveneers and Danen, 2009). Forced expression of VE-PTP thus likely enhances the activation of Rac and Cdc42 by integrins to promote cell spreading and lamellipodium formation. Activation of c-Src is implicated in the activation of Rac and Cdc42 downstream of integrins (DeMali et al., 2003; Brunton et al., 2004; Huveneers and Danen, 2009). One mechanism by which integrin-mediated activation of c-Src promotes activation of Rac and Cdc42 is through c-Src-mediated tyrosine phosphorylation of GEFs for these small GTPases (DeMali et al., 2003; Brunton et al., 2004; Huveneers and Danen, 2009). Indeed, we showed that Vav2 and FRG participate in VE-PTP-induced cell spreading and lamellipodium formation. Given that both of these GEFs are activated as a result of tyrosine phosphorylation by c-Src (Marignani and Carpenter, 2001; Miyamoto et al., 2003; Servitja et al., 2003), enhancement by VE-PTP of integrin-mediated activation of SFKs likely results in the tyrosine phosphorylation of Vav2 or FRG and the consequent activation of Rac or Cdc42.

The effects of VE-PTP overexpression on cell morphology, especially the induction of lamellipodium formation, were more pronounced in CHO-Ras cells, which express an active form of Ras, than in parental CHO cells. Although the mechanism by which an active form of Ras enhances these effects of VE-PTP remains unclear, Ras is thought to activate Rho family proteins such as Rac and Cdc42 through PI3K-dependent or -independent pathways (Bar-Sagi and Hall, 2000). Given that Ras is activated by various endothelial growth factors such as VEGF and angiopoietin, VE-PTP might promote the spreading and migration of endothelial cells in cooperation with integrins and Ras. However, forced expression of both VE-PTP and an active form of H-Ras failed to promote lamellipodium formation in HUVECs plated on FN (data not shown). The effects of coexpression of VE-PTP and Ras on cell morphology may thus differ among cell types.

We showed that forced expression of VE-PTP promoted cell spreading in HUVECs plated on FN. Conversely, depletion of endogenous VE-PTP by RNAi in HUVECs or bEnd.3 cells resulted in inhibition of cell spreading on FN. These results suggest that regulation of cell spreading on FN by VE-PTP occurs not only in fibroblasts but also in endothelial cells. Integrins are key regulators of endothelial cell proliferation, migration, morphogenesis, and survival, all of which are important for the formation and remodeling of blood vessels (Davis and Senger, 2005). Our results suggest that VE-PTP likely cooperates with integrins to regulate a variety of processes underlying the formation and remodeling of blood vessels. VE-PTP was previously shown to negatively regulate the actions of VEGFR-2 or Tie-2 in endothelial proliferation and tubule formation (Fachinger et al., 1999; Mellberg et al., 2009; Winderlich et al., 2009). In contrast, the integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ physically interact with VEGFR-2 and Tie-2, respectively (Soldi et al., 1999; Borges et al., 2000; Cascone et al., 2005), and such interactions are important for full activation of VEGFR-2 or Tie-2 on ligand stimulation (Somanath et al., 2009). VE-PTP thus

likely interacts with integrins as well as endothelial growth factor receptors and thereby regulates the actions of these molecules. Moreover, VE-PTP physically interacts with VE-cadherin and enhances cell–cell adhesion mediated by this molecule, resulting in regulation of cell-layer permeability (Nawroth et al., 2002; Nottebaum et al., 2008). VE-PTP thus appears to orchestrate the functions of vascular adhesion molecules as well as of endothelial growth factor receptors for proper regulation of angiogenesis.

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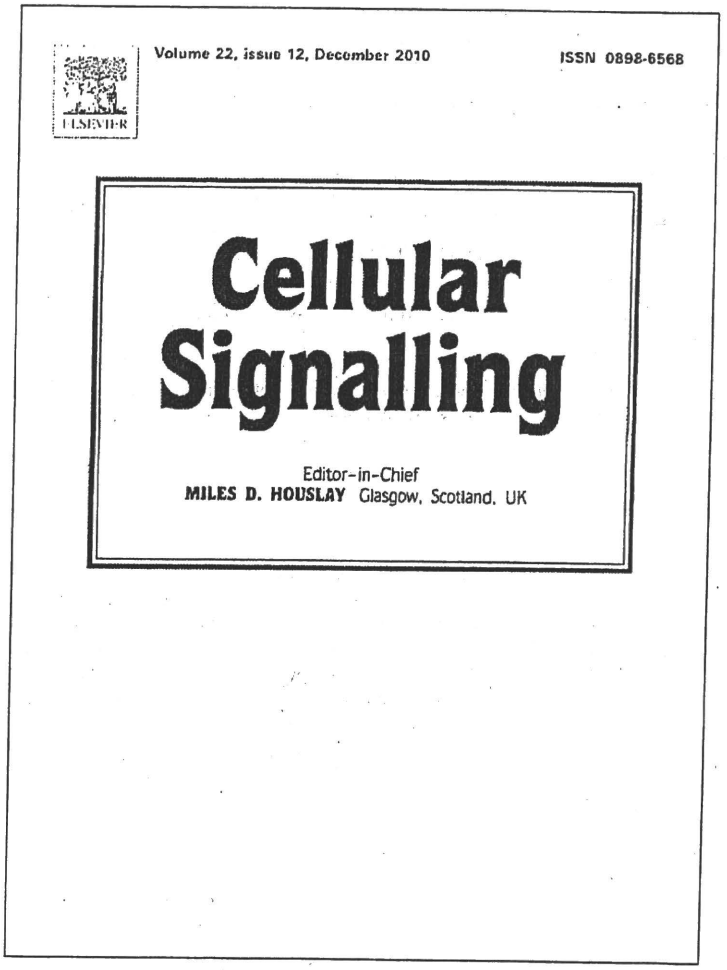

