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Biochemical and Biophysical Research Communications 301 (2003) 243–249
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Platelet–endothelial cell adhesion molecule-1 modulates endothelial migration through its immunoreceptor tyrosine-based inhibitory motif

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Received 10 December 2002

Abstract

Coordinated migration of endothelial cells models the remodeling of existing endothelia as well as angiogenesis and vasculogenesis. Platelet–endothelial cell adhesion molecule-1, PECAM-1, a transmembrane endothelial adhesion protein, binds and activates the tyrosine phosphatase SHP-2 via phosphotyrosines 663 and 686. PECAM-1 phosphorylation and recruitment of SHP-2 are regulated by cell–cell and cell–substrate adhesion. We found that PECAM-1 is dephosphorylated on tyrosine 686 during endothelial migration, resulting in diffuse dispersal of PECAM-1 and SHP-2. Overexpression of native PECAM-1 slowed, and nonphosphorylatable PECAM-1 increased, endothelial migration, implying that the SHP-2-regulatory phosphotyrosines negatively regulate migration. Using differentially phosphorylated recombinant proteins we found that phosphotyrosine 686 preferentially mediates binding and 663 mediates activation of SHP-2 by PECAM-1. In PECAM-1-null endothelial cells, SHP-2 bound and dephosphorylated an alternative set of phosphoproteins and its distribution to the cytoskeletal fraction was significantly decreased. Tyrosine phosphorylation of β-catenin and focal adhesion kinase was increased in endothelial cells overexpressing nonphosphorylatable PECAM-1. Thus homophilically engaged, tyrosine-phosphorylated PECAM-1 locally activates SHP-2 at cell–cell junctions; with disruption of the endothelial monolayer, selective dephosphorylation of PECAM-1 leads to redistribution of SHP-2 and promigratory changes in phosphorylation of cytoskeletal and focal contact components.

Keywords: SHP-2; Tyrosine phosphatase; CD31; Entothelium; Migration; ITIM

Platelet–endothelial cell adhesion molecule-1 or PECAM-1, is a homophilically adhesive transmembrane glycoprotein [1,2] which modulates endothelial migration, tube formation, and angiogenesis in vivo and in vitro [3–5]. Many of these functions localize to phosphotyrosines 663 and 686 within an Immunoreceptor Tyrosine-based Inhibitory Motif or ITIM [6], which bind and activate SH2-domain-containing proteins [7]. In endothelial cells the tyrosine phosphatase SHP-2 is a major PECAM-1 ITIM partner [8,9]. ITIM tyrosine 686 is dephosphorylated in endothelial cells upon engagement of β1 integrins [10] and during normal vasculogenesis [11]. Conversely PECAM-1 mutated at tyrosine 686 increases rates of endothelial migration [4]. Coordinated migration of endothelial cell monolayers requires loosening of adherens junctions and focal contact turnover. Linkage of VE-cadherin to the actin cytoskeleton via β-catenin is regulated by β-catenin tyrosine phosphorylation [12]. Endothelial PECAM-1 binds β-catenin [13], making it a potential target for dephosphorylation by ITIM-associated SHP-2. SHP-2 also influences tyrosine phosphorylation of focal adhesion kinase (FAK) and thus focal contact turnover and cell motility [14,15]. The actual role of PECAM-1-mediated modulation of SHP-2 tyrosine phosphatase activity in endothelial cell migration remains unclear. To this end we have studied the modulation of ITIM phosphorylation during migration, its role in SHP-2 recruitment and activation, and the subsequent phosphorylation changes that may account for changes in endothelial motility.

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doi:10.1016/S0006-291X(02)02982-0

