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PECAM-1 Isoform-Specific Activation of MAPK/ERKs and Small GTPases: Implications in Inflammation and Angiogenesis

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Abstract Platelet-endothelial cell adhesion molecule-1 (PECAM-1/CD31) is expressed on the surface of endothelial cells (EC) and leukocytes. PECAM-1 plays an important role in endothelial-leukocyte and endothelial-endothelial cell–cell interactions. The anti-PECAM-1 antibody-mediated blockade of these interactions inhibits transendothelial migration (TEM) of leukocytes and angiogenesis. PECAM-1 may accommodate these processes through the regulation of cell adhesive and migratory mechanisms. How PECAM-1 regulates these dynamic processes remain unknown. Here we show that PECAM-1 transduces outside-in signals, which activate MAPK/ERKs and small GTPases. This occurs through PECAM-1-mediated formation of intracellular-signaling complexes, Shc/Grb2/SOS1 and/or Crkl/C3G, which is initiated by PECAM-1 engagement on the surface of leukocytes and/or EC. Src, SHP2, and alternative PECAM-1 pre-mRNA splicing play a regulatory role in these signaling events. Our findings reveal that PECAM-1 engagement on the cell surface can transduce ''outside-in'' signals and activate MAPK/ERKs and small GTPases, impacting both cadherin-mediated cell–cell and integrin-mediated cell–matrix interactions. Thus, we propose PECAM-1 is an important mediator of vascular barrier and regulator of leukocyte and EC adhesion and migration. J. Cell. Biochem. 98: 451–468, 2006.

Key words: CD31; cell adhesion; transendothelial migration; endothelial cells; leukocytes

PECAM-1 is a cell adhesion glycoprotein with a six-Ig-like extracellular domain, a transmembrane domain, and a cytoplasmic domain. PECAM-1 is highly expressed on the surface of endothelial cells (EC) and at moderate levels on hematopoietic cells including leukocytes and lymphocytes. PECAM-1 plays an active role during transendothelial migration (TEM) of leukocytes and angiogenesis. Antibodies to PECAM-1 and/or soluble PECAM-1 inhibit TEM of leukocytes and angiogenesis, both in vivo and in vitro [Albelda et al., 1991; Muller, 1995; DeLisser et al., 1997; Liao et al., 1997; Sheibani et al., 1997; Sheibani and Frazier, 1999; Thompson et al., 2000; Aurrand-Lions et al., 2002; Schenkel et al., 2002; Mamdouh et al., 2003]. Furthermore, PECAM-1-deficient mice exhibit abnormalities in their inflammatory and angiogenic responses to foreign body challenges [Duncan et al., 1999; Solowiej et al., 2003; Schenkel et al., 2004]. Therefore, PECAM-1 activity is important for leukocyte and/or endothelial cell–cell interactions during inflammation and angiogenesis.

Predominant translocalization of PECAM-1 is observed at EC-leukocyte borders where leukocytes extravasate through the endothelial barrier [Mamdouh et al., 2003]. This occurs through special intracellular trafficking pathways connecting PECAM-1 reservoirs to the EC-leukocyte interacting borders. Engagement of PECAM-1 on the surface of leukocytes results in activation of integrins, thereby promoting their adhesion and migration [Berman and

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Muller, 1995; Berman et al., 1996; Reedquist et al., 2000]. Functional blockade of PECAM-1 on leukocytes and/or on EC prevents disruption of endothelial adherens junctions and inhibits TEM of leukocytes [Liao et al., 1997; Thompson et al., 2000; Schenkel et al., 2002]. These data suggest that PECAM-1 is a determinant of TEM of leukocytes, potentially through the regulation of endothelial adherens junctions, and leukocyte adhesion and migration.

The TEM of leukocytes is associated with a transient disruption of the VE-cadherin–catenin complexes at the site of, and coincident with, leukocyte migration [Allport et al., 2000; Shaw et al., 2001]. These events are regulated by transient activation of Rac1 and MAPK/ERKs. The MAPK/ERKs activity is also essential during leukocyte extravasation through the endothelium [Stein et al., 2003]. During TEM, activation of MAPK/ERKs and small GTPases may result in endocytosis of VE-cadherin and modulate cellular adhesion and migration of leukocytes [Allport et al., 2000; Shaw et al., 2001; Stein et al., 2003]. PECAM-1 homophilic interactions between leukocytes and EC during TEM coincide with an occurrence of temporal and spatial internalization of VE-cadherin, a major component of the EC adherens junctions [Mamdouh et al., 2003]. Therefore, these observations suggested that PECAM-1 homophilic interactions between EC and leukocytes may activate specific-intracellular-signaling pathways, which mediate rapid and reversible opening of the vascular barrier, leukocyte adhesion, and their migration through the endothelium.

Activation of MAPK/ERKs is also essential for EC proliferation and migration in response to various proangiogenic factors including VEGF and FGF-2 [D'Angelo et al., 1995]. The important role of this signaling pathway in disruption of cadherin-mediated cell–cell interactions, specifically during invasive phase of angiogenesis, was not previously appreciated. We showed that sustained activation of MAPK/ERKs in EC results in disruption of cadherin-mediated cell-cell interactions and enhanced cell migration [Wu and Sheibani, 2003]. PECAM-1-mediated endothelial cell–cell interactions are essential during angiogenesis in vivo and in vitro [DeLisser et al., 1997; Sheibani et al., 1997]. However, the role of these PECAM-1-mediated cell–cell interactions in activation of MAPK/ERKs and regulation of cadherin-mediated cell–cell interactions during angiogenesis remains largely unknown.

PECAM-1 cytoplasmic domain contains highly conserved-signaling motifs known as the intrinsic immunoreceptor-tyrosine inhibitory motifs (ITIMs), which are located in exons 13 and 14 of PECAM-1 cytoplasmic domain [Newman and Newman, 2003]. Although the PECAM-1 cytoplasmic domain lacks enzymatic activity, the ITIMs serve to mediate its association with several intracellular-signaling proteins. Phosphorylation of PECAM-1 ITIMs by protein tyrosine kinases creates sites for binding and activation of several cellular-signaling molecules, including protein tyrosine kinase Src and protein tyrosine phosphatase SHP2, through their Src homology 2 (SH2) domains. Src and SHP2 are linked to the small GTPase Ras and MAPK/ERKs signaling [Neel and Tonks, 1997; Kodama et al., 2001; Servitja et al., 2003]. Several studies have reported interaction of PECAM-1 with Src and/or SHP2 and their potential contribution to activation of MAPK/ERKs [Sheibani et al., 2000; Osawa et al., 2002; Ilan and Madri, 2003; Newman and Newman, 2003]. However, the molecular mechanisms that govern PECAM-1-mediated activation of MAPK/ERKs and small GTPases, and the consequences of these signaling events on cellular adhesive and migratory properties require further investigation.

Here we demonstrate that engagement of PECAM-1 on the cell surface transduces outside-in signals and activates MAPK/ERKs and small GTPases. The activation of MAPK/ERKs and small GTPases occurs through PECAM-1 ITIM-mediated formation of signaling complexes, Shc/Grb2/SOS1 and/or Crkl/C3G, and impacts cell adhesive and migratory properties. The alternative splicing of PECAM-1 and, SHP2 and Src activity play an important regulatory role in these signaling events. Thus, we propose PECAM-1 is an important mediator of vascular barrier and regulator of leukocyte and EC adhesion and migration.

MATERIALS AND METHODS

Cell Cultures

Human (HEL) and mouse (MEL) erythroleukemia cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD) and maintained as recommended by the supplier. Mouse brain EC (bEND$^{+/+}$), PECAM-1-deficient

bEND (bEND$^{-/-}$), and PECAM-1-transfected MDCK cells were maintained as described previously [Sheibani et al., 2000; Rothermel et al., 2005]. Transfected MDCK cells or those incubated with specific inhibitors, PP1 (Src, 10 μM), PD98095 (MEK-1, 50 μM), calpeptin [Schoenwaelder et al., 2000] (SHP2, 100 μM) (CalBiochem, San Diego, CA), or DMSO (dimethyl sulfoxide, solvent control), were maintained as described above. The cells were photographed by using a phase microscope in a digital format.

Co-Immunoprecipitation Assays

PECAM-1 interactions with intracellular-signaling proteins and αvβ3 integrin in selected cell lines were determined by using co-immunoprecipitation assays. MDCK cells with 90% confluency were serum-starved overnight and then incubated at 37°C with regular growth medium containing 0.5 mM Na_3VO_4 (phosphatase inhibitor) for 10 min to enhance PECAM-1 phosphorylation [Lu et al., 1996]. Cells were rinsed with ice-cold Dulbecco's phosphate-buffered saline (PBS) containing 0.5 mM Na_3VO_4 and lysed with 1 ml of lysis buffer containing 10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 25 mM NaF, 1 mM Na_3VO_4, 0.5% Triton X-100, 2 mM phenylmethlysulfonyl fluoride, and protease inhibitor cocktail (Roche, Mannhem, Germany). The cell lysates were centrifuged for 10 min at 4°C to remove insoluble debris. Protein concentrations were determined by the BCA protein assay kit (Pierce, Rockford, IL). Equal amounts (~800 μg) of protein lysates were used for immunoprecipitation. The lysates were precleared with magnetic beads (Dynal Biotech, Brown Deer, WI) for 1 h at 4°C. The precleared lysates were then subjected to immunoprecipitation with anti-mouse PECAM-1 antibody (MEC13.3, BD Bioscience, San Diego, CA) for 2 h at 4°C. Following incubation, 30 μl of secondary antibody-coated magnetic beads were added to each tube and incubated for an additional 1 h at 4°C.

For co-immunoprecipitation in bEND cells, an equal number of bEND$^{+/+}$ and bEND$^{-/-}$ cells were serum-starved overnight and incubated at 37°C with regular medium containing 0.5 mM Na_3VO_4 and anti-PECAM-1 antibody (5.0 μg/ml, MEC13.3, BD Bioscience) for 30 min. The control bEND$^{+/+}$ cells were lysed (without stimulation) by addition of the lysis buffer containing the anti-PECAM-1 antibody. All the bEND cell lysates were incubated at 4°C with rocking overnight. PECAM-1 was quantitatively immunoprecipitated by the addition of 30 μl of secondary antibody-coated magnetic beads and incubated for 1 h.