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Review

Expression, localization, and biological function of the R3 subtype of receptor-type protein tyrosine phosphatases in mammals

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ABSTRACT

The R3 subtype of receptor-type protein tyrosine phosphatases (RPTPs) includes VE-PTP, DEP-1, PTPRO, and SAP-1. All of these enzymes share a similar structure, with a single catalytic domain and putative tyrosine phosphorylation sites in the cytoplasmic region and fibronectin type III–like domains in the extracellular region. The expression of each R3 RPTP is largely restricted to a single or limited number of cell types, with VE-PTP and DEP-1 being expressed in endothelial or hematopoietic cells, PTPRO in neurons and in podocytes of the renal glomerulus, and SAP-1 in gastrointestinal epithelial cells. In addition, these RPTPs are localized specifically at the apical surface of polarized cells. The structure, expression, and localization of the R3 RPTPs suggest that they perform tissue-specific functions and that they might act through a common mechanism that includes activation of Src family kinases. In this review, we describe recent insights into R3-subtype RPTPs, particularly those of mammals.

Contents

1. Introduction

Protein tyrosine phosphatases (PTPs) are thought to play important roles in a variety of basic cellular functions such as proliferation, differentiation, and migration [1,2]. These enzymes are also important regulators in the central nervous system, the immune system, and many other organs. Deregulation of PTPs is associated with various disorders, with the result that many members of the PTP family are considered potential therapeutic targets [2–4]. Among 107 PTPs encoded by the human genome, the class I cysteine-based PTPs constitute the largest group and are further divided into 38 tyrosine-specific PTPs and 61 dual-specific phosphatases [1]. The tyrosine-specific PTPs are further classified into two groups on the basis of their structure and cellular localization: receptor-type PTPs (RPTPs) and non-receptor-type PTPs. RPTPs are further classified into eight subtypes (R1 to R8) according to their primary structure [1,5].

Abbreviations: PTP, protein tyrosine phosphatase; RPTP, receptor-type PTP; VE-PTP, vascular endothelial-PTP; DEP-1, density-enriched PTP-1; SAP-1, stomach cancer-associated PTP-1; SFK, Src family kinase; SH, Src homology; E, embryonic day; VEGF, vascular endothelial growth factor; VEGFR-2, VEGF receptor-2; MAPK, mitogen-activated protein kinase; CNS, central nervous system; APC, adenomatous polyposis coli.

