The protein tyrosine phosphatase TCPTP controls VEGFR2 signalling

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Summary
Vascular endothelial growth factor (VEGF) is a major angiogenic factor that triggers formation of new vessels under physiological and pathological conditions. However, the mechanisms that limit the VEGF responses in target cells and hence prevent excessive and harmful angiogenesis are not well understood. Here, our objective was to study whether T-cell protein tyrosine phosphatase (TCPTP, also known as PTN2), which we found to be expressed in human endothelial cells, could alter VEGF signalling by controlling phosphorylation of VEGFR2. We show that a TCPTP substrate-trapping mutant alters VEGF signalling by controlling phosphorylation of VEGFR2. Moreover, TCPTP dephosphorylates VEGFR2. We conclude that matrix-controlled TCPTP phosphatase activity can inhibit VEGFR2 signalling, and the growth, migration and differentiation of human endothelial cells.

Key words: T-cell protein tyrosine phosphatase (TCPTP), VEGF, VEGFR2, Endothelium, Integrin

Introduction
VEGF is a major growth factor for endothelial cells. It regulates endothelial cell function in the formation of new blood vessels both during development and in adults (e.g. during wound healing and in proliferative diseases such as cancer) (Coultas et al., 2005). In the vascular endothelium, VEGF binds to two high-affinity tyrosine-kinase receptors, VEGFR1 and VEGFR2. VEGFR2 appears to be the main mediator of VEGF-induced endothelial proliferation, survival, migration, tubular morphogenesis and sprouting in adults (Ferrara et al., 2003). The effects of VEGF are mainly controlled by its availability and by the activity of VEGFR signalling. Upon binding of VEGF, the receptors dimerize, become autophosphorylated, and recruit signal-transduction molecules such as PLC, PI3K and Ras (Olsson et al., 2006; Kerbel, 2008; Siekmann et al., 2008). By contrast, negative regulation of VEGFR2 signalling is equally important in limiting the response of VEGF in target cells. In fact, inhibition of neovascularization is a promising new way of treating diseases such as cancer and certain retinopathies (Carmeliet, 2005).

Angiogenesis is a multistep process that involves basement-membrane degradation by the endothelial cells, sprouting and migration, proliferation, structural organization, synthesis of new basement membrane, and returning to quiescence (Carmeliet, 2000; Yancopoulos et al., 2000; Avraamides et al., 2008). During the different steps of angiogenesis, the endothelial cell encounters dramatically different extracellular matrices, ranging from the mature basement membrane below the quiescent endothelial cells to the type-I-collagen-rich stroma around the sprouting cells. Integrin-mediated adhesion to the extracellular matrix (ECM) provides permissive signals to cells by supporting migration, cell survival, and signalling by receptor tyrosine kinases (RTKs) (Giancotti, 1997; Larsen et al., 2006). Different integrins have the capacity to form complexes with growth-factor receptors on the cell membrane, and regulate their activation and internalization. For example, integrin-mediated adhesion alone can activate growth-factor-receptor phosphorylation in the absence of ligands (Moro et al., 1998) and integrins bind directly to growth-factor-receptors such as EGFR and MET (Moro et al., 1998; Trusolino et al., 2001). Adhesion of the endothelial cells to ECM molecules also profoundly affects their angiogenic properties (Avraamides et al., 2008). For instance, binding of endothelial cells to vitronectin by α1β3 integrin positively regulates VEGFR2 activation via direct interaction between the two molecules (Borges et al., 2000), and expression of active integrin α9β1 induces secretion of VEGF (De et al., 2005). More recently, integrin α9β1 expressed in endothelial cells was shown to cooperate with VEGFR2 in angiogenesis (Vlahakis et al., 2007). It has also been known for a long time that collagenous matrix can inhibit endothelial growth (Form et al., 1986). However, the molecular nature of the matrix-dependent integrin-mediated signals that inhibit angiogenesis is not fully understood.

We recently reported that a collagen-binding integrin, α1β1, negatively regulates EGFR phosphorylation in malignant epithelial cells. This was the first demonstration of an inhibitory role for the ECM in signalling by RTKs. The effect of α1 integrin was mediated via coupling to the phosphatase TCPTP (Mattila et al., 2005). TCPTP is a 45-kDa protein that, despite its name, is expressed in...
several cell types (Alonso et al., 2004). TCPTP is prominently present in the nucleus, but it is capable of translocating to the cytoplasm in response to mitogenic stimuli or α1β1-integrin-mediated adhesion to collagen (Tiganis et al., 1998; Mattila et al., 2005). We studied here whether TCPTP would be present in endothelial cells and whether ligation of collagen-binding integrins or TCPTP activation could have biological effects in endothelial cells. We found that TCPTP dephosphorylates and silences VEGFR2, and inhibits biological responses to VEGF. We also devised a novel peptide from the cytoplasmic domain of integrin α1 that can be used as a tool to restrain VEGFR2 signalling via the activation of TCPTP. These data suggest that endothelial cell growth during different steps of angiogenesis can be regulated by microenvironmentally controlled phosphatase activities.