Experimental procedures

Materials. Polyclonal antibody (pAb) to human PECAM-1 and to PECAM-1 phosphotyrosines (PY) 663 or 686 [10,11] were generated in our laboratory. We purchased monoclonal antibody (mAb) to PY and SHP-2 and polyclonal antibody (pAb) to caveolin and PECAM-1 from Santa Cruz Biotechnology (Santa Cruz, CA), mAb to β-catenin and Focal Adhesion Kinase (FAK) from Transduction Labs (San Diego, CA), pAb to FAK PY397 from Upstate (Lake Placid, NY), and mAb to β-actin from Sigma–Aldrich (St. Louis, MO). pAPEX PECAM-1 and its Y663F mutant have been described [10]. An additional mutation at Y686F was made using the QuikChange Mutagenesis Kit (Stratagene, La Jolla, CA). The mutagenic primers were 5′ GGACAC AGAGACAGTGTCAGTGAAGTCCGG 3′ and its inverse; the changed nucleotide is underlined. pGEX2T-PECAM-1 cytoplasmic domain [16] was also mutated at Y686F and at Y663F with 5′ CCT CTGAACTCAGACGTGCAGTTCACGGAAGTTCAAGTGTCC 3′. pGEX2TSHP-2 construct was a gift of Ohnishi et al. [17]. A phosphatase-dead mutant (D425A) [18] was made using 5′ GGACCTGGC CAGCCCATGGCGTGCC 3′. pCDNA3-VCAM (Biogen, Cambridge, MA) and pIRES-EGFP (Clontech, Palo Alto, CA) were used in control transfections. Polypeptides were synthesized by the HHMI-Keck Foundation Lab at Yale University. Sequences were: for Y663, PLNSDVQY(phospho)TEVQVSS; and for Y686, KKDTET VY(phospho)SEVRKAV.

Cell culture, transfection, and immunostaining. Bovine aortic endothelial cells (BAECs) were cultured as previously described [19]. For the wound healing assay, confluent endothelial monolayers were scraped with a 15-well minigel comb. Endothelioma cell line 1uEnd.PECAM-1.1 (PecamKO) was established by retroviral transduction of primary endothelial cells with polyoma virus middle T-oncogene [20]. PecamKO cells retrovirally transduced with full-length murine PECAM-1 cDNA (PecamRC) [20,21] were cultured as described. Subconfluent PecamRC cells were transiently transfected using LipofectAMINE2000, Life Technologies (Grand Island, NY) according to the manufacturer's instructions. After 24 h cells were lightly trypsinized and replated to 60 mm fibronectin-coated dishes for wound-healing assays. Distance migrated was quantitated from pictures taken at 0 and 24 h with a Coolpix995 Digital Camera (Nikon, Tokyo Japan) on an Olympus IM light microscope (Melville, NY). Human umbilical vein endothelial cells were purchased from Dr. Jordan Pober (Yale Medical School) and cultured on gelatin as described [22]. For immunostaining, cells were cultured on 8-chamber culture slides (Falcon, Becton–Dickinson), fixed in Streck's Tissue Fixative (Streck Labs, La Vista, NE), permeabilized with 0.5% Triton X-100 in Tris-buffered saline (TBS), and stained with anti-PECAM-1 or SHP-2 1:100 followed by fluorescein- or rhodamine-conjugated secondary (Jackson Labs, West Grove, PA).

Recombinant protein production. GST-PECAM-1 cytoplasmic domain (GST-PEC) was expressed in BL21 bacteria (Pharmacia) induced with 0.5 mM isopropyl-β-D-thiogalactoside (Sigma) for 3 h at 37 °C. Bacteria were lysed with Bacterial Protein Extraction Reagent (Pierce) and Complete Protease Inhibitor (CPI) (Boehringer–Mannheim), the fusion protein was incubated with 50% glutathione agarose beads (Sigma) and washed in TBS + 1 mM ethylenediamenetetraacetic acid. PY GST-PEC was produced as directed in TKX1 bacteria (Stratagene). Concentration was determined using a standard curve of bovine serum albumin (Pierce) after SDS–polyacrylamide gel electrophoresis (PAGE) and Coomassie staining. GST-SHP-2 was produced essentially as described [23], but in BL21 bacteria (Pharmacia) and at 28 °C. Thrombin cleavage of SHP-2 (rSHP-2) from its GST tag was carried out as described [23]; concentrations were determined as with Bio-Rad Protein Assay (Hercules, CA).