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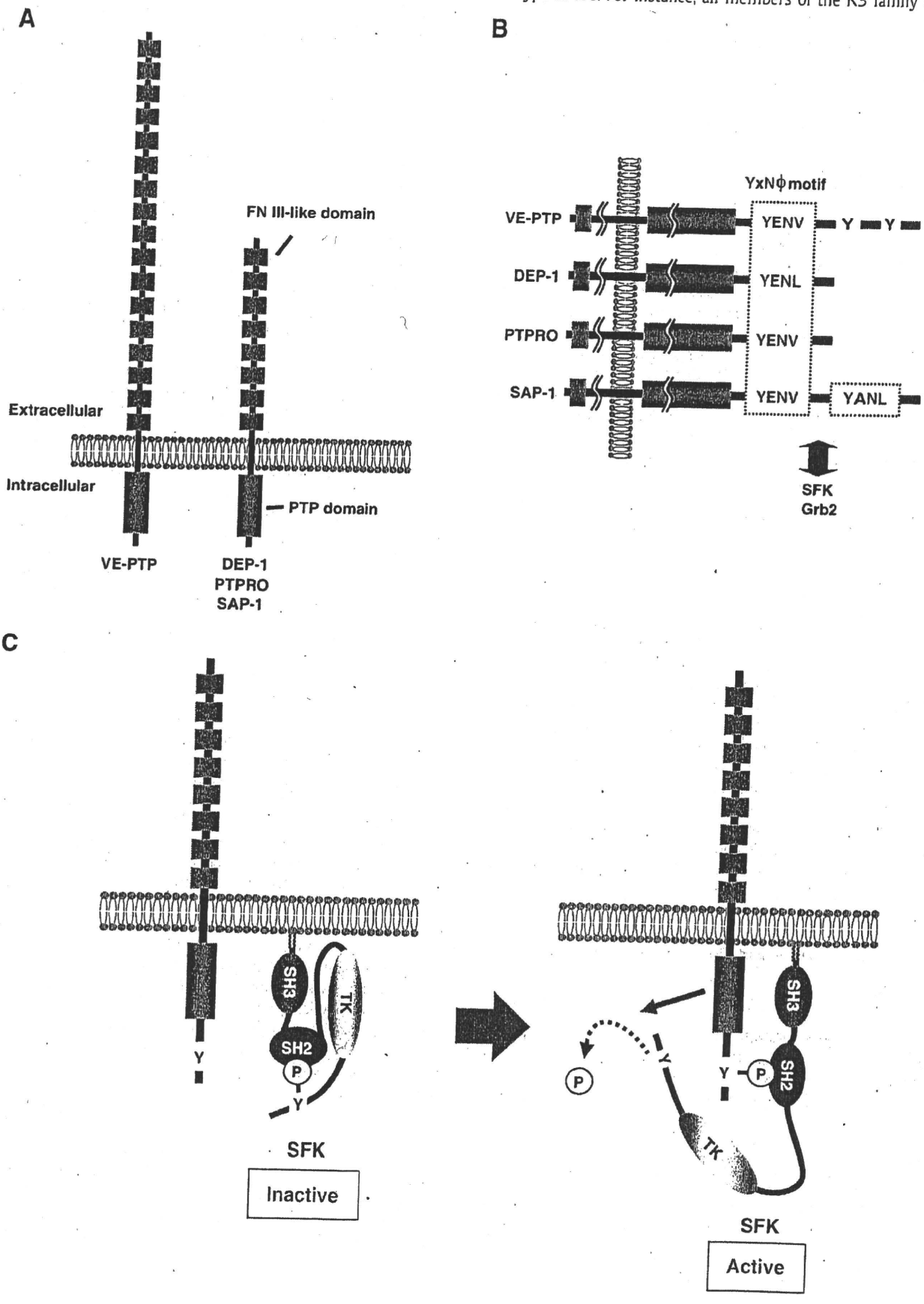
RPTPs of the R3 subtype include vascular endothelial–protein tyrosine phosphatase (VE-PTP) [6,7], density-enriched PTP-1 (DEP-1) [8,9], PTPRO [10,11], and stomach cancer–associated protein tyrosine phosphatase-1 (SAP-1) [12]. All of these enzymes share a similar structure, with a single catalytic domain in the cytoplasmic region and fibronectin type III–like domains in the extracellular region (Fig. 1A). Recent studies have revealed additional common features of these R3-subtype RPTPs. For instance, all members of the R3 family undergo



tyrosine phosphorylation in their COOH-terminal region, and such phosphorylation promotes the binding of Src family kinases (SFKs). The expression of R3 RPTPs is also restricted to a single or limited number of cell types. In addition, these RPTPs tend to be expressed specifically at the apical surface of polarized cells. All R3 RPTPs might therefore function through a common mechanism. In this review, we will describe recent progress in research on the expression, localization, and function of R3-subtype RPTPs.

2. Tyrosine phosphorylation of the COOH-terminal region as a posttranslational modification of R3-subtype RPTPs

With regard to posttranslational modification, all R3 RPTPs are highly glycosylated proteins that contain multiple N-glycosylation sites in the extracellular region [6,10,12]. SAP-1, PTPRO, and VE-PTP also each contain the amino acid motif YxNΦ (where Φ represents a hydrophobic amino acid and x any amino acid) in the COOH-terminal region [13] (Fig. 1B), and these YxNΦ motifs undergo tyrosine phosphorylation on exposure of cells to pervanadate. In addition, a global phosphoproteomic analysis suggested that the YxNΦ motif of VE-PTP is phosphorylated in human gastric cancer cells [14]. Given that a YxNΦ motif is also present in the COOH-terminal region of DEP-1 (Y^{1221}ENL in the mouse protein), it is likely that DEP-1 also undergoes tyrosine phosphorylation in the COOH-terminal region. SFKs contribute to the tyrosine phosphorylation of these RPTPs [13]. Moreover, the phosphorylated YxNΦ motifs serve as binding sites for the Src homology (SH) 2 domain of the SFK Fyn (Fig. 1C). Members of the R4 subtype of RPTPs [5], such as PTPα and PTPε, also undergo tyrosine phosphorylation in the COOH-terminal region, with the tyrosine phosphorylation site also providing a binding site for the SH2 domain of Src [15,16]. This binding results in disruption of the closed conformation formed by interaction between the SH2 domain and the COOH-terminal phosphorylated tyrosine residue of Src (Tyr^{527} of avian Src). The phosphorylation of this tyrosine residue is thought to negatively regulate the activity of c-Src [17,18]. Exposure of phosphorylated Tyr^{527} and its consequent dephosphorylation by PTPα or PTPε result in the activation of Src. Tyrosine phosphorylation of R3-subtype RPTPs thus also likely promotes the binding and consequent activation of SFKs in a manner similar to that apparent for PTPα and PTPε (Fig. 1C). Indeed, forced expression of VE-PTP in cells increases the activity of Src [19]. The phosphorylated YxNΦ motifs of R3 RPTPs also bind the adaptor molecule Grb2 [13], although the functional relevance of such complex formation remains unclear. Grb2 is implicated in regulation of clathrin-mediated endocytosis of the epidermal growth factor receptor through interaction of its SH2 domain with the receptor [20]. Interaction of tyrosine-phosphorylated R3 RPTPs with Grb2 might therefore also contribute to regulation of endocytosis of these RPTPs.