For co-immunoprecipitation in HEL cells, an equal number (1×10^7) of HEL cells were collected and resuspended in 0.5 ml of serum-free medium in a 1.5-ml centrifuge tube. The cells were incubated at 37°C with 30 μl of anti-human PECAM-1 antibody (Alexis Biochemicals, San Diego, CA) coated magnetic beads for 30 min. After incubation, cells were pelleted by quick centrifugation and lysed by adding 0.5 ml of lysis buffer to each tube. The control HEL cells were immediately lysed without incubation.

The immunocomplexes prepared above were washed once with 1 ml of lysis buffer (0.25% Triton X-100) and three times with 1 ml of lysis buffer (0.1% Triton X-100). Proteins from immunoprecipitation or cell lysates (20 μg protein) from all experiments were separated on a 4%–20% SDS–polyacrylamide gel (Invitrogen) and Western blotted with primary antibodies to mouse PECAM-1 (1369, made in our laboratory; [Sheibani et al., 1999]), human PECAM-1 (SEW16, a gift of Dr. Peter J. Newman), Src, Grb2, and Crkl (Santa Cruz Biotechnology, Santa Cruz, CA), SHP2 (Up States, Charlottesville, VA), Shc, SOS1, and C3G (BD Transduction Laboratory, San Diego, CA), active ERK1/2 and ERK1/2 (Cell Signaling, Beverly, MA), αv integrin (Chemicon, Temecula, CA), or β-catenin (BD Transduction, San Jose, CA).

GTPase Activation Assays

Ras, Rac1, and Rho activity assays were performed by pull-down as previously described [Reid et al., 1996; Sander et al., 1998]. Total cell lysates and affinity precipitates were analyzed on Western blots using appropriate antibodies against pan (H-, K-, and N-) Ras, Rac1 (BD Transduction Laboratories), R-Ras, and RhoA (Santa Cruz Biotechnology).

Isolation of PECAM-1 Positive (MEL$^+$) and PECAM-1 Negative (MEL$^-$) Cells

The MEL cells spontaneously generate two populations of cells during culture: those that express PECAM-1 or those that lack PECAM-1. PECAM-1 positive and negative MEL cells were separated by fluorescence cell sorting based on intensities of PECAM-1 staining. Both types of

MEL cells are maintained in the same medium and demonstrated similar morphology and rate of proliferation. Lack of PECAM-1 expression in the MEL^- cells was further confirmed by FACS analysis.

MAPK/ERKs Assays During Endothelial-Lymphocyte Interactions

PECAM-1 positive $bEND^{+/+}$ and MEL^+ cells were utilized for PECAM-1 homophilic interaction assays and were compared with the PECAM-1-deficient $bEND^{-/-}$ and MEL^- cells. $bEND^{+/+}$ or $bEND^{-/-}$ cells were plated, allowed to reach 90% confluency ($\sim 1 \times 10^7$ cells), and then incubated with MEL^+ or MEL^- cells (2×10^7). All cells were thoroughly washed with serum-free medium prior to initiation of the experiments. Adhesion of MEL cells to EC was monitored under a phase microscope. Furthermore, this adhesion was confirmed by staining for PECAM-1 in a co-culture of MEL and bEND cells. The cells were incubated for designated periods (Fig. 1) and lysed in 0.5 ml of lysis buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 25 mM NaF, 1 mM Na_3VO_4, 1% Triton X-100, 1% NP-40, 0.1% SDS, 2 mM phenylmethlysulfonyl fluoride, and protease inhibitors). Approximately equal amounts (20 μg) of protein lysates were utilized for analysis of active ERKs and ERKs proteins.

Indirect Immunofluorescence Analysis

MDCK Cells expressing a specific PECAM-1 isoform were incubated with PD98059 (a MEK1 inhibitor), calpeptin (a SHP2 inhibitor), or DMSO on glass coverslips and fixed in 4% paraformaldehyde in PBS (20 min). Cells were stained for αvβ3 integrin (LM609, Chemicon), PECAM-1 (MEC13.3, BD Pharminegen), β-catenin and E-cadherin (BD Bioscience). The primary antibodies were detected with specific FITC-conjugated secondary antibodies (Alexis Biochemicals). Coverslips were then mounted on slides and photographed using a Ziess microscope (Ziess, Germany) in a digital format.

Analysis of Integrin Expression and Cell Adhesion Assays

Expression of cell surface integrins, α2, α3, α5, α5β1, αvβ3, αvβ5, and β1, on MDCK cells was determined by FACS analysis. All the integrin antibodies were obtained from Chemicon. Monolayers of the transfected MDCK cells were washed once with PBS containing 0.04% EDTA,

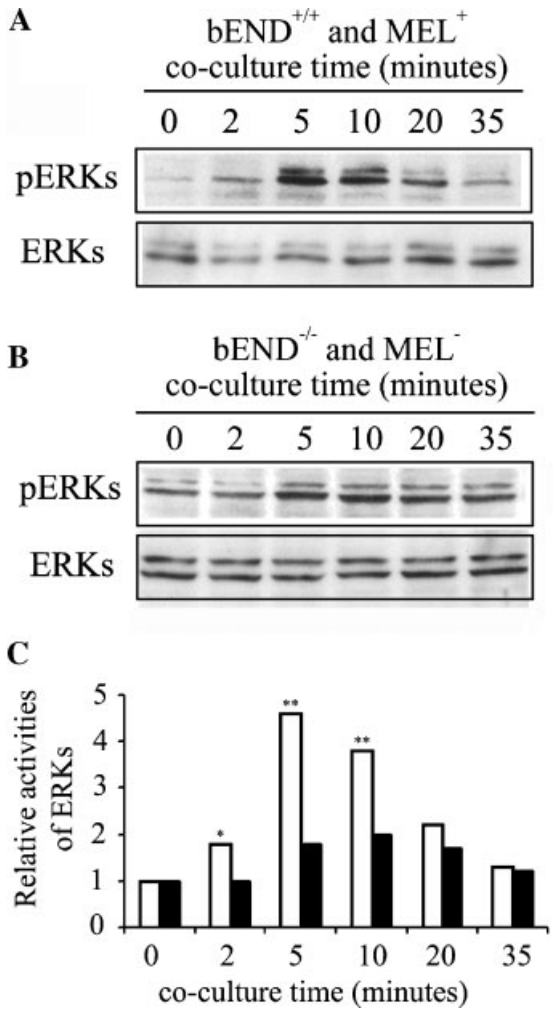

Fig. 1. Activation of MAPK/ERKs by PECAM-1 homophilic interactions. **A**: Co-culture of PECAM-1 positive $bEND^{+/+}$ and MEL^+ cells resulted in transient activation of MAPK/ERKs determined by Western blot analysis. Levels of the active ERKs (**upper panel**) and their proteins (**lower panel**) are shown separately. **B**: Co-culture of PECAM-1-deficient $bEND^{-/-}$ and MEL^- cells did not result in the activation of MAPK/ERKs. **C**: The average relative activities (fold activation) of the total ERKs from three independent experiments in **panel A** (the white box) and **panel B** (the black box). The significant differences in the ERKs activities between $bEND^{+/+}/MEL^+$ and $bEND^{-/-}$ and MEL^- co-cultures were observed at 2 ($^*P < 0.05$), 5, and 10 min ($^{**}P < 0.01$).

and incubated with 3 ml of cell dissociation solution (Sigma, St. Louis, MO) to remove the cells. Cells were fixed in 0.5 ml of 4% paraformaldehyde in TBS containing 0.1% Triton X-100 on ice for 30 min. Cells were washed three times with cold PBS and once with DMEM containing 10% FBS, blocked in 0.5 ml TBS with 1% goat serum for 20 min on ice, and incubated with antibodies to α2, α3, α5, α5β1, αvβ3, αvβ5, or β1, prepared in 0.5 ml of TBS with 1% BSA at

2 μg/ml for 30 min on ice. Following incubation, cells were washed twice with TBS containing 1% BSA, and then incubated with appropriate FITC-conjugated secondary antibody (1:200 dilution in 0.5 ml of TBS with 1% BSA; Pierce, Rockford, IL) for 30 min on ice. The stained cells were washed twice with TBS containing 1% BSA and resuspended in 0.5 ml of TBS with 1% BSA, and analyzed by FACScan caliber flow cytometer (Becton-Dickinson, Franklin Lakes, NJ).

Cell adhesion assays were performed in 96-well flat-bottom plates (Nunc Immunoplate Maxisorp, Fisher Scientific, Chicago, IL) coated with selected concentrations of matrix proteins or BSA as control. Extracellular matrix proteins, fibronectin (FN), type I collagen (Col), vitronectin (VN), and laminin (LN) (BD Biosciences) were diluted in TBS containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$ (TBS with Ca/Mg) and 50 μl (in triplicates) was used to coat the wells of the 96-well plate overnight at 4°C. The next day, plates were washed four times with 200 μl of TBS with Ca/Mg and then blocked with 200 μl of TBS with Ca/Mg containing 1% BSA for 1 h at room temperature. Plates were then washed once with 200 μl of TBS with Ca/Mg and incubated with 50 μl of TBS with Ca/Mg and 50 μl of cell suspension for 90 min at 37°C in a humidified incubator. Cell suspensions were prepared by removing the cells from tissue culture plates using 4 ml of 2 mM EDTA in TBS with 0.05% BSA, washed once with TBS, and resuspended in 25 mM HEPES pH 7.4, 150 mM NaCl, 4 mg/ml BSA at approximately 6×10^5 cells per milliliter.