Coprecipitations and fractionations. Cells were washed twice in PBS containing 1 mM orthovanadate. Wound-healing BAECs were lysed in 200 μl [50 mM Tris, pH 7.4, 100 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, a protease cocktail (CPI, Boehringer–Mannheim) and 0.2 mM orthovanadate]. PecamRC and PecamKO cells were lysed in 500 μl (20 mM Tris, pH 7.5, 10 mM NaCl, 10 mM EDTA, 1% NP-40, 1% deoxycholate, 0.1% SDS, 50 mM sodium fluoride, 1 mM sodium orthovanadate, and CPI). Lysates were centrifuged at 14,000*g* at 4 °C for 10 min, and supernatant concentration was determined using Bio-Rad Protein Assay. For Western blots 20 μg protein was boiled with 4× sample buffer (240 mM Tris, pH 6.8, 40% glycerol, 8% SDS, 0.002% bromophenol blue, and 0.002% β-mercaptoethanol). Triton X-100-soluble and insoluble fractions [24] and sucrose density gradient lipid raft fractionation [25] were carried out as described. For immunoprecipitation (IP), 200 μg protein was precleared with protein A/G–Sepharose (Santa Cruz); cleared supernatant was incubated with antibody and then precipitated with A/G–Sepharose. Beads were washed in lysis buffer and boiled in 4× sample buffer. For pulldowns, 10 μg of glutathione–agarose-bound GST-PEC and 5 μg rSHP-2 in 500 μl Pulldown Buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 0.5% Tween 20, 10 mM β-mercaptoethanol) were rotated for 30 min at 4 °C; the beads were washed in cold Pulldown Buffer. All samples were separated by SDS–PAGE; transferred to an Immobilon P filter (Millipore, Bedford, MA); and probed with anti-PECAM-1, FAK, SHP-2, β-catenin, PY, caveolin, or β-actin at 1:10,000, or anti-PECAM-1 663 PY, 686 PY, or FAK PY 397 at 1:1000 followed by horseradish peroxidase conjugated secondary (Promega, Madison, WI). Blots were developed using Chemiluminescent Reagent (Perkin–Elmer, Boston, MA), scanned on an Arcus II (Agfa, Mortsel, Belgium), and quantitated using BioMax 1D software (Kodak, Rochester, NY).

Phosphatase assays. To assess activation of rSHP-2 by GST-PEC, 3 μg bead-bound GST-PEC and 2 μg rSHP-2 were suspended in 45 μl Phosphatase Buffer (50 mM sodium acetate, pH 5.3, 50 mM NaCl, and 10 mM dithiothreitol) in 96-well plates (Falcon); at $t = 0.5$ μl of 500 mM *p*-nitrophenyl phosphate (pNPP) was added and the mixture was shaken for 10 min (adapted from [17]). A Wallac Victor Multilabel Counter attached to a PC laptop was used to record the absorbance at 405 nm. To demonstrate phosphatase activity of IPed SHP-2, cells were pretreated with pervanadate, SHP-2 was IPed in the presence of orthovanadate, and vanadate was washed out to disinhibit phosphatase activity, essentially as described [26]. Tyrosine phosphorylation of SHP-2-associated proteins was evaluated by Western blotting.

Results

PECAM-1 ITIM tyrosines contribute differentially to SHP-2 binding and activation

GST-PECAM-1 cytoplasmic domain fusion proteins were tyrosine-phosphorylated at one or both ITIM tyrosines by generating site-directed mutations at 663 and 686 (Fig. 1A). Western blotting with phosphospecific antibodies [10] confirmed selective tyrosine phosphorylation (Fig. 1B). Recombinant SHP-2 (rSHP-2) was cleaved from its GST tag (Fig. 1C). Pulldown assays using GST-PECAM-1 assessed the contribution of the respective ITIM phosphotyrosines to SHP-2 recruitment (Fig. 1D). PECAM-1 phosphorylated only at tyrosine 686 pulled down the same amount of SHP-2 as dually phosphorylated PECAM-1; lack of phosphorylation at 686 however significantly decreased SHP-2 binding. Engagement of the SH2 domains of SHP-2 serves not only to recruit the phosphatase but to activate it [23,27]. We used a colorimetric assay of dephosphorylation of pNPP to