3. Expression, localization, and biological function of R3 RPTPs

3.1. VE-PTP

VE-PTP (also known as PTPRB or PTPβ) contains 16 or 17 fibronectin type III–like domains in its extracellular region [6,21] (Fig. 1A). It was first identified by Krueger et al. in 1990 [6], but neither its expression nor its biological function was investigated in this initial study. The expression of VE-PTP was subsequently shown to be restricted to endothelial cells [7], and was found to be especially prominent in those of arteries and arterioles [7,22,23] (Fig. 2A). It first becomes apparent along the primitive aorta at embryonic day (E) 9 to E10 in mice, and then gradually increases in the entire vascular system in accordance with embryonic development [23]. During mammalian embryonic development, aggregation of endothelial cell precursors (angioblasts) results in the formation of early blood vessels and a primitive vascular plexus, a process termed vasculogenesis [24,25]. The primitive vasculature subsequently undergoes a remodeling process, termed angiogenesis, that includes endothelial cell growth and migration as well as vessel sprouting and pruning, resulting in the development of a functional vascular system [24,25]. Ablation of VE-PTP in mice resulted in embryonic death at E10 as a consequence of a variety of angiogenesis defects in both the embryo and yolk sac [22,23]. These defects included failure of remodeling of the vascular plexus into large veins and branched vascular networks, suggesting that VE-PTP is important for angiogenic processes such as remodeling and maintenance of blood vessels rather than for vasculogenesis [22,23]. However, the molecular basis for such a function of VE-PTP in development of the vascular system remains unclear.

Vascular endothelial growth factor (VEGF) and its receptors, in particular VEGF receptor-2 (VEGFR-2), are important for early vasculogenesis as well as later angiogenesis [25–27], whereas Tie-2, a receptor for angiopoietin, is thought to play a key role in angiogenesis [24,25,28]. Both VEGFR-2 and Tie-2 are receptor-type protein tyrosine kinases. VE-PTP is thought to bind to Tie-2 and to inactivate it by mediating its dephosphorylation [7,29], resulting in inhibition of the proliferation of endothelial cells [29]. VE-PTP was also shown to form a complex with VEGFR-2 and to negatively regulate its actions in the resting condition, but it dissociates from VEGFR-2 on ligation of the latter with VEGF [30]. VE-cadherin, an endothelial cell–specific cadherin, is essential for formation of stable contacts between endothelial cells that underlie development and maintenance of blood vessels [31]. VE-PTP physically interacts with VE-cadherin and enhances cell-cell adhesion mediated by this molecule, contributing to regulation of cell-layer permeability [21,32].

Integrins, through their interaction with extracellular matrix, are also thought to be important for maintenance of the structural stability of blood vessels, as well as for the proliferation, migration, and survival of endothelial cells, during both vasculogenesis and angiogenesis [33]. Although PTPs are generally considered to function as negative regulators on the basis of their ability to oppose the effects of protein tyrosine kinases, it was recently shown that VE-PTP, in cooperation with integrins, promotes the spreading and migration of cultured fibroblasts as well as endothelial cells [19]. These effects of VE-PTP were found to be dependent on its PTP activity, suggesting that they are mediated by tyrosine dephosphorylation of a substrate (or substrates) for this enzyme. They are likely attributable to enhancement of integrin-induced activation of SFKs [19]. Moreover, VE-PTP likely binds SFKs through its COOH-terminal region and thereby induces direct activation of these kinases in a manner similar to that apparent for PTPα and PTPε [13,19] (Fig. 2B). The integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ physically interact with VEGFR-2 and Tie-2, respectively [34–36], and such interactions are important for full activation of VEGFR-2 and Tie-2 on ligand stimulation [37]. VE-PTP thus likely interacts with integrins as well as with endothelial growth factor receptors and thereby regulates the actions of these molecules. Given that VE-PTP also

