Role of immunoreceptor tyrosine-based inhibitory motifs of PECAM-1 in PECAM-1-dependent cell migration

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Division of Pulmonary, Allergy and Critical Care, Department of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6160; and Department of Obstetrics and Gynecology, University of Crete, Heraklion, Greece

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O’Brien, Christopher D., Gaoyuan Cao, Antonis Makrigiannakis, and Horace M. DeLisser. Role of immunoreceptor tyrosine-based inhibitory motifs of PECAM-1 in PECAM-1-dependent cell migration. Am J Physiol Cell Physiol 287: C1103–C1113, 2004.—Platelet endothelial cell adhesion molecule (PECAM-1), a transmembrane glycoprotein, has been implicated in angiogenesis, with recent evidence indicating the involvement of PECAM-1 in endothelial cell motility. The cytoplasmic domain of PECAM-1 contains two tyrosine residues, Y663 and Y686, that each fall within an immunoreceptor tyrosine-based inhibitory motif (ITIM). When phosphorylated, these residues together mediate the binding of the protein tyrosine phosphatase SHP-2. Because SHP-2 has been shown to be involved in the turnover of focal adhesions, a phenomenon required for efficient cell motility, the association of this phosphatase with PECAM-1 via its ITIMs may represent a mechanism by which PECAM-1 might facilitate cell migration. Studies were therefore done with cell transfectants expressing wild-type PECAM or mutant PECAM-1 in which residues Y663 and Y686 were mutated. These mutations eliminated PECAM-1 tyrosine phosphorylation and the association of PECAM-1 with SHP-2 but did not impair the ability of the molecule to localize to intercellular junctions or to bind homophilically. However, in vitro cell motility and tube formation stimulated by the expression of wild-type PECAM-1 were abrogated by the mutation of these tyrosine residues. Importantly, during wound-induced migration, the number of focal adhesions as well as the level of tyrosine phosphorylated paxillin detected in cells expressing wild-type PECAM-1 were markedly reduced compared with control cells or transfectants with mutant PECAM-1. These data suggest that, in vivo, the binding of SHP-2 to PECAM-1, via PECAM-1’s ITIM domains, promotes the turnover of focal adhesions and, hence, endothelial cell motility.

Platelet endothelial cell adhesion molecule-1; endothelial cells; angiogenesis

PLATELET ENDOTHELIAL CELL ADHESION MOLECULE (PECAM-1) is a 130-kDa transmembrane glycoprotein member of the Ig superfamily that is expressed on endothelial cells, where it is enriched at intercellular junctions, as well as on leukocytes and platelets (9, 36). There is accumulating evidence that in addition to being an adhesive protein, PECAM-1 also participates in intracellular signaling cascades (20, 24, 37). Although the molecule itself does not possess any catalytic activity, its cytoplasmic domain contains two tyrosine residues (Y663 and Y686) that each fall within a conserved signaling sequence ([I/V/L/S]-X-YXX-[L/V]) known as the immunoreceptor tyrosine-based inhibitory motif (ITIM) (3, 49, 62). Receptors bearing these sequences were originally recognized on immune cells for their ability to attenuate activation signals generated by homologous receptors containing sequences (YXXL/I)<sub>[X<sub>6–8</sub>]YXXL/I</sub> that conform to an immunoreceptor tyrosine-based activation motif (ITAM) (3). As has been described for other ITIM-containing receptors, phosphorylation of these two tyrosine residues in PECAM-1 through the action of protein tyrosine kinases (e.g., Src, Csk, and Fer family kinases) (6, 27, 29) creates docking sites for the binding and activation of several cytosolic signaling molecules containing Src homology 2 (SH2) domains. Included among the SH2-containing molecules that associate with PECAM-1 are the protein tyrosine phosphatases (PTP) SHP-1 and SHP-2 (6, 11, 17, 22, 23, 33, 48, 52), the inositol phosphatase SHIP (48), and phospholipase C-γ (48). There is also evidence that PECAM-1 may associate with phosphoinositide 3-kinase (44) and β- or γ-catenin (18, 19). The ability of PECAM-1 to bind to these various cytosolic molecules enables it to potentially modulate the activity of intracellular signaling pathways.

In addition to its involvement in hemostasis (29, 43, 51), leukocyte recruitment at sites of inflammation (35, 60, 61), and T-cell activation (38, 47, 68), PECAM-1 has also been implicated in angiogenesis (5, 10, 34, 57, 69). Initial studies established that treatment with anti-PECAM-1 antibody inhibited vessel formation in rodent models of cytokine or tumor-induced angiogenesis (10, 69), while more recently, in a SCID mouse model of the human vasculature, antibody against human PECAM-1 limited the vascularization of tumors by human vessels (5). Further support for the involvement of PECAM-1 comes from Solowiej et al. (57), who noted reduced angiogenesis in a model of chronic inflammation in mice deficient in the expression of PECAM-1.

With respect to its specific roles in angiogenesis, it has been proposed that PECAM-1 promotes intercellular endothelial cell adhesion (1, 50). Consistent with this is the finding that PECAM-1 associates with β- and γ-catenins, two molecules that are critical to the assembly and stability of endothelial intercellular junctional complexes (18, 19). Recent evidence, however, indicates that PECAM-1 may also promote endothelial cell migration (5, 15). As reported by Cao et al. (5), antibody against human PECAM-1 inhibited the migration of human umbilical vein endothelial cells (HUVEC) through Matrigel-coated filters or during the repair of wounded cell monolayers. Involvement of PECAM-1 in these processes was confirmed by the finding that expression of PECAM-1 enhanced the motility of cellular transfectants in a PECAM-1-dependent manner.