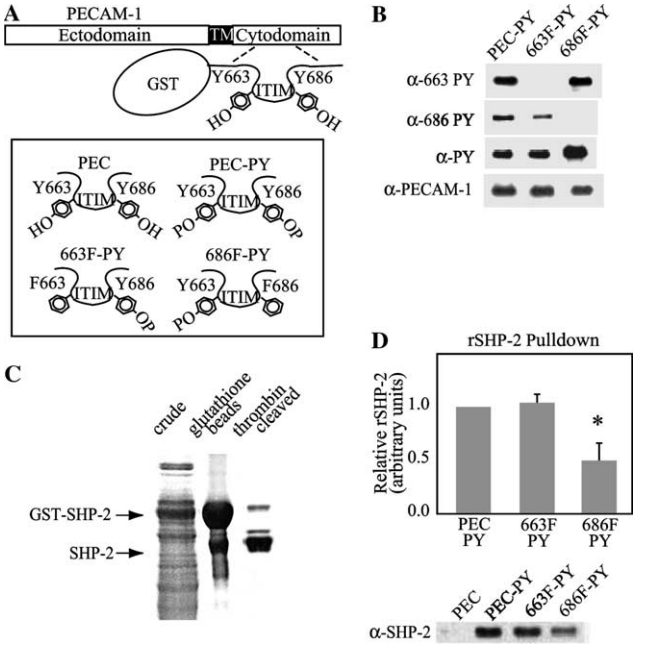

Fig. 1. Differential contribution of ITIM tyrosines to PECAM-1/SHP-2 association. (A) GST-PECAM-1 cytoplasmic domain fusion protein contains Y–F mutations in its ITIM. (B) GST-PECAM-1 phosphorylation was confirmed by Western blotting with phosphospecific antibodies. (C) GST-SHP-2 was captured on glutathione beads and then thrombin-cleaved to remove GST (Coomassie stain). (D) Pulldown assay using phosphorylated GST-PECAM-1 as bait. The 663F-PY pulls down as much SHP-2 as PEC-PY, but 686F-PY pulls down significantly less ($n = 5$, $^*p < 0.005$ vs PEC-PY).

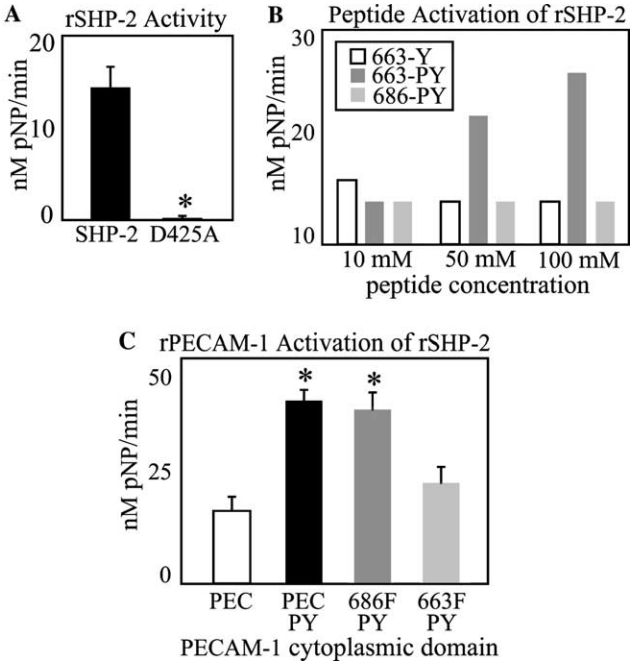

Fig. 2. Recombinant (r)SHP-2 is preferentially activated by ITIM phosphotyrosine 663. Phosphatase activity was measured using *p*-nitrophenyl phosphate (pNPP) as a substrate. (A) rSHP-2 dephosphorylates pNP at 15 nM/min at baseline; a loss of function mutant (D425A) is inactive ($n = 5$, $^*p < 0.005$). (B) Stimulation of SHP-2 activity by ITIM peptides, phosphorylated (663-PY, 686-PY) or not (663-Y). The 663-PY but not 663-Y or 686-PY stimulates rSHP-2 activity in a dosedependent manner. (C) Stimulation of rSHP-2 activity by rPECAM-1. PECAM-1 phosphorylated at both ITIM tyrosines (PEC-PY) or at 663 alone (686F-PY) but not 686 alone (663F-PY) significantly activated SHP-2 ($n = 6$, $^*p < 0.005$).

measure SHP-2 activity. Wildtype rSHP-2 had a baseline activity of 15 nM pNP/min, while rSHP-2 mutated in its catalytic acid [18] was inactive as expected (Fig. 2A). The responsiveness of rSHP-2 to engagement of its SH2 domains was tested using ITIM phosphopeptides. While phosphopeptide 663 produced a dose-dependent increase in SHP-2 activity, neither nonphosphorylated 663 nor phosphopeptide 686 did so (Fig. 2B). Activation was then tested in the context of the full PECAM-1 cytoplasmic domain (Fig. 2C). PECAM-1 phosphorylated at 663 alone activated SHP-2 as well as dually phosphorylated PECAM-1, whereas PECAM-1 phosphorylated at 686 caused very little activation over baseline. Thus the two individual phosphotyrosines of the PECAM-1 ITIM play individual and complementary roles in modulation of SHP-2 activity, with phosphotyrosine (PY) 686 recruiting and PY 663 activating the phosphatase.

