Shear Stress-induced Redistribution of Vascular Endothelial-Protein-tyrosine Phosphatase (VE-PTP) in Endothelial Cells and Its Role in Cell Elongation*

Vascular endothelial cells (ECs) are continuously exposed to shear stress (SS) generated by blood flow. Such stress plays a key role in regulation of various aspects of EC function including cell proliferation and motility as well as changes in cell morphology. Vascular endothelial-protein-tyrosine phosphatase (VE-PTP) is an R3-subtype PTP that possesses multiple fibronectin type III-like domains in its extracellular region and is expressed specifically in ECs. The role of VE-PTP in EC responses to SS has remained unknown, however. Here we show that VE-PTP is diffusely localized in ECs maintained under static culture conditions, whereas it undergoes rapid accumulation at the downstream edge of the cells relative to the direction of flow in response to SS. This redistribution of VE-PTP triggered by SS was found to require its extracellular and transmembrane regions and was promoted by integrin engagement of extracellular matrix ligands. Inhibition of actin polymerization or of Cdc42, Rab5, or Arf6 activities attenuated the SS-induced redistribution of VE-PTP. VE-PTP also underwent endocytosis in the static and SS conditions. SS induced the polarized distribution of internalized VE-PTP. Such an effect was promoted by integrin engagement of fibronectin but prevented by inhibition of Cdc42 activity or of actin polymerization. In addition, depletion of VE-PTP by RNA interference in human umbilical vein ECs blocked cell elongation in the direction of flow induced by SS.

Our results suggest that the polarized redistribution of VE-PTP in response to SS plays an important role in the regulation of EC function by blood flow.
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importance of protein-tyrosine kinases as mediators of SS-induced EC responses, protein-tyrosine phosphatases (PTPs), which counter protein tyrosine phosphorylation by protein-tyrosine kinases, are also likely to play a key role in these responses. The contribution of PTPs to the regulation of SS-evoked signaling pathways in ECs has remained largely unexplored, however.

Vascular endothelial-protein-tyrosine phosphatase (VE-PTP, also known as PTPRB or PTPRO) is specifically expressed in ECs, with its expression being especially prominent in those of arteries and arterioles (7–9). VE-PTP is a receptor-type PTP (RPTP) with a single catalytic domain in its cytoplasmic region and multiple fibronectin type III (FNIII)-like domains in its extracellular region (7, 10, 11), being classified as an RPTP of the R3 subtype (11). Ablation of VE-PTP in mice resulted in embryonic death at 10 days of gestation as a result of a variety of angiogenesis-related defects in both the embryo and yolk sac. These defects included failure of the vascular plexus to undergo remodeling into large veins and branched vascular networks, suggesting that VE-PTP is important for angiogenic processes such as remodeling and maintenance of blood vessels (8, 9). Despite these findings on the role of VE-PTP in angiogenesis, its function in EC responses to SS has remained unclear.

We recently showed that VE-PTP, in cooperation with integrins, promotes the spreading and migration of ECs through up-regulation of c-Src activity (12). VE-PTP was also shown to inactivate VEGFR2 by mediating its dephosphorylation, resulting in inhibition of EC proliferation and of the establishment of EC polarity and lumen formation (13, 14). In addition, VE-PTP is thought to form a complex with VE-cadherin and thereby to promote its adhesive function (15, 16). Given that integrins, VEGFR2, and VE-cadherin are key regulators of SS-induced responses in ECs (1, 3, 4), we have now investigated whether VE-PTP also contributes to such regulation.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—A rat mAb and rabbit polyclonal antibodies (pAbs) to VE-PTP (12) as well as rat mAbs to SAP-1 and to PTPRO (17, 18) were described previously. Mouse mAbs to the HA epitope tag (12CA5) and to β-tubulin were obtained from Roche Applied Science and Sigma, respectively. Rhodamine-conjugated phalloidin and Alexa Fluor 488-conjugated goat pAbs to rat or mouse IgG were from Invitrogen, and Cy3-conjugated goat pAbs to rat or mouse IgG as well as HRP-conjugated goat pAbs to rabbit, mouse, or rat IgG were obtained from Jackson ImmunoResearch (West Grove, PA). DAPI was from Nacalai Tesque (Kyoto, Japan); LY294002 and Y-27632 were from EMD Chemicals (Darmstadt, Germany); fibronectin, vitronectin, and poly-1-lysine were from Sigma; collagen-I and laminin were from BD Biosciences and Invitrogen, respectively; cytochalasin D and nocodazole were from Wako (Osaka, Japan).

Cell Culture—Mouse endothelioma bEnd.3 cells (American Type Culture Collection, Manassas, VA) and HEK293A cells (Invitrogen) were maintained in DMEM supplemented with 10% FBS (Invitrogen). Human umbilical vein endothelial cells (HUVECs) were obtained from Kurabo (Osaka, Japan) and were cultured in basal medium (HuMedia-EB2, Kurabo) supplemented with 2% FBS as well as with human EGF (10 ng/ml), human basic FGF (5 ng/ml), hydrocortisone (1 μg/ml), heparin (10 μg/ml), gentamicin (50 μg/ml), and amphotericin B (50 μg/ml), all of which were obtained from Kurabo.

