CRSBP-1/LYVE-1 ligands disrupt lymphatic intercellular adhesion by inducing tyrosine phosphorylation and internalization of VE-cadherin

Wei-Hsien Hou, I-Hua Liu, Cheng C. Tsai, Frank E. Johnson, Shuan Shian Huang and Jung San Huang

Journal of Cell Science 125, 246
© 2012. Published by The Company of Biologists Ltd
doi:10.1242/jcs.096065

There was an error published in J. Cell Sci. 124, 1231-1244.

Jun San Huang and Shuan Shian Huang had an equity position in Auxagen Inc. during the time the research was carried out and should have declared that there is a conflict of interest.

Part of the research described in the article was supported by NIH grants awarded to St Louis University (AA 019233 to J.S.H.) and to Auxagen Inc. (HL 087463-01 and DK 078438 to J.S.H.).

The authors apologise for this omission.
CRSBP-1/LYVE-1 ligands disrupt lymphatic intercellular adhesion by inducing tyrosine phosphorylation and internalization of VE-cadherin

Wei-Hsien Hou¹, I-Hua Liu¹, Cheng C. Tsai², Frank E. Johnson³, Shuan Shian Huang⁴,* and Jung San Huang¹,*

¹Departments of Biochemistry and Molecular Biology, St Louis University School of Medicine, Doisy Research Center, 1100 S. Grand Boulevard, St Louis, MO 63104, USA
²WCP Pathology Laboratories, Inc., 2326 Mallpark Drive, St Louis, MO 63043, USA
³Department of Surgery, St Louis University School of Medicine, St Louis, MO 63104, USA
⁴Auxagen Inc., 7 Pricewoods LN, St Louis, MO 63132, USA
*Authors for correspondence (huangjs@slu.edu; shuanh@gmail.com)

Accepted 12 November 2010
Journal of Cell Science 124, 1231-1244
© 2011. Published by The Company of Biologists Ltd
doi:10.1242/jcs.078154

Summary
Cell-surface retention sequence (CRS) binding protein (CRSBP-1) is a membrane glycoprotein identified by its ability to bind PDGF-BB and VEGF-A via their CRS motifs (clusters of basic amino acid residues). CRSBP-1 is identical to LYVE-1 and exhibits dual ligand (CRS-containing proteins and hyaluronic acid) binding activity, suggesting the importance of CRSBP-1 ligands in lymphatic function. Here, we show that CRSBP-1 ligands induce disruption of VE-cadherin-mediated intercellular adhesion and opening of intercellular junctions in lymphatic endothelial cell (LEC) monolayers as determined by immunofluorescence microscopy and Transwell permeability assay. This occurs by interaction with CRSBP-1 in the CRSBP-1–PDGFβR–β-catenin complex, resulting in localizing tyrosin phosphorylated β-catenin and p120-catenin from VE-cadherin, and internalization of VE-cadherin. Pretreatment of LECs with a PDGFβR kinase inhibitor abolishes ligand-stimulated tyrosine phosphorylation of VE-cadherin, halts the ligand-induced disruption of VE-cadherin intercellular adhesion and blocks the ligand-induced opening of intercellular junctions. These CRSBP-1 ligands also induce opening of lymphatic intercellular junctions that respond to PDGFβR kinase inhibitor in wild-type mice (but not in Crsbp1-null mice) as evidenced by increased transit of injected FITC–dextran and induced edema fluid from the interstitial space into lymphatic vessels. These results disclose a novel mechanism involved in the opening of lymphatic intercellular junctions.

Key words: VE-cadherin intercellular junctions, PDGF β-type receptor, Tyrosine phosphorylation, Endothelial cell permeability, Interstitial–lymphatic transit

Introduction
Cell-surface retention sequence (CRS) binding protein-1 (CRSBP-1) is a membrane glycoprotein first identified by its ability to mediate cell-surface retention (after synthesis and secretion) of the simian sarcoma virus (SSV) oncogene v-sis gene product (PDGF-BB) in SSV-transformed fibroblasts (Boensh et al., 1995; Boensh et al., 1999; Lokesherw, et al., 1990). CRSBP-1 forms complexes with the v-sis gene product (by binding its CRS motif near the C-terminus of the v-sis gene product) during trafficking from the endoplasmic reticulum (ER) to the plasma membrane, thus retaining it at the cell surface (Boensh et al., 1999). All members of the PDGF superfamly, including PDGF-AA, PDGF-BB, placentatal growth factor (PIGF) and vascular endothelial cell growth factor-A, -C and -D (VEGF-A, VEGF-C and VEGF-D), possess CRS motifs and exhibit cell-surface retention during secretion (Carmeliet et al., 1999; Houck et al., 1991; Joukov et al., 1996; Joukov et al., 1997; LaRochelle et al., 1991; Ostman et al., 1991). These CRS motifs contain clustered basic amino acid residues (Arg, Lys and His) and are evolutionarily conserved (Carmeliet et al., 1999; Joukov et al., 1997; LaRochelle et al., 1991; Ostman et al., 1991). Through cross-linking studies (Boensh et al., 1995; Boensh et al., 1999), CRSBP-1 was identified as a receptor that binds the synthetic oligopeptides containing the CRS motifs of PDGF-BB and VEGF-A. cDNA cloning, sequencing and expression of bovine CRSBP-1 (Huang et al., 2003) revealed that CRSBP-1 is a 120 kDa disulfide-linked homodimeric type I membrane glycoprotein with distinct dual ligand (CRS-containing growth factors and cytokines, and hyaluronic acid (HA)) binding activity. The deduced amino acid sequences of human and murine CRSBP-1 exhibit 61% and 56% identity, respectively, to that of bovine CRSBP-1 and are identical to those of human and murine lymphatic vessel endothelial HA receptor-1 (LYVE-1) (Banerji et al., 1999; Prevo et al., 2001; Huang et al., 2003). LYVE1 was cloned using CD44 sequence homology cloning (Banerji et al., 1999). Both CD44 and LYVE-1 are members of the Link protein superfamily that bind the large extracellular matrix glycosaminoglycan HA (Banerji et al., 1999; Teriete et al., 2004). LYVE-1 is primarily expressed in lymphatic endothelium and localized to both luminal and abluminal faces of lymphatic capillary vessels (Teriete et al., 2004). The primary localization of CRSBP-1/LYVE-1 to lymphatic endothelium has made it a suitable marker for studying lymphangiogenesis (Altalal and Carmeliet, 2002; Jackson, 2003; Jussila and Altalal, 2002). This also implies the likely importance of the dual ligand binding activity of CRSBP-1 in regulating the function of lymphatic vessels.

To define the in vivo role of CRSBP-1, we recently generated Crsbp1-null (Crsbp1−/−) mice (Huang et al., 2006). These mice are...
macroscopically normal. However, their lymphatic vessel lumens are constitutively distended compared with the irregularly shaped, collapsed lumens of lymphatic vessels often found in wild-type mice (Huang et al., 2006). The alteration in the shape of lymphatic vessel lumens is most prominent in the liver and intestine (Huang et al., 2006). High molecular weight FITC–dextran (average MW \(~2,000,000\) injected subcutaneously in the tail of \(Crsbp1^{+/−}\) mice is cleared from the tail more rapidly than in wild-type mice (Huang et al., 2006). Since high molecular weight FITC–dextran can only be cleared via lymphatic drainage, we reasoned that \(Crsbp1^{+/−}\) mice might have a constitutive increase in the transit of large molecules, fluids and cells from the interstitial space into the lumen of lymphatic capillaries, a process we call interstitial–lymphatic transit (Huang et al., 2006). To further define the role of CRSBP-1 in the regulation of interstitial–lymphatic transit, FITC–dextran was co-injected with and without PDGF-BB, a putative physiological CRSBP-1 ligand, into tails of wild-type mice. PDGF-BB enhanced the clearance of FITC–dextran near the injection site (Huang et al., 2006). Co-injection of HA, a known CRSBP-1 ligand, also enhanced the clearance of FITC–dextran near the injection site. PDGF-BB was more effective than HA in wild-type mice; however, in \(Crsbp1^{+/−}\) mice, neither PDGF-BB nor HA enhanced the clearance of the FITC–dextran (Huang et al., 2006).

