Platelet-endothelial cell adhesion molecule (PECAM-1, CD31) is a 130-kDa glycoprotein belonging to the Ig superfamily of cell adhesion molecules. PECAM-1 expression is restricted to cells of the vascular system platelets, monocytes, neutrophils, selected T cells, and endothelial cells (1). In the latter, PECAM-1 is localized to cell-cell borders of confluent monolayers and, in addition, to lumen-facing areas of blood vessels or tube-like endothelial structures formed in vitro (2). PECAM-1 becomes diffusely distributed on the cell surface of sparse cell cultures or at the leading fronts of migrating cells (3). PECAM-1 has been shown to be a key player in the adhesion cascade leading to extravasation of leukocytes during inflammation. Pretreatment of monocytes or neutrophils, as well as endothelial cells, with anti-PECAM-1 antibodies effectively inhibited transmigration in vitro (4) and in vivo (5), indicating that PECAM-1 molecules on both the endothelial cells and the leukocytes contribute to the transmigration process. This was further supported by a genetic approach in PECAM-1 knockout mice, in which leukocytes are transiently arrested between the vascular endothelium and the basement membrane of inflammatory sites (6). In addition, PECAM-1 knockout mice have been noted to suffer from prolonged bleeding times, which is at least in part due to disrupted endothelial-platelet interactions (53), supporting the role of PECAM-1 as mediator of cell adhesion/activation. PECAM-1 has been shown to be more than just a passive player in adhesive interactions and indeed is actively involved in signal transduction pathways. PECAM-1 was demonstrated to undergo phosphorylation on tyrosine residues following mechanical (7) or biochemical (8–10) stimulation. Specifically, an immunoreceptor tyrosine-based activation motif (ITAM) domain was recently identified in the cytoplasmic tail of PECAM-1 (11). Phosphorylation of specific tyrosine residues within the ITAM domain of PECAM-1 were found to mediate selective recruitment of adapter and signaling molecules. These include SH-2-containing protein-tyrosine phosphatase (SHP)-1 (12) and -2 (9), SHIP, phospholipase C-γ (13), and phosphoinositide 3-kinase (14). Another set of proteins found to associate with PECAM-1 is represented by β-catenin (15). Recently, we reported that PECAM-1/β-catenin association is regulated by β-catenin tyrosine phosphorylation (2). Moreover, PECAM-1 overexpression resulted in recruitment of β-catenin into cell-cell junctions and a decrease in β-catenin tyrosine phosphorylation levels, suggesting that PECAM-1 plays active roles as a β-catenin modulator (2). Here we present evidence that not only β- but also γ-catenin associates with PECAM-1. Interestingly, however, serine/threonine, rather than tyrosine, phosphorylation was found to be the major regulatory mechanism responsible for PECAM-1/γ-catenin association. We demonstrate a shift in γ-catenin localization from nuclear to cell-cell junctions upon stable PECAM-1 expression in SW480 colon carcinoma cells and, moreover, suggest that γ-catenin mediates recruitment of PECAM-1 into an insoluble, cytoskeletal fraction. These results confirm and further expand the concept of PECAM-1 being a binder and modulator of catenins.
MATERIALS AND METHODS

Cells and Cell Cultures—Human umbilical vein endothelial cells (HUVEC) were obtained from Jordan Pober (Yale Medical School) and were cultured in gelatin-coated flasks as described (2, 16). Hemangioendothelioma (EOMA) cells were obtained from Robert Auerbach (University of Wisconsin, Madison, WI) and were grown in complete Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics (17). MCP7 human breast adenocarcinoma cells were obtained from the American Type Culture Collection (HTB-22). The cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 10 μg/ml insulin (bovine; Sigma) and transiently transfected as described (2). SW480 human colon carcinoma cells stably expressing PECAM-1 cDNA were generated as described (2) and were grown in Dulbecco’s modified Eagle’s medium supplemented with 0.5% fetal bovine serum. Embryoid bodies of human embryonic stem cells were generated in a three-dimensional type-I collagen gel was performed as described (16).

For cell migration assays, HUVEC were grown to confluency in 100-mm dishes. A 15-well microblack was used as a rake in a circular pattern to scrape cells from the dish leaving concentric rings of cells separated by intermittent cell-free regions. Cells were then allowed to migrate for 2 days before analyzed.