Exposure of Cells to SS—Cells were exposed to laminar SS as described previously (19). In brief, bEnd.3 cells, HUVECs, or HEK293A cells were cultured on glass coverslips (24 by 45 mm; Matsunami, Osaka, Japan) coated with either fibronectin (20 μg/ml), collagen-I (50 μg/ml), vitronectin (1.1 μg/ml), laminin (50 μg/ml), or poly-1-lysine (50–100 μg/ml). The coverslips were loaded into a parallel flow chamber (Yasuhsa Koki, Tokyo, Japan) containing DMEM with or without 2% FBS, and SS was then applied to the cells. The SS τ value (dyne/cm²) was calculated from the volumetric flow rate (Q) with the use of the equation $\tau = 6\eta Q/ab^2$, where $\eta$ is the apparent viscosity of the medium (taken to be 0.76 centipoise), $a$ is the channel height (0.02 cm), and $b$ is the channel width (1.6 cm). All SS experiments were performed at 37 °C in a CO₂ incubator, with most being performed at 3 or 9 dyne/cm², both of which are within the physiological range of venous or arterial SS (20, 21).

Immunofluorescence Analysis—Cells were fixed with 4% paraformaldehyde for 10 min, incubated for 30 min with buffer G (PBS containing 5% goat serum) in the absence (nonpermeabilization) or presence (permeabilization) of 0.1% Triton X-100, 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 containing 0.1% Triton X-100, and observed with a laser-scanning confocal microscope (LSM 700; Zeiss, Oberkochen, Germany) or with a fluorescence microscope (BX51; Olympus, Tokyo, Japan).

Quantification of VE-PTP Distribution—For quantification of VE-PTP distribution, images of sheared or static cells were divided into quadrants (Q1, Q2, Q3, and Q4) as shown in Fig. 1B. Cells were scored for VE-PTP staining by visual inspection in a blinded fashion and assigned to one of the quadrants according to where the heaviest VE-PTP staining occurred. The percentage of cells assigned to Q4, which show a polarized distribution of VE-PTP to the downstream edge of the cells relative to the direction of flow, was then determined.

Plasmids—Expression vectors for mouse VE-PTP, SAP-1, or PTPRO were generated as described previously (12, 17, 18). For construction of an expression vector for the Δcyto mutant of VE-PTP, which lacks most of the cytoplasmic region of the protein, a DNA fragment corresponding to amino acids 1–1645 of mouse VE-PTP was amplified by PCR and subcloned into pEntr-CMV (12). An expression vector for the chimeric protein, VE-PTP-ex-SAP-1-cyto, which consists of the extracellular and transmembrane regions of VE-PTP fused to the cytoplasmic tail of SAP-1, was generated by PCR-ligation-PCR mutagenesis (22). For expression of enhanced green fluorescent protein (EGFP), the pEGFP-N3 vector was obtained from Clontech (Palo Alto, CA). Expression vectors for an HA-tagged dominant negative mutant of Rab5(S34N), an EGFP-tagged dominant negative mutant of Rac(T17N), and an EGFP-tagged CRIB domain of NWASP were described previously (23). Expression vectors for an HA-tagged DEP-1 and for an
EGFP-tagged dominant negative mutant of Arf6(T27N) were kindly provided by T. Takahashi (Vanderbilt University, Nashville, TN) and K. Nakayama (Kyoto University, Japan), respectively. Expression vectors for HA-tagged dominant negative mutants of Rab4(N121I) and Rab11(S25N) were kindly provided by T. Sasaki (Tokushima University, Japan). The sequences of all PCR products were verified by sequencing with an ABI3100 instrument (Applied Biosystems, Foster City, CA).

**Transfection and RNAi**—bEnd.3 cells, HEK293A cells, or HUVECs were transfected with expression vectors with the use of Lipofectamine2000 (Invitrogen) or FuGENE HD (Promega, Madison, WI) reagents. RNAi for endogenous human VE-PTP was performed with the siRNA sequences 5′-CCACAUACCUUCUAAUCCAA-3′ (VE-PTP siRNA#1) and 5′-CCUAGUUCAUUGGCGUUGU-3′ (VE-PTP siRNA#2). The MISSION siRNA universal negative control (Sigma) was also used. Cells were transfected with siRNAs with the use of Lipofectamine RNAiMAX (Invitrogen).