Fig. 1. Structure, COOH-terminal tyrosine phosphorylation sites, and mode of action of R3-subtype RPTPs. A. Structural organization of R3-subtype RPTPs. All of these enzymes share a similar structure, with a single catalytic (PTP) domain in the cytoplasmic region and fibronectin type III (FN III)–like domains in the extracellular region. VE-PTP contains 16 or 17 fibronectin type III–like domains in its extracellular region, whereas DEP-1, PTPRO, and SAP-1 contain eight or fewer such domains. B. Tyrosine phosphorylation sites in the COOH-terminal region of R3 RPTPs. All R3 RPTPs share the same YxNΦ motif (x, any amino acid; Φ, a hydrophobic amino acid) in the COOH-terminal region (YENV/L), whereas SAP-1 also contains another such motif (YANL). Tyrosine phosphorylation of these motifs provides binding sites for Src family kinases (SFKs) or Grb2. C. Model for the activation of SFKs by R3-subtype RPTPs. Tyrosine phosphorylation of the R3 RPTPs in the COOH-terminal region promotes the binding of the SH2 domain of an SFK. This binding disrupts the closed conformation formed by interaction between the SH2 domain and the COOH-terminal phosphorylated Tyr residue of the SFK. The phosphorylated Tyr of the SFK is consequently dephosphorylated by the RPTP, resulting in SFK activation. TK, tyrosine kinase domain.

physically interacts with VE-cadherin and enhances cell-cell adhesion mediated by this molecule [21,32], VE-PTP appears to orchestrate the functions of vascular adhesion molecules as well as endothelial growth factor receptors for proper regulation of angiogenesis (Fig. 2B).

3.2. DEP-1

DEP-1 (also known as PTPRJ, PTP-η, Byp, or CD148) is an R3 RPTP with eight fibronectin type III-like domains in its extracellular region