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The mechanism(s) by which PECAM-1 might promote cell migration is unclear. The interaction of cells with matrix results in the formation of discrete adhesion complexes known as focal adhesions (or contacts) (4, 13). Efficient cell motility requires the rapid turnover of these structures (28), a process that appears to require the activity of phosphatases (2). Thus one possible mechanism by which PECAM-1 might enhance cell motility may involve its ITIM domains (encompassing residues Y663 and Y686) and their ability, when tyrosine phosphorylated, to bind SH-2 domain-containing phosphatases. One such phosphatase is SHP-2, a cytosolic protein tyrosine phosphatase that has been shown to stimulate cell migration by promoting the turnover of cell-matrix adhesive interactions critical for cell motility (21, 32, 64, 67). Therefore, to define the involvement of the two ITIM domains of PECAM-1 in PECAM-1-dependent cell motility, we performed studies on REN cells (a mesothelioma cell line) expressing either wild-type human PECAM-1 (REN-HP) or human PECAM-1 mutants in which either the cytoplasmic domain was deleted (REN-HPTailless) or Y663 and Y686 were changed to phenylalanines (REN-HPY→F). We observed that the wounding of cell monolayers induced PECAM-1 tyrosine phosphorylation and SHP-2 binding, whereas the loss of Y663 and Y686 eliminated PECAM-1 tyrosine phosphorylation and the association of PECAM-1 with SHP-2. Furthermore, enhanced wound-induced migration and single-cell motility, stimulated by the expression of wild-type PECAM-1, were abrogated by removal of these tyrosines, and although REN cell transfectants expressing wild-type PECAM-1 formed tubelike structures on a Matrigel-collagen substrate, the REN-HPTailless and REN-HPY→F transfectants did not. During wound-induced migration, reduced numbers of focal adhesions and levels of tyrosine-phosphorylated paxillin were detected on migrating REN-HP cells compared with control REN cells and REN-HPY→F transfectant. Studies of REN-HPY→F demonstrated that the PECAM-1 Y→F mutant was still able to localize to cell-cell borders and bind homophilically, suggesting that the inhibition of PECAM-1-dependent tube formation resulting from the disruption of the ITIM domains is due to an inhibition of PECAM-1-dependent migration and not to a compromise of PECAM-1-mediated cell-cell interactions. These data suggest that in vivo, the binding of SHP-2 to PECAM-1, via PECAM-1’s ITIM domains, promotes the turnover of focal adhesions and, hence, endothelial cell motility.

EXPERIMENTAL PROCEDURES

Reagents and chemicals. All reagents and chemicals were obtained from Sigma (St. Louis, MO) unless otherwise specified.

Antibodies. The following antibodies were employed: MAb 4G6, a murine anti-PECAM-1 domain 6 antibody (a generous gift of Dr. Steven Albelda, University of Pennsylvania, Philadelphia, PA); MAb 1.3, a murine anti-PECAM-1 IgG (a generous gift of Dr. Peter Newman, Blood Center of Southeastern Wisconsin, Milwaukee, WI); murine anti-SHP-2 MAb (Santa Cruz Biotechnology, Santa Cruz, CA); MAb 4G10, a murine anti-phospho-tyrosine IgG (Upstate Biotechnology, Lake Placid, NY); and anti-paxillin antibody (BD Biosciences Transduction Laboratories, Lexington, KY).

Cell lines. The human mesothelioma cell line REN (56) was cultured in RPMI medium supplemented with 10% FBS, penicillin/streptomycin, and 2 mM l-glutamine. REN cell transfectants stably expressing wild-type human PECAM-1 (REN-HP), cytoplasmic domain-deleted PECAM-1 (REN-HPTailless), or the dual Y663F and Y686F mutant PECAM-1 (REN-HPY→F) have been previously described (25, 56) and were cultured in the same media with G418 (0.5 g/l; GIBCO BRL, Grand Island, NY). HUVEC (Clonetics, San Diego, CA) were cultured on tissue culture surfaces preincubated with 1% gelatin in medium 199 containing 15% FBS, 75 µg/ml endothelial growth factor, 100 µg/ml heparin, and 1 mM glutamine. HUVEC were used between passages 2 and 6.