**Antibody Labeling Assay**—bEnd.3 cells plated on fibronectin- or poly-L-lysine-coated glass coverslips were incubated with a mAb to VE-PTP (20 μg/ml) for 15 min on ice and washed with cold DMEM, after which they were either maintained under the static condition or exposed to SS at 3 dyne/cm² for 30 min. The cells were then washed with acid solution (0.2M acetic acid (pH 3.0), 0.5M NaCl) to remove the antibody bound to the cell surface and fixed with 4% paraformaldehyde. Fixed cells were incubated for 30 min with buffer G in the absence or presence of 0.1% Triton X-100 and further incubated with secondary antibodies. Images were acquired with a fluorescence microscope. For measurement of fluorescence intensity of anti-VE-PTP antibody-labeled VE-PTP in cells, acquired images of cells were analyzed using ImageJ software (National Institutes of Health).

**Immunoblot Analysis**—Cells were washed with ice-cold PBS and then lysed in SDS sample buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% SDS). The lysates were centrifuged at 17,500 × g for 20 min at room temperature, and the resulting supernatants were subjected to immunoblot analysis as previously described (24).

**Assay of Cell Elongation**—Cells were plated on glass coverslips coated with fibronectin (20 μg/ml) and cultured in DMEM for 1 h. They were then either maintained under the static condition or exposed to SS at 9 dyne/cm² for 30 min before fixation and staining with rhodamine-conjugated phalloidin. The cells were observed with a fluorescence microscope (Olympus BX51), and the captured images were analyzed with the use of ImageJ software (National Institutes of Health) for determination of the cell elongation index, defined as the ratio of the maximum length of a cell in the direction of the flow to that in the perpendicular direction. An index greater or less than 1 indicates elongation of a cell in the direction of the flow or in the perpendicular direction, respectively.

**Statistical Analysis**—Data are presented as the means ± S.E. and were analyzed by Student’s t test, by analysis of variance (ANOVA) followed by Tukey’s post hoc test, or by the Kruskal-Wallis test and Dunn’s post hoc test. A p value of <0.05 was considered statistically significant.

**RESULTS**

**Shear Stress-induced Redistribution of VE-PTP**

**SS Induces the Rapid Accumulation of VE-PTP at the Downstream Edge of ECs**—To investigate the role of VE-PTP in the regulation of EC function by SS, we first examined whether the subcellular localization of this enzyme is affected by SS in the mouse EC line bEnd.3, which was previously shown to express the endogenous protein (12). The cells were cultured on fibronectin-covered glass slides and either maintained under the static condition or exposed to SS at 3 dyne/cm² for 15 min in a parallel plate flow chamber before immunostaining with a mAb that recognizes the extracellular domain of VE-PTP. Under the static condition, bEnd.3 cells at either low or high cell density manifested a diffuse, dot-like staining pattern for VE-PTP (Fig. 1A). By contrast, in cells at low or high density exposed to SS, VE-PTP immunoreactivity showed marked accumulation at the region of the cells farthest from the source of flow (defined as “the downstream edge” of the cells) and a concomitant decrease in abundance at the region of the cells nearest to the source of flow (defined as “the upstream edge” of the cells) (Fig. 1A). The percentage of cells with such a polarized distribution of VE-PTP increased in a time-dependent manner after the onset of SS, achieving a plateau at 15 min (Fig. 1C). Furthermore, the SS-induced redistribution of VE-PTP was dependent on the level of SS, with ~40% of cells showing such a redistribution at 1 dyne/cm² and this value increasing to ~90% at 3 or 9 dyne/cm² (Fig. 1D). Together, these results suggested that SS induces a polarized redistribution of VE-PTP to the downstream edge of ECs relative to the direction of flow in a time- and stress level-dependent manner.

To determine whether VE-PTP that accumulates at the downstream edge of ECs in response to SS is associated with the cell surface, we subjected bEnd.3 cells to immunostaining without permeabilization. Under the static condition, VE-PTP immunoreactivity was distributed over the entire cell surface (Fig. 1E). After exposure of cells to SS, a marked increase in the level of VE-PTP immunoreactivity was still observed at the downstream edge of the nonpermeabilized cells (Fig. 1E), suggesting that the VE-PTP that accumulates in this region in response to SS is present at the cell surface. Moreover, VE-PTP that was exogenously expressed in either HUVECs or HEK293A cells also manifested accumulation at the downstream edge of the cells, with a concomitant decrease in its abundance at the upstream edge, in response to SS (Fig. 1F). The SS-induced redistribution of VE-PTP thus occurs not only in bEnd.3 ECs but also in other types of cells that overexpress VE-PTP.

**Effect of SS on the Subcellular Localization of Other R3-subtype PTPs**—VE-PTP, SAP-1, PTPRO, and DEP-1 are all R3-subtype PTPs that share similar structures characterized by the presence of FNIII-like domains in the extracellular region (10). We, therefore, examined whether SS regulates the subcellular localization of SAP-1, PTPRO, and DEP-1 as well as that of VE-PTP in HEK293A cells. The cells were transfected with expression vectors for either SAP-1, PTPRO, HA-tagged DEP-1, or VE-PTP, repleted on fibronectin-covered glass coverslips, cultured for 12 h, and then either maintained under the static condition or exposed to SS at 3 dyne/cm² for 15 min. Immunoreactivity for each R3-subtype PTP was diffusely local-
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On exposure to SS, neither SAP-1, PTPRO, nor DEP-1 accumulated at the downstream edge of HEK293A cells (Fig. 2). Consistent with these results, SS failed to induce a polarized redistribution of either SAP-1, PTPRO, or HA-tagged DEP-1 in transfected bEnd.3 cells (data not shown).