These results support our hypothesis that the \(Crsbp1\)-null mutation and binding of CRSBP-1 by its cognate ligands cause opening of lymphatic intercellular junctions, resulting in increased interstitial–lymphatic transit (Huang et al., 2006).

To define the molecular mechanism by which CRSBP-1 ligands regulate the function of lymphatic intercellular junctions, we determined the effects of several CRSBP-1 ligands on the structure (morphology) and function (permeability) of intercellular junctions in the monolayers of LECs, including SVEC4-10 cells and primary human dermal LECs (HDLECs). These CRSBP-1 ligands included putative physiological ligands (PDGF-BB, VEGF-A165 and hyaluronic acid) and two specific ligands (PDGF peptide and VEGF peptide, which are synthetic oligopeptides containing the CRS motifs of PDGF-BB and VEGF-A165, respectively and do not interact with PDGFr and VEGFR2) (supplementary material Fig. S1). SVEC4-10 cells are SV40-transformed LEC-like cells (supplementary material Fig. S2). We also determined the effects of these CRSBP-1 ligands on the function of lymphatic intercellular junctions by measuring the transit of intradermally injected high molecular weight FITC–dextran and \(\lambda\)-carrageenan-induced edema fluid from the interstitial space into lymphatic vessels in wild-type and \(Crsbp1\)-null mice. In this communication, we show that these CRSBP-1 ligands induce disruption of VE-cadherin-mediated intercellular adhesion and opening of intercellular junctions in a manner dependent on PDGFr activity in LEC monolayers (as evidenced by increased permeability) and in lymphatic vessels in wild-type mice (as evidenced by rapid clearance of injected FITC–dextran near the injection site and by attenuated \(\lambda\)-carrageenan-induced edema).

**Results**

**CRSBP-1 ligands induce disruption of VE-cadherin-mediated intercellular adhesion**

We hypothesized that CRSBP-1 is involved in the formation of overlapping intercellular junctions of lymphatic endothelial cells in lymphatic vessels and that CRSBP-1 ligands induce opening of the lymphatic intercellular junction (Huang et al., 2006). Baluk and colleagues (Baluk et al., 2007) demonstrated that VE-cadherin

---

**Fig. 1. CRSBP-1 ligands induce disruption of VE-cadherin–\(\beta\)-catenin-mediated intercellular adhesion in SVEC4-10 cells.** SVEC4-10 cells were seeded on coverslips and treated with vehicle only (control) (A–C), 10 \(\mu\)M VEGF peptide (D–F), 10 \(\mu\)M PDGF peptide (G–I), 100 ng/ml VEGF-A\(^{165}\) (J–L) or 100 \(\mu\)g/ml HA (M–O) for 1 hour. After incubation, cells were fixed with methanol at \(−20°C\) for 10 minutes. Cells were stained with anti-\(\beta\)-catenin antibody (green) (A,D,G,J,M) and anti-VE-cadherin antibody (red) (B,E,H,K,N) and visualized with a confocal microscope. Cells treated with vehicle showed colocalization of VE-cadherin and \(\beta\)-catenin on the plasma membrane (as indicated by an arrowhead in C). CRSBP-1 ligand treatment induced disruption of \(\beta\)-catenin distribution from a linear pattern to a zigzag pattern in the cell borders as indicated by arrows (F,I,L,O), diminished plasma membrane distribution of VE-cadherin (E,H,K,N) and decreased colocalization of VE-cadherin and \(\beta\)-catenin (F,I,L,O). CRSBP-1 ligand stimulation also enlarged gaps between cells (as indicated by asterisk in F,I,L,O). Scale bar: 10 \(\mu\)m.

---

is required for maintenance of lymphatic junctional integrity. To test the hypothesis that CRSBP-1 ligands cause opening of lymphatic intercellular junctions by compromising VE-cadherin and \(\beta\)-catenin-mediated intercellular adhesion, we analyzed the subcellular localization of VE-cadherin and its associated protein, \(\beta\)-catenin, in monolayers of SVEC4-10 cells treated with CRSBP-1 ligands using immunofluorescence confocal microscopy. Without stimulation, VE-cadherin colocalized with \(\beta\)-catenin at the plasma membrane (Fig. 1C). Upon stimulation with 10 \(\mu\)M VEGF peptide and 10 \(\mu\)M PDGF peptide for 1 hour, VE-cadherin levels diminished at the plasma membrane, presumably because of increased VE-cadherin endocytosis or internalization (Fig. 1E,H vs 1B). Additionally, \(\beta\)-catenin staining became disorganized into a zigzag pattern (Fig. 1D,G) and there was no colocalization of VE-cadherin and \(\beta\)-catenin at the plasma membrane (Fig. 1F,I). It has been shown that the change in \(\beta\)-catenin membrane distribution
from a linear pattern to zigzag pattern is associated with disassembly of the VE-cadherin–β-catenin complex and disruption of VE-cadherin-mediated cell–cell adhesion (Boccellino et al., 2005; Esser et al., 1998). Furthermore, when compared with cells treated with vehicle only, gaps and slits appeared between endothelial cells treated with CRSBP-1 ligand, which we believe is due to loss of cell–cell adhesion (Fig. 1F,I). Stimulation with physiological CRSBP-1 ligands (5 nM VEGF-A165 and 100 μg/ml HA) also induced decreased plasma membrane distribution of VE-cadherin and disorganization of distribution of β-catenin in the membrane from a linear pattern to a zigzag pattern (Fig. 1L,O). VEGF-A165 is a putative physiological CRSBP-1 ligand for LECs that express very little endogenous VEGFR2, which is a specific receptor for VEGF-A165 in blood vascular endothelial cells (Farrara, 2004). Taken together, these results suggest that CRSBP-1 ligands inhibit VE-cadherin trans interaction (Boccellino et al., 2005; Esser et al., 1998; Vincent et al., 2004) and impair cell–cell adhesion by inducing disassembly of the VE-cadherin–β-catenin complex at intercellular junctions in SVEC4-10 cells.

The effects of CRSBP-1 ligands on VE-cadherin–β-catenin intercellular junctions were also determined in HDLECs. HDLECs were stimulated with CRSBP-1 ligands, as described above. Similarly to SVEC4-10 cells, indirect immunofluorescence staining of unstimulated HDLECs using antibodies against VE-cadherin and β-catenin revealed colocalization of VE-cadherin and β-catenin at the plasma membrane along the cell borders. HDLEC-stimulated with CRSBP-1 ligands (10 μM PDGF peptide, 10 μM VEGF peptide, 5 nM VEGF-A165 and 100 μg/ml HA) exhibited disorganization of the membrane distribution of VE-cadherin–β-catenin at the intercellular junctions from a linear pattern to a zigzag pattern, with a diminished plasma membrane distribution of VE-cadherin and decreased colocalization of VE-cadherin and β-catenin (data not shown). PDGF and VEGF peptides induced more disorganization of VE-cadherin and β-catenin than was observed with PDGF-BB and HA (data not shown). Additionally, gaps between cells in HDLEC monolayers stimulated with PDGF and VEGF peptides, and VEGF-A165 were increased (data not shown).

Furthermore, the linear and zigzag staining patterns of VE-cadherin–β-catenin were quantified using the method described previously (Vincent et al., 2005). The VE-cadherin–β-catenin immunofluorescence staining of cell borders in HDLECs treated with vehicle only exhibited mainly linear patterns. Treatment with PDGF peptide (10 μM), VEGF peptide (10 μM), VEGF-A165 (5 nM) and HA (100 μg/ml) resulted in increased zigzag patterns in the plasma membrane distribution of VE-cadherin–β-catenin (n=10) (20±3%, 27±3%, 17±4% and 11±3%, respectively). PDGF-BB (2 nM) had moderate effects on the staining patterns of VE-cadherin–β-catenin (6±2% zigzag patterns) compared with those in vehicle-treated cells. This is consistent with the concept that PDGF-BB weakly increases opening of VE-cadherin–β-catenin intercellular junctions in SVEC4-10 cells. This is probably due to the fact that the PDGF-BB (Abcam ab73229) used in these experiments is not the full-length protein and lacks the CRS motif near the C-terminal end of the protein. PDGF-BB is believed to slightly induce opening of intercellular junctions, mainly via interaction with PDGFRβ.