PECAM-1 is selectively dephosphorylated at tyrosine 686 during endothelial migration

PECAM-1 localizes to cell–cell contacts in confluent endothelial cells [1]; homophilically engaged PECAM-1 is tyrosine-phosphorylated and able to bind SHP-2 [28]. Accordingly PECAM-1 and SHP-2 colocalized to cell–cell borders of confluent human umbilical vein endothelial cells, whereas migrating cells displayed diffuse PECAM-1 and SHP-2 staining (Fig. 3A). To assess PECAM-1 PY during migration, confluent bovine aortic endothelial cells (BAECs) were wounded to leave concentric circles of migrating endothelium at 24 h, which reachieve confluence by 72 h. Immunoprecipitation revealed decreased total PECAM-1 PY, and consequently, SHP-2 association, in migrating BAECs 24 h post-wounding; at 72 h, the newly confluent BAECs reachieve baseline PECAM-1 PY (Fig. 3B). Analysis with phosphospecific antibodies revealed that relative dephosphorylation at tyrosine 686 (74%) was much greater than that at tyrosine 663 (21%) (Fig. 3C). Thus, dephosphorylation of the PECAM-1 ITIM during migration occurs preferentially at the site that recombinant protein pulldown experiments implicate in recruiting SHP-2.

PECAM-1 targets SHP-2 to particular subcellular locations and to target phosphoproteins

Immortalized lung endothelial cells derived from the PECAM-1 knockout mouse (PecamKO) and reconstituted with full-length PECAM-1 (PecamRC) form

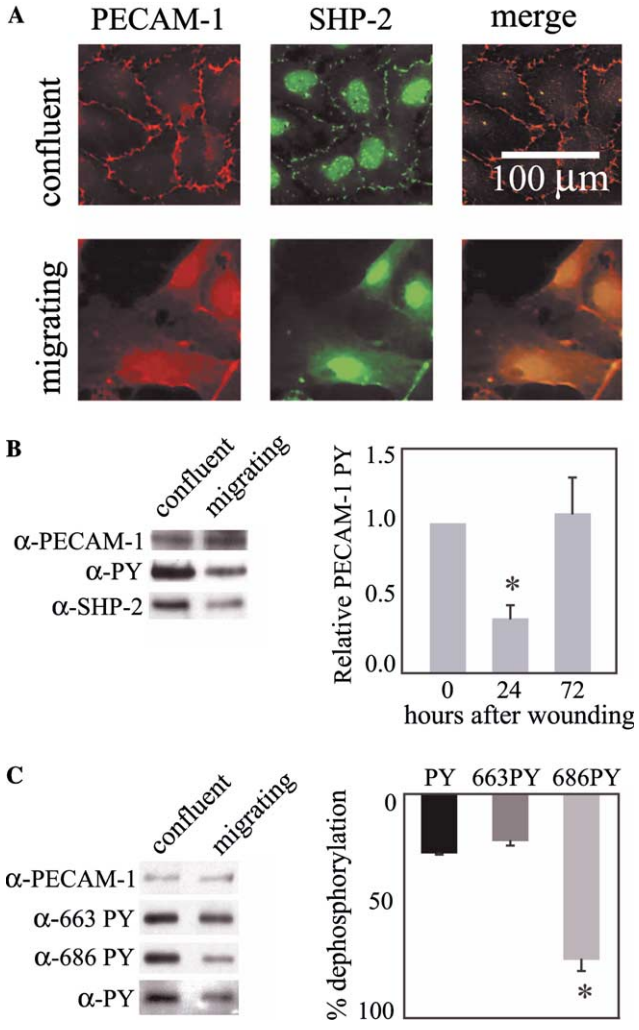

Fig. 3. PECAM-1 is differentially dephosphorylated on its ITIM during wound healing migration. (A) Immunofluorescence reveals cell–cell border localization of both PECAM-1 (upper right) and SHP-2 (upper middle) in confluent human umbilical vein endothelial cells, with colocalization in yellow (upper left). In migrating cells, both PECAM-1 and SHP-2 are diffusely localized. (B) Immunoprecipitation of PECAM-1 reveals decreased tyrosine phosphorylation (PY) and SHP-2 association in migrating bovine aortic endothelial cells. Quantitation reveals a >50% decrease in PECAM-1 PY at 24 h wound healing, with baseline PY restored at confluence in 72 h ($n = 3$, $^*p < 0.05$ vs 0 h). (C) Blotting with phosphospecific antibodies to the PECAM-1 ITIM reveals greater dephosphorylation at tyrosine 686 than at 663 ($n = 2$, $^*p < 0.05$ vs total PY).