Importance of the Extracellular and Transmembrane Regions of VE-PTP for Its SS-induced Redistribution—To determine which regions of VE-PTP are responsible for its SS-induced redistribution, we generated expression vectors encoding two mutant proteins: VE-PTP(ΔCyto), a mutant of VE-PTP that lacks almost the entire cytoplasmic region of the protein, and

FIGURE 1. SS induces the rapid accumulation of VE-PTP at the downstream edge of ECs. A, bEnd.3 cells were cultured on fibronectin-coated glass coverslips at low (panels a–c) or high (panels d–f) cell density and were either maintained under the static condition (panels a and d) or exposed to SS at 3 dyne/cm² for 15 min (panels b, c, e, and f). The cells were then subjected to immunofluorescence staining with a mAb to VE-PTP (green). The boxed regions in panels b and e are shown at higher magnification in panels c and f, respectively. Arrowheads indicate accumulation of VE-PTP at the downstream edge of the cells relative to the direction of flow indicated by the arrow. Scale bars, 50 μm in panel e and 20 μm in panel f. B, for quantification of VE-PTP distribution, images of sheared or static cells were divided into quadrants (Q1, Q2, Q3, and Q4). C, bEnd.3 cells were exposed to SS at 3 dyne/cm² for the indicated times and then subjected to immunostaining as in A. The percentage of cells showing a polarized distribution of VE-PTP was determined. Data are the means ± S.E. from three separate experiments, with 20 cells being examined for each condition in each experiment. **, p < 0.01 (one-way ANOVA and Tukey’s test) versus the static condition (0 min). N.S., not significant. D, bEnd.3 cells were exposed to SS at the indicated levels for 15 min and then examined for the percentage of cells showing a polarized distribution of VE-PTP as in C. **, p < 0.01 (one-way ANOVA and Tukey’s test) versus the static condition (0 dyne/cm²). E, bEnd.3 cells were cultured at low density and either maintained under the static condition or exposed to SS as in A. The cells were then subjected to immunostaining without cell permeabilization with the mAb to VE-PTP (green), which recognizes the extracellular domain of the protein, after which they were permeabilized and stained with rhodamine-phalloidin to visualize F-actin (red). Arrowheads indicate accumulation of VE-PTP. Scale bar, 50 μm. F, HUVECs or HEK293A cells were transfected with an expression vector for VE-PTP, plated on fibronectin-coated glass coverslips, and cultured for 12 h, after which they were either maintained under the static condition or exposed to SS as in A. They were then subjected to immunostaining for VE-PTP (green) and to staining of nuclei with DAPI (blue). Arrowheads indicate accumulation of VE-PTP in response to SS. Scale bars, 20 μm. Results in A, E, and F are representative of at least three separate experiments.
VE-PTP-ex-SAP-1-cyto, a chimera that consists of the extracellular and transmembrane regions of VE-PTP and the cytoplasmic tail of SAP-1 (Fig. 3A). The expression of WT VE-PTP, VE-PTP(ΔCyto), and VE-PTP-ex-SAP-1-cyto in HEK293A cells transfected with the corresponding expression vectors was confirmed by immunoblot analysis (Fig. 3B). The transfected cells were plated on fibronectin-coated glass coverslips and then either maintained under the static condition (panels a–e) or exposed to SS at 3 dyne/cm² for 15 min (panels f–j). The cells were then subjected to immunostaining (green) with mAbs to VE-PTP (panels a, d, f, and i), to SAP-1 (panels b and g), to PTPRO (panels c and h), or to HA (panels e and j) under nonpermeabilized (panels a, c, e, i, and j) or permeabilized (panels d, f, and h) conditions (the mAbs to VE-PTP, to SAP-1, and to PTPRO each recognize the extracellular domain of the corresponding PTP). Nuclei were also stained with DAPI (blue). Arrowheads indicate accumulation of VE-PTP at the downstream edge of cells in response to SS. Scale bar, 20 μm. B, the percentage of cells with a polarized distribution of exogenously expressed RPTP after exposure to SS was determined for HEK293A cells transfected and treated as in A. Data are the means ± S.E. from three separate experiments, with 20 cells being examined for each condition in each experiment.