CRSBP-1 ligands increase permeability in LEC monolayers in a CRSBP-1-dependent manner

As described above, CRSBP-1 ligands induce opening of VE-cadherin–β-catenin intercellular junctions. This finding raises the possibility that CRSBP-1 ligands are capable of stimulating the permeability of LEC monolayers by inducing opening of intercellular junctions. Using a Transwell system, we determined the permeability of SVEC4-10 cell and HDLEC monolayers stimulated with CRSBP-1 ligands by measuring the passage of FITC–dextran (MW ~40,000) from the upper chamber to the lower chamber. Stimulation of SVEC4-10 cells with 10 μM VEGF (Fig. 2Ba) and 10 μM PDGF peptide (Fig. 2Ab) significantly increased transit of FITC–dextran across the cell monolayer when compared with results obtained with the vehicle alone. Treating SVEC4-10 cell monolayers with 100 μg/ml HA slightly enhanced the transit of FITC–dextran to the lower chamber (Fig. 2Ac). The same experiment was performed using HDLECs. VEGF peptide also increased permeability in HDLEC monolayers as determined using the Transwell permeability assay (Fig. 2Ad). These results indicate that specific CRSBP-1 ligands (VEGF and PDGF peptides) are capable of increasing permeability in SVEC4-10 cell and HDLEC monolayers, presumably by inducing opening of VE-cadherin–β-catenin intercellular junctions.

To test the hypothesis that CRSBP-1 ligands act specifically with CRSBP-1 to exert its biological activity, we performed gene-specific downregulation of CRSBP-1 by transfection of SVEC4-10 cells with Crsbp1 siRNA. At 48 hours after siRNA transfection, about 80% of CRSBP-1 protein was downregulated compared with levels in cells transfected with control siRNA (Fig. 2Ba). SVEC4-10 cells transfected with control siRNA or Crsbp1 siRNA for 48 hours were used for the Transwell permeability assay. Cells transfected with Crsbp1 siRNA exhibited increased transit of FITC–dextran from the upper chamber to the lower chamber compared with cells transfected with control siRNA (Fig. 2Bb), indicating increased permeability in Crsbp1 siRNA-transfected cells. However, cells transfected with control siRNA exhibited increased permeability upon VEGF peptide stimulation (Fig. 2Bc). Interestingly, the increase in permeability in ligand-stimulated control cells was similar to that observed in cells transfected with Crsbp1 siRNA. In addition, downregulation of CRSBP-1 expression by transfection of cells with Crsbp1 siRNA abolished VEGF-peptide-stimulated permeability of transfected cell monolayers (Fig. 2Bd). These results suggest that CRSBP-1 is required for mediation of CRSBP-1-ligated-stimulated permeability in SVEC4-10 cell monolayers. These results are consistent with the fact that Crsbp1-null mice exhibit constitutively increased interstitial–lymphatic transit (Huang et al., 2006). We hypothesize that this results from constitutive opening of intercellular junctions in lymphatic capillary vessels.

CRSBP-1 ligands induce internalization of VE-cadherin and dissociation of β-catenin and p120-catenin from VE-cadherin

As described above, confocal microscopy of intercellular junction proteins (VE-cadherin and β-catenin) reveals that CRSBP-1 ligand stimulation results in decreased cell-surface staining of VE-cadherin, decreased colocalization of VE-cadherin and β-catenin at the plasma membrane and opening of VE-cadherin intercellular junction in LEC monolayers. These results suggest that CRSBP-1 ligands induce internalization of cell-surface VE-cadherin, because internalized cell-surface proteins are resistant to trypsin digestion. Following treatment of SVEC4-10 cells with vehicle only, 10 μM PDGF peptide and 10 μM VEGF peptide, cells were untreated (for analysis of the total
VE-cadherin) or treated with trypsin to remove cell-surface proteins. The lysates of cells untreated and treated with trypsin were subjected to western blot analysis using anti-VE-cadherin antibodies. Lysates of SVEC4-10 cells, which were not subjected to further incubation with trypsin, contained 130 kDa VE-cadherin (Fig. 3A, lane 1). This represented the total VE-cadherin in the cells. In these cells, a fraction (~30%) of VE-cadherin was estimated to be trypsin resistant, representing intracellular or internalized VE-cadherin (Fig. 3A, lane 2). This finding suggests that VE-cadherin undergoes internalization and recycling without stimulation by exogenous CRSBP-1 ligands. However, treatment with PDGF and VEGF peptides increased the amount of trypsin-resistant VE-cadherin to ~80% of the total VE-cadherin (Fig. 3A, lanes 3 and 4 vs lane 1), suggesting that these peptides stimulate internalization of cell-surface VE-cadherin ~2.7-fold. Similar results were also obtained from the experiments measuring trypsin-resistant cell surface (biotinylated) VE-cadherin following treatment of SVEC4-10 cells with VEGF peptide (data not shown).

In blood vascular endothelial cells, internalization of VE-cadherin might result from the dissociation of β-catenin or p120-catenin from VE-cadherin. To determine the interaction between VE-cadherin and β-catenin or p120-catenin in LECs, SVEC4-10 cells were stimulated with vehicle only, 10 μM VEGF peptide or 10 μM PDGF peptide for 30 minutes. Stimulated cell lysates were subjected to immunoprecipitation using anti-VE-cadherin antibody followed by SDS-PAGE and immunoblot analysis using anti-β-catenin antibody or anti-p120-catenin antibody. In vehicle-treated cells, VE-cadherin and β-catenin or p120-catenin were found to be co-immunoprecipitated (Fig. 3B, lane 1). Upon stimulation by VEGF and PDGF peptides, there was a decrease in the amount of β-catenin (~50%) and p120-catenin (~90%) co-immunoprecipitated with VE-cadherin (Fig. 3B, lanes 2 and 3 vs lane 1). These results suggest that CRSBP-1 ligands stimulate permeability in SVEC4-10 cell monolayers and HDLEC monolayers in a CRSBP-1-dependent manner. (A) CRSBP-1 ligands stimulate permeability in SVEC4-10 cell (a–c) and HDLEC (d) monolayers. Cells (1.5×10^5) were plated onto 6.5 mm Transwell (collagen-coated, 3 μm pore PTFE) membrane inserts and grown overnight. Cell monolayers were treated with vehicle only (control) (a–d), 10 μM VEGF peptide (a,d), 10 μM PDGF peptide (b) and 100 μg/ml HA (c). FITC–dextran (MW 40 kDa; 1 mg/ml) was then added to the upper chamber. At each time period (0, 60, 120, 180, 240, 300 and 360 minutes), an aliquot was taken from the lower chamber to measure its fluorescence intensity (A.U.). *P<0.05 compared with vehicle-only control. (B) Downregulation of CRSBP-1 expression by transfection with Crsbp1 siRNA abolishes CRSBP-1-ligand-stimulated permeability in SVEC4-10 cells. Cells were transfected with control or Crsbp1 siRNA for 48 hours. Following transfection, the amounts of CRSBP-1 protein and β-actin (as a loading control) were determined by western blot (a). SVEC4-10 cells (1.5×10^5) transfected with control or Crsbp1 siRNA were plated onto 6.5 mm Transwell (collagen-coated, 3 μm pore PTFE) membrane inserts and grown overnight. Cell monolayers were treated with vehicle only or 10 μM VEGF peptide. FITC–dextran (MW 40 kDa; 1 mg/ml) was then added to the upper chamber. At each time point, an aliquot was taken from the lower chamber to measure its fluorescence intensity (A.U.). *P<0.05 compared with vehicle-only control. Monolayers of cells transfected with Crsbp1 siRNA exhibited greater permeability than monolayers of cells transfected with control siRNA (b). Monolayers of cells transfected with control siRNA showed VEGF-peptide-increased permeability of the cell monolayer (c), whereas in monolayers of cells transfected with Crsbp1 siRNA, VEGF-peptide-increased permeability of the cell monolayer was abolished (d). *P<0.05, compared with control siRNA or vehicle-only control.
CRSBP-1 forms complexes with PDGFβR and β-catenin