Results

TCPTP is present in human endothelial cells

We hypothesized that human endothelial cells might express TCPTP and that the collagen-binding integrin α1 could regulate the activity of TCPTP in these cells. We found α1β1 integrin in early-passage human umbilical vein endothelial cells (HUVECs) by immunoblotting (Fig. 1A). FACS analyses further showed that α1 integrin is expressed on the surface of HUVECs (supplementary material Fig. S1A). Immunoblotting experiments clearly revealed that TCPTP protein is present in HUVECs (Fig. 1A). In fact, the amount of TCPTP protein in these endothelial cells was almost comparable to that seen in T lymphocytes in peripheral blood (Fig. 1B). Immunocytochemistry further showed that TCPTP is present both at the membrane and in the cytoplasm in HUVECs. TCPTP staining was specific, because it could be competed out with excess of recombinant soluble TCPTP (Fig. 1C).

TCPTP was also found in intact human endothelium in vivo. In frozen sections of human umbilical cords, TCPTP, α1 integrin and VEGFR2 were all found to be expressed in the endothelial layer of the veins (Fig. 1D). Also, in inflamed tonsils, specific staining with anti-TCPTP antibody was evident in endothelial cells (supplementary material Fig. S1B). In tonsils, the intensity of TCPTP staining in endothelial cells was lower than in many resident leukocytes, but it clearly exceeded that of some leukocytes and many other cell types. To our knowledge, this is the first indication of TCPTP expression in the vascular endothelium and suggests that TCPTP plays a role in vascular biology.

TCPTP activity regulates responsiveness of endothelial cells to VEGF

To study the possible biological effects of TCPTP activity in endothelial cells, we silenced or induced TCPTP activity and studied the behaviour of HUVECs in two different models. For cell-proliferation experiments, we silenced endogenous TCPTP in HUVECs using siRNA. Silencing of TCPTP was efficient and it led to a modest, but statistically significant, increase (28±6%, P<0.05) in VEGF-induced proliferation when compared with control cells (Fig. 2A). No difference in proliferation was seen between TCPTP-silenced and control cells in the absence of exogenous VEGF (not shown).

Wild-type 45-kDa TCPTP protein (TC45) exists in a partially auto-inhibited state involving the C terminus of the protein, and mitogenic stimulus or interaction with α1β1 integrin is needed for full activity (Hao et al., 1997; Mattila et al., 2005). To circumvent the need for TCPTP activation, we used constitutively active 37-kDa TCPTP (TC37), which is a truncation mutant lacking the C-terminal part of the 45-kDa molecule (Hao et al., 1997). The effect of enhanced TCPTP activity on motility of HUVECs was then analyzed in cell-migration assays. We found that chemotaxis of vector-transfected HUVECs was stimulated by VEGF by more than twofold. Strikingly, expression of constitutively active TCPTP rendered the cells completely unable to migrate towards VEGF (Fig. 2B). Together, these functional studies showed that decreased TCPTP activity enhances and increased TCPTP activity inhibits...
VEGFR2, dephosphorylates it in a phosphosite-specific manner.

Phosphorylation of VEGFR2 controls the internalization of VEGFR2 or that TCPTP targets VEGFR2 in a phosphorylation-site-specific manner.

VEGFR2 contains several critical tyrosine residues that are autophosphorylated or that bind distinct effector molecules. Some of the most important ones are: tyrosines 1054 and 1059 (Tyr1054/1059), which are located in the activation loop of the tyrosine-kinase domain and are required for maximal activation of the kinase activity of the receptor (Claesson-Welsh, 2003) and for VEGF-A-induced sprouting angiogenesis in vitro (Kawamura et al., 2008); tyrosine residue 1175 (Tyr1175), which binds to PLCγ and Shb; and Tyr1214, which triggers the p38 cascade via unknown intermediates (Olsson et al., 2006). Our phosphorylation assays showed that TCPTP was able to target specific tyrosines in VEGFR2. The autophosphorylation sites Tyr1054/1059 and Tyr1214 were dephosphorylated by TCPTP (Fig. 4B). Tyr996, the functional significance of which is currently uncertain (Olsson et al., 2006), was a TCPTP target as well. Tyr1175, by contrast, remained phosphorylated and was not a TCPTP target (Fig. 4B).