Immunoprecipitation and immunoblotting. HUVEC, REN cells, and REN transfectants were grown to confluence. Cells were maintained for 12 h in low-serum medium to deplete constitutive tyrosine phosphorylation. Cell monolayers were treated for 3 h with control medium or in medium containing 0.5 mM sodium orthovanadate at 37°C before the placement of the wounds. At various time points, the cells were then washed with iced PBS and lysed in TNC (0.1 M Tris-acetate, pH 8.0, 0.5% NP-40, 0.5 mM Ca2+) with 1 mM vandate, 10 mM aprotinin, 10 µM leupeptin, and 2 mM PMSF for 20 min at 4°C. Extracts were clarified at 14,000 rpm for 10 min, the supernatant protein concentration was quantified, and 100-µg aliquots were incubated with 2 µg of 4G6 anti-PECAM-1 or anti-paxillin antibody for 1 h at 4°C. Protein G-Sepharose beads (Pharmacia, Piscataway, NJ) were added and incubated for an additional 2 h. After immunoprecipitation, beads were washed five times with DOC wash (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 5% deoxycholate, and 1% SDS), dissolved in sodium dodecyl sulfate, and resolved on NuPAGE 4–12% Bis-Tris gels (Invitrogen, Carlsbad, CA). Proteins were then transferred to polyvinylidene difluoride (PVDF) membranes, blocked for 1 h in 4% BSA-PBS-0.02% azide, incubated in 1 µg/ml anti-phospho-tyrosine antibody 4G10 for 1 h, washed, and then counterstained for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody. Blots were developed with chemiluminescence reagent according to the manufacturer’s instructions (NEL Life Sciences, Boston, MA). The membranes were then stripped and reprobed with MAb 1.3 or anti-SHP-2 for the PECAM-1 associations or with anti-paxillin for the paxillin studies, followed by counterstaining with HRP-conjugated goat anti-mouse antibody. Densitometric analysis was performed using computer-assisted image analysis with subsequent determination of the relative intensities of the SHP-2 and phospho-PECAM-1 bands compared with the intensities of PECAM-1 band.

In vitro wounding assay. REN cell wounding was modified from previously published procedures (5). Twenty thousand endothelial cells were added to twenty-four-well tissue culture plates and allowed to grow to confluence. Linear defects were then made in the monolayer. The wounded culture was washed with PBS and then incubated for 24 h in medium (with 1% serum). Using computer-assisted image analysis and Image Pro Plus software (Media Cybernetics, Silver Spring, MD), we obtained images immediately after wounding and then 24 h later, and the distance migrated by cells at the wound edge was determined. For each cell type, three to five wounds were analyzed.

Single-cell migration. Time-lapse video microscopy was performed as previously described (31). For these studies, slides obtained from LabTek (Micro Video Instruments, Avon, MA), which were sealed with a mixture of petroleum jelly and paraffin (20:1) to maintain pH of the medium, were coated with fibronectin (10 µg/ml) for 1 h at 37°C and blocked with 2% BSA for 1 h at 37°C. The cells were plated on the slides at a density of 0.5 × 104 cells and then placed in a 37°C humidified Plexiglas microscope culture chamber (Nikon, Tokyo, Japan). A field containing several REN cells was selected and observed under phase contrast for 24 h. Motile activity was studied by measuring the total distance covered by the migrating cells using time-lapse video microscopy. Sequential images were collected at 1-h intervals. A minimum of 70 cells were studied in each experiment for each 0.1 h time increment.

Immunofluorescence staining and confocal imaging. Cells were cultured on fibronectin-coated chamber slides and subsequently wounded as described in In vitro wounding assay. The slides were
then washed in PBS, fixed with 3% paraformaldehyde for 10 min, and then permeabilized with ice-cold 0.5% NP-40 for 1 min. After being washed, cells were subjected to immunofluorescence staining with anti-paxillin antibody followed by FITC-conjugated goat anti-murine IgG secondary antibody to identify focal contacts. The slides were viewed by confocal microscopy with a Zeiss phase-epifluorescence microscope using a ×60 fluorescence lens. Confocal microscopic images were obtained using a computer-interfaced laser scanning microscope (Leica TCS 4D) in the Confocal Core Facility at the Children’s Hospital of Philadelphia. Simultaneous wavelength scanning allowed superimposition of fluorescent labeling with FITC at wavelengths of 488 nm. Laser power was fixed at 75% for all image acquisition. Image output was at 1.024 × 1.024 pixels.

In vitro tube formation assay. In vitro tube formation was studied using previously described procedures (5, 69). REN cells and REN cell transfectants were plated on a mixture of Matrigel (Collaborative Biomedical Products, Bedford, MA) and rat tail type I collagen (Collaborative Biomedical Products), which was prepared as follows: 2 volumes of Matrigel (10 mg/ml) were mixed with 1 volume of a collagen solution prepared by mixing rat tail collagen type I (stock concentration 3–4 mg/ml), NaHCO₃ (1.76 mg/ml), and 10× DMEM together in a ratio (by volume) of 7:2:1, respectively. Fifty microliters of solution were added to each well of a 96-well plate and allowed to form a gel at 37°C for 30 min. Cells (20,000) in 200 μl of complete medium were subsequently added to each well and incubated for 8 h at 37°C in 5% CO₂. The wells were washed, and the gel and its cells were fixed with 3% paraformaldehyde. Total tube length per well was determined by computer-assisted image analysis using the Image Pro Plus software.

In vitro cell proliferation assay. Cells were cultured for 24 h in 96-well plates, and the number of viable cells was determined using a commercially available nonradioactive colorimetric assay according to the manufacturer’s instructions (CellTiter 96 AQueous One nonradioactive cell proliferation assay; Promega, Madison, WI).

Fluorescence-activated cell sorting analysis. The various cell lines used in this study were treated with the indicated primary antibodies for 1 h at 4°C. The primary antibody was then removed, the cells were washed with PBS, and a 1:200 dilution of FITC-labeled goat anti-mouse or anti-rat secondary antibody (Cappell ICN Pharmaceuticals, Aurora, OH) was added for 30 min at 4°C. After washing in PBS, flow cytometry was performed using an Ortho Cytofluorograph 50H cell sorter equipped with a 2150 data handling system (Ortho Instruments, Westwood, MA).