Role of Integrins in the SS-induced Redistribution of VE-PTP—Integrins are key mediators of EC responses to SS (3, 4). SS is thought to activate integrins by promoting their interaction with extracellular matrix (ECM) proteins including fibronectin (3, 4). We, therefore, examined whether integrins contribute to the SS-induced redistribution of VE-PTP in ECs. bEnd.3 cells were plated on glass coverslips coated with either fibronectin, vitronectin, laminin, collagen-I, or poly-L-lysine, cultured in serum-free medium for 2 h, and then exposed to SS at 3 dyne/cm² for 15 min. The cells were found to be well spread on fibronectin, vitronectin, laminin, or collagen-I compared with those on poly-L-lysine (Fig. 4A). After exposure to SS, immunoreactivity for VE-PTP accumulated markedly at the downstream edge of cells plated on laminin or vitronectin as well as of those plated on fibronectin, whereas this effect was much less pronounced for the cells plated on poly-L-lysine (Fig. 4A). Indeed, the percentage of cells that exhibited a polarized distribution of VE-PTP after exposure to SS was significantly higher for the cells plated on each of these three ECM ligands than for those plated on poly-L-lysine (Fig. 4B). The percentage of cells with a polarized distribution of VE-PTP after exposure to SS on

FIGURE 3. Importance of the extracellular and transmembrane regions of VE-PTP for its SS-induced redistribution. A, a schematic representation of WT and mutant forms of mouse VE-PTP. Ex, extracellular domain; TM, transmembrane domain; Cyto, cytoplasmic domain; PTP, protein-tyrosine phosphatase domain. B, HEK293A cells transfected with expression vectors for the indicated VE-PTP proteins were plated on fibronectin-coated glass coverslips, cultured for 12 h, and then either maintained under the static condition (panels a–c) or exposed to SS at 3 dyne/cm² for 15 min (panels d–f). The cells were then subjected to immunostaining with the mAb to VE-PTP (green) and to staining of nuclei with DAPI (blue). Arrowheads indicate accumulation of the WT or mutant forms of VE-PTP at the downstream edge of cells relative to the direction of flow. Scale bar, 20 μm. C, HEK293A cells were transfected and treated as in C, after which the percentage of transfected cells showing a polarized distribution of either WT or mutant VE-PTP in response to SS was determined. Data are the means ± S.E. from three separate experiments, with 20 cells being examined for each condition in each experiment.

FIGURE 2. Effect of SS on the subcellular localization of other R3-subtype PTPs. A, HEK293A cells transfected with expression vectors for either VE-PTP (panels a, d, f, and i), SAP-1 (panels b and g), PTPRO (panels c and h), or HA-tagged DEP-1 (panels e and j) were plated on fibronectin-coated glass coverslips, cultured for 12 h, and then either maintained under the static condition (panels a–e) or exposed to SS at 3 dyne/cm² for 15 min (panels f–j). The cells were then subjected to immunostaining (green) with mAbs to VE-PTP (panels a, d, f, and i), to SAP-1 (panels b and g), to PTPRO (panels c and h), or to HA (panels e and j) under nonpermeabilized (panels a, c, e, i, and j) or permeabilized (panels d, f, and h) conditions (the mAbs to VE-PTP, to SAP-1, and to PTPRO each recognize the extracellular domain of the corresponding PTP). Nuclei were also stained with DAPI (blue). Arrowheads indicate accumulation of VE-PTP at the downstream edge of cells in response to SS. Scale bar, 20 μm. B, the percentage of cells with a polarized distribution of exogenously expressed RPTP after exposure to SS was determined for HEK293A cells transfected and treated as in A. Data are the means ± S.E. from three separate experiments, with 20 cells being examined for each condition in each experiment.
Vlam, vitronectin, laminin, collagen-I, or poly-L-lysine, cultured for 2 h in serum-free medium, and then exposed to SS at 3 dyne/cm² for 15 min. The cells were then subjected to immunostaining with a mAb to VE-PTP (green) as well as to staining with rhodamine-phalloidin to visualize F-actin (red). Arrowheads indicate accumulation of VE-PTP at the downstream edge of cells relative to the direction of flow. Scale bar, 20 μm. B, the percentage of cells with a polarized distribution of VE-PTP was determined for cells treated as in A. Data are the means ± S.E. from three separate experiments, with 20 cells being examined for each condition in each experiment. *, p < 0.05; **, p < 0.01 (one-way ANOVA and Tukey’s test) versus cells plated on poly-L-lysine.

collagen-I was also higher than that for those on poly-L-lysine, but it was lower than that for those on the other ECM proteins (Fig. 4B). These results suggested that the engagement of integrins by ECM proteins is required for the efficient redistribution of VE-PTP in ECs exposed to SS.