Phosphorylation of VEGFR2 controls the internalization of the receptor (Dougher and Terman, 1999; Lampugnani et al., 2006). This prompted us to use the internalization as a biological read-out for the activation status of VEGFR2. In microscopic assays, transfection of TC37 into subconfluent HUVECs strongly inhibited the internalization of VEGFR2 to endosome-resembling vesicles (Fig. 4C). In biochemical analyses, we used cell-surface labelling with cleavable biotin, which allows the quantification of the internalized, biotinylated VEGFR2 that is protected when any biotinylated receptors remaining on the cell surface are cleaved (Ivaska et al., 2002). We found that VEGF stimulation increased VEGFR2 internalization in sparse mock-transfected HUVECs. This was evident from the increased amount of biotinylated VEGFR2 protected from cleavage (Fig. 4D). In cells expressing TC37 or wild-type TCPTP (TC45), the internalization of VEGFR2 was clearly inhibited (Fig. 4D). Together, these experiments revealed that TCPTP binds to VEGFR2, dephosphorylates it in a phosphosite-specific manner and alters its biological activity.
The cytoplasmic domain of α1 integrin activates TCPTP and inhibits VEGFR2 activity

We have demonstrated that, in malignant epithelial cells, a cell-permeable TAT-fusion peptide from the cytoplasmic tail of integrin α1, but not from that of integrins α2, α5, α10 or α11, specifically activates TCPTP (Mattila et al., 2005). Therefore, we studied whether this peptide could be used as a tool to trigger TCPTP-dependent VEGFR2 dephosphorylation in normal endothelial cells.

We observed that the FITC-labelled α1-TAT fusion peptide and scrambled control peptide (ScrTAT) entered HUVECs with the same efficiency within 30 minutes, and, for both, the fluorescence signal remained high for over 16 hours (supplementary material Fig. S2C). The peptides had no obvious effects on cell morphology or survival (not shown). VEGF-induced phosphorylation on VEGFR2 Tyr1214 was markedly inhibited by the α1-TAT peptide (Fig. 5C). The inhibition was specific, because the peptide was not able to induce dephosphorylation of VEGFR2 at Tyr1175, which is in line with the in vitro phosphatase assay (Fig. 4B). Remarkably, α1-TAT peptide also abolished the kinase activity of VEGFR2 in VEGF-treated HUVECs (Fig. 5D). Thus, the α1-TAT peptide can be used to dephosphorylate and inactivate VEGFR2 in HUVECs.

To further increase the stability of TAT-fusion peptides in cells, we designed novel TAT peptides with D amino acids instead of normal L isomers. In the in vitro phosphatase assay, α1-D-TAT highly significantly activated TCPTP (P<0.01) (Fig. 5E). Again, the specificity of the TCPTP activation was evident, inasmuch as the ScrTAT fusion made of D isomers had no effect.

TCPTP activation by the cytoplasmic tail of integrin α1 inhibits endothelial proliferation, migration and morphological changes

Capillary morphogenesis requires the adhesion, migration, proliferation and differentiation of endothelial cells, and VEGFR2 plays a major role in all of these steps (Ferrara et al., 2003; Avraamides et al., 2008; Kerbel, 2008). The α1-TAT peptide inhibited VEGF-induced morphological changes by 60% compared with non-treated cells and by 40% compared with control peptide-treated cells in a fibrin-gel assay (Fig. 6A). These results show that activation of TCPTP leads to inhibition of VEGF-driven changes in endothelial morphology.

We next determined the chemokinesis and chemotaxis of HUVECs in response to the peptide treatment. When the motility of HUVECs on gelatin substrate was measured for 3 hours using time-lapse imaging, chemokinesis was induced by VEGF by 84±21% (Fig. 6B). In this assay, α1-TAT treatment inhibited VEGF-induced chemokinesis by 61±4%, whereas ScrTAT had no significant effect. It was also clear from the images that neither TAT peptide was harmful to the cells, because all cells were viable and mitosis was entered occasionally (not shown). VEGF-induced chemotaxis of HUVECs was also inhibited by treatment with α1-TAT peptide, but not with the ScrTAT peptide (Fig. 6C).