Murine Conceptuses—Harvesting and in vitro culturing of murine conceptuses was performed as described previously (22, 23). Briefly, conceptuses were collected from timed pregnant mice (CD1, Charles River, Wilmington, MA) using a dissecting microscope. Yolk sacs and embryos were separated in day 9.5 p.c. p.c. Groups of 50–60 embryos of day 7.5 p.c. concepts and 15 for day 9.5 p.c. concepts were analyzed.

For glucose treatment, embryos were collected at day 7.5 p.c. and cultured in rat serum and in the absence or presence of 25 mM D-glucose (C14) antibodies were purchased from Santa Cruz Biotechnology. Polyclonal antibodies to desmoplakin 1 and 2 were purified as described (2). Given the structural and functional similarities to β-catenin, we asked whether β-catenin and γ-catenin antibodies as described (2). For migration cell staining, cells were grown on the coverslip to confluency and scraped in the middle in a cross-like pattern. Cells were allowed to migrate for 2 days before staining. All experiments were repeated at least twice with similar results.

Data Base Search—The PECAM-1 cytoplasmic domain sequence was examined for consensus PKC substrate sites using the ScanProsite-Pitch program against PROSITE program (ISREC bioinformatics server, Lausanne, SW).

RESULTS

γ-Catenin Co-immunoprecipitates with PECAM-1 and Tyrosine Phosphorylation Is Not Important for This Interaction—We had recently reported that PECAM-1 functions as a reservoir for and a modulator of tyrosine phosphorylated β-catenin (2). Given the structural and functional similarities between β-catenin and γ-catenin (plakoglobin), we asked whether γ-catenin would similarly associate with PECAM-1 and investigated potential mechanisms that might be involved in the regulation of such an interaction. Previously, we found that PECAM-1/β-catenin interactions correlate with β-catenin tyrosine phosphorylation as illustrated in Fig. 1, β-catenin association with PECAM-1 is higher in EMAH compared with HUVEC, whereas no association was detected in transfected MCF7 cells (Ref. 2 and Fig. 1A). Interestingly, in the same cell culture model system, PECAM-1/γ-catenin association showed the exact opposite phenotype, being highest in HUVEC (Fig. 1A). This would suggest that not only β-catenin but also γ-catenin is a PECAM-1 partner and that PECAM-1 interaction with the two catenins is differentially regulated. It
EOMA cells were IP for PECAM-1, followed by used after stripping and reblotting. Interestingly, although γ-catenin was mainly present in the pellet fraction, β-catenin was unable to interact with PECAM-1 and was mainly detected in the supernatant fraction (Fig. 1C, second panel), suggesting that under these conditions γ-catenin but not β-catenin is the major PECAM-1 partner, and in agreement with our previous IP experiments (Fig. 1A).

PECAM-1/γ-Catenin Association Is Modulated by PECAM-1 Serine/Threonine Phosphorylation—Although much of the recent interest in PECAM-1 function arose from its tyrosine-based ITAM domain (11), PECAM-1 was initially characterized to be phosphorylated mainly on serine residues (20). However, the role of serine/threonine phosphorylation for PECAM-1 function at the cellular or molecular level has not yet been reported. Incubation of our GST-PECAM-1 fusion protein with purified PKC enzyme resulted in significant PECAM-1 phosphorylation (Fig. 2A, bottom panel), whereas no 32P incorporation was observed with GST alone (data not shown). To our knowledge, this is the first evidence that PKC can directly phosphorylate PECAM-1. Pull-down experiments with the PKC-derived phosphorylated and nonphosphorylated GST-PECAM-1 fusion protein and endothelial cell lysates indicated a significant decrease (more than 6-fold based on densitometric analysis, Fig. 2B) in the ability of γ-catenin to bind PECAM-1 (Fig. 2A, top panel). In agreement with our previous finding (Fig. 1C), β-catenin was only detected in the supernatant fractions (Fig. 2A, second panel), suggesting that PKC exclusively modulates γ-catenin but not β-catenin interactions with PECAM-1.