Following incubation, non-adherent cells were removed by gently washing the wells with 200 μl of TBS with Ca/Mg until no cells were left in the BSA coated wells. For quantitative assessment of the number adherent cells the level of intracellular acid phosphatase was measured by lysing the adherent cells in 100 μl of lysis buffer (50 mM sodium acetate pH 5.0, 1% Triton-X100, 6 mg/ml *p*-nitrophenyl phosphate) and incubating at 4°C overnight. The next day the reaction was neutralized by adding 50 μl of 1 M NaOH and the absorbance at 405 nm was determined using an ELISA plate reader.

Densitometry Analysis

The relative intensities of protein bands were determined using the Molecular Dynamic Storm 860 Scanner and Image Quant Software (Amersham, New Piscataway, NY). Standard curves were run to ensure the data obtained were in the linear range of the curve. In addition, all values were normalized to their respective loading control lanes. The fold activation in each assay was calculated through the value of each sample or each time point relative to their control value; that is, vector control or zero time point.

Statistical Analysis

Data are expressed as means ± SE of n determinations. Results were analyzed by two-tailed Student's *t*-test. *P*-values <0.05 were considered significant.

RESULTS

PECAM-1 Engagement-Mediated Signaling in EC and Hematopoietic Cells

To address whether PECAM-1 homophilic interactions can activate MAPK/ERKs, we utilized PECAM-1-deficient ($bEND^{-/-}$) and wild-type ($bEND^{+/+}$) mouse brain EC, and murine erythroleukemia (MEL) cells (a B lymphocytic cell line). B lymphocytes express ligands of inflammatory adhesion molecules on their surface and extravasate through the endothelium. Adhesion of B lymphocytes to EC is normally dependent upon chemical stimulations, such as cytokine stimulation, which influences the intracellular-signaling pathways [Price and Bos, 2004]. The MEL cells spontaneously generate two populations of cells in culture, those that are PECAM-1 positive and those that are not. We have established a MEL^+ cell line, which expresses PECAM-1, and a MEL^- cell line, which lacks PECAM-1 expression. We have observed that the MEL cells with or without PECAM-1 similarly adhere to EC. This indicates involvement of special adhesion molecules, including PECAM-1, in MEL cells adhesion to EC.

We next determined whether interaction of EC and MEL cells activates MAPK/ERKs. Activation of MAPK/ERKs in these cells was determined by Western blotting with phosphorylated ERK-specific antibodies. The MAPK/ERKs activation was detectable within 2 min following the co-incubation of PECAM-1 positive EC ($bEND^{+/+}$) and MEL cells (MEL^+), reaching maximum levels at 5 min and returning to basal levels after 20 min (Fig. 1A). Under similar conditions, interactions between PECAM-1-deficient EC

(bEND$^{-/-}$) and lymphocytes (MEL^{-}) resulted in adhesion of the MEL^{-} cells to bEND$^{-/-}$ cells, but in contrast, did not significantly activate MAPK/ERKs (Fig. 1B). Quantification of the results indicated that the activation of MAPK/ERKs was significantly enhanced in the bEND$^{+/+}$/MEL^{+} co-cultures compared to the bEND$^{-/-}$/MEL^{-} co-cultures at 2 min ($^{*}P < 0.05$), 5 min ($^{**}P < 0.01$), and 10 min ($^{**}P < 0.01$) (Fig. 1C). Other co-culture combinations of these cells failed to significantly activate MAPK/ERKs (similar to Fig. 1B; not shown). Thus, these results indicated that interaction of these cells, perhaps through PECAM-1 homophilic interactions, activates MAPK/ERKs, presumably in both cell types.

To further elucidate the mechanism of PECAM-1-mediated MAPK/ERKs activation in EC and hematopoietic cells, we set out to identify the potential-signaling molecules that associate with PECAM-1. We hypothesized that engagement of PECAM-1 on the cell surface (Fig. 2A) may result in recruitment of intracellular-signaling molecules containing SH2 domains, such as Src and SHP2 [Newman and Newman, 2003], which regulate the tyrosine phosphorylation of PECAM-1. Phosphorylation of PECAM-1 ITIMs by Src may create active docking sites for sequential associations of other proteins containing SH2 domains, including Shc, Grb2, and Crkl, which are involved in the activation of MAPK/ERKs-signaling pathways [Buday and Downward, 1993; Pawson, 1995; de Vries-Smits et al., 1995; Lamorte et al., 2002].

To address these possibilities, serum-starved bEND cells were stimulated with the regular growth medium containing anti-PECAM-1 antibodies and Na_3VO_4 (a phosphatase inhibitor). Serum stimulation elevates the levels of

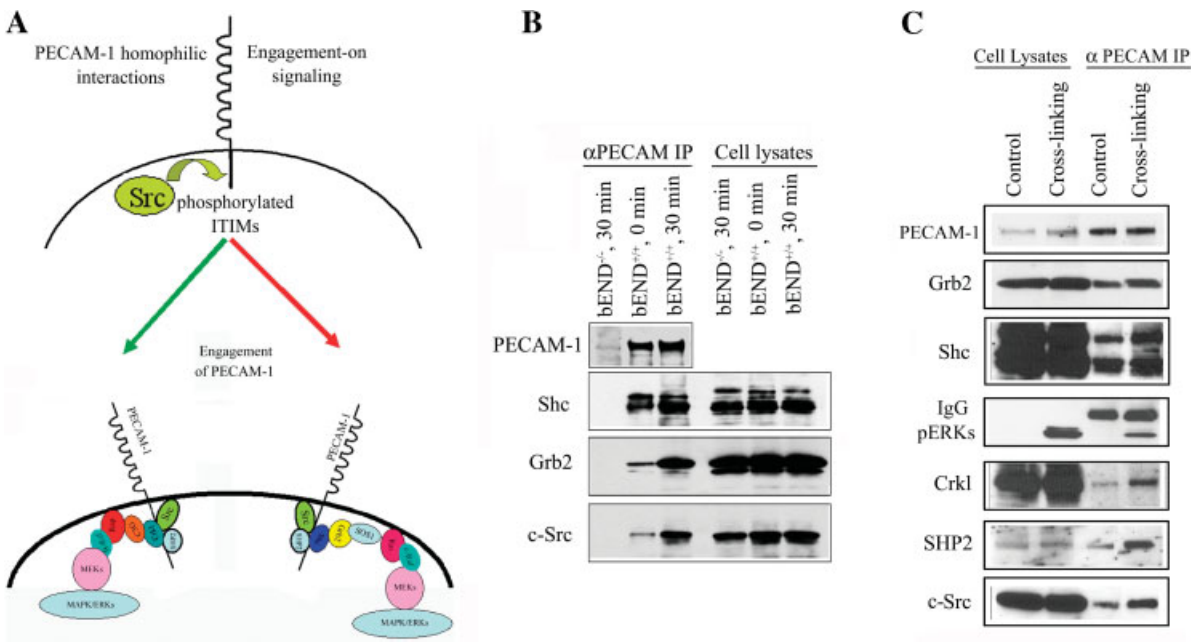

Fig. 2. PECAM-1-mediated ''engagement-on'' signaling in hematopoietic and endothelial cells. **A**: Diagram depicting the mechanisms by which engagement of PECAM-1 mediates activation of MAPK/ERKs. PECAM-1 homophilic interactions between monocyte and EC recruit Src, which triggers sequential molecular associations. PECAM-1 may activate Ras through its interactions with Shc and Grb2, recruiting SOS1 and/or activate Rap through its interaction with Crkl, recruiting C3G. The active Ras and Rap can further result in activation of MAPK/ERKs in EC and hematopoietic cells. **B**: PECAM-1 can interact with the SH2 domain-containing proteins in EC. Engagement of PECAM-1 on the surface of EC by anti-PECAM-1 antibody enhanced its association with the intracellular proteins Src, Shc, and Grb2, which were assayed by co-immunoprecipitation. The serum-starved bEND cells were lysed without stimulation (bEND$^{+/+}$, 0 min) or incubated with regular medium containing anti-PECAM-1 antibody (MEC13.3) in the presence of Na_3VO_4 (bEND$^{+/+}$, 30 min or bEND$^{-/-}$, 30 min). Blots of cell lysates (right panel) show similar levels of all proteins under each experimental condition. **C**: PECAM-1 can interact with the SH2 domain-containing proteins in hematopoietic cells. Cross-linking of PECAM-1 on the surface of human HEL cells by anti-PECAM-1 antibody (Alexis) coated magnetic beads enhanced its association with the intracellular proteins, Src, SHP2, Grb2, Shc, pERKs, Crkl, and active ERK2. The cells were incubated with the magnetic beads for 0 (control) or 30 (cross-linking) min. The PECAM-1-associated proteins were examined by co-immunoprecipitation assays. The Western blot results were analyzed on the same membrane. These experiments were repeated three times with identical results.

active tyrosine kinases such as Src. PECAM-1 antibody promotes further PECAM-1 engagement on the surface of EC. The Na_3VO_4 inhibits phosphatase activities and prevent dephophorylation of PECAM-1 [Lu et al., 1996], thereby stabilizing PECAM-1-signaling complexes. These conditions, therefore, promote PECAM-1 tyrosine phosphorylation within its intrinsic ITIM domains [Osawa et al., 2002] initiating PECAM-1 signaling. Our co-immunoprecipitation assays under these conditions showed that Shc, Grb2, and Src associate with PECAM-1 in the wild-type, but not PECAM-$1^{-/-}$, bEND cells (Fig. 2B). This association was enhanced by incubation of the bEND cells with anti-PECAM-1 antibody in the presence of Na_3VO_4 and serum as expected.