siRNA silencing was finally used to further confirm that the effect of α1-TAT on VEGF-induced HUVEC migration is mediated via TCPTP. As shown in Fig. 6D, α1-TAT had no inhibitory effects on the VEGF-induced chemotaxis of cells treated with TCTP siRNA, whereas α1-TAT effectively inhibited VEGF-induced migration of cells treated with scrambled siRNA. Together, these data indicate that TCPTP is the target of the cytoplasmic peptide of α1 integrin in HUVECs and that decreased TCPTP activity results in augmented VEGFR2-mediated proliferation and migration of endothelial cells.
Fig. 4. TCPTP dephosphorylates VEGFR2 in a phosphosite-specific manner and controls its activity. (A,B) HEK293 cells were transfected with VEGFR2, treated with VEGF and immunoprecipitated (IP) with anti-VEGFR2 antibody. Immunoprecipitates were incubated in the presence of recombinant TCPTP (TC45) or buffer control, and immunoblotted using total phosphotyrosine antibody (A) or phospho-specific VEGFR2 antibodies (B). Total VEGFR2 was blotted as a control. Representative blots out of three experiments with similar results are shown. The values under the blots represent VEGFR2 phosphorylation relative to the buffer controls (mean ± s.e.m.; n=3). (C,D) TCPTP activity controls the internalization of VEGFR2 in endothelial cells. (C) Subconfluent HUVECs were transfected with vector control (pCG) or constitutively active TCPTP (TC37), stimulated (+) or not (–) with VEGF, and stained for VEGFR2 (red) and nuclei (blue) after fixation and permeabilization. Arrows indicate representative VEGFR2 vesicles. (C, bottom) The number of VEGFR2-positive vesicles (mean ± s.e.m.; ***P<0.005) was quantitated using image analysis. The perinuclear bright Golgi-resembling staining was excluded in the analysis. Scale bar: 10 μm. (D) HUVECs were transfected with TC37, TC45 or vector (pCG), or left untransfected, and surface-labelled with cleavable biotin. VEGFR2 was allowed to internalize for 15 minutes in the presence or absence of VEGF (100 ng/ml). Biotin remaining on the cell surface was cleaved and VEGFR2 was immunoprecipitated using anti-VEGFR2 antibody. Internalized VEGFR2 was detected by blotting for biotin. Because biotin is cleavable by reducing reagents, non-reducing gel was used, and this alters the mobility of the proteins compared to a reducing gel. The numbers below the blot show levels of internalized VEGFR2 (mean ± s.d., two individual experiments).
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**Discussion**

The signalling pathways that downregulate and terminate VEGF action are incompletely understood, although they are of paramount importance in controlling physiological and pathological angiogenesis. In this study we describe a novel phosphatase-mediated inhibition of endothelial cell proliferation, migration and sprouting. We show that TCPTP binds to VEGFR2 and dephosphorylates its tyrosines in a phosphosite-specific manner. TCPTP activation diminishes the kinase activity of VEGFR2 and blocks its internalization from the cell surface. We also demonstrate that, in endothelial cells, the cytoplasmic domain of integrin α1 integrin activates TCPTP and that a cell-membrane-permeable form of the peptide efficiently attenuates VEGFR2-driven functions such as proliferation, migration, sprouting angiogenesis and tube formation in actively growing cells. Together, these data suggest TCPTP as a crucial regulator of the growth and differentiation of endothelial cells.
We show here that TCPTP dephosphorylates specific phosphorylation sites of VEGFR2, such as Tyr1054/1059. Phosphorylation of these residues has been shown to be required for maximal kinase activity and for sprouting angiogenesis, and VEGF-induced internalization of VEGFR2 is delayed when these two sites have been mutated to phenylalanine (Dougher and Terman, 1999; Kawamura et al., 2008). This correlates well with our observation that TCPTP activation impairs sprouting angiogenesis and VEGFR2 internalization in HUVECs. Tyr1175 is an important autophosphorylation site, which is not influenced by TCPTP. This site mediates activation of the PI3K and PLCγ pathways, and plays a role in cell proliferation (Takahashi et al., 2001), although VEGF does not seem to be a powerful mitogen for endothelial cells (Olsson et al., 2006). The lack of TCPTP-mediated dephosphorylation of Tyr1175 is most probably the reason why activation of TCPTP has only modest effects on VEGF-induced proliferation. TCPTP also targeted Tyr1214 of VEGFR2. Phosphorylation of this site has been implicated in VEGF-induced actin remodelling and could thus play a role in endothelial cell migration (Lamalice et al., 2004). Our findings that TCPTP controls VEGF-driven chemotaxis and chemokinesis of HUVECs might thus be due to dephosphorylation of the Tyr1214 site [although Tyr1175 can also influence motility (Olsson et al., 2006)].