contact-inhibited confluent monolayers and maintain expression of endothelial markers such as VE-cadherin [20,21]. To evaluate the role of PECAM-1 in localizing SHP-2 to potential target phosphoproteins, we assessed the relative recruitment of SHP-2 to a Triton X-100 insoluble fraction. Less than half as much SHP-2 was found in the Triton-insoluble pellet in PecamKO as PecamRC endothelial cells; FAK was by comparison found in comparable amounts, regardless of endothelial PECAM-1 expression status (Fig. 4A). The Triton-insoluble pellet represents actin cytoskeleton-associated proteins, but lipid raft components are also Triton-insoluble. Sucrose density gradient fractionation of the low-density, caveolin-positive lipid raft fraction [25] however failed to demonstrate any significant partitioning of SHP-2 to rafts in either PecamKO or PecamRC endothelial cells (Fig. 4B); SHP-2 instead localized to the higher density fractions along with β-actin. Thus in the absence of PECAM-1, SHP-2 is inefficiently localized to an actin cytoskeletal fraction, and consequently, likely has access to an alternate set of target phosphoproteins. To test this hypothesis we immunoprecipitated SHP-2 from PecamKO and PecamRC endothelial cells in the presence of orthovanadate to inhibit tyrosine phosphatase activity. Orthovanadate was then washed out and SHP-2 allowed to dephosphorylate any coprecipitating phosphoproteins (Fig. 4C). SHP-2 coprecipitated with different major phosphoproteins of ~125 and 140 kDa, respectively, from PecamKO and PecamRC cells, and these phosphoproteins were substrates for the phosphatase activity of SHP-2. Thus PECAM-1 is instrumental in localizing SHP-2 to the actin cytoskeletal fraction of endothelial cells and in directing it to target phosphoproteins.

PECAM-1 ITIM domain slows migration and controls tyrosine phosphorylation of adherens junction and focal contact components

To assess the role of PECAM-1 ITIM tyrosine phosphorylation in endothelial wound healing migration, PecamRC endothelial cells were transiently transfected with either wildtype PECAM-1 (PEC) or with PECAM-1 mutated in its ITIM domain (PEC 2F). Interestingly PEC transfection slowed and PEC 2F transfection increased wound healing migration (Fig. 5A). Thus the functional PECAM-1 ITIM domain, and its associated SHP-2, acts in a dose-dependent manner to retard migration; the ITIM-deficient PECAM-1 construct acts as a dominant negative, increasing migration. It is not the case that an overall increase in SHP-2 activity retards migration; in fact overexpression of a loss of function SHP-2 mutant slows migration (data not shown). We hypothesized that decreased ITIM phosphorylation and thus PECAM-1-mediated SHP-2 activation should lead to decreased dephosphorylation of SHP-2 targets. Adherens junction component β-catenin dissociates from the actin cytoskeleton, when tyrosine phosphorylated, as in response to shear stress [12]. Immunoprecipitation revealed significantly increased β-catenin tyrosine phosphorylation in PEC 2F transfected endothelial cells (Fig. 5B). FAK is a target of SHP-2 [15]; FAK kinase activity in turn is required for endothelial cell migration [14]. Western blotting with phosphospecific antibodies for the src-activating autophosphorylated tyrosine 397 of FAK [29] revealed increased FAK PY 397 in PEC 2F transfected endo-