To further demonstrate that PECAM-1-mediated signaling events also occur in hematopoietic cells, we used human erythroleukemia (HEL) cells to characterize the association of the SH2 domain-containing proteins with PECAM-1. HEL cells express PECAM-1 isoforms with intact-ITIM domain (containing exons 13 and 14) at higher frequencies compared to mouse EC [Sheibani et al., 1999] and hematopoietic cells including MEL^+ (Wang and Sheibani [2002]; our unpublished data). This would enhance association of PECAM-1 with SH2 domain containing signaling molecules. PECAM-1 was engaged on the surface of hematopoietic cells by using anti-PECAM-1 antibody-coated magnetic beads. Antibody cross-linking of PECAM-1 mimics the mechanical force produced by EC and leukocyte interactions during TEM. As indicated above, these conditions promote PECAM-1 tyrosine phosphorylation within its intrinsic ITIM domains [Osawa et al., 2002] and initiates PECAM-1 signaling. Antibody engagement of PECAM-1 on the surface of HEL cells enhanced PECAM-1 association with the SH2 domain-containing proteins, Shc, Grb2, Crkl, Src, and SHP2, and resulted in MAPK/ERKs activation (Fig. 2C). Interestingly, active ERK2 also associated with PECAM-1 in the HEL cells (Fig. 2C).

Induction of Epithelial-Mesenchymal Transition (EMT) by PECAM-1-Mediated Activation of MAPK/ERKs

Activation of MAPK/ERKs is a downstream consequence of the formation of PECAM-1-signaling macrocomplexes and occurs in both EC and hematopoietic cells. This activation of MAPK/ERKs may occur through interactions of PECAM-1 cytoplasmic domain with Shc-Grb2-SOS/Crkl-C3G and activation of small GTPases (Fig. 2A). PECAM-1-mediated signaling in endothelial and hematopoietic cells is dependent upon its engagement (Figs. 1 and 2), thereby occurring in a spatiotemporal manner. Furthermore, PECAM-1 signaling in these cells may be influenced by the relative presence of alternatively spliced PECAM-1 isoforms, with and without an intact-ITIM domain. Therefore, the study of PECAM-1 signaling in EC and hematopoietic cells has been very difficult. To overcome these difficulties, we had utilized Madin–Darby canine kidney (MDCK) epithelial cells as a model system to study PECAM-1 isoform specific-mediated MAPK/ERKs activation [Sheibani et al., 2000]. The MDCK cells do not express PECAM-1. The most notable feature of MDCK cells is that activation of MAPK/ERKs in these cells results in epithelial to mesenchymal transition [EMT; Klemke et al., 1997; Sheibani et al., 2000]. EMT is a fundamental process governing morphology, adhesion, and migration during development. Therefore, MDCK cells may provide a suitable system to study PECAM-1-mediated activation of MAPK/ERKs and its impact on cell adhesion and migration.

Exon 15 of PECAM-1 cytoplasmic domain interacts with the mesenchymal transcription factor, Stat5a and β-catenin [Ilan and Madri, 2003], and may impact EMT in MDCK cells. The main focus of this work was to dissect the signaling function of PECAM-1 intact-ITIM domain containing exons 13 and 14. Therefore, to exclude the potential effects of exon 15 in PECAM-1 signaling events we used Δ15 PECAM-1 isoform with an intact-ITIM domain instead of full-length PECAM-1 (contains exon 15). Furthermore, full-length PECAM-1 normally represents a small percentage of PECAM-1 isoforms detected in murine endothelium [Sheibani et al., 1999]. MDCK cells were stably transfected with the two predominant mouse PECAM-1 isoforms detected in vivo [Sheibani et al., 1999, 2000; Wang and Sheibani, 2002], the Δ14&15 (lacks exons 14 and 15) and Δ15 (lacks exon 15) (Fig. 3A). Exons 13 and 14 comprise the intact PECAM-1 ITIM domain, which provide docking sites for selected SH2-containing signaling molecules including SHP2 and Src. The Δ15 PECAM-1 isoform contains the intact-ITIM domain while the Δ14&15

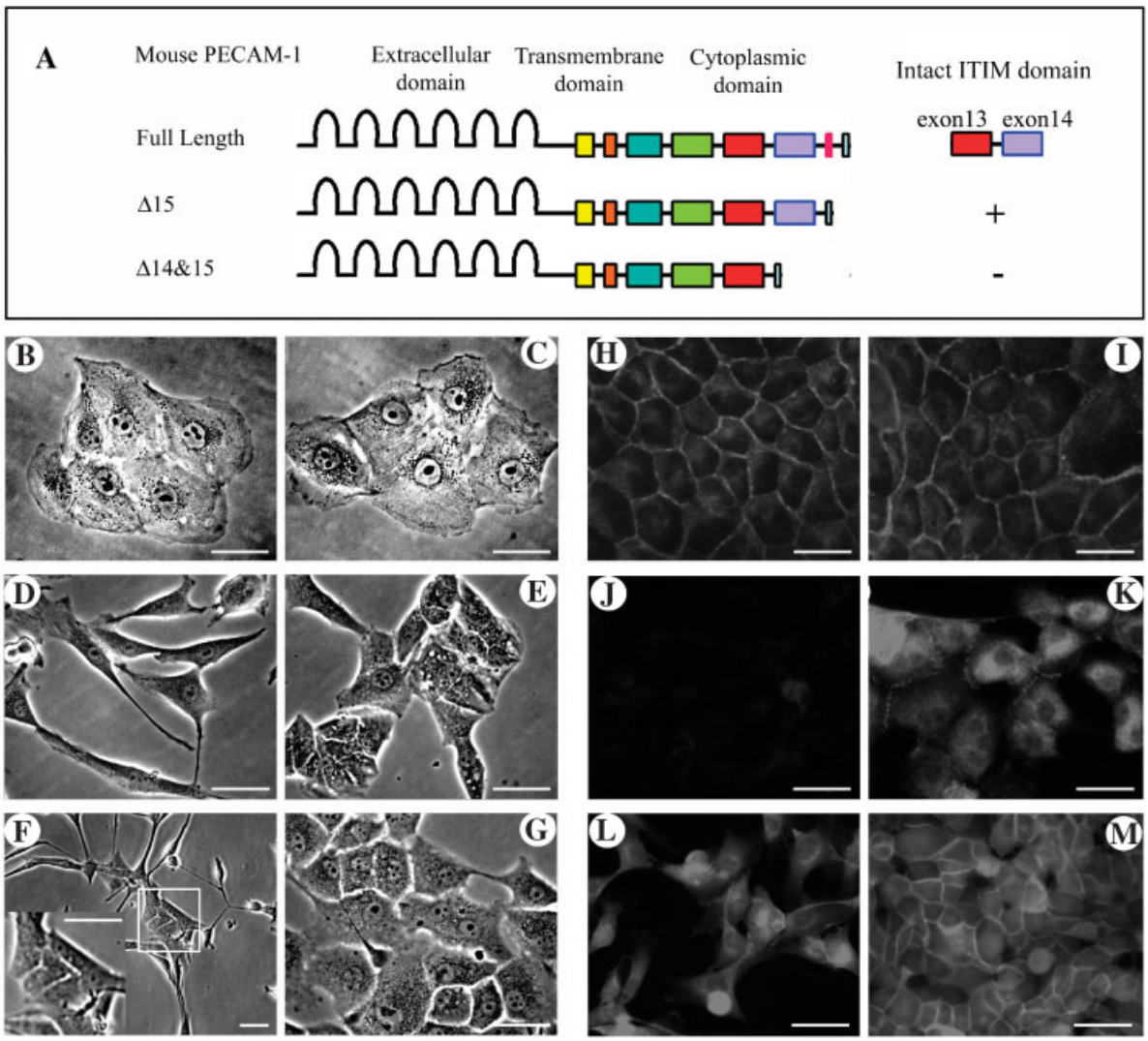

Fig. 3. Characteristics of the MDCK cells expressing a specific PECAM-1 isoform. **A**: Schematic representation of mouse PECAM-1 isoforms, including the full-length PECAM-1 and alternatively spliced PECAM-1 isoforms, Δ15 lacking exon 15 (pink), and Δ14&15 lacking exons 14 (blue) and 15 (pink). The PECAM-1 ITIM domain includes exons 13 (red) and 14 (blue). Therefore, the Δ15 PECAM-1 isoform contains intact ITIM domain, while the Δ14&15 PECAM-1 does not. Figures B, C, and D are phase micrographs of the MDCK cells stably transfected with vector (**B**), Δ14&15 PECAM-1 (**C**), or Δ15 PECAM-1 (**D**). The scattering phenotype was observed in the Δ15 PECAM-1 cells. **Panels E**, **F**, and **G** are phase micrographs of the Δ15 PECAM-1 cells incubated with MEK1 inhibitor PD98059 (50 μM, E), Src inhibitor PP1 (10 μM, F), SHP2 inhibitor calpeptin (100 μM, G), or DMSO solvent control (no change, not shown). Partial image in the middle of Figure 3F was also shown in higher magnification (40×). The phase micrographs were captured at 96 h after the addition of various inhibitors. Scale bars (—) for B–G, 30 μm. **Panels H**, **I**, **J**, and **K** are the vector (H), Δ14&15 PECAM-1 (I), and Δ15 PECAM-1 cells incubated with solvent DMSO (J) or calpeptin (K), and stained for E-cadherin localization by indirect immunofluorescence. **Panels L** and **M** are the Δ15 PECAM-1 cells incubated with solvent DMSO (L) or PD98059 (M), and stained for β-catenin localization by indirect immunofluorescence. Please note that the parental morphology of the Δ15 PECAM-1 cells is restored by incubation with PD98059, calpeptin, or PP1. Bar: 40 μm. These experiments were repeated at least five times with identical results.

PECAM-1 isoform (lacks exon 14) does not (Fig. 3A).