CRSBP-1 is known to form complexes with PDGFβR in SSV-transformed cells (Huang et al., 2006). We determined whether this complex formation also occurs in SVEC4-10 cells by immunoprecipitation with rabbit anti-CRSBP-1 serum or non-immune serum followed by immunoblot analysis with rabbit anti-PDGFβR serum. PDGFβR was present in the immunoprecipitates using anti-CRSBP-1 serum (Fig. 3C, lane 2) but not non-immune serum (Fig. 3C, lane 1). Interestingly, ~10% of the total PDGFβR was found to form complexes with CRSBP-1 (Fig. 3C, lane 2 vs lane 3). Because CRSBP-1 ligands stimulate dissociation of β-catenin from VE-cadherin, we hypothesized that the CRSBP-1–PDGFβR complex interacts with VE-cadherin or β-catenin in the absence of CRSBP-1 ligands. To test this hypothesis, SVEC4-10 cell lysates were immunoprecipitated using pre-immune serum, anti-CRSBP-1 serum or anti-PDGFβR serum, followed by immunoblot analysis with anti-β-catenin antibody or anti-VE-cadherin antibody. The immunoprecipitates using anti-CRSBP-1 serum (Fig. 3D, lane 2) or anti-PDGFβR serum (Fig. 3D, lane 4) contained β-catenin but not VE-cadherin (data not shown). These results suggest that CRSBP-1 forms complexes with PDGFβR and β-catenin and that the CRSBP-1–PDGFβR complex might associate with β-catenin to form ternary complexes. These results also suggest that PDGFβR is involved in mediating CRSBP-1-ligand-induced disassembly and opening of VE-cadherin–β-catenin intercellular junctions.

CRSBP-1 ligands stimulate tyrosine phosphorylation of PDGFβR

As described above, CRSBP-1 forms complexes with PDGFβR and β-catenin in SVEC4-10 cells. It is possible that CRSBP-1 ligands induce disruption of VE-cadherin- and β-catenin-mediated intercellular adhesion by stimulating the protein tyrosine kinase activity of PDGFβR in the CRSBP-1–PDGFβR complex and subsequent tyrosine phosphorylation of VE-cadherin and β-catenin (probably by stimulated PDGFβR). It has recently been reported that VEGF-A stimulates serine phosphorylation of VE-cadherin, resulting in disruption of intercellular adhesion and opening of intercellular junctions in blood vascular endothelial cells (Gavard and Gutkind, 2006). To test a similar possibility, we determined the levels of tyrosine phosphorylation of several protein species in SVEC4-10 cells stimulated with 10 μM VEGF peptide or vehicle only using western blot analysis with anti-phosphotyrosine antibody. Upon VEGF peptide stimulation, there was a remarkable increase in tyrosine phosphorylation of a protein at ~180 kDa, which coincides with the molecular mass of PDGFβR (Hellberg et al., 2010). Based on this preliminary result and the finding of the complex formation of CRSBP-1 and PDGFβR in SVEC4-10 cells and other cell types expressing both CRSBP-1 and PDGFβR (Boensch et al., 1999), we hypothesized that CRSBP-1 ligands can stimulate tyrosine phosphorylation of PDGFβR by activation of PDGFβR in the CRSBP-1–PDGFβR complex. To test this hypothesis, we determined the level of tyrosine phosphorylation of PDGFβR by immunoprecipitation with anti-PDGFβR serum, followed by immunoblotting for phosphorysotyrosine in SVEC4-10 cells stimulated by PDGF peptide, VEGF peptide, VEGF-A165 or PDGF-BB. CRSBP-1 ligands, including 10 μM PDGF peptide, 10 μM VEGF peptide, 5 nM VEGF-A165 and 5 nM PDGF-BB all stimulated tyrosine phosphorylation of PDGFβR after 30 minutes of stimulation in SVEC4-10 cells (Fig. 4A, lanes 2–5, respectively). By contrast, in NIH3T3 cells, which are fibroblasts that express a very low level of endogenous CRSBP-1 but a high level of endogenous PDGFβR, 10 μM concentrations of these CRSBP-1 ligands did not significantly stimulate tyrosine phosphorylation of PDGFβR (Fig. 4B, lanes 2 and 3). However, PDGF-BB is a potent
stimulator of tyrosine phosphorylation of PDGFR in both cell types (Fig. 4A, lane 5 and Fig. 4B, lane 4). PDGF-BB-stimulated tyrosine phosphorylation of PDGFR was ~tenfold greater than that stimulated by PDGF peptide or VEGF peptide (Fig. 4A, lane 5 vs lanes 2 and 3). These results are consistent with the observation that only ~10% of the total PDGFR forms complexes with CRSBP-1. CRSBP-1 ligands appear to stimulate PDGFR in the CRSBP-1–PDGFR complex.

Because VEGF and PDGF peptides are rich in basic amino acid residues, we determined whether poly-L-lysine is also capable of activating PDGFR. SVEC4-10 cells were treated with 10 μM VEGF peptide, 10 μM PDGF peptide or 20 μM poly-L-lysine (average molecular mass ~40 kDa) for 30 minutes and the level of tyrosine phosphorylation in PDGFR was determined as described above. Poly-L-lysine was only capable of slightly increasing tyrosine phosphorylation of PDGFR when compared with vehicle-only treatment in SVEC4-10 cells (Fig. 4C, lanes 1 and 4). CRS-containing peptides (PDGF peptide and VEGF peptide; Fig. 4C, lanes 2 and 3, respectively), which contain a cluster of basic amino acid residues (Boensch et al., 1995), elicited a stronger effect on

Fig. 4. See next page for legend.
CRSBP-1 regulates intercellular junctions

PDGFR tyrosine phosphorylation than poly-L-lysine did (Fig. 4C, lane 4). This suggests that certain amino acid sequences in the CRS motifs, which are rich in basic amino acid residues, are required for the optimal activity of CRSBP-1 ligands. The dose-response relationship of CRSBP-1-ligand-stimulated tyrosine phosphorylation of PDGFR was further dissected by treating SVEC4-10 cells with VEGF peptide and PDGF peptide at 0, 0.1, 2, 10, 20 μM (Fig. 4D). VEGF peptide (Fig. 4Da) and PDGF peptide (Fig. 4Db) stimulated tyrosine phosphorylation in a dose-dependent manner with EC_{50} values of ~2 μM.

To determine whether CRSBP-1 ligands stimulate and activate PDGFR, SVEC4-10 cells were pretreated with the specific PDGFR kinase inhibitor Tyrophostin AG 1296 (Kovalenko et al., 1997) before they were stimulated with CRSBP-1 ligands. Pretreatment of cells with 1 μM Tyrophostin abolished PDGFR and VEGF peptide-stimulated tyrosine phosphorylation of PDGFR (Fig. 4E, lanes 5 and 6 vs lanes 2 and 3). These results indicate that this specific PDGFR kinase inhibitor abolishes CRSBP-1-ligand-stimulated tyrosine phosphorylation and activation of PDGFR. These results also suggest that CRSBP-1 ligands probably stimulate activation and tyrosine phosphorylation (most likely autophosphorylation) of PDGFR through interaction with CRSBP-1 in the CRSBP-1–PDGFR complex. This explanation is supported by other observations: (1) CRSBP-1 ligands fail to stimulate tyrosine phosphorylation of PDGFR in cells expressing very low levels of CRSBP-1 (e.g. NIH3T3 cells), and (2) specific CRSBP-1 ligands stimulate tyrosine phosphorylation of PDGFR ~10% as efficiently as PDGF-BB in SVEC4-10 cells. In these cells, ~10% of the total PDGFR form complexes with CRSBP-1 as determined by immunoprecipitation and immunoblot analysis.

