Platelet/endothelial cell adhesion molecule-1 (PECAM-1) is a homophilic adhesion receptor that mediates leukocyte/endothelial cell interactions that take place during transendothelial migration. Recent reports have shown that the binding of certain anti-PECAM-1 antibodies results in up-regulation of integrin function on the surface of leukocytes and platelets, suggesting that PECAM-1 may be capable of transmitting information into the cell following its engagement. PECAM-1 isolated from resting or activated but nonaggregated platelets was phosphorylated predominantly on serine residues; however, PECAM-1 derived from activated, aggregated platelets was strongly phosphorylated on tyrosine. Synthetic tyrosine-phosphorylated peptides derived from five different regions within the cytoplasmic domain of PECAM-1 were screened for their ability to associate with cytoplasmic signaling molecules. The protein-tyrosine phosphatase SHP-2 was found to interact specifically with two different PECAM-1 phosphopeptides containing highly conserved phosphatase-binding motifs on PECAM-1 with the sequences VQpY663TEV and TVpY686SEV. More important, SHP-2 bound not only PECAM-1 phosphopeptides, but also became associated with full-length cellular PECAM-1 during the platelet aggregation process, and this interaction was mediated by the amino-terminal Src homology 2 domains of the phosphatase. Since SHP-2 normally serves as a positive regulator of signal transduction, its association with activated PECAM-1 suggests a number of potential mechanisms by which PECAM-1 engagement might be coupled to integrin activation in vascular cells.

PECAM-1 is a 130-kDa member of the immunoglobulin gene superfamily that is expressed on platelets and leukocytes and is also present at high concentration at the intercellular junctions of endothelial cells (for a review, see Refs. 1 and 2). Because of its presence on the surface of these vascular cells, PECAM-1 has been implicated in mediating a number of cell/cell interactions, including those that take place during cell migration (3), transendothelial migration of monocytes and neutrophils (4), and following antigenic stimulation of transmigrating lymphocytes (5). Although multiple mechanisms have been proposed for PECAM-1-mediated adhesion events, recent studies suggest that PECAM-1 molecules on adjacent cells are able to interact homophilically with each other, utilizing amino-terminal Ig homology domains 1 and 2 to effect these cellular associations (6, 7).

In addition to serving as a cell adhesion receptor, several lines of evidence suggest that PECAM-1 may be capable of transmitting signals into the cell following its engagement. Tanaka et al. (8) were the first to show that antibody-mediated engagement of PECAM-1 on the surface of lymphocytes could lead to up-regulation of integrin function, a finding that has been reproduced in lymphokine-activated killer cells (9), CD34+ hematopoietic progenitor cells (10), monocytes and neutrophils (11), and natural killer cells (12). Although stimulation of Fc receptors by bound antibody may be contributing to cellular activation in some of these studies, it is likely that PECAM-1 dimerization itself may be capable of transducing as yet undefined signals into the cell, a process that could be mimicked by homophilic PECAM-1/PECAM-1 interactions that are thought to occur between leukocytes and endothelial cells during the process of transendothelial migration.

The mechanism by which PECAM-1 engagement might lead to downstream signaling events is unknown. Previous studies have shown that, following cellular activation, PECAM-1 becomes phosphorylated (13, 14), but the target for this phosphorylation event appeared to be serine residues within the cytoplasmic domain (13). A more recent report by Modderman et al. (15) showed that PECAM-1 derived from resting platelets that had been preincubated with the membrane-permeable tyrosine phosphatase inhibitor pervanadate became tyrosine-phosphorylated. Precisely when this might occur, if at all, during normal platelet physiology remained to be determined, but it was clear from their studies that the balance of intracellular kinases and phosphatases was likely to play a role in regulating both the stoichiometry of PECAM-1 phosphorylation and the residues that might become phosphorylated during different stages of cellular activation.

The purpose of this investigation was to determine whether PECAM-1 could become tyrosine-phosphorylated in response to
agonist-induced cellular activation and subsequent cell/cell interactions. In addition, we sought to identify cytoplasmic signaling molecules that might associate with the cytoplasmic domain of PECAM-1 and to provide a mechanistic link between PECAM-1 engagement and the subsequent activation of cell-surface integrins that had been observed in previous investigations. Using human platelets as a model, we show that PECAM-1 becomes tyrosine-phosphorylated during the platelet aggregation process and that this creates docking sites for the protein-tyrosine phosphatase SHP-2. The interaction between SHP-2 and PECAM-1 is dependent upon Igg-mediated platelet/platelet interactions and occurs via the Src homol-
genic domain (SH2) domains of the phosphatase and highly conserved phosphatase-binding motifs encompassing phosphotyrosines 663 and 686 within the cytoplasmic domain of PECAM-1.