Expression of these two PECAM-1 isoforms had significantly different effects on the morphology and adhesive properties of MDCK cells (Fig. 3B,C,D; Sheibani et al. [2000]). Cells expressing the Δ15 PECAM-1 isoform (Fig. 3D) lacked the closely packed cobblestone epithelial morphology observed in parental or vector control cells (Fig. 3B,C). These cells were also more disorganized and scattered. In contrast, the Δ14&15 cells (Fig. 3C) had morphology similar to the vector or parental cells. Incubation of the Δ15 PECAM-1 cells with the MEK-1

inhibitor PD98059 was sufficient to restore parental morphology and cell–cell adhesions (Fig. 3E). In addition, incubation of the Δ15 PECAM-1 cells with PP1 (a Src inhibitor) resulted in an elongated cell morphology and enhanced cell–cell contacts (Fig. 3F). Inhibition of SHP2 by calpeptin in Δ15 PECAM-1 cells also restored the parental cell morphology (Fig. 3G). As expected, the Δ15 PECAM-1 cells lost cadherin-mediated cell–cell interactions, compared to the Δ14&15 PECAM-1 and vector cells (Fig. 3H,I,J). Inhibition of protein tyrosine phosphatase SHP2 (Fig. 3K) or MAPK/ERKs (Fig. 3L,M) resulted in partial or complete restoration of cadherin/catenin-mediated cell–cell interactions. Incubation of vector or Δ14&15 PECAM-1 cells with these inhibitors had no effect on their morphology (not shown). Therefore, Src, SHP2, and MAPK/ERKs activities are essential for the PECAM-1-mediated disruption of adherens junctions in an isoform-specific manner.

Association of PECAM-1 With Intracellular-Signaling Molecules and Activation of Small GTPases and MAPK/ERKs

To reconfirm that expression of Δ15 PECAM-1 in MDCK cells results in the activation of MAPK/ERKs, we examined the levels of active MAPK/ERKs in the MDCK cells. Western blot analysis showed that activation of MAPK/ERKs was dramatically upregulated in the Δ15 PECAM-1 cells (Fig. 4A; Sheibani et al. [2000]). In the vector and Δ14&15 PECAM-1 cells, the active MAPK/ERKs were significantly lower. To further delineate the down-stream effectors of PECAM-1 signaling, we utilized co-immunoprecipitation assays to identify the signaling molecules that associate with PECAM-1 (Fig. 4B). We found that Shc and Grb2 associated with Δ15 PECAM-1 and recruited SOS1 in the Δ15 PECAM-1-transfected cells (Fig. 4B), as shown in EC and hematopoietic cells (Fig. 2B,C). These associations were abrogated in the Δ14&15 PECAM-1-expressing cells (Fig. 4B), suggesting that the formation of Δ15 PECAM-1-Shc-Grb2-SOS1 complexes requires amino acids encoded by exon 14. The Δ15 PECAM-1-mediated molecular interactions further resulted in activation of Ras GTPases (4.6-fold increase, Fig. 4C). The Ras family is comprised of four main members, K-, H-, N-, and R-Ras. Elevation of GTP-Ras level was observed in the Δ15 PECAM-1 cells compared to the Δ14&15 PECAM-1 or vector control cells. The Ras pan-antibody, used in Figure 4C for Western blotting, reacts with K-, H-, and N-Ras but not with R-Ras. Using an R-Ras-specific antibody revealed that the GTP-R-Ras level was slightly decreased in the Δ15 PECAM-1 cells compared to the Δ14&15 PECAM-1 or vector control cells (Fig. 4D).

SOS1 is also a guanine nucleotide-releasing factor for Rac1 GTPase [Innocenti et al., 2002]. Therefore, its association with Δ15 PECAM-1 may impact Rac-signaling pathways. We next examined whether PECAM-1 influences Rac1 GTPase activity in MDCK cells by determining active GTP-Rac1 protein levels. Western blot analysis showed that GTP-Rac1 level was threefold higher in the Δ15 PECAM-1 cells compared to the Δ14&15 PECAM-1 or the vector control cells (Fig. 4E). The total level of Rac1 was not significantly altered. Activation of Rac1 is known to mediate downregulation of RhoA activity [Sander et al., 1999]. We found that the activation of Rac1 in Δ15 PECAM-1 cells was associated with a fivefold decrease in RhoA activity (Fig. 4F).

PECAM-1 engagement on EC activates Rap GTPases, thereby regulating integrin activities [Reedquist et al., 2000]. However, the events that lead to activation of Rap and integrins by PECAM-1 remain unknown. Given the role of Crkl and C3G in regulation of cell adhesive and migratory properties [Reedquist et al., 2000; Ohba et al., 2001; Lamorte et al., 2002; Voss et al., 2003; Price and Bos, 2004], it was reasonable to speculate that these molecules may be involved in PECAM-1-mediated signaling. We found an association between PECAM-1 and Crkl in HEL cells (Fig. 2B). Likewise, in the transfected MDCK cells, co-immunoprecipitation studies demonstrated that Crkl and C3G associated with Δ15, but not Δ14&15 PECAM-1 (Fig. 4B). These data suggest that PECAM-1 modulates integrin activities potentially through its recruitment of Crkl-C3G and activation of Rap GTPases.

Regulatory Roles of Src and SHP2 in PECAM-1 Signaling

Upregulation of Src or SHP2 activities results in MAPK/ERKs activation [Neel and Tonks, 1997; Kodama et al., 2001; Servitja et al., 2003]. Src and SHP2 associate with PECAM-1 (Figs. 2 and 4) and may impact PECAM-1 signaling, thereby resulting in the morphological changes

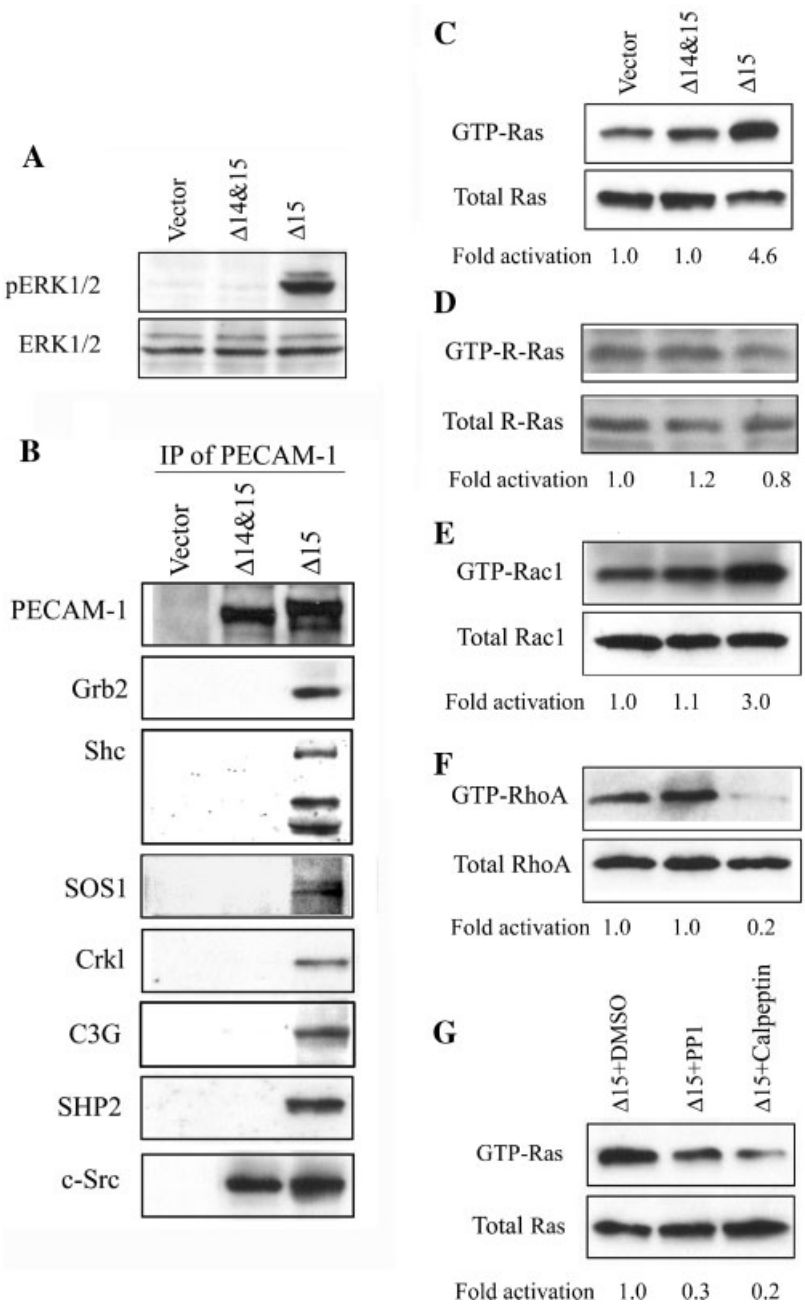

Fig. 4. PECAM-1 ITIM-mediated signaling and molecular interactions. **A**: Levels of active ERKs in MDCK cells were analyzed by Western blot. **B**: Formation of PECAM-1 isoform-dependent adapter protein complexes. PECAM-1-associated proteins were assayed by co-immunoprecipitation. The precipitates were analyzed by Immunoblotting. The Western blot results were analyzed on the same membrane. Therefore, these results suggest that Δ15 PECAM-1 recruits Grb2, Shc, SOS1, Crkl, C3G, SHP2, and Src to form macromolecular complexes. **C**: Levels of GTP-Ras (pan antibodies to K-, H-, and N-Ras) in MDCK cells were analyzed by Western blot. **D**: Levels of GTP-R-Ras in the MDCK cells were analyzed by Western blot using a R-Ras-specific antibody. **E**: Levels of GTP-Rac1 in the MDCK cells were analyzed by Western blot. **F**: Levels of GTP-RhoA in the MDCK cells were analyzed by Western blot. **G**: Western blot showed that levels of GTP-Ras in the Δ15 PECAM-1 cells were influenced by inhibition of Src (PP1) and SHP2 (calpeptin), respectively. Please note that activation of Ras by PECAM-1 was inhibited by PP1 and calpeptin, compared to solvent control. These experiments were repeated at least three times with identical results. The fold activations were determined as described in Materials and Methods and is indicated in the bottom of appropriate panels.

(Fig. 3F,G). Since little is known regarding the role of Src and SHP2 in PECAM-1 signaling, we were interested in understanding their functions in PECAM-1-mediated signal transduction. Association of Src with both Δ14&15 and Δ15 PECAM-1 suggested that Src association with PECAM-1 is exon 14-independent (Fig. 4B). In contrast, SHP2 associated with Δ15 PECAM-1 but not with Δ14&15 PECAM-1 (Fig. 4B). The closely related phosphatase SHP1 did not associate with Δ15 PECAM-1 in these cells (results not shown). Therefore, PECAM-1 preferably associates with SHP2 in an exon 14-dependent manner, while its association with Src is exon 14 independent.