CRSBP-1 ligands stimulate tyrosine phosphorylation of VE-cadherin and β-catenin

The adhesive state of the VE-cadherin–β-catenin intercellular junction can be regulated via tyrosine phosphorylation of VE-cadherin and β-catenin (Piedra et al., 2003; Potter et al., 2005). In SVEC4-10 cells and HDLCEs stimulated with or without CRSBP-1 ligands, we determined the levels of tyrosine phosphorylation of VE-cadherin and β-catenin by western blot analysis and indirect immunofluorescence staining using tyrosine-phosphorylation-site-specific anti-VE-cadherin and anti-β-catenin antibodies. In SVEC4-10 cells, specific CRSBP-1 ligands (VEGF peptide and PDGF peptide) and putative physiological CRSBP-1 ligands (VEGF-A165 and PDGF-BB) stimulated tyrosine phosphorylation of VE-cadherin at Tyr658 and Tyr731 ~two- to threefold (Fig. 4F, lanes 2–5) more efficiently than the control vehicle treatment (Fig. 4F, lane 1). These CRSBP-1 ligands stimulated tyrosine phosphorylation of β-catenin at Tyr142 ~1.6-fold. In HDLCEs, the same CRSBP-1 ligands also stimulated tyrosine phosphorylation of VE-cadherin at Tyr658 and Tyr731 ~two- to threefold (Fig. 4G, lanes 2–5) more efficiently than the control vehicle treatment (Fig. 4G, lane 1). The other CRSBP-1 ligand HA at 100 μg/ml only slightly stimulated tyrosine phosphorylation of VE-cadherin at Tyr731, but had no effect on tyrosine phosphorylation of VE-cadherin at Tyr658 (Fig. 4G, lane 6). CRSBP-1 ligands (VEGF peptide, PDGF peptide, VEGF-A165 and PDGF-BB) stimulated tyrosine phosphorylation of β-catenin at Tyr142 by ~1.4-fold (Fig. 4G, lanes 2–5), whereas HA stimulation had no effect on β-catenin tyrosine phosphorylation (Fig. 4G, lane 6). The effects of CRSBP-1 ligands on tyrosine phosphorylation of VE-cadherin and β-catenin were also determined with indirect immunofluorescence.
staining using tyrosine-phosphorylation-site-specific antibodies to VE-cadherin and β-catenin. In SVEC4-10 cells, VEGF peptide stimulation increased the localization of tyrosine-phosphorylated VE-cadherin and β-catenin at the plasma membrane in a discontinuous punctate distribution (Fig. 4Hb,d,f vs 4Ha,c,e). These results indicate that CRSBP-1 ligands (PDGF peptide, VEGF peptide, PDGF-BB and VEGF-A165) stimulate tyrosine phosphorylation of VE-cadherin and β-catenin in SVEC4-10 cells, whereas HA, which is also a CRSBP-1 ligand, only stimulates limited tyrosine phosphorylation of VE-cadherin and does not affect β-catenin tyrosine phosphorylation in these cells. Similar results were also obtained when HDLECs were used for the same set of experiments (data not shown). Because phosphorylation of intercellular junction proteins VE-cadherin and β-catenin has a pivotal role in regulation of permeability in endothelial cell monolayers (Boccellino et al., 2005; Esser et al., 1998; Gavard and Gutkind, 2006; Piedra et al., 2003; Potter et al., 2005; Vincent et al., 2004), these results might explain why HA is weaker than other CRSBP-1 ligands in stimulating permeability in LEC monolayers and interstitial–lymphatic transit in mice (Huang et al., 2006).

PDGFβR mediates CRSBP-1-ligand-stimulated tyrosine phosphorylation of VE-cadherin
To determine whether PDGFβR is involved in CRSBP-1-ligand-stimulated tyrosine phosphorylation of VE-cadherin, we determined the effects of CRSBP-1 ligands on tyrosine phosphorylation of VE-cadherin in SVEC4-10 cells pretreated with and without Tyrphostin using SDS-PAGE and western blot analysis. SVEC4-10 cells were pretreated with or without 1 μM Tyrphostin for 30 minutes and then treated with vehicle only, 10 μM VEGF peptide, 10 μM PDGF peptide or 100 μg/ml HA. PDGF peptide (Fig. 5A, lane 2) and VEGF peptide (Fig. 5A, lane 3) stimulated phosphorylation of VE-cadherin at Tyr658 and Tyr731 ~1.6-fold and ~2.5-fold, respectively, when compared with vehicle-only treatment, whereas HA stimulated phosphorylation of VE-cadherin at only Tyr731 (Fig. 5A, lane 4). Pretreatment of SVEC4-10 cells with Tyrphostin abolished CRSBP-1-ligand-stimulated tyrosine phosphorylation of VE-cadherin (Fig. 5A, lanes 5 and 6 vs lanes 2 and 3). Tyrphostin also attenuated ligand-untimulated tyrosine phosphorylation of VE-cadherin in these cells (Fig. 5A, lane 5 vs lane 1).

PDGFβR kinase activity is crucial in CRSBP-1 ligand-induced opening of VE-cadherin–β-catenin intercellular junctions
As described above, the binding of specific CRSBP-1 ligands to CRSBP-1 triggers the molecular disassembly of VE-cadherin–β-catenin intercellular junctions and formation of microscopically visible intercellular gaps, leading to increased permeability of LEC monolayers. Because CRSBP-1 ligands stimulate tyrosine phosphorylation and activation of PDGFβR, we wished to determine whether PDGFβR is the crucial signaling protein that mediates CRSBP-1-ligand-stimulated effects in LECs. SVEC4-10 cell monolayers were pretreated with or without 1 μM Tyrphostin, followed by stimulation with vehicle only, 10 μM VEGF peptide (Fig. 5B) or 10 μM PDGF peptide (data not shown). In vehicle-stimulated cells without Tyrphostin pretreatment, VE-cadherin and β-catenin colocalized at the plasma membrane and displayed linear patterns in the plasma membrane distribution (Fig. 5Bc). Stimulation with VEGF peptide (Fig. 5Bd,e,f) and PDGF peptide (data not shown) resulted in decreased plasma membrane
distribution of VE-cadherin and disorganization of β-catenin from a linear pattern to a zigzag pattern (Fig. 5Bi vs 5Bc). However, pretreatment of SVEC4-10 cells with 1 μM Tyrophostin abolished VEGF-peptide-induced (Fig. 5Bi vs 5Bf) and PDGF peptide-induced (data not shown) disorganization (formation of zigzag patterns) of VE-cadherin–β-catenin intercellular junctions. In the control experiments, pretreatment of SVEC4-10 cells with 1 μM Tyrophostin only did not alter the distribution of either VE-cadherin or β-catenin when compared with cells without inhibitor pretreatment (Fig. 5Bi vs 5Bc). These results suggest that Tyrophostin effectively abolishes VEGF-peptide- and PDGF-peptide-induced opening of VE-cadherin–β-catenin intercellular junctions.