To further evaluate the role of PKC on PECAM-1/β-γ-catenin interactions in the context of a cell system, we took advantage of the cell culture model presented in Fig. 1A. High levels of PECAM-1/γ-catenin association in HUVEC may be due to low PKC activity in these cells. Indeed, exposure of HUVEC to a physiologic PKC inducer, diacylglycerol analog, significantly decreased PECAM-1/γ-catenin interactions (Fig. 2C), more than 3-fold based on densitometric analysis (Fig. 2D). Stauroporine, a potent PKC inhibitor, did not affect PECAM-1/γ-catenin interactions (Fig. 2C), further supporting the concept that PKC activity in HUVEC is low. In contrast, exposure of HUVEC to bisindolylmaleimide GF-109203x (bis), a potent and selective PKC inhibitor (21), resulted in a substantial increase in PECAM-1/γ-catenin association (Fig. 2E, top panel). Interestingly, the increase in γ-catenin binding was accompanied by a comparable decrease in β-catenin association with PECAM-1 (Fig. 2E, middle panel). This lends further support to our pull-down (Figs. 1C and 2A) and IP (Fig. 1A) experiments in which γ-catenin had a higher affinity for PECAM-1. In contrast, the PECAM-1/SHIP-2 interaction remained unchanged (Fig. 2E, third panel), suggesting that although β-catenin and γ-catenin may compete for a common binding site/domain, the SHP-2-binding site is different (tyrosine residues 663/686 in the ITAM domain) and is not influenced by the catenins binding on PECAM-1. Taken together, our in vitro model systems support the concept of an inverse correlation between PECAM-1 serine/threonine phosphorylation and its ability to associate with γ-catenin and, moreover, may indicate that PECAM-1 is an in vivo PKC substrate.

Differential PECAM-1/γ-Catenin Association during Vascularization of the Marine Conceptus—Given the ex vivo and in vitro ability of PECAM-1 to bind γ-catenin, we sought an analogous interaction in vivo. Significant increases in extraembryonic and embryonic vasculogenesis, with a concomitant de-
Regulation of γ-Catenin/PECAM-1 Interactions

fig. 2. PECAM-1/γ-catenin association is modulated by PKC-derived PECAM-1 serine/threonine phosphorylation. A, PECAM-1/GST fusion protein was incubated without (+) or with (-) nPKCε purified enzyme in the presence of [32P]ATP, and one-half of the reaction was analyzed for PECAM-1 phosphorylation by autoradiography (bottom panel). Pull-down experiments with phosphorylated (cold) or nonphosphorylated PECAM-1/GST fusion protein were performed with HUVEC as described in Fig. 1C, followed by immunoblotting for γ-catenin (top panel), β-catenin (second panel), and PECAM (third panel). B, densitometric analysis of PKC-derived PECAM-1 phosphorylation and PECAM-1/γ-catenin co-IP (average of two independent experiments) indicates a 6-fold decrease following PECAM-1 phosphorylation. C, HUVEC were left untreated (Con) or treated with diacylglycerol (DAG), a PKC inducer, 5 μM or staurosporine (St), a PKC inhibitor, 0.1 μM) for 45 min. Total cell lysates were IP for PECAM-1 followed by immunoblotting for γ-catenin (top panel), stripped, and reprobed for PECAM-1 (bottom panel). D, densitometric analysis of PECAM-1/γ-catenin co-IP after diacylglycerol and staurosporine treatments (an average of two independent experiments). Note the 3-fold decrease in PECAM-1/γ-catenin association after PKC activation. E, EOMA cells were left untreated (Con.) or treated with the PKC inhibitor bisindolylmaleimide GF 109203x (bis., 1 μM) for 60 min. Total cell lysates were IP for PKC-1 followed by γ-catenin (γ-cut, top panel), β-catenin (β-cut, middle panel), and SHP-2 (bottom panel) immunoblotting. The same membrane was used for all antibodies after stripping and reblotting. Note the reciprocal association of β- and γ-catenin with PECAM-1 upon PKC inhibition.