To determine the important role of Src and SHP2 in PECAM-1-mediated Ras activation, we utilized PP1 (Src) or calpeptin [Schoenwaelder et al., 2000] (SHP2) inhibitors to determine their effect on the levels of active Ras. Both PP1 and calpeptin significantly inhibited activation of Ras in Δ15 PECAM-1 cells by threefolds and fivefolds, respectively (Fig. 4G). Inhibition of MAPK/ERKs, SHP2, or Src activity in these cells restored cell–cell adhesions (Fig. 3F,G,K). Together, these data suggest that activities of Src and SHP2 are essential for PECAM-1-mediated activation of Ras and MAPK/ERKs pathways.

Regulation of Integrin-Mediated Cell Adhesive Properties by PECAM-1 Signaling

Since PECAM-1 engagement upregulates integrin activities in leukocytes and EC [Berman and Muller, 1995; Berman et al., 1996; Reedquist et al., 2000; Wong et al., 2000], we were interested in understanding the role of PECAM-1 signaling in modulation of integrin-mediated cell–matrix adhesion in our model system (Fig. 5). Cell–matrix adhesion assays showed that expression of Δ15 PECAM-1 in the MDCK cells altered their ability to adhere to selected ligands. In contrast, Δ14&15 PECAM-1-transfected cells showed adhesive properties similar to those of control cells (Fig. 5A–D, and Table I). Adhesion of Δ15 PECAM-1 cells to

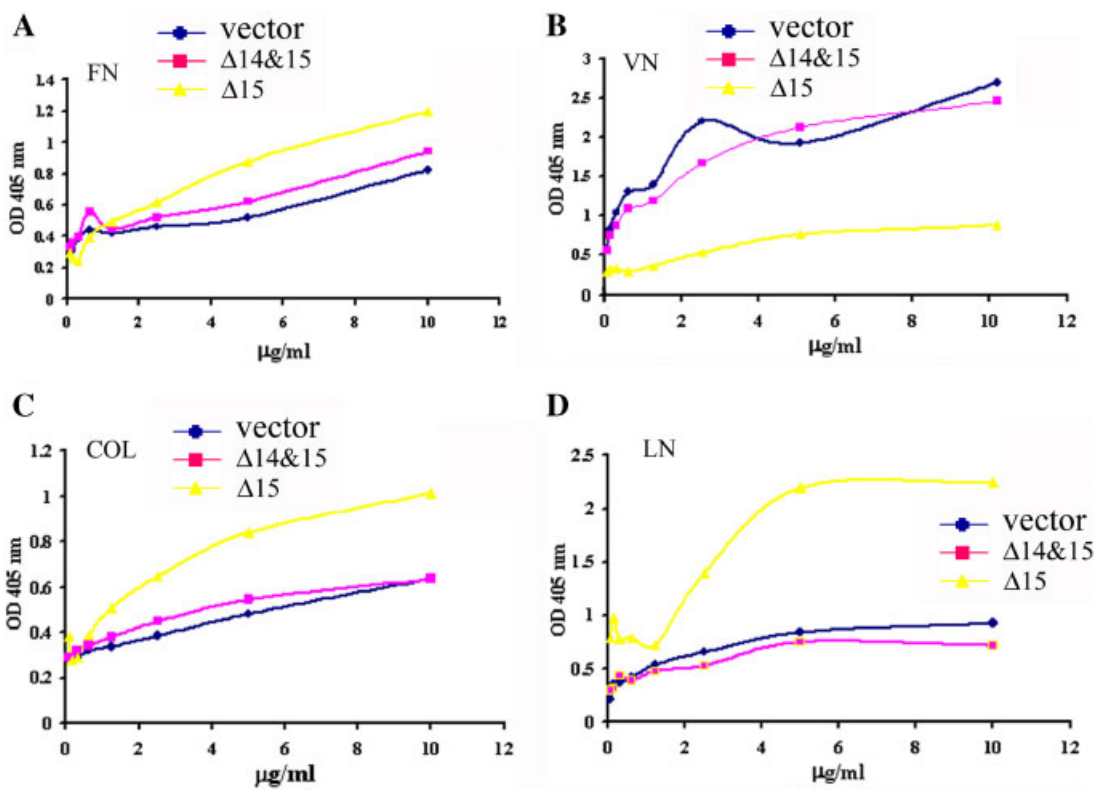

Fig. 5. Alterations in cell adhesion and integrin expression of MDCK cells expressing a specific PECAM-1 isoform. Adhesion assays were used to illustrate the effects of specific PECAM-1 isoforms on MDCK cell adhesion to matrix proteins, fibronectin (FN; **A**), vitronectin (VN; **B**), collagen (Col; **C**), or laminin (LN; **D**). These experiments were repeated five times with identical results. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE I. Adhesion of the MDCK Cells Expressing a Specific PECAM-1 Isoform to Various Matrix Proteins

Cell lines	LN	FN	VN	Col
Vector	+	++	+++++	++
Δ15	+++++	+++	+	++++
Δ14&15	+	++	+++++	++

Summary of the adhesion assays from Figure 5 (+ very weak to +++++ very strong). Please note that the vector and Δ14&15 PECAM-1 cells adhered strongly to vitronectin (VN) but not other substrates, while Δ15 PECAM-1 adhered strongly to fibronectin (FN), collagen (Col), and laminin (LN), but not VN.

fibronectin (a ligand of α5β1), collagen (a ligand of α2β1 and α1β1), and laminin (a ligand of α3β1 and α6β1) was enhanced. Flow cytometry analysis of surface integrin expression revealed that the expression of Δ15 PECAM-1 in MDCK cells did not dramatically alter the expression level of integrins with the exception of α3 and αvβ3 integrins (Table II). Δ15 PECAM-1 cells expressed relatively lower levels of α3 integrin. However, this decreased expression did not result in a reduction in its laminin binding activity in the Δ15 PECAM-1 cells. In contrast, Δ15 PECAM-1 cells expressed twice as much αvβ3 integrin on their surface, but poorly adhered to vitronectin (the main αvβ3 ligand).

The αvβ3 integrin interacts with PECAM-1. However, the significance of these interactions remains unknown. Therefore, the ability of PECAM-1 isoforms to selectively inhibit αvβ3 integrin interaction with vitronectin was further investigated. We examined the distribution of αvβ3 integrin in MDCK cells, which express a specific PECAM-1 isoform. In vector control and Δ14&15 PECAM-1 cells, αvβ3 integrin localized to focal adhesions at the migrating fronts, as well as at the peripheral sites of cell–cell contacts, where PECAM-1 is also known to localize (Fig. 6A, upper and middle panels, and Fig. 6C). In contrast, the distribution of αvβ3 integrin was altered in cells expressing Δ15 PECAM-1 (Fig. 6A, the lowest panel). The αvβ3 integrin colocalized apically with PECAM-1 and was absent from focal adhesions, even though these cells formed numerous vinculin-positive focal adhesions (Fig. 6B). Inhibition of MAPK/ERKs by MEK1 inhibitor PD98059 resulted in restoration of cell–cell adhesions (Fig. 3C,M) and junctional localization of PECAM-1 in the Δ15 PECAM-1 cells (Fig. 6D). This also facilitated the relocalization of αvβ3 integrin to focal adhesions in Δ15 PECAM-1 cells (results not shown).

TABLE II. Expression of Integrins in the MDCK Cells Expressing a Specific PECAM-1 Isoform

Integrins	Vector	Δ15	Δ14&15
α2	19 ± 3	18 ± 2	23 ± 2
α3	72 ± 6	23 ± 2	56 ± 4
α5	36 ± 4	34 ± 3	30 ± 2
α5β1	9 ± 1	7 ± 0.5	10 ± 0.5
αvβ3	64 ± 4	134 ± 6	70 ± 4
αvβ5	11 ± 1	12 ± 1	11 ± 1
β1	129 ± 8	110 ± 6	101 ± 6

The levels of α2, α3, α5, α5β1, αvβ3, αvβ5, and β1 integrins on the MDCK cells were analyzed by FACS analysis. The mean fluorescence intensities from three separate experiments are shown. Please note that expression levels of most integrins are similar among the cell lines with the exception of α3 and αvβ3 integrins. The Δ15 PECAM-1 cells express approximately twice as much αvβ3 integrin and half as much α3 integrin on their cell surface compared to the Δ14&15 PECAM-1 or vector control cells.

The PECAM-1 isoform-specific interaction with αvβ3 integrin was further demonstrated by co-immunoprecipitation assays (Fig. 6E). The Δ15, but not Δ14&15 PECAM-1, associated with the αv integrin, and potentially with β3 integrin. In addition, Western blot analysis showed that the Δ15 PECAM-1 cells expressed higher levels of αv integrin compared to the vector or Δ14&15 cells (Fig. 6F). This is consistent with our FACS results, which showed that Δ15 PECAM-1 cells expressed higher levels of αvβ3 integrin (Table II). Therefore, isoform-specific association of PECAM-1 with αvβ3 integrin may regulate its affinity and/or avidity for vitronectin.