Specific CRSBP-1 ligands stimulate interstitial–lymphatic transit in mice in a CRSBP-1-dependent manner

We previously demonstrated that PDGF-BB and HA increase entry of large molecules from the interstitial space into lymphatic vessels (interstitial–lymphatic transit) in wild-type mice but not Crsbp1−/− mice (Huang et al., 2006), as determined by intradermal injection of high molecular weight FITC–dextran in mouse tails and by monitoring the decay in fluorescence intensity near the injection site. The results from these studies indicate that CRSBP-1 mediates PDGF-BB- and HA-induced increase in interstitial–lymphatic transit. However, it is possible that, in addition to CRSBP-1, PDGFβR is also involved in PDGF-BB-stimulated interstitial–lymphatic transit. To test this possibility, we determined the effect of PDGF peptide, a specific CRSBP-1 ligand which does not bind to PDGFβR, on interstitial–lymphatic transit, as determined by using the FITC–dextran tail injection assay (Huang et al., 2006). PDGF peptide (5 μg/20 μl) was capable of stimulating interstitial–lymphatic transit (Fig. 6Aa). It appeared to accelerate the egress of FITC–dextran more than PDGF-BB at their optimal concentrations (Fig. 6Ab). The reason that PDGF peptide is more potent than PDGF-BB is unknown. This might be due to the fact that PDGF-BB is unknown. This might be due to the fact that PDGF–BB used in the experiments is not a full-length protein and lacks the putative CRS motif. PDGF peptide and PDGF-BB might exert their different effects by binding to CRSBP-1 and PDGFβR, respectively.

To further define the effects of specific CRSBP-1 ligands on interstitial–lymphatic transit, we used the system of λ-carrageenan-induced paw edema. The system has been well characterized (Otterness and Moore, 1988; Wise et al., 2007). An acute inflammatory response induced by λ-carrageenan is characterized by an increase in vascular permeability and inflammatory cell infiltration, leading to edema formation, as a result of extravasation of fluid and protein accumulation at the inflammatory site. It can provide a quantitative measurement of alterations in the transit of fluid from the interstitial space into the lumen of lymphatic capillaries. For example, the degree of the attenuation of λ-carrageenan-induced paw edema corresponds to the magnitude of the opening of intercellular junctions in lymphatic vessels and increased interstitial–lymphatic transit of fluid. Using this assay, Crsbp1−/− mice exhibited attenuated swelling (or edema) compared with wild-type mice (Fig. 6Ba). Significant attenuation of paw swelling was observed 1 hour after injection of λ-carrageenan. A maximal attenuation of ~40% was observed 3 hour after λ-carrageenan injection (Fig. 6Ba). This suggests that constitutively increased interstitial–lymphatic transit of fluid results in attenuation of edema in Crsbp1−/− mice. This result is consistent with the observation of constitutively increased interstitial–lymphatic transit of fluid in Crsbp1−/− mice as determined using the FITC–dextran tail injection assay (Huang et al., 2006). Furthermore, specific CRSBP-1 ligands (VEGF peptide and PDGF peptide) attenuated paw swelling by ~30% in wild-type mice (Fig. 6Bb,c). By contrast, these peptides did not affect λ-carrageenan-induced swelling in Crsbp1−/− mice (Fig. 6Bd,e). The histological analysis of paw edema (Fig. 6C) revealed that PDGF peptide and VEGF peptide effectively reduced fluid accumulation in paw edema lesions in wild-type mice (Fig. 6Cb,c vs 6Ca), whereas these peptides did not significantly affect λ-carrageenan-induced paw edema in Crsbp1−/− mice (Fig. 6Ce,f vs 6Cd). PDGF peptide, VEGF peptide, HA and VEGF-A165 decreased the paw edema in a dose-dependent manner (Fig. 6Da–d, respectively). It is important to note that HA was not as potent as PDGF peptide, VEGF peptide and VEGF-A165 in decreasing paw edema at their optimal doses. These results indicate that Crsbp1−/− mice and wild-type mice treated with CRSBP-1 ligands exhibit attenuated λ-carrageenan-induced swelling in mouse paws compared with wild-type mice and wild-type mice treated with vehicle only, respectively and that this attenuation is due to opening of intercellular junctions in lymphatic vessels and increased interstitial–lymphatic transit of fluid in the paw tissues of Crsbp1−/− mice and wild-type mice treated with CRSBP-1 ligands. These results also support the contention that Crsbp1−/− mutation and CRSBP-1 ligand stimulation can cause opening of intercellular junctions in lymphatic vessels, resulting in increased interstitial–lymphatic transit of fluid.

PDGFβR kinase activity is required for CRSBP-1-ligand-stimulated interstitial–lymphatic transit

As described above, the PDGFβR kinase activity is pivotal in CRSBP-1-ligand-induced opening of lymphatic intercellular junctions. To define the role of the PDGFβR kinase activity in CRSBP-1-ligand-stimulated interstitial–lymphatic transit in wild-type mice, we determined the effect of the PDGFβR kinase activity inhibition on interstitial–lymphatic transit in mice using the system of λ-carrageenan-induced mouse paw edema. Hind paws of wild-type mice were injected with 1% λ-carrageenan and either Tyrophostin or Tyrophostin + VEGF peptide. In this experiment, two additional groups of mice were injected with 1% λ-carrageenan and 1% λ-carrageenan + VEGF peptide, as negative and positive controls, respectively. VEGF peptide stimulation was capable of attenuating λ-carrageenan-induced paw edema compared with vehicle-treated animals (Fig. 6E). Treatment with Tyrophostin alone enhanced paw edema when compared with the vehicle control; it is likely that this abolished the action of endogenous CRSBP-1 ligands under the experimental conditions. Moreover, treatment with Tyrophostin abolished VEGF-peptide-induced attenuation of paw edema (Fig. 6E). Together with the results from the cell experiments, these results suggest that inhibition of PDGFβR protein kinase activity can effectively abolish CRSBP-1-ligand-induced opening of lymphatic intercellular junctions in lymphatic vessels.

Discussion

Here, we demonstrate that CRSBP-1 forms complexes with PDGFβR in LECs. We also demonstrate that pretreatment or co-injection with the PDGFβR inhibitor abolishes the CRSBP-1-ligand-induced effects in cultured LECs and in animals. Based on our results and results published by others (Knudsen et al., 1995; Piedra et al., 2003; Potter et al., 2005; Dejana et al., 2008; Hartscock et al., 2008), we propose a model (Fig. 7) to illustrate the mechanism by which CRSBP-1 ligands induce opening of
Fig. 6. See next page for legend.
intercellular adherens junctions in LECs. This model stipulates that, in the unstimulated state, VE-cadherin mediates intercellular adhesion by forming trans homodimers with VE-cadherin from adjacent cells. VE-cadherin-mediated adhesion is maintained through the interaction of the VE-cadherin cytoplasmic tail with β-catenin and p120-catenin. These catenin interactions maintain localization of VE-cadherin at the plasma membrane. Furthermore, β-catenin couples VE-cadherin to the actin cytoskeleton by interacting with α-catenin. Our model proposes that CRBP-1, a 120 kDa disulfide-linked homodimeric membrane protein that forms complexes with the monomeric form of PDGFβR, has an important role in lymphatic vessel biology. Upon ligand binding to CRBP-1, the PDGFβR in the CRBP-1 complex is induced to form dimers or oligomers, resulting in activation and tyrosine phosphorylation (autophosphorylation) of the PDGFβR. Activated PDGFβR or PDGFβR downstream effectors (cytoplasmic protein tyrosine kinases) (Adam et al., 2010) are responsible for tyrosine phosphorylation of VE-cadherin at Tyr658 and Tyr731 and of β-catenin at Tyr142. Tyrosine phosphorylation of VE-cadherin at these residues prevents β-catenin and p120-catenin from binding to VE-cadherin. β-catenin and p120-catenin then become dissociated from VE-cadherin, resulting in destabilization and internalization of VE-cadherin. Furthermore, tyrosine phosphorylation of β-catenin hinders the association with α-catenin, causing VE-cadherin to uncouple from the actin cytoskeleton. Consequently, VE-cadherin-mediated intercellular adhesion is disrupted, leading to opening of intercellular junctions with resulting modulation of properties of lymphatic vessels (Fig. 7). This model describes how CRBP-1 mediates ligand-stimulated interstitial–lymphatic transit through regulation of VE-cadherin intercellular junctions.