**Importance of Cdc42 and the Actin Cytoskeleton in the SS-induced Redistribution of VE-PTP**—Integrins are thought to mediate the activation of small GTPases of the Rho family, including Rho, Rac, and Cdc42, in response to SS (25–27). Rho family GTPases regulate reorganization of the actin cytoskeleton and the establishment of cell polarity, both of which play important roles in the polarized trafficking and distribution of membrane proteins (28–30). To investigate whether the SS-induced redistribution of VE-PTP requires the activation of Rac or Cdc42, we transfected bEnd.3 cells with expression vectors for either EGFP or EGFP-tagged forms of a dominant negative mutant of Rac(T17N) or the CRIB domain of NWASP (NWASP-CRIB), the latter of which binds specifically to the GTP-bound (active) form of Cdc42 and thereby inhibits its activity (31). The transfected cells were then replated on fibronectin-coated glass coverslips and cultured for 12 h before exposure to SS at 3 dyne/cm² for 15 min. They were then subjected to immunostaining with a mAb to VE-PTP (green). EGFP fluorescence was monitored to identify transfected cells (green). Arrowheads or arrows indicate cells with or without accumulation of VE-PTP at their downstream edge relative to the direction of flow, respectively. Scale bar, 20 μm. B, the percentage of EGFP-positive cells with a polarized distribution of VE-PTP was determined for cells treated as in A. Data are the means ± S.E. from three separate experiments, with 20 cells being examined for each condition in each experiment. **, p < 0.01 (one-way ANOVA and Tukey’s test). N.S., not significant. C, bEnd.3 cells plated on fibronectin-coated glass coverslips were incubated with either DMSO (0.2%, vehicle) or Y-27632 (5 μM) for 30 min and then exposed to SS at 3 dyne/cm² for 15 min. They were then subjected to immunostaining with a mAb to VE-PTP (green). Arrowheads indicate accumulation of VE-PTP at the downstream edge of cells relative to the direction of flow. Scale bar, 50 μm. Results in A and C are representative of at least three separate experiments.

Rac, is important for the SS-induced redistribution of VE-PTP. In addition, treatment of bEnd.3 cells with Y-27632, an inhibitor of Rho kinases (also known as ROCKs, a key downstream effector of Rho), failed to prevent the accumulation of VE-PTP at the downstream edge of cells exposed to SS (Fig. 5C), indicating that Rho kinases are not important for this process. Given that Rho regulates a variety of cell functions through Rho kinases, Rho is unlikely involved in the regulation of VE-PTP localization by SS. We further examined whether either the actin or microtubule cytoskeleton is implicated in a polarized redistribution of VE-PTP in response to SS. Cytochalasin D, an inhibitor of actin polymerization, induced the disruption of actin stress fibers and inhibited the SS-induced redistribution of VE-PTP (Fig. 6, A and B), whereas the disruption of microtubule cytoskeleton by the treatment with nocodazole, an inhibitor of microtubule polymerization, had no effect on SS-induced changes in VE-PTP subcellular localization (Fig. 6C). Reorganization of the actin cytoskeleton appears to be required for the accumulation of VE-PTP at the downstream edge of ECs in response to SS.
We also examined the effect of the inhibition of PI3K that is shown to be activated by SS (1, 3, 4) and a regulator for actin cytoskeleton reorganization and cell polarity (32). However, treatment of bEnd.3 cells with LY294002, a PI3K inhibitor, failed to prevent the SS-induced redistribution of VE-PTP. It is thus unlikely that PI3K contributes to the regulation of VE-PTP subcellular localization by SS (Fig. 6D).

Role of Rab5 and Arf6 in the SS-induced Redistribution of VE-PTP—Endocytosis and endocytic recycling processes play an important role in the polarized localization of transmembrane proteins such as integrins (33). To investigate whether VE-PTP undergoes endocytic processes, VE-PTP on cell surface of bEnd.3 cells was labeled with a mAb to VE-PTP. The cells were then either maintained under static condition or exposed to SS at 3 dyne/cm² for 30 min, after which the antibodies bound to the cell surface were removed by acid treatment, and the cells were subjected to immunostaining to visualize antibody-labeled VE-PTP, which is thought to be internalized. The percentage of cells with a polarized distribution of VE-PTP was determined for cells treated as in panel A, **p < 0.01 (Student’s t test). C, the fluorescence intensity of internalized VE-PTP per cell was measured for cells transfected and treated as in panel A, ***, p < 0.001 (Student’s t test). Scale bar, 50 μm. Results in all panels are the means ± S.E. from at least three separate experiments.
internalized into the cells. When the cells were treated with acid solution, antibody-labeled VE-PTP on the cell surface was barely observed (Fig. 7A). Under the static condition, internalized VE-PTP were diffusely distributed in bEnd.3 cells, whereas after SS exposure, the internalized VE-PTP was localized near the downstream edge of the cells relative to the direction of flow (Fig. 7, A and B). Moreover, the fluorescence intensity of internalized VE-PTP tended to increase in bEnd.3 cells exposed to SS compared with that apparent in the static condition; such an increase was not statistically significant, however (Fig. 7, A and C). These results thus suggested that endocytosis of VE-PTP from the cell surface occurs in the static and SS conditions and that SS induces polarized distribution of internalized VE-PTP in ECs.