decrease in PECAM-1 tyrosine phosphorylation levels have been characterized between days 7.5 and 9.5 p.c. of the developing murine conceptus (22). Lysates, made from the whole conceptus at days 7.5 and 9.5 p.c. or yolk sac lysates from day 9.5 p.c. embryos were IP for PECAM-1, followed by γ-catenin immunoblotting. γ-Catenin was noted to be associated with PECAM-1 at all stages (Fig. 3A, top panel). However, an increase in PECAM-1/γ-catenin association occurred between days 7.5 and 9.5 p.c., a stage during which there is an increase in yolk sac blood island formation and simultaneous formation of embryonic vasculature. Interestingly, immunoblot analysis of the same lysate samples revealed a marked decrease in phospho PKC reactivity (Fig. 3A, third panel), whereas PKC expression levels were similar (Fig. 3A, fourth panel). As a control, the same membrane was stripped and reprobed with an antibody for the phosphorylated MAPK. No changes in phosphorylated MAPK levels were detected between days 7.5 and 8.5 p.c. (Fig. 3A, fifth panel), suggesting that the observed decrease in P-PKC reactivity is specific. Densitometric analysis of PECAM-1/γ-catenin co-IP and P-PKC reactivity during these stages of murine conceptuses development are summarized in Fig. 3B. These observations further support the ex vivo/in vitro inverse correlation between PECAM-1 serine/threonine phosphorylation and its ability to bind γ-catenin.

We have recently reported that hyperglycemia causes yolk sac and embryonic vasculopathy in cultured murine conceptuses (23). Moreover, PECAM-1 was found to be hyper-phosphorylated on tyrosine residues in hyperglycemic day 9.5 p.c. yolk sacs compared with control embryos (23). Other reports have documented a glucose-induced PKC activity, and PECAM-1 phosphorylation in cultured endothelial cells (24). To evaluate the potential glucose effect on PECAM-1/γ-catenin interactions, control, or glucose-treated day 9.5 p.c. yolk sac samples were IP for PECAM-1 followed by γ-catenin immunoblotting (Fig. 3C). A two-fold decrease in PECAM-1/γ-catenin association was noted in the glucose-treated samples (Fig. 3C, top panel). In contrast, a significant increase in PECAM-1/SHP-2 association was detected after glucose exposure (Fig. 3C, middle panel), in agreement with the glucose-induced increase in PECAM-1 tyrosine phosphorylation levels previously reported (23). In addition, glucose treatment induced a 4 fold increase in PKC phosphorylation, as judged by anti-phospho PKC immunoblotting (Fig. 3C, bottom panel), while PKC expression profile was similar (not shown). Thus, our ex vivo, in vitro, and in vivo studies all confirm the ability of γ-catenin to be associated with PECAM-1 and point to PECAM-1 serine/threonine phosphorylation, mediated, at least in part, by PKC as a major regulatory mechanism.

PECAM-1 recruits γ-catenin to areas of cell-cell junctions in transfected SW480 cells—Having demonstrated the PECAM-1/γ-catenin interaction biochemically, we were interested in the possible function of such an association. We have previously documented that stable expression of PECAM-1 in colon carcinoma SW480 cells results in recruitment of β-catenin to cell-cell junctions (2). Immunofluorescent staining of these SW480 cells confirmed similar findings for γ-catenin as well (Fig. 4A). In the Vo cells, areas of cell-cell interaction were completely devoid of γ-catenin, which seemed to be mainly localized to the nucleus (Fig. 4A, panel a). In contrast, upon PECAM-1 expression, γ-catenin was found mainly at cell-cell junctions (Fig. 4A, b), co-localizing with PECAM-1 (Fig. 4A, panel d). Thus, one possible function of the PECAM-1/γ-catenin association is to maintain γ-catenin localization to cell-cell junctions and to prevent its nuclear translocation. It has been recently reported (25, 26) that one downstream target of the β-catenin/lymphoid enhancer binding factor (LEF) complex is up-regulation of cyclin D1 expression. Interestingly, stable PECAM-1 expression in SW480 cells (Fig. 4, A, panel c, and B, top panel) significantly attenuated cyclin D1 protein expression (Fig. 4B, fourth panel). In contrast, neither cyclin D3 (Fig. 4B, bottom panel), β-catenin, or γ-catenin (Fig. 4B, second and third panels) expression levels were changed, suggesting that the decrease in cyclin D1 levels may be specific for inhibition of catenin-regulated transcription. Therefore, PECAM-1-mediated recruitment of both β- (2) and γ-catenin (Fig. 4A, panels b and d) to cell-cell junctions decreases their nuclear accumulation and gene regulation.