Statistical analysis. Differences among groups were analyzed using one-way analysis of variance. Results are presented as means ± SE. When statistically significant differences were found (P < 0.05), individual comparisons were made using the Bonferroni/Dunn test.

RESULTS

Absence of Y663 and Y686 results in loss of PECAM-1 tyrosine phosphorylation and its association with SHP-2 in REN cells. To study the role of PECAM-1 in endothelial cell function, we made use of the REN human mesothelioma cell line (56) and transfectants of these cells expressing human PECAM-1 (REN-HP). For the purposes of our studies, REN cells are appealing because they do not express PECAM-1, yet they have several relevant endothelial cell surface molecules (e.g., α/β₁ ICAM-1, VCAM-1, and VEGF receptors) and form cobblestone cell monolayers reminiscent of endothelial cells. More importantly, for both endothelial cells and REN-HP, 1) PECAM-1 concentrates at cell-cell junctions (58); 2) tubelike structures form on a Matrigel-collagen substrate (see Fig. 7 and Ref. 5); 3) H₂O₂ activates a calcium-permeant, nonselective cation current (25); and 4) wound-induced cell migration (see Fig. 2) and shear stress (data not shown) are associated with PECAM-1 tyrosine phosphorylation and SHP-2 association. Furthermore, monolayers of PECAM-1 REN cell transfectants support cytokine-mediated neutrophil transmigration and thus have provided a useful model system for characterizing PECAM-1-dependent neutrophil transcellular migration (39). Together, these features indicate that the expression of PECAM-1 in REN cells represents a relevant system for modeling the function of EC PECAM-1.

In several other cell systems including platelets, T cells, and endothelial cells, PECAM-1 has been found to associate with SHP-2 when both Y663 and Y686 are phosphorylated (20, 23, 49). There has also been in vivo demonstration of a SHP-2-PECAM-1 interaction during murine conceptus development (45, 46). We therefore sought to confirm that patterns of PECAM-1 tyrosine phosphorylation and protein–protein interaction in transfected REN cells occurred in the same manner as observed in endothelial cells and heterologous overexpression systems (33). Untransfected REN cells or REN cells expressing wild-type PECAM-1 (REN-HP), cytoplasmic domain-deleted PECAM-1 (REN-HPTvailless), or mutant PECAM-1 in which tyrosines residues 663 and 686 were changed to phenylalanines (REN-HPY₆₆₃P-Y₆₈₆P) were treated with sodium orthovanadate, a nonspecific tyrosine phosphatase inhibitor, and immunoprecipitated with anti-PECAM-1 antibody 4G6 (Fig. 1). Resultant gels were transferred to PVDF membranes and immunoblotted with anti-PECAM-1 MAb 1.3, anti-phosphotyrosine MAb 4G10, and anti-SHP-2 antibody. In cells treated with 0.5 mM vanadate for 3 h, only full-length PECAM-1 in REN-HP supported tyrosine phosphorylation (Fig. 1, lane 1). The lack of a tyrosine phosphorylation signal in vanadate-treated REN-HPY₆₆₃P-Y₆₈₆P cells confirms that the PECAM-1 cytoplasmic domain is selectively tyrosine phosphorylated on these two residues (Fig. 1, lane 7). SHP-2 immunoprecipitation was similarly detected only on tyrosine-phosphorylated wild-type PECAM-1, confirming that the cytoplasmic domain, and the Y663/Y686 motif in particular, is required for SHP-2 association. The pattern of reaction seen with REN-HP was similar to that seen with HUVEC (see Fig. 2).

Wound-induced migration by PECAM-1-expressing cells is associated with PECAM-1 tyrosine phosphorylation and SHP-2 association. A number of physical and chemical stimuli have been reported to induce tyrosine phosphorylation of PECAM-1 in endothelial cells (16, 41, 42). However, it is unknown whether the wounding of monolayers of these cells and the subsequent initiation of cell migration is also associated with PECAM-1 tyrosine phosphorylation. We observed that the placement of linear wounds in monolayers of HUVEC or REN-HP stimulated PECAM-1 tyrosine phosphorylation and SHP-2 association (Fig. 2). Significantly, given its membrane location, these data suggest that in the context of wound-induced migration, PECAM-1 either recruits SHP-2 to the membrane and/or sequesters membrane-associated SHP-2. Wound-induced migration stimulated by the expression of PECAM-1 does not occur if Y663 and Y686 are absent. We (6) recently showed that expression of PECAM-1 in REN cells increases the rate of closure of defects made in confluent monolayers of these cells. However, the requirement of PECAM-1’s cytoplasmic domain, and in particular intracellular sequences such as its ITIMs, in this process is not known. We therefore investigated cell migration induced by the placement of linear wounds in confluent monolayers of REN-HP,
REN-HP Tailless or REN-HP Y\textsubscript{663F}. For monolayers of REN-HP, the rate of wound closure was two- to threefold higher than for control REN cells (Fig. 3). In contrast, the rate of wound closure for REN-HP Tailless or REN-HP Y\textsubscript{663F} did not differ significantly from that for controls. Cell proliferation for REN-HP was comparable to that for the REN cells and the REN-HP Tailless and REN-HP Y\textsubscript{663F} transfectants (Table 1), and thus the differences in wound closure are not explained by differences in cell proliferation. These data suggest that the cytoplasmic domain and presence of the Y663 and Y686 residues are essential to the ability of PECAM-1 to promote wound-induced cell migration.