DISCUSSION

Transmembrane adhesion molecules can both physically bridge and biochemically transduce signals among interacting cells. PECAM-1 (CD31) is involved in endothelial-endothelial and endothelial-leukocyte cell–cell interactions during angiogenesis and inflammation. Cell surface engagement of PECAM-1 can generate both outside-in and inside-out signals through recruitment of signaling proteins. PECAM-1 also potentiates the calcium burst of EC in response to oxidative stress [Ji et al., 2002] and modulates integrin affinity and avidity [Reedquist et al., 2000]. Here we demonstrate that PECAM-1 engagement on the cell surface can activate MAPK/ERKs and small GTPases-signaling pathways through formation of isoform-specific signaling complexes. The specific

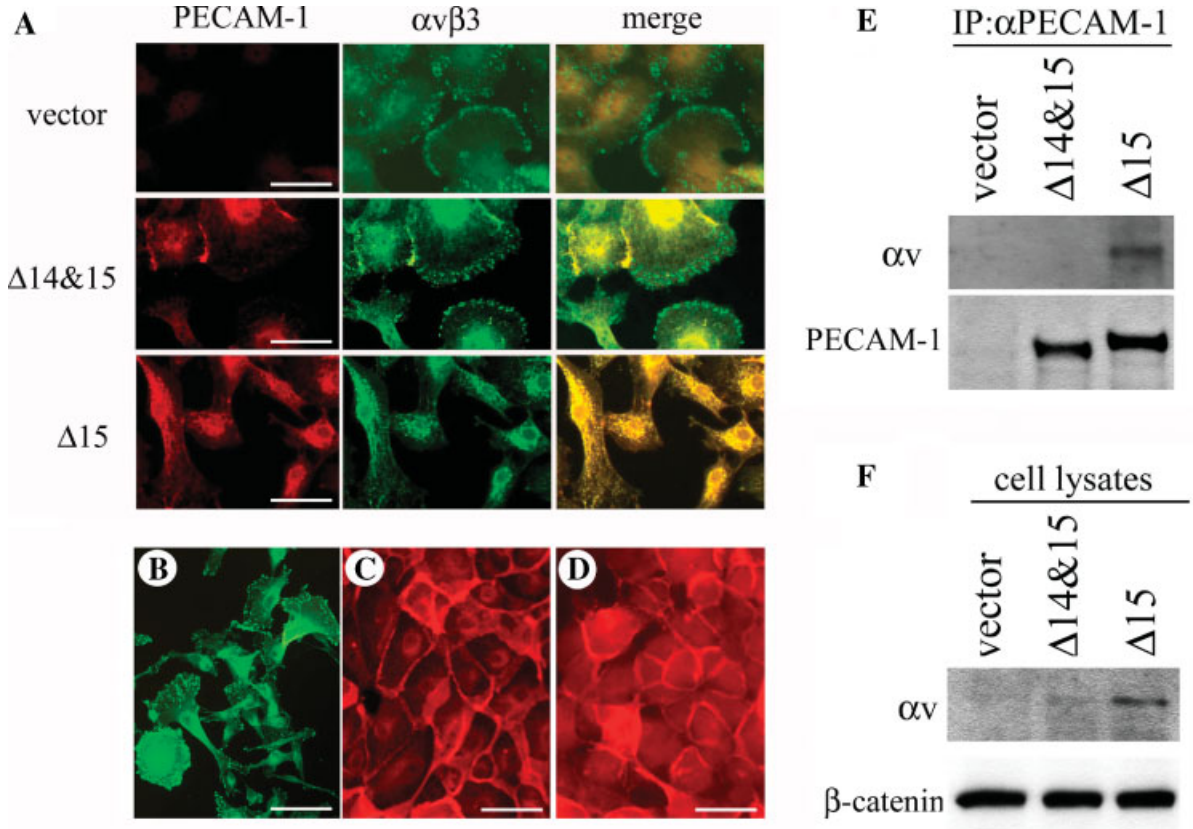

Fig. 6. Effects of Δ15 PECAM-1 on the function of αvβ3 integrin. **A**: Co-localization of Δ15 PECAM-1 with integrin αvβ3 in the MDCK cells was determined by double staining of the MDCK cells with antibodies to PECAM-1 and αvβ3 integrin. Please note that Δ15 PECAM-1 alters the distribution of αvβ3 integrin. Bar: 25 μm. **B**: Distribution of focal adhesions in the Δ15 PECAM-1 cells stained for the focal adhesion-associated protein vinculin. **C**: Junctional localization of Δ14&15 PECAM-1 in the MDCK cells. **D**: Junctional localization of Δ15 PECAM-1 upon incubation with the MEK1 inhibitor PD98059. PD98059 can restore cadherin-mediated cell–cell interactions (Fig. 3E,M) and PECAM-1 junctional localization (Fig. 6D) in the Δ15 PECAM-1 cells. Bar: 40 μm (B–D). **E**: αvβ3 integrin immunoprecipitated with Δ15 PECAM-1 in the MDCK cells. Please note we failed to detect β3 integrin levels in this assay because commercially available β3 integrin antibodies do not cross-react with canine β3 integrin. **F**: Upregulation of αv integrin expression in the Δ15 PECAM-1 MDCK cells. These experiments were repeated at least three times with identical results.

activation of these signaling pathways in EC and/or leukocytes may regulate their adhesive and migratory properties during inflammation and angiogenesis, as depicted in Figures 2A and 7.

PECAM-1 Modulates Activities of MAPK/ERKs and Small GTPases

Our studies demonstrate that PECAM-1 activates MAPK/ERKs during its homophilic interaction on endothelial and hematopoietic cells (Fig. 1), its cross-linking on the surface of EC and hematopoietic cells (Fig. 2), or potentially by its heterophilic interaction with αvβ3 integrin (Figs. 6A,E and 7A). We also demonstrate that expression of the Δ15 PECAM-1 isoform in MDCK cells has a dramatic effect on their adhesive and migratory properties (Fig. 3D,J,L) in a MAPK/ERKs activation-dependent manner (Fig. 3E,M). The Δ15 PECAM-1 cells lack cadherin mediated cell–cell interactions (Fig. 3J) and undergo EMT (Fig. 3D). Previous studies have shown that EMT occurs in a MAPK/ERKs activation-dependent manner in MDCK cells incubated with hepatocyte growth factor/scatter factor (HGF/SF) or in cells expressing a dominant active MEK1 [Klemke et al., 1997]. We recently demonstrated that expression of a dominant active MEK1 in EC is also sufficient to disrupt adherens junctions [Wu and Sheibani, 2003]. Therefore, activation of MAPK/ERKs, both in epithelial and EC, results in disruption of cadherin-mediated cell–cell interactions and promotion of cell migration.

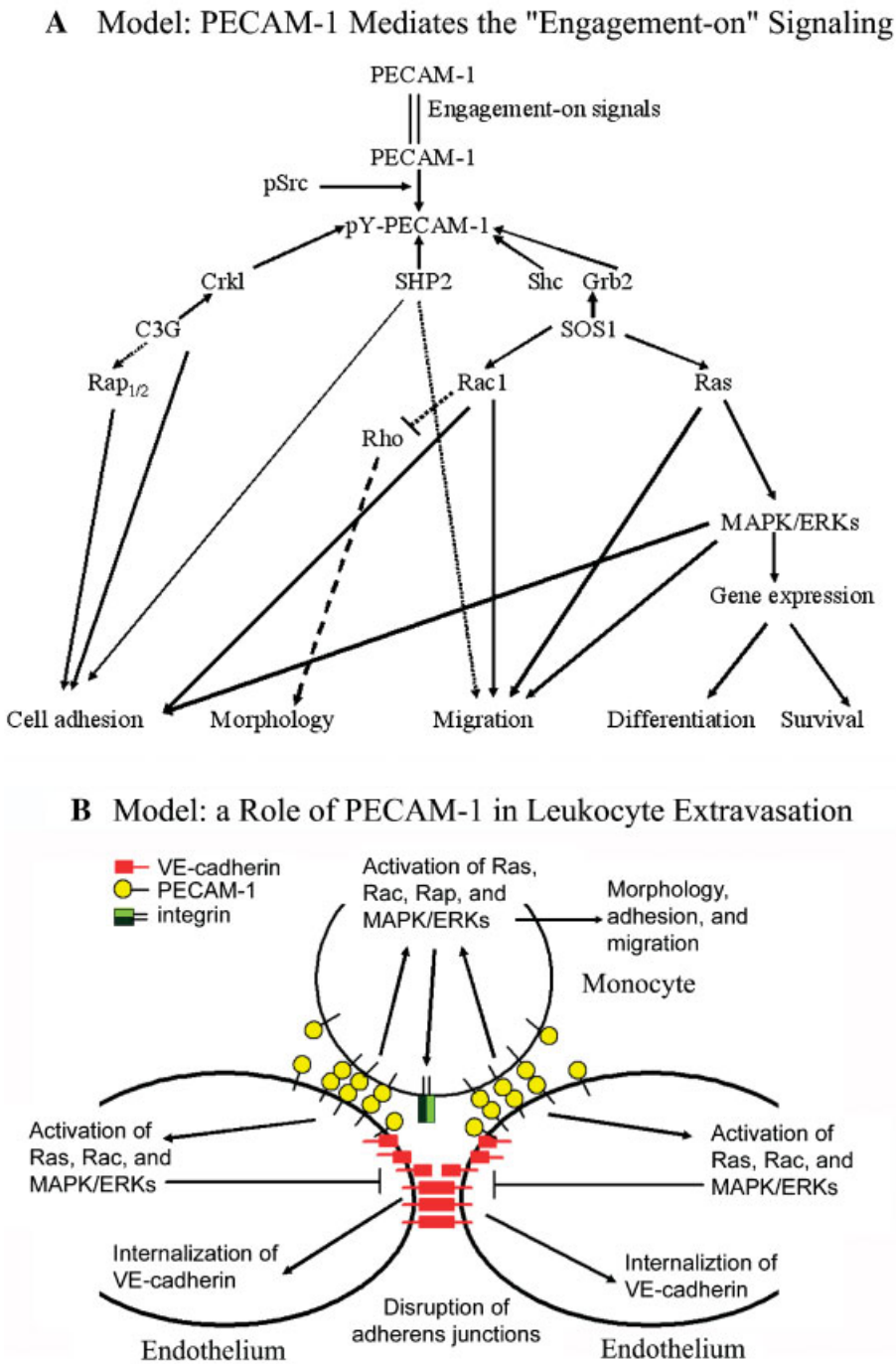

Fig. 7. PECAM-1-mediated signals and their functional implication. **A**: Diagram for PECAM-1 ''engagement-on'' signals regulating activities of MAPK/ERKs and small GTPases. The figure depicts potential PECAM-1-mediated signaling pathways that control cellular morphology, adhesion, motility, differentiation, and survival. **B**: Model illustrating the role of PECAM-1 in TEM. PECAM-1 homophilic interaction during TEM of leukocytes activates MAPK/ERKs and small GTPases regulating EC adherens junctions, and monocyte morphology, adhesion, and motility. The PECAM-1-mediated temporal spatial signals result in disruption of adherens junctions, thereby accommodating leukocyte extravasation.