PECAM-1/γ-Catenin Association, a Possible Link to the Cytoskeleton—Platelet activation was reported to induce PECAM-1 serine phosphorylation and PECAM-1 redistribution from the soluble to a cytoskeletal insoluble fraction (20). Frac-
migrate. For example, a significant amount of $\alpha$-catenin was shifted to the soluble fraction (Fig. 5A, top panel, Mig), suggesting less cadherin-actin interactions and hence decreased cell adhesion, as would be expected under migratory conditions (28). Interestingly, however, PECAM-1 expression exhibited the opposite trend and was now equally distributed between soluble and insoluble fractions (Fig. 5A, third panel). Moreover, under migration conditions, PECAM-1/$\gamma$-catenin association was mainly detected in the insoluble fraction (Fig. 5A, fourth panel), whereas overall $\gamma$-catenin distribution between the two fractions did not change (Fig. 5A, second panel, compare Con with Mig). Densitometric analysis of PECAM-1/$\gamma$-catenin association under confluency or during HUVEC migration is summarized in Fig. 5B. Thus, although the overall amount of PECAM-1-associated $\gamma$-catenin was not significantly changed, the distribution shifted from a ratio of 80% (soluble) to 20% (insoluble) in confluent cultures to a ratio of 35% (soluble) to 65% (insoluble) during migration.

The increase in insoluble PECAM-1 fraction may be explained by interaction with the actin-based cytoskeleton or with intermediate filaments, vimentin in the case of endothelial cells. Both cytoskeletal components utilize $\gamma$-catenin as a molecular linker (29, 30). Co-immunoprecipitation of PECAM-1 with desmoplakin (Fig. 1B, fifth panel), a component involved in connecting desmosomal cadherins, via $\gamma$-catenin, to intermediate filaments (30), suggests that PECAM-1, in part, may become associated with vimentin. Immunofluorescent staining indicated a significant rearrangement of PECAM-1, as well as $\alpha$-catenin, during migration (Fig. 5C). At confluency, PECAM-1 and vimentin are localized to two distinct cellular compartments that do not seem to interact with each other at this level of resolution (Fig. 5C, panels a–c, and Ref. 31). However, during migration, PECAM-1 is diffusely localized on the cell surface (Fig. 5C, panel d), whereas vimentin filaments are present up to the leading edges of the migrating cells (Fig. 5C, panel e, and Ref. 32). Under these conditions, partial PECAM-1/vimen-
Regulation of γ-Catenin/PECAM-1 Interactions

Fig. 5. PECAM-1/γ-catenin association is mainly detected in the insoluble fraction during endothelial cell migration. A, lysate samples from soluble (S) and insoluble (I) fractions of control (Con) or migrating (Mig) HUVECs were analyzed by immunoblotting for the expression of α-catenin (α-cat, top panel) and γ-catenin (γ-cat, second panel). The same samples were IP for PECAM-1, followed by immunoblotting for PECAM-1 (third panel), stripping, and reprobing for γ-catenin (γ-cat, bottom panel). B, densitometric analysis (an average of two independent experiments) of PECAM-1/γ-catenin co-IP distribution between soluble (Sol) and insoluble (IS) fraction in confluent versus migrating HUVECs. C, confluent (panels a–c) or migrating (panels d–i) HUVECs were stained for PECAM-1 (panels a, d, and g), vimentin (panels b and e) and desmoplakin (panel h). The merged staining of PECAM-1 and vimentin is shown in panels c and f, and that of PECAM-1 and desmoplakin is shown in panel i. Note partial PECAM-1/vimentin co-localization in migrating but not confluent HUVECs and partial PECAM-1/desmoplakin co-localization at the cell edges. 

Fig. 6. PECAM-1/vimentin co-localization in HUVEC three-dimensional cultures correlates with a dramatic increase in PECAM-1/γ-catenin association. A, HUVECs were grown in three-dimensional collagen gels for 1 day, formalin-fixed, and embedded in paraffin. 5-μm sections were double-stained for PECAM-1 (panel a) and vimentin (panel b). The merged images are shown in panel c, demonstrating co-localization of both proteins. Scale bar, 50 μm. B, HUVEC were grown to confluency on gelatin-coated flasks (2D) or embedded in collagen gels (3D) for 1 day. Lysate samples were IP for PECAM-1 and blotted for γ-catenin (γ-cat, top panel), stripped, and reprobed for PECAM-1 (bottom panel). C, densitometric analysis (an average of two independent experiments) demonstrating a 7-fold increase in PECAM-1/γ-catenin association when grown under three-dimensional conditions.