Random single-cell migration stimulated by expression of PECAM-1 does not occur if Y663 and Y686 are absent. Studies of wound-induced migration involving molecules, such as PECAM-1, that mediate intercellular adhesion must be interpreted with caution. Although unlikely, the expression of PECAM-1 could stimulate an increase in wound-induced migration by disrupting cell-cell interactions and thereby facilitate the release of individual cells from their neighbors.

REN-HP Tailless or REN-HP Y\textsubscript{663F}.

![Image 1](http://ajpchem.physiology.org/)

**Fig. 1.** Platelet endothelial cell adhesion molecule (PECAM-1) immunoprecipitation, phospho-tyrosine immunoblotting, and SHP-2 coimmunoprecipitation. Untransfected REN cells (A) and REN cells expressing wild-type PECAM-1 (REN-HP) (B), cytoplasmic domain-deleted PECAM-1 (REN-HP Tailless) (C), and the dual Y663F and Y686F mutant PECAM-1 (REN-HP Y\textsubscript{663F}) (D) were subjected to fluorescence-activated cell sorting (FACS) analysis with anti-PECAM-1 antibody. REN cells lacked expression of PECAM-1, whereas PECAM-1 transfectants demonstrated comparable PECAM-1 expression. E: lysates from REN-HP (lanes 1 and 2), REN cells (lanes 3 and 4), REN-HP Tailless (lanes 5 and 6), and REN-HP Y\textsubscript{663F} (lanes 7 and 8) were immunoprecipitated with anti-PECAM-1 MAb 4G6 and then immunoblotted with anti-PECAM-1 MAb 4G6 and anti-phospho-tyrosine (middle), and anti-SHP-2 antibodies (top). Cells were exposed to medium alone (−) or medium with 0.5 mM vanadate (+) for 3 h. PECAM-1 expression is equivalent between treatment and nontreatment groups. Only wild-type PECAM-1 supports tyrosine phosphorylation and associates with SHP-2 (lanes 1 and 2).

**Fig. 2.** PECAM-1 tyrosine phosphorylation and SHP-2 association during wound-induced migration. A: control (C) human umbilical vein endothelial cells (HUVEC) and REN-HP cells (lanes 1 and 3) and cells migrating 60 min after the wounding (W) of confluent monolayers of these cells (lanes 2 and 4) were immunoprecipitated with anti-PECAM-1 antibody and then immunoblotted with anti-PECAM-1 (bottom), anti-phospho-tyrosine (middle), and anti-SHP-2 antibodies (top). Densitometric analysis was performed in HUVEC (B) and REN-HP cells (C) using computer-assisted image analysis with subsequent determination of the relative intensities of the SHP-2 and phospho-PECAM-1 bands compared with the intensities of PECAM-1 bands. Wounding was associated with increased PECAM-1 tyrosine phosphorylation and SHP-2 binding. Data are presented as means ± SE; n = 3. *P < 0.05 compared with control.
adhesion complexes. Given the involvement of PECAM-1 in cell migration, we determined the effect of expression of PECAM-1 on focal adhesions in REN cells (Fig. 5). In resting monolayers of cells, comparable numbers of focal adhesions (indicated by staining for paxillin) were noted in REN, REN-HP, and REN-HP Y→F cells (Fig. 5, A–C). Reduced numbers of focal adhesions, however, were detected on migrating REN-HP cells compared with control REN cells during wound-induced migration (Fig. 5, D–F). Importantly, the number of focal contacts on migrating REN and REN-HP Y→F were similar. Staining for vinculin, another focal adhesion constituent, yielded comparable results (data not shown). These findings suggest that during wound-induced cell migration, the expression of PECAM-1 (with intact ITIM domains) promotes the turnover of focal adhesions, and hence cell motility. This increased turnover of focal adhesions could be related to decreased tyrosine phosphorylation of the protein elements of focal adhesions, because the level of tyrosine-phosphorylated paxillin at baseline and during wound-induced migration was significantly less in the REN-HP transfectants compared with the REN and REN-HP Y→F cells (Fig. 6).

Tube formation stimulated by PECAM-1 expression is inhibited if Y663 and Y686 are absent. A critical step in the formation of new vessels is the organization of proliferating and migrating endothelial cells into stable tubular structures (7). With respect to the elaboration of tubular networks by endothelial cells and cell transfectants expressing PECAM-1, we and others have shown that PECAM-1 is involved in this process (6, 10, 34, 55, 66, 69). Given the role of cell motility in tube formation and the requirement of intact ITIM domains for PECAM-1-dependent migration (see Figs. 3 and 4), we hypothesized that disruption of the ITIM domains of PECAM-1 would also compromise PECAM-1-dependent tube formation. To evaluate this, we plated control REN cells or REN cell transfectants on a mixture of type 1 collagen and Matrigel. On this substrate, the expression of PECAM-1 (REN-HP) induced a three- to fourfold increase in the formation of tube/cordlike structures compared with untransfected REN cells.