The CRBP-1-mediated regulation of interstitial–lymphatic transit demonstrated here might have an important role in interstitial–lymphatic transit or trafficking of immune and carcinoma cells during immune responses and lymphatic metastasis, respectively. Immune cells such as dendritic cells initiate immune responses after they acquire antigens from the interstitial space and migrate to lymphatic vessels and lymph nodes where they activate naive T-cells. The primary tumor can be spread to other tissues and organs through blood and lymphatic systems (Ji, 2009). Lymphatic vessels appear to be used often by certain carcinomas such as breast cancer to metastasize to other tissues (Eccles et al., 2007). These immune and carcinoma cells in the interstitial space might migrate into the lumen of lymphatic vessels by secreting or using CRBP-1 ligands to open intercellular junctions in lymphatic vessels, which results in increased interstitial–lymphatic transit. The interstitial–lymphatic transit of immune and carcinoma cells has been shown to require physical contact between these cells and lymphatic vessels. These cells are also known to produce CRBP-1 ligands, such as members of the PDGF superfamily and CRS-containing cytokines (Cao, 2005; Eccles et al., 2007; Robbiani et al., 2000). The expression levels of PDGF-BB in tumors are correlated with their tendency to produce lymphatic metastasis (Cao, 2005; Cao et al., 2006). VEGF-C produced by tumors promotes tumor-cell invasion by increasing lymphatic metastasis (He et al., 2005). Antibodies against CCL19, which contains a typical CRS motif and is a putative CRBP-1 ligand, block accumulation of dendritic cells in the lymph nodes (Robbiani et al., 2000).

A well-known mechanism that governs interstitial–lymphatic flow is interstitial fluid (hydrostatic) pressure (IFP) dynamics (Aukland and Reed, 1993). In blood vascular capillary beds, plasma fluid and protein are filtrated into the interstitial compartment. Increased fluid in the interstitial compartment results in an increased hydrostatic pressure gradient between the interstitial space and the lymphatic capillary lumen. As interstitial fluid volume and hydrostatic pressure increase, the anchoring filaments that connect LECs to the collagen and elastin fibers are stretched, resulting in opening of intercellular junctions and blind ends of lymphatic capillaries as well as distention of lumens of lymphatic capillaries (Leak, 1976; Skobe and Detmar, 2000). Consequently, increased interstitial fluid enters lymphatic capillaries, thus increasing interstitial–lymphatic transit. This IFP-regulated interstitial–lymphatic transit appears to be involved in fluid homeostasis in tissues, but is not likely to be responsible for driving immune and

Fig. 6. CRBP-1 ligands stimulate interstitial–lymphatic transit in wild-type mice but not in CrsBP1–/– mice. (A) Specific CRBP-1 ligand stimulated egress of high molecular weight FITC–dextran injected intracutaneously into tails of wild-type mice. 20 μl FITC–dextran (8 mg/ml) were intradermally co-injected with vehicle only (control), human PDGF-BB (0.25 μg), or PDGF peptide (5 μg) into tails of mice (female, 6 weeks old). Five mice were used in each experimental group. After 10 minutes, photographs were taken under UV illumination. A representative sample of five mice is shown (a). The arrow indicates the injection site of FITC–dextran and PDGF-BB or PDGF peptide. FITC–dextran fluorescence near the injection site was measured (b). The fluorescence intensity near the site co-injected with vehicle only was taken as 100%. Bars represent mean ± s.d. (n=5). *P<0.05 compared with control. Scale bar: 10 μm. (B) Specific CRBP-1 ligand-induced attenuation in β-catenin-regulated paw edema in wild-type mice (a–c) but not in CrsBP1–/– mice (a,d). Wild-type and CrsBP1–/– (CrsBP1-null) mice (female, 6 weeks old) received subplantar injection of 25 μl of 1% β-catenin and vehicle only (a). Mice received subplantar injection of 25 μl of 1% β-catenin with vehicle only (b–c), 1.5 μg VEGF peptide (b,d) or PDGF peptide (c,e). After injection of β-catenin ± PDGF peptide or VEGF peptide, paw thickness was measured using calipers (Mitatoyo, Japan) at 1, 2, 3, 4, and 5 hours after the injection. The increase in paw thickness was determined by the difference between the paw volume at each time point and the basal volume. Bars represent mean ± s.d. (n=5). *P<0.05 compared with control and wild-type mice. (C) Histological analysis of mouse paw edema induced by β-catenin in the presence or absence of PDGF peptide or VEGF peptide in wild-type mice (a–c) and CrsBP1–/– mice (d–f). CrsBP1–/– mice and wild-type mice received subplantar injection of 25 μl of 1% β-catenin with vehicle only (a,d), PDGF peptide (b,e) or VEGF peptide (c,f). After 2 hours, mice were killed and paws were fixed in formalin and embedded in paraffin. The tissue slides were stained with hematoxylin and eosin (H&E). Edema was measured as increased thickness between muscularis and epidermal layers. The black line in the figure indicates the thickness of the edema. Scale bar: 2 mm. (D) Dose-dependence of CRBP-1-ligand-induced attenuation in β-catenin-regulated paw edema in wild-type mice. Wild-type mice received subplantar injection of 25 μl of 1% β-catenin alone or with varying amounts of PDGF peptide (a), VEGF peptide (b), HA (c) and VEGF-A165 (d). At 2 hours, paw thickness was measured by caliper. CRBP-1 ligand treatment attenuated paw edema. Bars represent mean ± s.d. (n=5). *P<0.05 compared with control. (E) Tyrophin abolishes CRBP-1-ligand-attenuated β-catenin-regulated paw edema in wild-type mice. Wild-type C57BL/6J mice (female, 6 weeks old and five mice for each experimental group) were separated into groups. Hind paws of mice received subplantar injection of 25 μl of β-catenin (1% in H2O). Hind paws of mice were also co-injected with vehicle only (control) (C), 0.2 μg VEGF peptide (O), 10 nmol Tyrophitin (Δ), or 10 μmol Tyrophitin with 2 μg VEGF peptide (▲). VEGF peptide treatment attenuated β-catenin-regulated paw edema. Tyrophitin treatment alone increased β-catenin-regulated paw edema. When co-injected with VEGF peptide, Tyrophitin abolished the VEGF-peptide-attenuated paw edema.
carcinoma cells into lymphatic vessels during immune responses and lymphatic metastasis. The mechanisms regulating interstitial–lymphatic transit other than that regulated by IFP are not yet clear (Aukland and Reed, 1993). Here, we provide evidence indicating a novel ligand-regulatory mechanism of interstitial–lymphatic transit.

According to the model (Fig. 7), PDGFβR in the CRSBP-1–PDGFβR complex has an important role in mediating CRSBP-1–ligand-stimulated permeability in LECs and interstitial–lymphatic transit in whole animals. This model raises several questions, including: (1) is the signaling pathway mediated by the CRSBP-1–PDGFβR complex different from that mediated by free PDGFβR (which contributes 90% of the total PDGFβR in LECs)? (2) If so, are the cellular responses also different? The signaling and cellular responses mediated by CRSBP-1–PDGFβR and by free PDGFβR appear to be different, as evidenced by the observation that PDGF-β (without a putative CRS motif) is much weaker than other CRSBP-1 ligands (PDGF peptide, VEGF peptide and VEGF-A165) in stimulating permeability in LEC monolayers and interstitial–lymphatic transit in mice. Apparently, alterations in the morphology (distended lumens) and function (constitutively increased interstitial lymphatic-transit) of lymphatic capillary vessels cannot be compensated for, or rescued by, the presence of PDGFβR and PDGF-BB in Crsbp1−/− mice. Similarly to other CRSBP-1 ligands, PDGF-BB (Abcam ab73229), which a proteolytic product of PDGF-BB lacking a typical CRS motif, is unable to effectively stimulate interstitial–lymphatic transit in Crsbp1−/− mice as determined by high molecular weight FITC–dextran mouse tail assay (Huang et al., 2006). This suggests that PDGF-BB, which lacks a typical CRS motif, stimulates interstitial–lymphatic transit through interaction with PDGFβR in the CRSBP-1–PDGFβR complex in wild-type mice.