TCPTP-deficient mice show normal intrauterine development, but they die soon after birth because of an overwhelming inflammatory phenotype (You-Ten et al., 1997). This phenotype suggests that TCPTP is not absolutely necessary for vasculogenesis. However, the postnatal angiogenetic response of these mice has not been studied to our knowledge. There are many examples of molecules that are not needed for vasculogenesis but can still modulate angiogenesis. For instance, deficiency of placental growth factor or its inhibition with an antibody does not affect embryonic blood-vessel development, but reduces pathological angiogenesis during ischemia, wound healing and cancer (Carmeliet et al., 2001; Fischer et al., 2007). Unfortunately, the role of the TCPTP targets of VEGFR2, Tyr1054/1059, has not been studied in transgenic mice. Notably, there are major differences in TCPTP between mice and humans, which makes it relevant to study the role of TCPTP in...
primary endothelial cells of human origin. The TCPTP transcript is alternatively spliced resulting in the expression of different isoforms in human and mouse. In human cells, TCPTP is expressed in two isoforms (48 kDa and 45 kDa), each with a distinct subcellular localization, whereas in murine cells only mRNA coding for the 45-kDa isoform is detected (Ibarra-Sanchez et al., 2000). Thus, the fact that TCPTP dephosphorylates Tyr1054/1059, which control the kinase activity of VEGFR2, suggests that TCPTP could modulate angiogenesis also in vivo.

VEGF upregulates expression of the α1β1 and α2β1 integrins in microvascular endothelial cells. In addition, a combination of function-blocking anti-integrin-α1 and -α2 antibodies inhibit VEGF-driven angiogenesis in mouse skin (Senger et al., 1997) and in tumor xenografts (Senger et al., 2002). We have previously shown that cross-linking of α1 integrin with the exactly same function-blocking monoclonal antibody leads to activation of TCPTP in malignant epithelial cells (Mattila et al., 2005). Therefore, the inhibitory effects of anti-integrin-α1 antibodies on vascular growth in vivo might be due to antibody-triggered activation of TCPTP in addition to blocking the interaction of integrin α1β1 with collagen.

Anti-VEGF antibodies are in clinical use and VEGFR2 kinase inhibitors are being developed for blocking neovascularization in several diseases. Our data suggest that inhibition of VEGFR2 activity by inducing TCPTP could be a novel strategy for antagonizing VEGF activity. Because TCPTP-dependent VEGFR2 inactivation was only seen in subconfluent but not in confluent (supplementary material Fig. S2A,B) cells, its modulation might spare the normal vessels. Because TCPTP activation also directly inhibits other RTKs that control cell growth (Tiganis et al., 1999; Galic et al., 2003; Persson et al., 2004; Simoncic et al., 2006), it might simultaneously target both the malignant cells and the vessels nourishing them. To obtain suitable tools to test this hypothesis in vivo, we have started to screen for novel small-molecule TCPTP activators.

Upon VEGF stimulation, VEGFR2 is rapidly internalized and the rate of internalization is governed by phosphorylation (Dougher and Terman, 1999; Labrecque et al., 2003). Contrary to the earlier dogma, internalized cell-surface receptors maintain their signalling activity in the endosomes and may even recruit specific subset of downstream effectors (Di Fiore and De Camilli, 2001; Kermorgant et al., 2004; Lampugnani et al., 2006). Endothelial cells in nascent vessels have elaborate cell-cell contacts under homeostatic conditions and their turnover is very slow. Under these conditions, VEGFR2 is kept inactive by the VE-cadherin–β-catenin complex, which targets it to the vicinity of DEP1 (also known as PTPRJ) phosphatase, which dephosphorylates the receptor and prevents its internalization (Kroll and Waltenberger, 1997; Mitola et al., 2006). Once internalized, the receptor is actively signalling and is phosphorylated at least on tyrosines 1175 and 1214. SHP2 has also been shown to interact with VEGFR2, presumably via its SH2 domain, but there are contradictory reports about its ability to dephosphorylate the receptor (Mitola et al., 2006). Nevertheless, SHP2 has been reported to enhance internalization of VEGFR2 (Bhattacharya et al., 2005; Ikeda et al., 2005). By contrast, we show here that, in subconfluent cells, TCPTP activation dephosphorylates VEGFR2 and inhibits its internalization.