We demonstrate for the first time that Δ15 PECAM-1 recruits Shc-Grb2-SOS1 and Crkl-C3G, which form macrocomplexes to regulate Ras, Rac1, Rap, and MAPK/ERKs-signaling pathways (Figs. 1, 2, 4, and 7A). Active Src potentially phosphorylate PECAM-1 at tyrosine residues on its ITIMs and initiates assembly of Δ15 PECAM-1-mediated signaling complexes.

Phosphorylation of Δ15 PECAM-1 ITIMs creates sites for binding and activation of SHP2 which, in turn, provides docking sites for downstream adapter proteins, such as Shc, Grb2, and Crkl [Buday and Downward, 1993; Pawson, 1995; de Vries-Smits et al., 1995; Neel and Tonks, 1997; Lamorte et al., 2002; Newman and Newman, 2003]. In addition, inhibition of Src or SHP2 activities resulted in inhibition of Δ15 PECAM-1-mediated Ras activation (Fig. 4G), suggesting that protein tyrosine kinase Src and phosphatase SHP2 are involved in Δ15 PECAM-1-mediated signaling, potentially through regulation of the PECAM-1 ITIMs phosphorylation. Although the formation of Δ15 PECAM-1 signaling complexes in MDCK cells, which express a specific PECAM-1 isoform, is similar to those observed in EC and hematopoietic cells (Figs. 1 and 2), the consequences of activated signaling events on EC adhesion and migration remains to be determined and is currently under investigation in our laboratory.

Most interestingly, our results demonstrate that the presence of the exon 14 is essential for the ability of Δ15 PECAM-1 to activate MAPK/ERKs (Fig. 4A) and associate with its ligand αvβ3 integrin (Fig. 6A). Therefore, alternative splicing can regulate PECAM-1 signaling and ligand binding properties through inclusion or exclusion of exon 14. To further demonstrate the importance of Δ15 PECAM-1 ITIM domain in the activation of MAPK/ERKs, we have mutated the tyrosine residue in exon 14 to a phenylalanine (Y F). The MDCK cells that expressed the Y F Δ15 PECAM-1 isoform behaved similarly to the MDCK cells that expressed the Δ14&15 PECAM-1 isoform [Sheibani et al., 2000]. Therefore, the tyrosine residue in exon 14 is essential for the ability of Δ15 PECAM-1 to activate MAPK/ERKs.

Regulation of Endothelial Adherens Junctions by PECAM-1-Mediated Signaling

Our results further implicate PECAM-1-mediated MAPK/ERKs activation may play a regulatory role in disruption of vascular barrier during TEM and angiogenesis. PECAM-1 is directly targeted to regions of the cell border during TEM, and this occurs through PECAM-1-PECAM-1 homophilic interactions between leukocytes and EC [Mamdouh et al., 2003]. PECAM-1 engagement may be necessary to propagate a signal, such as MAPK/ERKs activation (Fig. 1A), to spatiotemporally disrupt endothelial adherens junctions [Thompson et al., 2000; Schenkel et al., 2002], and modulate both endothelial and leukocytes morphology [Tanaka et al., 1999] to accommodate leukocytes to extravasate. PECAM-1-mediated activation of MAPK/ERKs and small GTPase-signaling pathways is "engagement-on" dependent (Figs. 1 and 2). These signals are spatiotemporal in EC that interact with leukocytes. Thus, PECAM-1 signaling, particularly activation of MAPK/ERKs and Rac1 (Figs. 1 and 4), may result in endocytosis of VE-cadherin and spatiotemporal disruption of the vascular barrier, where leukocyte extravasation is occurring (Fig. 7B). Inhibition of MAPK/ERKs results in inhibition of leukocyte migration through endothelial barrier [Stein et al., 2003]. The opening of the endothelial adherens junctions during TEM is restricted to a limited region, which may be controlled by PECAM-1-mediated leukocyte-endothelial cell interactions. In addition, regulation of VE-cadherin-mediated cell–cell adhesion by VEGF is critical for EC migration and initiation of angiogenesis [Potter et al., 2005]. EC in particular are maintained in a mesenchymal state during the cell invasion phase of angiogenesis through tyrosine phosphorylation of VE-cadherin cytoplasmic domain. This prevents the formation of cadherin-mediated cell–cell adhesion promoting cell migration [Potter et al., 2005]. Thus, given our finding that PECAM-1 engagement activates Ras/MAPK/ERKs, we speculate that regulation of the cell–cell adhesion by PECAM-1 and its signaling, may also involve the maintenance of the mesenchymal state of EC during angiogenesis.

Regulation of Integrin Activities by PECAM-1 and/or its Signaling

Leukocyte extravasation and angiogenesis are integrin-dependent processes [Rainger et al., 1999; Thompson et al., 2000; Eliceiri and Cheresh, 2001; Price and Bos, 2004]. PECAM-1 engagement regulates activities of integrins in hematopoietic and EC [Berman and Muller, 1995; Berman et al., 1996; Reedquist et al., 2000; Newman and Newman, 2003], thereby suggesting a critical role for PECAM-1 in leukocyte extravasation and angiogenesis. Using MDCK cells expressing a specific PECAM-1 isoform, we examined how Δ15 PECAM-1 and its signaling regulate integrin-ligand binding activities. The adhesion of Δ15 PECAM-1 cells to fibronectin,

collagen, and laminin was greatly enhanced compared to the Δ14&15 PECAM-1 or vector cells (Fig. 5A,C,D). Loss of exon 14 in Δ14&15 PECAM-1 abrogated the effect of PECAM-1 on cell adhesion, suggesting that regulation of cell adhesion to the examined matrix proteins by PECAM-1 requires the intact PECAM-1 ITIM domain. SHP2, Src, Ras/Rac1, and MAPK/ERKs have important roles in regulation of integrin functions [Keely et al., 1997; Klemke et al., 1997; D'Souza-Schorey et al., 1998; Manes et al., 1999; von Wichert et al., 2003]. SHP2 localization to focal adhesions modulates integrin-mediated cell adhesion signals to stimulate or inhibit cell migration by regulated phosphorylation of focal adhesion kinase [Manes et al., 1999; von Wichert et al., 2003]. The ability of the Δ15 PECAM-1 isoform to bind SHP2 and its proximity to focal adhesions may enhance focal adhesion turnover and stimulate cell migration. The activation of Ras/Rac1/MAPK/ERKs (Figs. 1, 2 and 4) may also facilitate the migration of monocytes [Keely et al., 1997; Klemke et al., 1997; D'Souza-Schorey et al., 1998].

Rap1 is necessary for upregulation of integrin function during adhesion and migration of leukocytes and EC [Reedquist et al., 2000; Ohba et al., 2001; Lamorte et al., 2002; Voss et al., 2003; Price and Bos, 2004]. Given the results of activation of Crkl/C3G/Rap1 [Ohba et al., 2001; Voss et al., 2003], we postulate that engagement of PECAM-1 spatiotemporally activates Rap1 signaling. This, in turn, regulates the cycling of integrins at the leading edge of leukocytes and EC where PECAM-1-mediated homophilic interactions are occurring.

Interestingly, we found that Δ15 PECAM-1 associates with the αvβ3 integrin (Fig. 6). The association of PECAM-1 with αvβ3 integrin altered its distribution on MDCK cells and inhibited adhesion to vitronectin (Figs. 5B, 6A,B). However, once the Δ15 PECAM-1 cells reestablish cell–cell junctions (Figs. 3M and 6D), such as upon incubation with PD98059, Δ15 PECAM-1 localizes to the site of cell–cell contacts (Fig. 6D) and no longer interacts with αvβ3 integrin. This allows αvβ3 integrin to re-localize to focal adhesions and function independently (results not shown). Therefore, the association of Δ15 PECAM-1 with αvβ3 integrin on the surface of MDCK cells (Fig. 6) may inhibit αvβ3 integrin ligand binding activity (Fig. 5B). The αvβ3 integrin is a ligand for PECAM-1 [Wong et al., 2000], and both are important components of monocyte extravasation and angiogenesis [Brooks et al., 1994; Rainger et al., 1999; Thompson et al., 2000]. Although PECAM-1 binding to αvβ3 integrin occurs in both EC and hematopoietic cells (Wong et al. [2000]; our unpublished dada), the physiological importance of these interactions, both in EC and hematopoietic cells, remain to be determined. Our results reveal that PECAM-1 may be a regulator of αvβ3 integrin ligand binding activities through their reversible association during TEM and angiogenesis.

In summary, PECAM-1 plays an active role during TEM of leukocytes and angiogenesis. Adhesion of leukocyte to vascular endothelium results in reversible disruption of vascular barrier, which allows leukocytes to extravasate. PECAM-1-PECAM-1 homophilic interactions between EC and leukocyte are essential for disruption of vascular barrier and leukocyte extravasation [Schenkel et al., 2002]. PECAM-1-mediated endothelial cell–cell interactions also play an important role during angiogenesis [DeLisser et al., 1997; Sheibani et al., 1997]. Our findings reveal that PECAM-1 engagement on the surface of EC and/or leukocytes can transduce "outside-in" signals and activate MAPK/ERKs and small GTPases, thereby impacting both cadherin-mediated cell–cell adhesion and integrin-mediated cell–matrix adhesion (Fig. 7). We propose PECAM-1 is an important mediator of vascular barrier and regulator of leukocyte and EC migration. These findings help to further advance our understanding of the regulatory roles PECAM-1 plays during TEM and angiogenesis.

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