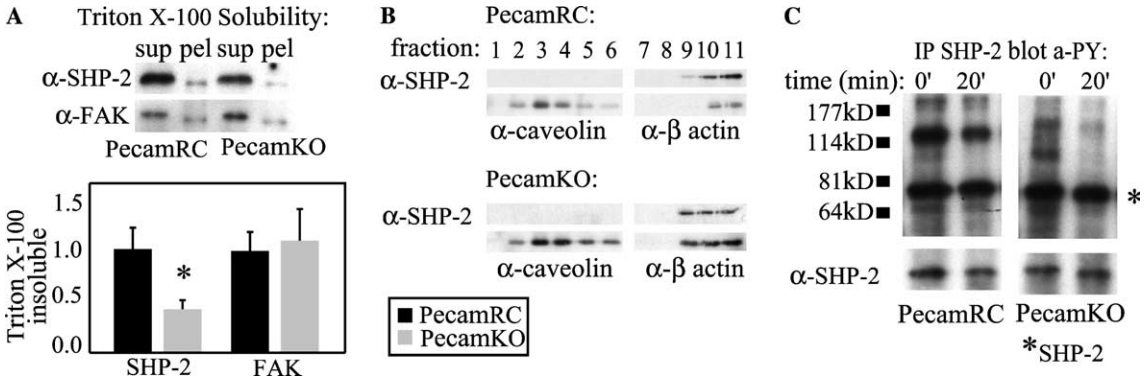

Fig. 4. PECAM-1 modulates subcellular distribution and phosphoprotein association of SHP-2 in endothelial cells. (A) PecamKO cells have significantly lower levels of SHP-2 but not FAK in the Triton X-100 insoluble fraction ($n = 6$, $^*p < 0.05$ vs PecamRC). (B) Sucrose density gradient fractionation separates caveolin-positive lipid rafts (fractions 3–4) from nonraft proteins in β-actin-positive fractions 9–11; SHP-2 is not present in rafts. (C) SHP-2 was immunoprecipitated, dephosphorylation was allowed to proceed for 0 or 20 min, and coprecipitates were immunoblotted for PY. SHP-2 associates with and is capable of dephosphorylating a distinct set of PY-proteins in PecamRC vs PecamKO endothelial cells.

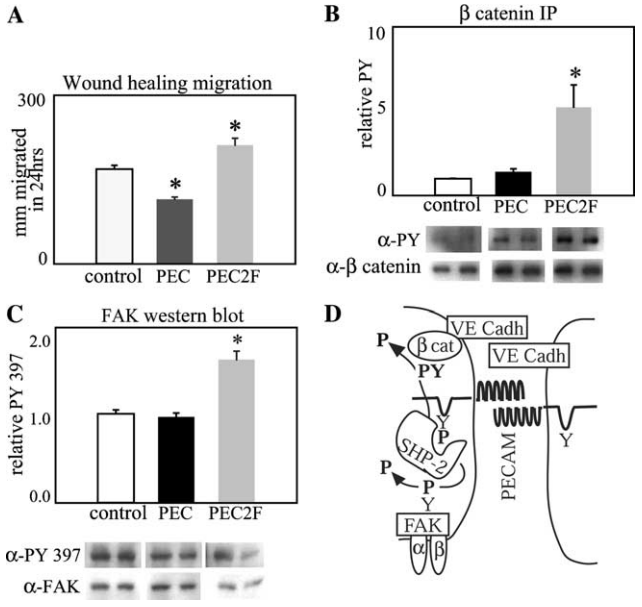

Fig. 5. Role of Pecam ITIM domain in wound healing migration. PecamRC cells were transiently transfected with wildtype (PEC) or ITIM-defective (PEC 2F) PECAM-1. (A) Wound healing migration was slowed with PEC and increased with PEC 2F transfection ($n = 8$, $^*p < 0.01$). (B) Immunoprecipitation of β-catenin reveals significantly increased PY with PEC 2F transfection ($n = 4$, $^*p < 0.05$). (C) Western blotting reveals increased FAK PY 397 in PEC 2F-transfected cells ($n = 3$, $^*p < 0.005$). (D) Model of modulation of adherens junction and focal adhesion dynamics through the phosphorylated PECAM-1 ITIM (see Discussion).

thelial cells, pointing to a potential role for PECAM-1 in modulating focal contact turnover.

Discussion

The role of PECAM-1 in coordinated endothelial cell behaviors appears paradoxical. As a whole, PECAM-1 plays a positive role in migration and angiogenesis [3,5]. Yet expression of ITIM-defective PECAM-1 increases migration in bovine aortic endothelial cells [4], implying a negative regulatory role for PECAM-1 signaling in coordinated motility. Restoration of wound healing migration to PecamKO endothelial cells by PECAM-1 is independent of signaling via the ITIM domain (D. Gratzinger, B. Engelhardt, J.A. Madri, unpublished data). In PecamRC endothelial cells, on the other hand, expression of ITIM-functional PECAM-1 slows and ITIM-defective PECAM-1 speeds wound healing migration (Fig. 5A). Thus PECAM-1 is required at baseline to support migration, while ITIM-mediated signaling provides a damp or brake to modulate that migration. In migrating endothelial cells, loss of cell–cell contacts releases PECAM-1 from the homophilic engagement that supports tyrosine phosphorylation and recruitment of SHP-2 [28]. Accordingly we observed a diffuse relocalization of both PECAM-1 and SHP-2 (Fig. 3A) as well as decreased overall tyrosine phosphorylation and SHP-2 association with immunoprecipitated PECAM-1 (Fig. 3B) in migrating endothelial cells. Using phosphospecific antibodies we determined that dephosphorylation was overwhelmingly at the second ITIM tyrosine, Y686, rather than Y663 (Fig. 3C).