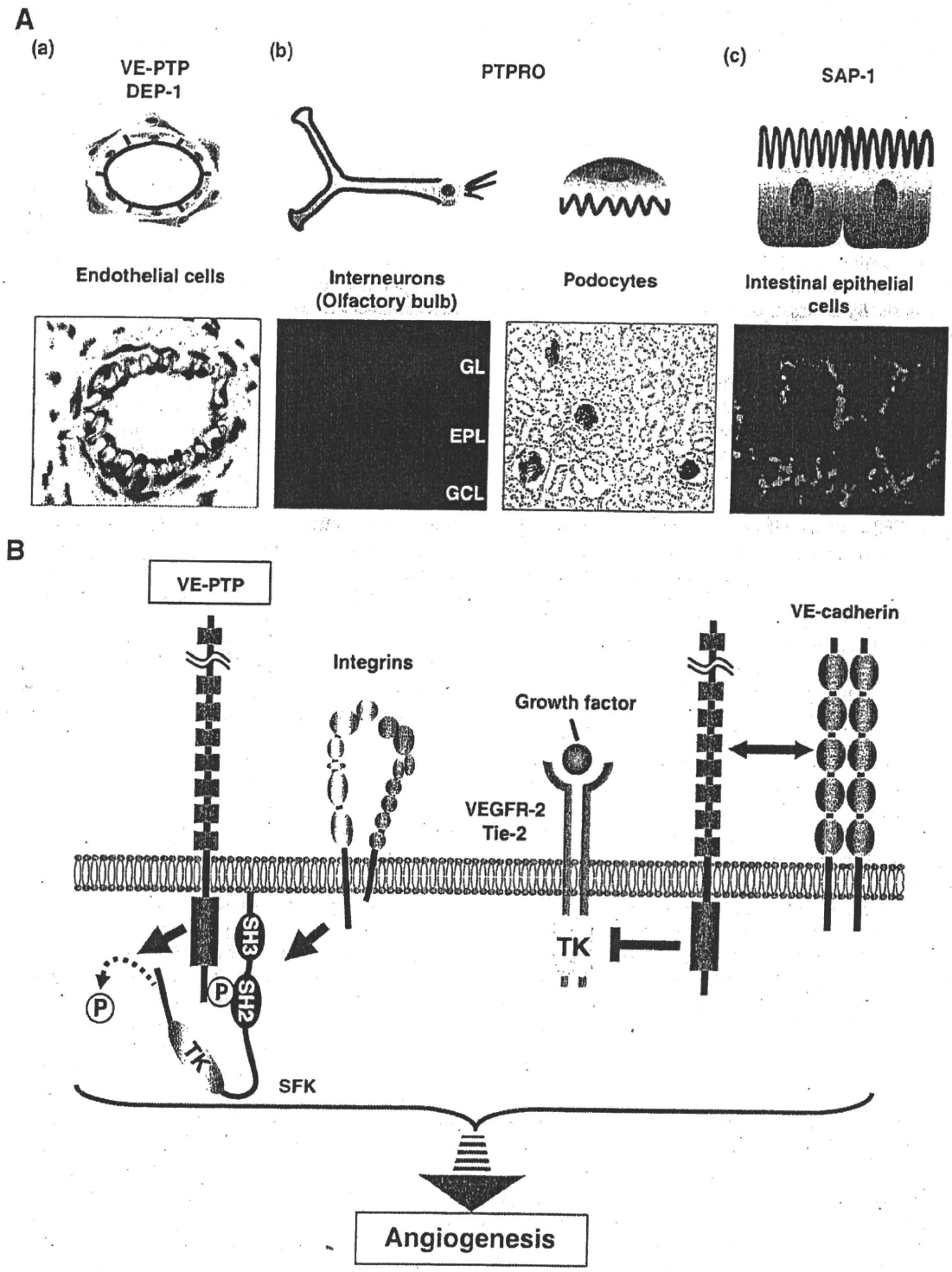


Fig. 2. Expression, localization, and biological function of R3-subtype RPTPs. A. Restricted expression and localization of R3-subtype RPTPs. The upper panels show schematic representations of the subcellular localization (red lines) of the indicated R3 RPTPs. The lower panels shown immunostaining of tissue sections for each R3 RPTP: The expression of VE-PTP (brown) is detected in arterial endothelial cells of the adult mouse kidney, with nuclei being stained blue (a); PTPRO is expressed in interneurons of the olfactory bulb in the adult mouse (red in left panel of (b)), with staining being most prominent in the external plexiform layer (EPL) but also present in the glomerular layer (GL) and granule cell layer (GCL); PTPRO is also expressed in podocytes of the glomeruli in mouse kidney (brown in right panel of (b)); and SAP-1 (red) is localized at the apical membrane of intestinal epithelial cells of the adult mouse, whereas β-catenin (green) is present at the basolateral membrane. B. Model for the molecular mechanism by which VE-PTP regulates angiogenesis. VE-PTP enhances integrin-induced activation of SFKs as well as directly activates SFKs in endothelial cells, with the latter effect being achieved through interaction of VE-PTP with the SFK and consequent dephosphorylation by VE-PTP of the COOH-terminal Tyr residue of the SFK. VE-PTP also physically interacts with VEGFR-2 and Tie-2 and negatively regulates these receptors. In addition, VE-PTP physically interacts with VE-cadherin and enhances VE-cadherin-mediated cell-cell adhesion.

[8,9,38,39] (Fig. 1A). It is most abundant in endothelial cells (Fig. 2A) and various types of hematopoietic cells [39–41], but it is also expressed in epithelial cells and fibroblasts [8,42]. The expression level of DEP-1 is directly related to cell density, suggestive of a regulatory role for this RPTP in cell contact–mediated growth inhibition [8]. The expression of DEP-1 in T lymphocytes is also increased by cell activation through cross-linking of CD3 [39], suggesting that the expression of this RPTP is dynamically regulated in accordance with cell condition.

With regard to its expression in endothelial cells, DEP-1 tends to be localized at sites of cell-cell contact, overlapping with the localization of VE-cadherin [43]. Ablation of DEP-1 in vivo was found to result in mouse embryonic death at E11.5 that was accompanied by vascularization failure. This defect was characterized by increased endothelial cell numbers and enlarged primitive vessels, indicative of a problem with vascular remodeling and branching [44]. DEP-1 was thus proposed to be indispensable for the development of blood vessels during embryogenesis. By contrast, DEP-1–deficient mice generated by other groups are viable and do not manifest any gross abnormalities [45,46]. The mechanism by which DEP-1 might regulate endothelial function remains unclear. DEP-1 contributes to cadherin-mediated contact inhibition through dephosphorylation of VEGFR-2 and attenuation of the activation of mitogen-activated protein kinase (MAPK) [47]. Consistent with this finding, depletion of DEP-1 in endothelial cells resulted in increased phosphorylation of VEGFR-2 at all major autophosphorylation sites [48]. However, DEP-1 depletion did not enhance VEGFR-2–mediated signaling, whereas it reduced the level of tyrosine phosphorylation of Gab-1 as well as the association of Gab-1 with phosphoinositide 3-kinase, and it reduced the activation level of the protein kinase Akt. Indeed, DEP-1 has been shown to be important for dephosphorylation of the negative regulatory COOH-terminal Tyr^{527} of Src and consequent activation of Src in endothelial cells [48]. These observations thus suggest that DEP-1, similar to VE-PTP, plays a positive role in regulation of endothelial function.