Rab and Arf family GTPases, including Rab4, Rab5, Rab11, and Arf6, are key regulators of endocytic and recycling processes (34, 35). To examine the possible role of these small GTPases in the SS-induced redistribution of VE-PTP, we transfected bEnd.3 cells with expression vectors for EGFP or for dominant negative mutants of Rab5(S34N), Rab4(N121I), Rab11(S25N), or Arf6(T27N). Expression of either HA-tagged Rab5(S34N) or EGFP-tagged Arf6(T27N) markedly inhibited the SS-induced accumulation of VE-PTP at the downstream edge of cells compared with that apparent in cells expressing EGFP alone, whereas expression of HA-tagged Rab4(N121I) or Rab11(S25N) had no such effect (Fig. 7, D and E). These results thus suggested that Rab5 and Arf6 are important for the SS-induced redistribution of VE-PTP in ECs.

Role of Integrins, Cdc42, and the Actin Cytoskeleton in the SS-induced Redistribution of Internalized VE-PTP—We further tried to examine whether integrins are also important for the redistribution of internalized VE-PTP in ECs exposed to SS. bEnd.3 cells were cultured on fibronectin- or poly-L-lysine-coated glass coverslips and incubated with a mAb to VE-PTP, after which the cells were exposed to SS at 3 dyn/cm² for 30 min. Immunoreactivity for internalized VE-PTP was observed in the cells plated on either poly-L-lysine or fibronectin. Such immunoreactivity markedly accumulated near the downstream edge of cells plated on fibronectin, whereas this effect was much less pronounced for the cells plated on poly-L-lysine (Fig. 8, A and B). In addition, we examined the effect of inhibition of either Cdc42 activity or actin polymerization. Forced expression of EGFP-tagged NWASP-CRIB as well as treatment with cytochalasin D markedly attenuated polarized distribution of internalized VE-PTP in bEnd.3 cells plated on fibronectin in response to SS (Fig. 8, C–F). Taken together, these results suggested that integrins, Cdc42, and the actin cytoskeleton contribute to the redistribution of internalized VE-PTP in ECs exposed to SS.

Participation of VE-PTP in SS-induced Cell Spreading and Elongation—Exposure of ECs to SS induces cell spreading and elongation along the direction of flow (36), and we have previously reported that SS-induced Cell Spreading and Elongation...
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siRNA was not (Fig. 9C). There was no significant difference in the cell elongation index between control siRNA- and VE-PTP siRNA-transfected HUVECs under the static condition. These results thus suggested that VE-PTP plays an important role in the regulation of EC morphology by SS.

DISCUSSION

We have here shown that VE-PTP is diffusely localized in ECs under static culture conditions, whereas SS induces the accumulation of VE-PTP at the downstream edge of the cells relative to the direction of flow. This polarized redistribution of VE-PTP occurs rapidly and depends on the level of SS. We also found that this change in VE-PTP localization was enhanced by plating of ECs on ECM proteins such as fibronectin, vitronectin, laminin, or collagen-I compared with that apparent for cells plated on poly-L-lysine, suggesting that integrin activation is indispensable for this process. Given that SS promotes the engagement of integrins with ECM proteins and thereby induces integrin activation (3, 4), our results are consistent with the notion that integrins act as mechanotransducers to promote the subcellular redistribution of VE-PTP in response to SS.

Our results also indicate that Cdc42 is important for the SS-induced redistribution of VE-PTP in ECs. Rho family GTPases such as Cdc42 and Rac are thought to be activated by SS as a result of integrin activation (37). In addition, activation of Cdc42 occurs at the downstream edge of cells exposed to SS (27). Given that activation of Cdc42 is thought to play an important role in the polarized localization of various transmembrane proteins (38), the polarized activation of Cdc42 by SS likely mediates the accumulation of VE-PTP at the downstream edge of cells exposed to SS. We also found that treatment of cells with cytochalasin D greatly inhibited the SS-induced redistribution of VE-PTP, suggesting that reorganization of the actin cytoskeleton is required for this effect of SS. Indeed, Cdc42 or Rac is thought to regulate reorganization of the actin cytoskeleton triggered by integrin activation (39). The reorganization of the actin cytoskeleton in the SS-induced redistribution of VE-PTP is likely to contribute to the regulation of endocytic processes of VE-PTP, as described below.