Table 1. Cell proliferation of REN cells and REN cell transfectants

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>REN</th>
<th>REN-HP</th>
<th>REN-HP Tailless</th>
<th>REN-HP Y→F</th>
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<tr>
<td>Absorbance (490 nm)</td>
<td>0.76±0.01</td>
<td>0.71±0.01</td>
<td>0.75±0.02</td>
<td>0.83±0.02</td>
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Values are means ± SE. The proliferation REN cells and REN cell transfectants cultured for 24 h in the presence of serum was assessed using a colorimetric assay (see MATERIALS AND METHODS) and measurement of the absorbance of the reaction mixture at 490 nm. The proliferative response of the REN-HP transfectants was comparable to that of the other cells.
cells (Fig. 7). In contrast, the ability of REN-HP Tailless or REN-HP Y<sup>3F</sup> to form these tube/cordlike networks was significantly limited and was only marginally greater than that of the untransfected control REN cells. These data suggest that the enhanced ability of PECAM-1-expressing REN cells to organize into tubular structures arises at least in part from enhanced cell migration mediated by the expression of PECAM-1, the activity of which requires the conservation of its ITIM sequences.

Mutant PECAM-1 expressed in REN-HP Y<sup>3F</sup> localizes to cell-cell borders and binds PECAM-1/IgG. Given the established role of PECAM-1 in mediating cell adhesion (55, 56), the failure of REN-HP Y<sup>3F</sup> to demonstrate enhanced tube/cord formation may also result from a disruption of required cell-cell interactions mediated by PECAM-1. However, this is unlikely to be the case for two reasons. First, we observed that the PECAM-1 Y<sup>3F</sup> mutant expressed in REN-HP Y<sup>3F</sup> , like wild-type PECAM-1, concentrated at cell-cell borders (Fig. 8). Second, the binding of PECAM-1/IgG (a reagent that exclusively mediates PECAM-1-dependent homophilic adhesion; Ref. 58) to REN-HP Y<sup>3F</sup> was comparable to that of REN-HP (data not shown). These data suggest that the inability of the REN-HP Y<sup>3F</sup> mutant to stimulate the formation of tubes/cords results from its inability to enhance cell motility and not from a disruption of PECAM-1-dependent intercellular adhesion.

DISCUSSION

To define the involvement of the two ITIM domains of PECAM-1 in PECAM-1-dependent migration, we performed studies on REN cells expressing wild-type PECAM-1 (REN-HP) or PECAM-1 mutants in which either the cytoplasmic domain was deleted (REN-HP Tailless) or Y663 and Y686 were...
changed to phenylalanines (REN-HP Y\textsubscript{3F}). The elimination of these two tyrosine residues resulted in the loss of PECAM-1 tyrosine phosphorylation and the association of PECAM-1 with the protein tyrosine phosphatase SHP-2. In contrast, wound-induced migration stimulated PECAM-1 tyrosine phosphorylation and SHP-2 association. Enhanced wound-induced migration and single-cell motility by REN cells resulting from the expression of wild-type PECAM-1 were abrogated if Y663 and Y686 were not present. Furthermore, although REN cell transfectants expressing wild-type PECAM-1 formed tube/cordlike structures on a Matrigel-collagen substrate, the REN-HP\textsubscript{Tailless} and REN-HP Y\textsubscript{3F} transfectants did not. During wound-induced migration, markedly reduced numbers of focal adhesions were detected on migrating REN-HP cells compared with control REN cells, whereas the number of focal adhesions on migrating REN and REN-HP Y\textsubscript{3F} cells were similar. Also, the level of tyrosine-phosphorylated paxillin detected in REN-HP transfectants at baseline and after wounding was significantly less than that observed in REN and REN-HP Y\textsubscript{3F}.

The PECAM-1 Y\textsubscript{3F} mutant expressed in REN-HP Y\textsubscript{3F} transfectants was still able to localize to cell-cell borders and bind homophilically, suggesting that the inhibition of PECAM-1-dependent tube formation observed after the disruption of the molecule’s ITIMs results from an inhibition of PECAM-1-dependent migration and not a compromise of PECAM-1-mediated cell-cell interactions. These data thus provide further evidence of the role of PECAM-1 in cell migration, possibly by enhanced turnover of focal adhesions, mediated by a SHP-2-dependent dephosphorylation of constituents of these adhesion complexes. The data also suggest that in vivo, PECAM-1 via its ITIMs regulates the formation of vascular tubes, in part, through regulation of endothelial cell motility.

Initial evidence for the involvement of PECAM-1 during in vivo angiogenesis came from studies demonstrating that treatment with anti-PECAM-1 antibody inhibited vessel formation in rodent models of cytokine or tumor-induced angiogenesis (10, 69). However, more recently, PECAM-1 was also implicated in tumor-induced human angiogenesis. In a model of the human vasculature in which human skin was transplanted on immunodeficient mice, antibody against human PECAM-1 limited the vascularization by human vessels of tumors grown in the human skin grafts (5). The conclusions from these studies done with antibody reagents have been confirmed by the finding that angiogenesis in models of chronic inflammation (57) and tumor growth (DeLisser H, unpublished obser-

Fig. 7. Tube formation by PECAM-1-expressing REN cell transfectants. Representative photographs show REN cells (A), REN-HP (B), REN-HP\textsubscript{Tailless} (C), or REN-HP Y\textsubscript{3F} (D) that were plated on a 2:1 mixture of Matrigel and collagen type I in 96-well plates. On this substrate, nontransfected REN cells did not form tubular structures (A), whereas REN cell transfectants expressing PECAM-1 (B) were able to organize into tubular/cordlike networks. The ability of PECAM-1 to promote tube formation was substantially lost if the cytoplasmic domain was deleted (C) or if Y663 and Y686 were mutated (D). Magnification, ×100. E: quantitation of tube formation for a representative experiment. Data are presented as means ± SE and are representative of at least 3 experiments done in triplicate; n = 3, *P < 0.01 compared with REN cells.