Materials and Methods

Materials

Na[125I] (17 Ci/mg) was obtained from ICN Biochemicals (Irvine, CA). PDGF peptide (a 19-mer peptide containing the amino acid sequence of YVVRPRPGKHKHRKFKKS) and VEGF peptide (a 25-mer peptide containing the same amino acid composition of VEGF peptide but with a scrambled amino acid sequence: KSKVVRSSSKGKGRKSYGKRQOR) were synthesized by C. S. Bio Co. (Menlo Park, CA). PDGF-BB, which lacks the putative CRS motif, was obtained from Abcam (Cambridge, MA). VEGF-A165 was a gift from Daniel T. Connolly (Monsanto, St Louis, MO). FITC–dextran (molecular weights ~40,000 and ~2,000,000), DMEM, α-carrageenan, chloramine-T and other biochemical reagents were obtained from Sigma (St Louis, MO). PDGF-BB, anti-phosphotyrosine, anti-cadherin, anti-β-catenin, anti-α20-catenin, anti-VE-cadherin, anti-β-catenin, anti-p120-catenin from VE-cadherin (which trans dimerizes between cells) and subsequent internalization of VE-cadherin. α-Catenin becomes dissociated from β-catenin, leading to uncoupling of VE-cadherin junctions from actin cytoskeleton, disruption of VE-cadherin-mediated intercellular adhesion and opening of intercellular junctions in LECs and increased interstitial–lymphatic transit in intact mice. Under the conditions of CRSBP-1 downregulation or null mutation, VE-cadherin-mediated intercellular adhesion (VE-cadherin trans-dimerization) is compromised.
CRSBP-1 regulates intercellular junctions

Adam, A. F., Sharenko, A. L., Pumiglia, K. and Vincenat, P. A. (2010). Src-induced tyrosine phosphorylation of VE-cadherin is not sufficient to decrease barrier function of endothelial monolayers. *J. Biol. Chem.* 285, 7045-7055.

Allhallo, T. and Carmeliet, P. (2002). Molecular mechanisms of lymphangiogenesis in health and disease. *Cancer Cell* 1, 219-227.

Ankland, K. and Reed, R. K. (1993). Intercellular-lymphatic mechanisms in the control of extracellular fluid volume. *Am. J. Physiol.* 264, C739-C750.

Barchi, M., Friedrich, G., Fazio, M., Ferriero, L., Calderazzo, V., Baleschieri, C. and Quaglini, L. (2005). Platelet-activating factor regulates cadherin-catenin adhesion system expression and beta-catenin phosphorylation during Kaposi's sarcoma cell motility. *Am. J. Pathol.* 166, 1515-1522.

References
Journal of Cell Science 124 (8)

Boesch, C., Kuo, M. D., Connolly, D. T., Huang, S. S. and Huang, J. S. (1995). Identification, purification, and characterization of cell-surface retention sequence-binding proteins from human SK-Hep cells and bovine liver plasma membranes. J. Biol. Chem. 270, 1807-1816.

Boesch, C., Huang, S. S., Connolly, D. T. and Huang, J. S. (1999). Cell surface retention sequence binding protein-1 interacts with the v-sis gene product and platelet-derived growth factor beta-type receptor in simian sarcoma virus-transformed cells. J. Biol. Chem. 274, 10582-10589.

Cao, R., Bjornsdal, M. A., Beliga, P., Clasper, S., Garvin, S., Galter, D., Meister, B., Ikomi, F., Tritias, K., Dissing, S. et al. (2006). PDGF-BB induces intratumoral lymphangiogenesis and promotes lymphatic metastasis. Cancer Cell 6, 333-345.

Cao, Y. (2005). Direct role of PDGF-BB in lymphangiogenesis and lymphatic metastasis. Cell Cycle 4, 228-230.

Carmeliët, P., N. Toya, S., N. Kalkkinen, N. and Alitalo, K. (1996). A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. J. Biol. Chem. 271, 1853-1865.

Dejana, E., Orsenigo, F. and Lampugnani, M. G. (2008). The role of adherens junctions and VE-cadherin in the control of vascular permeability. J. Cell Sci. 121, 2115-2122.

Eccles, S., Paon, L. and Sleeman, J. (2007). Lymphatic metastasis in breast cancer: importance and new insights into cellular and molecular mechanisms. Clin. Exp. Metastasis 24, 619-636.

Esser, S., Lampugnani, M. G., Corada, M., Dejana, E. and Risau, W. (1998). Vascular endothelial growth factor induces VE-cadherin tyrosine phosphorylation in endothelial cells. J. Cell Sci. 111, 1853-1865.

Ferrara, N. (2000). Vascular endothelial growth factor: basic science and clinical progress. Endocr. Rev. 25, 581-611.

Gavard, J. and Gutkind, J. S. (2006). VEGF controls endothelial-cell permeability by promoting the beta-arrestin-dependent endocytosis of VE-cadherin. Nat. Cell Biol. 8, 1223-1234.

Hartsock, A. and Nelson, W. J. (2008). Adherens and tight junctions: structure, function and connections to the actin cytoktoskeleton. Biochem. Biophys. Acta 1778, 660-669.

He, Y., Rajantie, I., Pajusola, K., Jeltsch, M., Holopainen, T., Yla-Herttuala, S., Huang, S. S., Tang, F. M., Huang, Y. H., Liu, I.-H., Hsu, S. C., Chen, S. T. and Huang, J. S. (2005). Tyrosine phosphorylation of VE-cadherin and VE-cadherin in the control of vascular permeability. J. Biol. Chem. 280, 1863-1871.

Potter, M. D., Barbero, S. and Chernes, D. A. (2005). Tyrosine phosphorylation of VE-cadherin prevents binding of p120- and beta-catenin and maintains the cellular mesenchymal state. J. Biol. Chem. 280, 31906-31912.

Prevo, R., Banerji, S., Ferguson, D. J., Clasper, S. and Jackson, D. G. (2001). Mouse LYVE-1 is an endocytic receptor for hyaluronan in lymphatic endothelium. J. Biol. Chem. 276, 19420-19430.

Robbiano, D. F., Finch, R. A., Jager, D., Muller, W. A., Sartorelli, A. C. and Randolph, G. J. (2000). The leukotriene C4 transporter MRP1 regulates CCL19 (MIP-3beta, ELC)-dependent mobilization of dendritic cells to lymph nodes. J. Immunol. 165, 3898-3911.

Skobe, M. and Detmar, M. (2000). Structure, function, and molecular control of the skin lymphatic system Expression of vascular endothelial growth factor induces an invasive phenotype in human squamous cell carcinomas. J. Invest. Dermatol. Symp. Proc. 5, 14-19.

Teriete, P., Banerji, S., Noble, M., Blandell, C. D., Wright, A. J., Pickford Lowe, E., Mahoney, D. J., Tammi, M. I., Kahlmann, J. D., Campbell, I. D. et al. (2004). Structure of the regulatory hyaluronan binding domain in the inflammatory leukocyte homing receptor CD44. Mol. Cell 13, 483-496.

Vincent, L. S., Larkmans, L., Cheng, J., Zhang, F., Shibdo, K., Lam, G., Bompais-Vincent, H., Zhu, Z., Hicklin, D. J. et al. (2005). Combretastatin A4 phosphate induces rapid regression of tumor neovessels and growth through interference with vascular endothelial-cadherin signaling. J. Clin. Invest. 115, 2992-3006.

Vincent, P. A., Xiao, K., Buckley, K. M. and Kovalczyk, A. P. (2004). VE-cadherin: adhesion at arm’s length. Am. J. Physiol. Cell Physiol. 286, C987-C997.

Wise, L. E., Cannavaccio, R., Cravatt, B. F., Martin, B. F. and Lichtman, A. H. (2007). Evaluation of fatty acid amidases in the carrageenan-induced paw edema model. Neuropearmacology 54, 181-188.