We found that VE-PTP on the cell surface undergoes endocytosis in the static and SS conditions. We also found that Rab5 and Arf6 likely participate in the polarized redistribution of VE-PTP in ECs exposed to SS. Rab and Arf family GTPases are thought to be key regulators of endocytosis and endocytic recycling pathways, both of which play an important role in the internalization of plasma membrane proteins and in their return to the cell surface (34, 35, 40). Rab5 orchestrates the initial trafficking steps of the endocytic pathway (35), whereas Arf6 is implicated together with various effector molecules and other small GTPases in endocytosis and endocytic recycling (34). Moreover, VE-PTP was shown to be localized to the recycling endosome compartment in bEnd.3 cells cultured at low cell density (16). It is, therefore, possible that Rab5 and Arf6 mediate endocytosis and endocytic recycling of VE-PTP, resulting in the polarized distribution of VE-PTP in ECs subjected to SS. Of note, integrins show a polarized distribution at the leading edge of migrating cells as a result of their endocyto-

ous shown that VE-PTP regulates EC spreading and migration (12). We, therefore, next tested whether VE-PTP participates in the SS-induced spreading and elongation of HUVECs, which express endogenous VE-PTP (12), with the use of RNAi. HUVECs were transfected with either a control siRNA or siRNAs specific for human VE-PTP. Immunoblot analysis showed that the abundance of endogenous VE-PTP was markedly reduced in cells transfected with either of two different VE-PTP siRNAs compared with that apparent for cells transfected with the control siRNA (Fig. 9A). The siRNA-transfected cells were plated on fibronectin-coated glass coverslips at low density, cultured in serum-free medium for 1 h, and then either maintained under the static condition or exposed to SS at 9 dyne/cm² for 30 min. Staining of the cells with rhodamine-phalloidin to visualize cell shape. Scale bar, 50 μm. C, cell elongation index (ratio of the maximum length of a cell in the direction of flow to that in the perpendicular direction) for HUVECs transfected and treated as in B. Data are the means ± S.E. for a total of 150 cells for each condition in three separate experiments. ***p < 0.001 (Kruskal-Wallis test and Dunn’s post hoc test). N.S., not significant.

FIGURE 9. Participation of VE-PTP in SS-induced cell spreading and elongation. A, HUVECs transfected with control or VE-PTP (#1 or #2) siRNAs were subjected to immunoblot analysis with pAbs to VE-PTP and a mAb to β-tubulin (loading control). B, HUVECs transfected with the indicated siRNAs were plated on fibronectin-coated glass coverslips, cultured in serum-free medium for 1 h, and then either maintained under the static conditions or exposed to SS at 9 dyne/cm² for 30 min. The cells were then subjected to staining with rhodamine-phalloidin to visualize cell shape. Scale bar, 50 μm. C, cell elongation index (ratio of the maximum length of a cell in the direction of flow to that in the perpendicular direction) for HUVECs transfected and treated as in B. Data are the means ± S.E. for a total of 150 cells for each condition in three separate experiments. ***p < 0.001 (Kruskal-Wallis test and Dunn’s post hoc test). N.S., not significant.
sis and endocytic recycling, processes that are regulated by Rab21 and Arf6 (33, 41). These findings also support the notion that the polarized localization of VE-PTP in cells exposed to SS is dependent on internalization of the protein and its recycling back to the cell surface. In addition, we show that activation by ECM proteins of integrins enhances the polarized distribution of internalized VE-PTP in response to SS. Such polarized distribution of internalized VE-PTP are also markedly prevented by the inhibition of either Cdc42 activity or actin polymerization in ECs exposed to SS. Given that activation of Cdc42 as well as reorganization of the actin cytoskeleton are thought to participate in endocytosis and endocytic recycling of membrane-associated proteins (38, 42, 43), integrin activation in response to SS is likely to promote activation of Cdc42 and reorganization of actin cytoskeleton, thereby promoting the endocytic trafficking of internalized VE-PTP.

Analysis of the subcellular localization of VE-PTP mutants revealed that the extracellular and transmembrane regions of VE-PTP are sufficient for its accumulation at the downstream edge of cells in response to SS. Although other R3-subtype RPTPs, including SAP-1, PTPRO, and DEP-1, also contain FNIII-like domains in the extracellular region, SS did not induce a redistribution of these proteins similar to that observed with VE-PTP. VE-PTP possesses 16 or 17 FNIII-like domains in its extracellular region, whereas other R3-subtype RPTPs contain only 6–8 such domains (10). The increased number of FNIII-like domains in the extracellular region of VE-PTP might thus be an important determinant of its polarized redistribution in response to SS. In addition, the efficient recycling of the internalized transmembrane protein endolyn back to the apical cell surface was found to require N-glycosylation of its extracellular domain in polarized Madin-Darby canine kidney cells (44). Given that VE-PTP is also a highly N-glycosylated transmembrane protein (15, 45), such glycosylation of VE-PTP might contribute to the regulation of its subcellular localization by SS. Further studies will be necessary, however, to understand the detailed molecular mechanism underlying the SS-induced accumulation of VE-PTP at the downstream edge of ECs.

Although VE-PTP has been implicated in angiogenesis, the physiological role of this protein in ECs exposed to SS has remained unclear. SS induces the alignment and elongation of ECs in the direction of flow (36, 37), with these responses thought to be adaptive in that they reduce the mechanical stress produced by flow. It is also possible that VE-PTP acts as a mechanotransducer and thereby contributes to other responses of ECs to SS.

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