Fig. 8. Immunofluorescence staining of REN-HP and REN-HP Y\textsubscript{3F} transfectants. REN-HP or REN-HP Y\textsubscript{3F} were plated on fibronectin and then subjected to immunofluorescence staining with anti-PECAM-1 antibody. The characteristic localization of PECAM-1 at the cell-cell borders of adjacent cells (arrows) was comparable in both cell types. Magnification, ×40.
The mechanism of PECAM-1's involvement in angiogenesis has been the subject of investigation. Although initial attention centered on the role of PECAM-1 in mediating endothelial cell-cell interactions (1, 10), in recent studies we have focused on PECAM-1 as a mediator of endothelial cell migration (5). In these investigations, anti-PECAM-1 antibody inhibited the migration of HUVEC through Matrigel-coated filters or during the repair of wounded cell monolayers. The involvement of PECAM-1 in these processes was further confirmed by the finding that the expression of PECAM-1 in cellular transfectants enhanced cell motility that was inhibited by anti-PECAM-1 antibody.

Cell motility involves three coordinated steps: membrane protrusion, cell traction and deadhesion, and tail retraction (28). Adhesion at the leading edge (dependent on the formation of focal adhesions) and deadhesion at the rear portions of the cell (requiring the disassembly of focal adhesions) are essential for protrusion and tail retraction, respectively. Consequently, the dynamic turnover of focal adhesions, a process that is regulated by the coordinated activity of distinct tyrosine kinases and phosphatases, is very critical to cell spreading and migration.

One of the phosphatases that may be important in cell locomotion is SHP-2, a widely expressed cytoplasmic PTP that contains two tandem SH2 domains at the NH2 terminus (12, 63). The in vivo importance of SHP-2 is reflected in the fact that the loss of expression of functional SHP-2 in developing mice results in death around days 8.5–10.5 of gestation with multiple defects in mesodermal patterning (53). Evidence of its involvement in regulating cell spreading, migration, and cytoskeletal organization has come from a number of sources. It has been reported that the fibroblasts derived from SHP-2−/− mice have lower levels of motility and spread more slowly than wild-type cells, defects that are rescued by reintroduction of wild-type SHP-2 protein into the SHP-2-deficient cells (21, 32, 64, 67). In a breast cancer model of cell migration, stimulation with insulin-like growth factor (IGF)-I concurrently increased tumor cell migration and promoted tyrosine dephosphorylation of focal adhesion kinase (FAK) and paxillin through the recruitment of the SHP-2 phosphatase (32). These effects of IGF-1 were lost if SHP-2 catalytic activity was neutralized by the expression of a dominant negative mutant. Significantly, the dominant negative mutants displayed a larger number of focal adhesions compared with control cells. In another study, overexpression of a catalytically inactive dominant negative SHP-2 mutant in Chinese hamster ovary cells resulted in substantial increases in the numbers of actin stress fibers and focal contacts and impaired migration on fibronectin and vitronectin (21). Together, these data suggest that SHP-2 may promote cell motility by regulating the remodeling and turnover of focal adhesions.

The putative involvement of SHP-2 (and other phosphatases) in cell migration provides a possible mechanism by which PECAM-1 might promote cell motility. As noted above, the cytoplasmic domain of PECAM-1 contains two tyrosine residues (Y663 and Y686), each of which falls within a conserved signaling sequence known as an ITIM, a motif that was initially noted in receptors that regulate immune function (3, 49, 62). Phosphorylation of these two tyrosine residues in PECAM-1 through the action of protein tyrosine kinases (e.g., Src, Csk, and Fes family kinases) (6, 27, 28) results in a docking site that mediates the binding and activation of several cytosolic signaling molecules containing SH2 domains, including SHP-2. Our finding that SHP-2 associates with PECAM-1 in REN cell transfectants and that this association is lost if tyrosine phosphorylation of the PECAM-1 ITIMs is eliminated (Fig. 1) is therefore consistent with previous studies that have reported a PECAM-1-SHP-2 interaction in other cell types including endothelial cells (6, 11, 17, 22, 23, 33, 48, 52).

Wounding of monolayers of PECAM-1-expressing cells stimulates SHP-2 binding to PECAM-1 (Fig. 2), and the loss of this association of SHP-2 with PECAM-1 appears to be significant in that the enhanced motility noted after expression of PECAM-1 was not seen in transfectants in which Y663 and Y686 were not present (Figs. 3 and 4). The observation that expression of PECAM-1 by migrating REN cells diminishes the number of focal contacts (Fig. 5) leads to the hypothesis that PECAM-1 in the context of a stimulus for migration may recruit SHP-2 to the membrane, where it facilitates a more rapid and efficient turnover of focal adhesions and thus promotes cell migration. These putative effects of SHP-2 could be mediated by the dephosphorylation of protein constituents of focal adhesions such as paxillin (Fig. 6).