In the different steps of angiogenesis there are endothelial cells with distinct phenotypes. These are, for example, the tip cells of sprouts (which are migratory, display filopodia and lead the growing sprout through the connective tissue surrounding the vessel) and the non-sprouting stalk cells (which form the vessel lumen) (Siekmann et al., 2008). The tip cells have been shown to upregulate the expression of VEGFR2 and PDGF-B (Gerhardt et al., 2003). We show here that TCPTP dephosphorylates VEGFR2 and that activation of TCPTP inhibits sprouting angiogenesis. Furthermore, PDGF-receptor signalling is upregulated in fibroblasts lacking TCPTP (Persson et al., 2004). Taken together, it is possible that TCPTP plays an important role in regulating sprouting angiogenesis because of its ability to modulate phosphorylation of several
signalling molecules that are relevant for the function of the tip cells.

ECM proteins have been shown to positively regulate VEGFR2 activity in a few cases. For instance, ligation of integrin αvβ3 supports VEGF-induced biological responses in endothelial cells in vitro and integrin αvβ3 associates with VEGFR2 in response to VEGF treatment (Borges et al., 2000). Here, we define the first molecular pathway that leads to matrix-guided inhibition of endothelial cell responsiveness to VEGF. This pathway is triggered by binding of endothelial cells to collagenous matrix through integrin α1, which leads to activation of TCPTP and inhibition of VEGFR2 signalling. Our findings, together with other reports on the role of two other phosphatases in controlling VEGFR2, allow us to propose a model of sequential phosphatase-regulated steps in angiogenesis. (1) In intact endothelium, cells elaborate cell-cell contacts and are unable to respond to VEGF. This is regulated by the VE-cadherin–β-catenin complex, which targets VEGFR2 to cell-cell contacts and results in VEGFR2 dephosphorylation by DEP1, and possibly by other junctional phosphatases. Ligation of integrin α1β1 to basement-membrane collagen IV and subsequent TCPTP activation would not have any additional effect in the endothelial cells in nascent vessels because VEGFR2 is already functionally inactivated through DEP1 in cells with cell-cell contacts (supplementary material Fig. S2A,B). (2) Upon induction of angiogenesis, basement membrane is degraded and the sprouting endothelial cells will become surrounded by stroma ECM and be ligated, most likely via integrins αvβ3, αvβ1 and α2β1 (which bind with high affinity to vitronectin, fibronectin and type-I collagen, respectively). The cells are sensitive to VEGF, and VEGFR2 is fully phosphorylated and actively signalling to drive vessel sprouting. (3) Upon formation of a new basement membrane, integrin α1β1 (binding to collagens IV and I) will activate TCPTP, and possibly SHP2, which begin to attenuate proliferation and migration of the endothelial cells until they return to quiescence upon maturation of the new vessel (Carmeliet, 2000; Conway et al., 2001). Based on this model, different phosphatases would inhibit VEGF signalling at different steps of angiogenesis.

Materials and Methods

Antibodies, plasmids and reagents

Antibodies against TCPTP (mAb CF4, Calbiochem; polyclonal Ab, BD PharMingen; 3E2 from Michel Tremblay, McGill University, Canada), total VEGFR1, total VEGFR2 (55B11), phosphoryrosines 986, 1175 and 1214 of VEGFR2 (Cell Signalling Technology), autophosphorylation phosphoryrosines 1054/1059 (Sigma), vinculin (Sigma), phosphorytosine (PY20 Transduction Laboratories), αt integrin (mAb 1973 (clone FB12) and Ab1934, Chemicon), β1 integrin (mAb 2252, Chemicon), β3 integrin (Serotec), αt-tubulin (Hybonoida Bank), and control mouse IgG were used. HRP- and Alexa-Flour-488/555/647-conjugated second-stage reagents were used, as appropriate.

A plasmid containing the 37-kDa constitutively active form of TCPTP [TC37 in pCDNA3.1(−)] was generated from the GST-TC37 expression construct described previously (Mattiola et al., 2005). Wild-type TCPTP (TC45), a substrate-trapping mutant of TCPTP pCG-Tc45-D182A, the control pCG-vector and VEGFR2 expression plasmid have also been described previously (Klingler-Hoffmann et al., 2001; Matsumoto et al., 2005).

siRNA against TCPTP has previously been shown to be specific to TCPTP and to have identical effects to another TCPTP-targeting oligo (Mattiola et al., 2005). TCPTP siRNA and a control siRNA were from Ambion. Peptides containing the cysteine-rich tails of the α1 or α2 integrins, the integrin-α1 tail fused to the 11-amino-acid-long TAT peptide, and scrambled TAT peptides were synthesized by Innovagen. Human recombinant VEGF165 was purchased from Peprotech. Recombinant TCPTP was produced and purified as a GST-fusion protein in Escherichia coli and cleaved from the GST by using PreScission according to the manufacturer’s instructions (BD Biosciences).