We assessed the effect of this dephosphorylation event on SHP-2 binding and activation in vitro using recombinant SHP-2 and PECAM-1 cytoplasmic domain. Lack of phosphorylation at tyrosine 686 significantly decreased SHP-2 binding, whereas phosphotyrosine 663 was dispensable for recruitment (Fig. 1D). In contrast, phosphotyrosine 686 did not contribute to SHP-2 activation, whereas PECAM-1 phosphorylated at tyrosine 663 activated SHP-2 as well as did fully phosphorylated PECAM-1 (Fig. 2C). Engagement of the N-SH2 domain of SHP-2 allosterically disinhibits the phosphatase active site [27]. Interestingly Biacore experiments using PECAM-1 ITIM phosphopeptides have indicated that it is

phosphotyrosine 663 that engages the N-SH2 domain [8], consistent with a model of activation of PECAM-1-bound SHP-2 by phosphotyrosine 663. Given the concentration of PECAM-1 at cell–cell borders and its association with SHP-2, the likeliest candidate for dephosphorylating tyrosine 686 would seem to be SHP-2 itself. Phosphotyrosine 663 could help backpropagate the signal at the wound edge, sustaining dephosphorylation of PECAM-1 at remaining cell–cell borders as nonengaged PECAM-1 diffuses over the cell surface. Since phosphorylation at tyrosine 686 sustains homophilic adhesion via PECAM-1 [30], dephosphorylation on one cell could be communicated to adjoining cells via decreased PECAM-1 engagement and consequent loss of phosphorylation.

In addition to any potential effects of SHP-2 on PECAM-1 phosphorylation and homophilic adhesion, we hypothesized that engaged, tyrosyl-phosphorylated PECAM-1 directs SHP-2 to target phosphoproteins involved in maintaining a stable, confluent as opposed to a loose, migrating endothelial monolayer. Using detergent-insolubility and sucrose-gradient fractionation techniques (Figs. 4A and B) we determined that SHP-2 is inefficiently targeted to the actin cytoskeleton in PECAM-1 deficient endothelial cells. Furthermore, SHP-2 associates with and is capable of dephosphorylating an alternate set of phosphoproteins in the absence of PECAM-1 (Fig. 4C). We then transiently transfected endothelial cells with ITIM-defective PECAM-1 (PEC 2F) to interfere with their dephosphoylation. β-Catenin links adherens junction component VE-cadherin to the actin cytoskeleton via β-catenin; tyrosine phosphorylation of β-catenin releases this linkage at the level of the β-catenin/β-catenin interaction [12]. PECAM-1 binds tyrosylphosphorylated β-catenin [13]; ITIM-regulated SHP-2 activity could modulate phosphorylation of membrane-associated β-catenin. We found that PEC 2F transfection increases overall β-catenin tyrosine phosphorylation without effecting VE cadherin association (Fig. 5B), potentially loosening adherens junction-actin cytoskeletal interactions and favoring migration. Focal adhesion kinase (FAK) activity contributes to endothelial cell migration [14] and FAK is a target for SHP-2 phosphatase activity [15]. SHP-2 but not FAK partitioning to the actin cytoskeletal fraction is decreased in the absence of PECAM-1 (Fig. 4A); we hypothesized that PECAM-1 might modulate SHP-2-mediated dephosphorylation of FAK. Indeed PEC 2F transfection increased phosphorylation of FAK at src-activating tyrosine 397 [29]; thus phosphorylated PECAM-1 in confluent endothelial cells may serve to downregulate FAK activity and stabilize β-catenin/VE-cadherin interactions in the absence of a migratory stimulus, while selective dephosphorylation of the PECAM-1 ITIM domain may serve to potentiate FAK activity and sequester tyrosine phosphorylated β-catenin away from VE-cadherin during endothelial migration (Fig. 5D). Thus PECAM-1 appears to play a key regulatory role in coordinated endothelial migration.

Acknowledgments

We thank B. Engelhardt for the kind gift of PECAM-1-deficient and reconstituted immortalized lung endothelial cells. We thank H. Ohnishi and S. Sano for the pGEX2TSHP2 construct. We thank S. Davis for subcloning the pCDNA3-VCAM construct. This work was supported by USPHS Grants USPHS R37-HL-28373 and P01-DK38979 to J.A.M., Medical Scientist Training Program and Anna Fuller MD/PhD Predoctoral Fellowship in Oncology Grants to D.G., and USPHS Grants TE-DK-07556 and F32-HL-09864 to M.B.

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