In a recent report, von Wichert et al. (64) noted that the loss of SHP-2 in fibroblasts resulted in a spreading defect characterized by immature focal adhesion complexes, hyperphosphorylated FAK, and an impairment in the stabilization of initial integrin-cytoskeleton linkages. Their results suggest that the initial maturation of focal complexes requires a SHP-2-dependant downregulation of FAK activity. PECAM-1 may therefore recruit SHP-2 to participate in this phase of the maturation process. However, subsequent focal adhesion maturation and cycling were found to require FAK activity. Thus, alternatively, PECAM-1 may also sequester SHP-2 away from FAK to permit FAK activity in promoting the latter stages of focal adhesion maturation. This suggests that the involvement of PECAM-1 in endothelial cell motility is likely to be complex and regulated.

Expression of PECAM-1 not only enhanced cell migration but also stimulated in vitro tube formation, an effect that was lost if phosphorylation of Y663 and Y686 was eliminated (Fig. 7). We previously proposed (69) that during in vivo blood vessel formation, PECAM-1 promotes endothelial intercellular adhesion and the stabilization of endothelial cell junctions. Our observations that the PECAM-1V−→F mutant localized to cell-cell junctions (Fig. 8) and retained the ability to bind homophilically (data not shown) indicate that failure of this mutant to stimulate tube formation was more likely the result of an inability to enhance endothelial cell motility and not a disruption of PECAM-1-dependent endothelial intercellular interactions. This suggests that during in vivo angiogenesis, PECAM-1 may also participate in the migration behavior of endothelial cells prior to and essential for the organization of these cells into patent vascular channels. However, these data do not exclude potential roles for PECAM-1 in the organization of junctional complexes and the assembly of tubular structures. Consistent with this is our observation that tube formation by the tailless mutant, which does not localize to intercellular junctions or bind homophilically, remains impaired even after the period of tube formation is extended from
8 to 24 h to compensate for the compromised cell motility (data not shown).

We note that our previous (5) and present findings differ from those of some earlier studies in which expression of PECAM-1 in murine fibroblasts or the ECV304 tumor line resulted in decreased migration (26, 54). Furthermore, Kim et al. (26) reported that expression in bovine aortic endothelial cells of mutant PECAM-1 in which Y686 was mutated to a phenylalanine actually stimulated cell migration. Subsequently, Gratzinger et al. (14) from this group observed that the wound-healing migration of an immortalized murine endothelial cell line expressing murine PECAM-1 was decreased after expression of wild-type human PECAM-1, whereas expression of an ITIM defective mutant actually increased the motility of these cells. The apparent conclusion from these studies was that with respect to endothelial cell migration, PECAM-1 was inhibitory. In their most recent study, however, Gratzinger et al. (15) reported that wound-induced migration was increased by the expression of PECAM-1 in PECAM-1-null murine endothelial cells or inhibited in HUVEC by treatment with PECAM-1 antisense oligonucleotides, findings that are consistent with the present study (see Fig. 3) and our previous report (5).

In contrast, Gratzinger et al. (15) also reported that single-cell motility across fibronectin-coated transwell filters in the absence of a directional signal was actually enhanced by the loss or absence of PECAM-1. In terms of single-cell migration, as described previously (5), we observed that anti-PECAM-1 antibody inhibited the migration of HUVEC through transwell filters coated with Matrigel. Furthermore, expression of PECAM-1 in REN cells stimulated cell motility in the Matrigel transwell assay, as well as in an assay of random single-cell motility on fibronectin-coated slides (see Fig. 4). Together, these studies suggest, in contrast to Gratzinger et al. (15), that PECAM-1 promotes single-cell endothelial cell motility. The reasons for these discrepancies are still unclear but may be due to differences in the assays and experimental approaches that were used and/or the cells that were employed in the transfections. With respect to the latter possibility, we believe for the reasons cited above that REN cells represent an appropriate system for modeling endothelial cells (5, 25, 39, 58). We further note that our finding that PECAM-1 promotes cell migration is readily consistent with the studies of PECAM-1-deficient mice in which the loss of PECAM-1 function results in impaired angiogenesis (see Ref. 57; DeLisser H, unpublished observations).

When considered in light of previous studies, our data suggest that the cell context may determine whether the ITIMs of PECAM-1 mediate inhibitory or stimulatory responses. On T lymphocytes, PECAM-1, like other ITIM-containing members of the Ig superfamily, appears to be able to function as an inhibitor protein tyrosine kinase-mediated signal transduction triggered by the engagement of the T-cell receptor (TCR) (38). Specifically, in Jurkat T lymphocytes, coligation of PECAM-1 and the TCR induces PECAM-1 tyrosine phosphorylation, the binding of SHP-2, and attenuation of the TCR-induced calcium mobilization. Our findings, however, suggest that the effect of PECAM-1 on other cell types may be activating rather than inhibitory. This should not be viewed as too surprising, because tyrosine phosphorylation may also be a negative regulator of intracellular signaling as has been shown for the COOH terminus of Src family kinases (8). Consequently, ITIM-mediated recruitment of phosphatases such as SHP-2 may stimulate intracellular signaling by eliminating inhibitory phosphorylation events (53).

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