Cells

HEK293 and HeLa cells (ATCC) were maintained in DMEM, 10% FBS, 2 mM L-glutamine, 5% CO2. Primary HUVECs were freshly isolated as described (Koskinen et al., 2004) and cultured in endothelial cell growth medium (PromoCell). Only HUVECs’ passages ≤ three times were used in this study. They were transfected with DNA plasmids or siRNA duplexes (100 nM) by electroporation using Bio-Rad Gene Pulser Xcell (Ambion).

Immunofluorescence stainings and FACS analyses

The cell-surface expression of integrins in HUVECs was analyzed using a previously described protocol (Pellinen et al., 2006). In brief, detached cells were incubated with the indicated antibodies followed by an Alexa-Flour-488/555/647-labelled second-step reagent. The cells were analyzed using FACScalibur.

For immunocytochemistry, HUVECs were plated on type-IV collagen and fixed with 4% paraformaldehyde. VEGFR2, TCPTP and αt integrin were visualized using indirect immunofluorescence stainings of 0.1% Triton-X-100-permeabilized cells in PBS containing 10% horse serum, 1% BSA and 0.01% azide. The samples were mounted in Vectashield with DAPI (Vector Laboratories, CA) and analyzed using a confocal microscope (Axioplan 2 with LSM 510; Carl Zeiss Microimaging). Frozen sections from human umbilical cord were fixed and stained as above. The samples were analyzed using an Axiosvert 200M inverted microscope and 20× objective.

Immunoprecipitations

TCPTP-D182A- and VEGFR2-encoding plasmids were co-transfected into HEK293 cells using Lipofectamine 2000. After 1 day, the cells were serum-starved (overnight), stimulated with VEGF (50 ng/ml, 5 minutes), washed with ice-cold PBS and lysed in the presence or absence of 100 μM sodium orthovanadate. Pre-cleared lysates were subjected to immunoprecipitation with anti-TCPTP, anti-VEGFR2 or control mouse IgG in the presence or absence of 100 μM sodium orthovanadate, resolved on SDS-PAGE and subjected to immunoblotting. Aliquots of the original lysates (5% from input of immunoprecipitations) were analyzed in parallel to control for protein expression.

Phosphatase and kinase assays

HUVECs were plated onto collagen- or gelatin-coated plates and allowed to adhere for 1 hour. TCPTP, SHP2 and control immunocomplexes were isolated from cell lysates, resuspended in the phosphate-assay buffer and assayed for phosphatase activity using 6,8-difluoro-4-methylumbelliferone phosphate (diFMUP, Molecular Probes) as a substrate in the presence of a serine/threonine-phosphatase-inhibitor cocktail (Sigma). Thereafter, the antibodies from the beads were used for immunoblotting. The phosphatase activities were always normalized to immunoblotted proteins. The phosphatase assays with purified recombinant TCPTP protein were performed using diFMUP as a substrate.

VEGFR2 kinase activity was studied from HUVECs, which were plated on gelatin, serum-starved overnight, treated with ω-tat-TAT or ScTAT peptides (400 nM) and stimulated with VEGF (100 ng/ml, 15 minutes) using Kinase-Glo Plus kinase assay kit according to the manufacturer’s protocol (Promega).

For the in vitro dephosphorylation experiments, HEK293 cells transfected with VEGFR2 were treated with VEGF as described above. VEGFR2 was immunoprecipitated from the lysates (50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 2.5 mM EDTA, 1% NP-40, Complete-protease-inhibitor cocktail, Sigma PPASE inhibitor cocktails I and II). The beads were washed with the phosphate-assay buffer (10 mM HEPES, 150 mM NaCl, 1 mM DTT) and exposed to recombinant TCPTP (10 μg/ml) or buffer only for 10 minutes at +37°C. Immunoblotting was used to detect total VEGFR2 and specific phosphophosphorysines.

Cell-proliferation and survival assays

The number of viable HUVECs was analyzed with WST-1 reagent (Roche). Cell proliferation was measured with BrdU assay (Cell Proliferation ELISA Biotrak system, Amersham) from HUVECs grown in RPMI1640 + 1% FBS or Ham’s EBM2. Cell-proliferation and survival assays are described in detail elsewhere (Korff et al., 2004) and cultured in endothelial cell growth medium (PromoCell). Only HUVECs’ passages ≤ three times were used in this study. They were transfected with DNA plasmids or siRNA duplexes (100 nM) by electroporation using Bio-Rad Gene Pulser Xcell (Ambion).