Forced expression of DEP-1 in T cells inhibited cell function and signal transduction [49,50]. By contrast, cross-linking of DEP-1 enhanced CD3-mediated T cell activation [39,41]. Moreover, ablation of DEP-1 in mice resulted in a partial block of B cell development, a B cell phenotype similar to that of CD45-deficient mice [46]. Mice deficient in both DEP-1 and CD45 manifested substantial defects in B and myeloid lineage development and immunoreceptor signaling, including hyperphosphorylation of the COOH-terminal tyrosine of the SFK Lyn. Together, these observations suggest that DEP-1 might regulate the functions of hematopoietic and endothelial cells through dephosphorylation of the negative regulatory COOH-terminal tyrosine of SFKs [46]. DEP-1 was also recently shown to function as a positive regulator of platelet activation and thrombosis by promoting SFK activation [51,52].

3.3. PTPRO

Mammalian PTPRO (also known as GLEPP1, PTP-BK, or PTPΦ) is an R3 RPTP with seven or eight fibronectin type III–like domains in its extracellular region [10,53,54] (Fig. 1A). Indeed, five spliced isoforms of this PTP have been described [11]. Two transmembrane-type isoforms are highly expressed in the brain [11,53] and podocytes of renal glomeruli [55,56], whereas three truncated isoforms (PTPΦ or PTPROt), which lack the extracellular domain, are expressed predominantly in macrophages, B cells, or osteoclasts [57–59]. We here focus on the expression, localization, and function of PTPRO in neurons and podocytes.

Most RPTPs, with the exception of CD45 (R1 subtype), are expressed in the central nervous system (CNS) and have been implicated in the regulation of neuronal adhesion as well as axon growth and guidance during CNS development [60,61]. Indeed, PTPRO is thought to participate in the regulation of axon growth and guidance during embryonic neurogenesis. In the chick brain, maximal expression of cPTPRO (previously named CRYP2), the chick ortholog of mammalian PTPRO, is apparent between E8 and E13, coincident with pervasive axonal extension and tract formation in the chick CNS [62,63]. The expression of cPTPRO in retinal projection neurons was also found to be concentrated in axons and growth cones [64]. Depletion of cPTPRO by RNA interference in developing motor neurons resulted in aberrant guidance of motor axons [65]. Furthermore, biochemical analysis revealed that the receptor tyrosine kinase EphB is a substrate for the phosphatase activity of cPTPRO [66]. cPTPRO regulates the sensitivity of retinal axons to ephrins and thereby plays a key role in establishment of retinotectal projections in the chick [66]. By contrast, the localization and biological function of mammalian PTPRO in the CNS have been less well characterized. PTPRO is widely expressed throughout the mouse brain from E16 to postnatal day 1, but the level of such expression decreases thereafter [11,67], similar to the temporal pattern of cPTPRO mRNA level. The expression of PTPRO was recently shown to be largely confined to the olfactory bulb and olfactory tubercle in the adult mouse brain [67] (Fig. 2A). In the olfactory bulb, PTPRO is expressed predominantly in the external plexiform layer, the granule cell layer, and the glomerular layer, and it is most abundant in interneurons such as γ-aminobutyric acid– or calretinin-positive granule cells [67]. Costaining of PTPRO with other neuronal markers suggested that PTPRO is localized to the dendrites or dendritic spines of these olfactory interneurons. Although the functional role of PTPRO in interneurons of the olfactory bulb remains unknown, PTPRO might participate in regulation of dendritic morphology or synapse formation. PTPRO-deficient mice did not show any abnormality with regard to hematoxylin-eosin staining or immunostaining for various olfactory bulb markers [67], suggesting that other RPTPs thought to be expressed in the olfactory bulb, such as RPTPσ, RPTPξ, or LAR, might be able to compensate for the loss of PTPRO [67]. In contrast, the number of peptidergic nociceptive neurons in dorsal root ganglia was found to be reduced in adult PTPRO-deficient mice [68]. In addition, spinal pathfinding by both peptidergic and proprioceptive neurons was abnormal in these mice, suggesting that PTPRO is required for peptidergic differentiation and process outgrowth in sensory neurons [68].

In addition to its expression in neurons, PTPRO is abundant in the highly polarized visceral glomerular epithelial cells (podocytes) of the renal glomerulus, being localized in particular at the apical surface of foot processes of these cells [55,69,70] (Fig. 2A). Podocytes contribute to the kidney filter by extending their primary processes onto the capillary surface [71,72]. They form fine secondary foot processes that interdigitate with those of neighboring podocytes. This interdigitation results in the formation of a 40-nm-wide slit between foot processes that contains a porous ultrafilter known as the slit diaphragm. Protein tyrosine phosphorylation is thought to play an important role in regulation of the morphology and function of the slit diaphragm. For instance, nephrin, an immunoglobulin-like transmembrane protein that bridges the distance between interdigitating podocyte foot processes, contains several tyrosine phosphorylation sites in its cytoplasmic region for the binding of SH2 domain–containing molecules [71,72]. Indeed, nephrin binds Fyn, which in turn phosphorylates nephrin [73]. In addition, nephrin binds Nck, an adaptor molecule that contains SH2 and SH3 domains and is thought to regulate reorganization of the actin cytoskeleton [74,75]. PTPRO thus likely plays a role in maintaining the structure and function of the slit diaphragm. Indeed, the ablation of PTPRO resulted in a transformation of the normal "octopoid" structure of podocytes to an "amoeboid" structure, with foot processes that were shorter and broader than normal [56]. Podocyte dysfunction due to mutation or deletion of slit diaphragm components such as nephrin is thought to result in leakiness of the glomerular filter, which manifests clinically as proteinuria and the nephritic syndromes [71]. However, PTPRO-deficient mice did not manifest leakiness of the glomerular filter; they