DISCUSSION

Based on morphological and biochemical criteria two major groups of cell-cell junctions are commonly distinguished: desmosomes and adherens junctions. These two cellular structures are biochemically distinct and have only one component in common, i.e. γ-catenin. Morphologically, endothelial cell-cell junctions primarily consist of an extended adherens junctional zone in which tight junctions and gap junctions are inserted (36). Endothelial cells do not have structures similar to desmosomes or hemidesmosomes, nor do they express desmosomal cadherins (37). A third category of adherens junction has been described (complexus adherens), which occurs in certain vascular endothelia and was found to be negative for desmosomal cadherins, α-actinin and vinculin but rich in γ-catenin and desmoplakin (35). Recent studies have demonstrated that the

PECAM-1/vimentin co-localization was detected (Fig. 5C, panel f). Moreover, under migratory conditions we were able to detect partial co-localization of PECAM-1 with desmoplakin (Fig. 5C, panels g–i), mainly at the cell periphery/edges. In addition, a significant co-localization of PECAM-1 and vimentin was observed when HUVEC were grown in type I, three-dimensional, collagen gels (Fig. 6A). These conditions are thought to provide an environment more closely mimicking in vivo conditions compared with cells grown on tissue culture plastic (2D). Indeed, a robust increase in PECAM-1/γ-catenin association was observed under three-dimensional conditions (Fig. 6B), more than 7-fold, based on densitometric analysis (Fig. 6C). However, desmoplakin expression levels in endothelial cells are low (Ref. 31 and data not shown) and, in addition, mainly exhibit a diffuse cytoplasmic localization (Ref. 31 and Fig. 5C, panels g–i). Therefore, to better study the possible PECAM-1/desmoplakin co-localization, we stably expressed PECAM-1 in HEK 293 cells (Fig. 7). PECAM-1 expression was noted to be at levels
Indeed, we could not detect co-localization of PECAM-1 with vimentin (Fig. 5C, panels a–c) or desmoplakin (not shown) under the same conditions. However, partial PECAM-1 co-localization with vimentin (Fig. 5C, panels d–f) and desmoplakin (Figs. 5C, panels g–i, and 7, panels a–c) was observed in HUVEC migration or transfected HEK 293 cells and, most importantly, in HUVEC three-dimensional cultures (Fig. 6A). The latter was accompanied by a dramatic increase in PECAM-1/γ-catenin association (Fig. 6, B and C). Thus, based on biochemical recruitment into an insoluble fraction (Fig. 5, A and B) and partial co-localization with vimentin (Figs. 5C, panels d–f, and 6A, panels a–c) and desmoplakin (Figs. 5C, panels g–i, and 7, panels a–c), we suggest that one possible role for PECAM-1/γ-catenin association is to mediate PECAM-1 interaction with a cytoskeletal element, presumably vimentin. An increase in insoluble PECAM-1 fraction was observed following transforming growth factor-β1 treatment of promonocytic U-937 cells (40). Interestingly, cytochalasin B, an inhibitor of actin polymerization, treatment had no effect on PECAM-1 recruitment into the insoluble cytoskeletal-associated fraction, supporting the notion that vimentin, rather than actin, is the relevant cytoskeletal element. Therefore, PECAM-1 may be a dynamic part of the endothelial complexus adherens. The role for such possible PECAM-1/vimentin interactions is not yet known, but it may mediate a physical link between the cell surface and the nuclear envelope. This may transmit mechanical or biochemical signals that may modulate the migratory phenotype of the endothelial cells. PECAM-1 overexpression has been shown to inhibit migration of endothelial (41) and nonendothelial (3) cells and was attributed to an increase in cell adhesion. Our present results may suggest an additional mechanism, i.e. that possible PECAM-1/γ-catenin/desmoplakin/vimentin interactions may transmit signal(s) that regulate cell migration.