Tube-formation assay

In the fibrin-gel in vitro angiogenesis assay (Chemicon), HUVECs were plated onto a fibrin gel and overlaid with a second fibrin gel after an overnight culture. The endothelial cell growth medium with or without VEGF (50 ng/ml) was added in the presence or absence of ω-tat-TAT or scrambled TAT peptides (400 nM). At the 24-hour end-point, the formation of cellular networks was quantified from three randomly selected fields from each well. Scores 0–5 were assigned by a naïve observer to each field based on cellular aligning, capillary-tube formation, sprouting and formation of polycagons according to the manufacturer’s instructions.

Sprouting assay

Sprouting assays from spheroids were based on the method described earlier (Korff and Augustin, 1999). Briefly, HUVECs or PAE cells were divided into round-bottomed non-treated 96-well plates (Greiner Bio-One), 3000 cells per well, in EB2M or Ham’s F12 GlutaMAX Gibco medium with 0.25% methyl cellulose (Sigma). After overnight incubation at +37°C, five spheroids were treated with VEGF, wounded and resuspended in spheroid medium (40% FBS, 0.5% methyl cellulose, plain medium). Collagen gel (20 mM HEPES, 1× DMEM (Sigma), PureCol collagen [Vitrogen]) was added 1:1 to cell suspension and the mix transferred to 48-well plates. After
polimerization, medium with or without 25 ng/ml VEGF, and 400 nM ScTrAT or α-TAT-peptide vehicle. Cumulative length of the sprouts around spheroids or the number of sprouts formed from spheroids was quantified using 10× magnification after 24 hours of incubation.

**VEGFR2-internalization assays**

For immuno/fluorescence analysis, HUVECs were transfected with control plasmid or with the constitutively active truncated 37-42a TCPTP mutant (TC37), grown on gelatin and stimulated with VEGF. VEGFR2 was stained as above and VEGFR2-positive endosome-like structures were scored from microscopic images. For biochemical analysis, 48 hours after transfection, vector (pCG empty vector), TC45 or TC37-transfected semi-confluent HUVECs were surface-biotinylated with EZ-Link Sulfo-NHS-SS-Biotin (Pierce) on ice for 30 minutes. After the biotinylation, an aliquot of the cells was immediately lysed after cleaving the biotin on the cell surface with a reducing agent, sodium 2-mercaptopethanesulfonate (MesNa, Fluka), to verify the efficacy of the cleavage reaction (not shown). For the remaining cells, biotinylated proteins were allowed to internalize at +37°C in the presence or absence of VEGF for 15 minutes. Thereafter, the plates were transferred on ice and any remaining biotin on the surface was cleaved with MesNa (leaving only the internalized proteins biotinylated). The cells were then lysed, after which VEGFR2 was immunoprecipitated and run on non-reducing SDS-PAGE. Internalized VEGFR2 was blotted with anti-biotin-HRP and the total immunoprecipitated VEGFR2 with anti-VEGFR2 antibody. Lysates were also blotted for TCPTP to verify TCPTP transfection efficiency.

**Time-lapse chemokinesis assays**

HUVECs were plated on gelatin-coated wells and allowed to attach for 1 hour in the presence or absence of 200 nM α-TAT or ScTrAT peptides and 20 ng/ml VEGF. Migration of individual cells was recorded at 5-minute intervals with an Axiosvert 200M inverted microscope with atmosphere control unit for 4 hours using a 20× objective and digital video recording. The migration pathway of at least ten randomly picked cells was tracked using the Metamorph program. The cumulative migration distances were also plotted.

**Chemosat assays**

HUVECs were treated with 200 nM α-TAT or ScTrAT, or left untreated for 1 hour. Transwell filters (8-μm pore size, Costar, Cambridge, MA) were coated with gelatin, and medium (RPMI1640, 4 mM L-glutamine, 10% FBS) and VEGF (100 ng/ml) were added to the lower chamber. HUVECs were added to Transwell inserts (4×10^5 cells/well) in RPMI1640 with 0.2% soybean trypsin inhibitor. Chemotaxis was allowed to proceed for 4 hours (+37°C, 5% CO2). The inserts were removed, and the non-migrated cells from the top of the insert were removed by scraping. The inserts were fixed in 4% paraformaldehyde, cells stained with 0.5% crystal violet and the number of migrating cells was counted using a microscope.

**Statistical analyses**

Statistical analyses were performed using Student’s t-test.

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