instead exhibited reduced glomerular filtration and a tendency to develop hypertension. PTPRO is therefore important for homeostasis of the relation between glomerular pressure and filtration rate through maintenance of podocyte structure and function [56]. However, a monoclonal antibody to PTPRO was recently shown to increase glomerular albumin permeability [76].

3.4. SAP-1

SAP-1 (also known as PTPRH) was originally identified as a PTP expressed in a human stomach cancer cell line [12]. It contains six (mouse) or eight (human) fibronectin type III–like domains in its extracellular region [12] (Fig. 1A), and its expression is largely restricted to the gastrointestinal tract [77]. Moreover, SAP-1 localizes to the microvilli of the brush border in the small intestine and colon as well as to the stomach (Fig. 2A). Given that the expression of VE-PTP is restricted to endothelial cells, with VE-PTP being localized at the apical surface of these cells, and that PTPRO is specifically expressed in and localized at the apical surface of foot processes of podocytes, which are highly polarized glomerular epithelial cells, the expression of RPTPs of the R3 subtype may be restricted to a single or limited number of cell types and be localized specifically at the apical surface of polarized cells. The predominant expression of SAP-1 in gastrointestinal epithelial cells and its localization to the microvilli of these cells suggest that SAP-1 might play a role in maintenance of microvillus architecture. However, SAP-1–deficient mice manifested no marked changes in the morphology of intestinal epithelial cells, including that of microvilli or of tight or adherens junctions between these cells [77]. Consistent with this finding, the expression of SAP-1 in the intestine was shown to be minimal during embryonic development and to increase markedly after birth [77], suggesting that SAP-1 is not important for determination of cell architecture in the intestinal epithelium.

Forced expression of SAP-1 was shown to inhibit the proliferation of cultured cells through attenuation of growth factor–induced activation of MAPK or through induction of caspase-dependent apoptosis [78,79]. SAP-1 is also implicated in regulation of the reorganization of the actin-based cytoskeleton in cultured cells through dephosphorylation of several focal adhesion–associated proteins, including focal adhesion kinase, p130cas, and paxillin [78]. By contrast, SAP-1 ablation inhibited tumorigenesis in $Apc^{Min/+}$ mice, which harbor a heterozygous mutation of the adenomatous polyposis coli (APC) gene, with results suggesting that SAP-1 might contribute to tumor expansion but not to the initial transformation of normal epithelial cells into dysplastic cells [77]. Functional impairment of the APC protein in $Apc^{Min/+}$ mice is thought to result in stabilization and marked accumulation of β-catenin, which initiates transformation of normal epithelial cells as a result of constitutive activation of the β-catenin–transcription factor 4 (TCF4) transcriptional pathway [80]. However, SAP-1 likely does not regulate this pathway itself [77]. Instead, given that VE-PTP and DEP-1 are thought to activate SFKs [19,48,51,52], SAP-1 likely promotes intestinal cell proliferation also through activation of SFKs. The expression of SAP-1 is markedly increased in human colon and pancreatic cancers [12,81], supporting the notion that SAP-1 promotes the tumorigenic potential of intestinal epithelial cells.

4. Concluding remarks

Although the physiological functions of VE-PTP and DEP-1 have been recently clarified, those of PTPRO in the brain and SAP-1 in the intestine require further investigation. Although each RPTP of the R3 subtype shows a distinct pattern of tissue-specific expression, the structural similarities of these enzymes suggest that they might function through common mechanisms. One such mechanism is likely the activation of SFKs through dephosphorylation of the negative regulatory COOH-terminal tyrosine, as has been demonstrated for VE-PTP and DEP-1. Further study will be necessary to elucidate whether PTPRO and SAP-1 might also act through such a mechanism. Another important issue waiting to be addressed is whether R3 RPTPs have specific ligands for their extracellular regions, which are potentially important for regulation of their phosphatase activities. Given the structural similarity of the extracellular regions of R3-subtype RPTPs, one might expect that such ligands would also be structurally similar. A more complete understanding of the physiological functions of R3 RPTPs as well as the identification of cognate ligands may also provide a basis for the development of new therapeutic agents for various medical disorders.

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