A structural role for PECAM-1/γ-catenin association is, however, only one aspect for such an interaction. γ-Catenin, like β-catenin, interacts with a multitude of proteins, including classical cadherins, α-catenin, fascin, axin, adenomatous polyposis coli (APC), and lymphoid enhancer binding factor/T cell factor (LEF/TCF) transcriptional factors (42). Elevated levels of β- and γ-catenin were observed when the ubiquitin-proteasome degradation system was inhibited, and this was followed by nuclear accumulation of both catenins (44). Indeed, recent studies have provided compelling evidence that catenins can play a central role in signal transduction and the regulation of gene expression (42, 43). The downstream gene targets were recently found to include c-Myc (45), the metalloproteinase matrilysin (46), the AP-1 transcription complex components c-Jun and Fra-1, urokinase-type plasminogen activator receptor, ZO-1 (47), and cyclin D1 (25, 26). Stable PECAM-1 expression in SW480 cells recruited β- (2) and γ-catenin to areas of cell-cell junctions (Fig. 4A) and prevented their nuclear accumulation. Interestingly, this correlated with decrease in cyclin D1, but not cyclin D3, expression levels (Fig. 4B). Thus, PECAM-1 apparently functions to maintain β- and γ-catenin localization at areas of cell-cell borders and prevent their signaling abilities, properties that were previously noted for E-cadherin (48), N-cadherin, or α-catenin (49). In endothelial cells, cadherin-based adherens junctions comprise a dynamic compartment, and its composition rapidly changes according to the functional state of the cells (28). For example, when cells are released from tight confluence and migrate, VE-cadherin is mostly linked to p120 and β-catenin, and only small amount of the complex is bound to the actin cytoskeleton (50). Once the junctions stabilize, p120 and β-catenin tend to detach from the complex and are substituted by γ-catenin (28). Therefore, such

Fig. 7. PECAM-1 colocalizes with desmoplakin in HEK 293 cells expressing PECAM-1. Stable PECAM-1 expression elicits a partial co-localization with desmoplakin in HEK 293 cells. PECAM-1-expressing 293 cells were double stained for desmoplakin (panel a) and PECAM-1 (panel b). A punctate pattern of desmoplakin staining, showing colocalization with PECAM-1 is shown in the merged images (panel c). Scale bar, 50 μm.
an increase in the free γ-catenin pool during cell migration and a decrease in cytoplasmic as well as membrane-associated PKC activity during cell migration both may account for PECAM-1/γ-catenin complex recruitment into an insoluble fraction (Fig. 5, A and B). Thus, although PECAM-1 has not been considered to be part of adherens junctions (27), its physical close proximity and its function as a catenin binder and modulator may suggest common features of PECAM-1 and VE-cadherin in endothelial cell adhesion. Interestingly, the observed decrease in cyclin D1 expression did not attenuate cell proliferation, suggesting that catenin-mediated transcription by itself is not responsible or sufficient for SW480 or other epithelial cell growth regulation (51).

β-Catenin and γ-catenin have a high degree of homology, especially in their central domain, the so-called armadillo repeats, and share some overlapping, as well as distinct, properties. Importantly, the mechanisms responsible for β- and γ-catenin binding to PECAM-1 were significantly different. We could not detect association of PECAM-1 with the third Arm repeats-containing protein-p120, suggesting that the catenin binding to PECAM-1 is specific and is not mediated by the Arm repeats per se. Although PECAM-1/β-catenin association is primarily due to β-catenin tyrosine phosphorylation state (Fig. 1B, fourth panel, and Ref. 2), PECAM-1/γ-catenin association was found to be mainly regulated by PECAM-1 serine/threonine phosphorylation. This was demonstrated directly (Fig. 2, A and B) and indirectly (Fig. 2, C–E) in ex vivo, in vitro, and in vivo systems. An increase in PECAM-1 serine/threonine phosphorylation was observed in glucose-treated (24) and CoCl2-treated (as an hypoxia mimetic; Ref. 52) HUVEC, in thrombin-stimulated platelets (20), and in tumor growth factor-β1-stimulated promonocytic U-937 cells (40). Such increases in PECAM-1 serine/threonine phosphorylation could be inhibited by PKC inhibitors (GF109203x; Refs. 24 and 52) and was therefore attributed to PKC activation. However, no direct evidence for PECAM-1 phosphorylation by PKC has been documented to date. The ability of nPKCe (Fig. 2A, bottom panel), as well as cPKC (α, β) isomers (data not shown) to directly phosphorylate GST-PECAM-1 fusion protein is therefore novel. Interestingly, scanning the PECAM-1 cytoplasmic domain for PKC substrate consensus sites using the ScanProsite program resulted in the identification of a consensus PKC phosphorylation site at residue S674 in the sequence 764SHK, which is located in the intervening sequence between the two ITAM tyrosines Tyr663 and Tyr698. Moreover, we found PKC-mediated PECAM-1 phosphorylation to play a key role in modulating PECAM-1/γ-catenin association. This is best demonstrated in our pull-down experiment (Fig. 2, A and B). In addition, the ability to regulate PECAM-1/γ-catenin association by PKC modulators in HUVEC (Fig. 2, C and D) and EOMA (Fig. 2E) cells is consistent with the notion that PECAM-1 is an in vivo PKC-substrate. We initially observed PECAM-1 to preferentially bind β-catenin in EOMA cells and correlated it with hyper β-catenin tyrosine phosphorylation levels (2). Interestingly, PKC inhibition in EOMA cells resulted in an increase in γ-catenin and a comparable decrease in β-catenin association with PECAM-1 (Fig. 2E), suggesting that γ-catenin has a higher affinity for PECAM-1 that would compete-off β-catenin. The higher affinity of γ-catenin toward PECAM-1 was supported by our pull-down experiments (Figs. 1C and 2, A and B). Experiments using surface plasmon resonance are currently underway aimed to quantify and compare the affinity of catenin toward PE-

![Fig. 8. A schematic representation of our current working model for PECAM-1 interaction with SHP-2, β-catenin, and γ-catenin. A, EOMA cells are known to have a relatively high degree of β-catenin and PECAM-1 tyrosine phosphorylation (2) and therefore exhibit robust PECAM-1/β-catenin and SHP-2/PECAM-1 interactions (upward arrows). In contrast, EOMA cells exhibit only modest γ-catenin/PECAM-1 interactions. B, when EOMA cells are treated with a PKC inhibitor, PECAM-1 serine/threonine phosphorylation is reduced, resulting in an increased PECAM-1/γ-catenin interaction (thick upward arrow). Although SHP-2 binding does not appear to be perturbed (Φ), an increased γ-catenin/PECAM-1 interaction is noted (thick upward arrow), and this appears to inhibit and reduce β-catenin/PECAM-1 interactions (thick downward arrow). C, in contrast, HUVEC cells are known to have a lower degree of β-catenin and PECAM-1 tyrosine phosphorylation and thus exhibit low β-catenin/PECAM-1 and SHP-2/PECAM-1 interactions (thin downward arrows) and a robust γ-catenin/PECAM-1 interaction (thick upward arrow). D, when HUVEC cells are treated with a PKC inducer PECAM-1 serine/threonine phosphorylation is increased, resulting in a decreased γ-catenin/PECAM-1 interaction (thick downward arrow) with no apparent changes in β-catenin/PECAM-1 or SHP-2/PECAM-1 interactions (Φ).](http://www.jbc.org/content/276/24/21442)

CAM-1. Taken together, the results presented herein support and further expand the concept that PECAM-1 is not only involved in cell adhesion but is intimately involved in signaling, for example by binding and modulating β- and γ-catenin localization. Our results also point to protein phosphorylation as the major regulatory mechanism responsible for the different sets of PECAM-1 interactions, suggesting dynamic tyrosine and serine/threonine phosphorylation/dephosphorylation events.

Fig. 8 is our current working hypothesis. In hemangioma-derived EOMA cell cultures, PKC activity is high (as evident by low PECAM-1/γ-catenin association and modulation by PKC inhibitor), and β-catenin as well as PECAM-1 tyrosine phosphorylation levels are also high. This results in the interaction of PECAM-1 with β-catenin and SHP-2 and β-catenin dephos-
phorylation, whereas γ-catenin association is minimal (Fig. 8A). However, exposure of EOMA cells to PKC inhibitor would cause an increase in γ-catenin association with PECAM-1, a decrease in β-catenin association with PECAM-1, but no change in SHP-2 association with PECAM-1, suggesting that γ-catenin is capable of competing off even tyrosine-phosphorylated β-catenin (Fig. 8B). In primary HUVEC cultures, PKC activity as well as β-catenin tyrosine phosphorylation levels are low, and γ-catenin is the major PECAM-1 partner (Fig. 8C). Exposure of HUVEC cultures to a PKC inducer causes increased PECAM-1 serine/threonine phosphorylation, which results in decreased PECAM-1/γ-catenin interactions (Fig. 8D).

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