EXPERIMENTAL PROCEDURES

Preparation of Washed Platelets—Platelets were obtained from 100 ml of blood obtained from volunteers who had not taken any antiplatelet medication in the preceding 10 days. Whole blood was anticoagulated with acid citrate/dextrose, pH 4.6, in the presence of 50 mg/ml prostaglandin E1, according to previously described methods (13). Platelet-rich plasma was obtained by centrifugation at 250 x g for 5 min at room temperature, and platelets were obtained by sedimentation at 1500 x g for 15 min. The pellet was washed twice in Ringer citrate/dextrose buffer (2% Triton X-100, 10 mM EGTA, 15 mM HEPES, 145 mM NaCl, 0.1 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 2 mM sodium orthovanadate, pH 7.4), and the lysate was slowly rocked for 1 h at 4°C. Triton-soluble and -insoluble (cytoskeletal) fractions were separated by centrifugation at 15,000 x g for 5 min at 4°C as described previously (13). In some experiments, samples were lysed by the addition of an equal volume of ice-cold 2 x radioligand immunoprecipitation assay buffer (final concentration = 20 mM Tris, pH 7.4, 1 mM EDTA, 150 mM NaCl, 0.1% (w/v) sodium deoxycholate, 1 mM Na3VO4, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1% (v/v) Triton X-100).

Immunoprecipitation Analysis—Following fractionation of the 15,000 x g Triton-soluble platelet supernatant, lysates were precleared with 50 μl of a 50% slurry of CNBr-activated Sepharose for 30 min at 4°C and then centrifuged at 4000 rpm for 5 min. Precleared lysates were incubated overnight with the following: (a) normal mouse IgG1 (10 μg/ml), (b) normal rabbit IgG (10 μg/ml) that does not cross-react with SHP-1. Horseradish peroxidase-conjugated goat anti-rabbit IgG (1:25,000). Immunoreactivity was then determined by chemilumi-
nescence detection according to the manufacturer's instructions (Am-
ershalm Life Science, Inc.).

Peptide Synthesis—PECAM-1 peptides were synthesized using a Model 9050 Pepsynthesizer (Millipore Corp.) with Fmoc chemistry as described previously (16). Minor modifications to the procedure included the incorporation of phosphotyrosine residues during peptide synthesis. Fmoc-Tyr(P(OBu))-OH (Quality Controlled Biochemicals, Hopkinton, MA) was side-chain-protected with a t-butyldiphenylsilyl group and was introduced as the free acid. It was activated with 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate and 0.1 M N,N,N',N'-

dimethylacetamide containing 20% 4-dimethylaminopyridine to facili-
tate cleavage from the resin. Peptides were biotinylated at the amino terminus by incubation with a 1.5-mold molar excess of NHS-LC-biotin (Pierce) dissolved in dimethylacetamide containing 20% 4-dimethylaminopropionic acid to facil-
tate coupling. All peptides were >85% pure and were analyzed by electrospray mass spectroscopy (Protein Structure and Carbohydrate Fac-
tory, University of Michigan, Ann Arbor, MI) to confirm the ex-
pected molecular mass for both the nonphosphorylated and phos-
phorylated PECAM cytoplasmic tail peptides. Lysylphosphorylated biotinylated tep-

Pepitide Binding Studies—Peptides (10 μg/ml) were incubated with 1.5 x 109 platelets/ml in 138 mM NaCl, 2.7 mM KCl, 5 mM glucose, and 50 mM HEPES, pH 7.4, and incubated at 37°C with constant mixing. Fifty μl of streptavidin-agarose beads were then added to the peptide mixture and incubated for an additional hour at 4°C. The beads were washed five times in immunoprecipitation buffer by centrifugation at 4000 rpm for 5 min. Bound proteins were eluted in SDS sample buffer, boiled 10 min, and resolved by 12.5% SDS-PAGE.

Phosphoamino Acid Analysis—Washed platelets were resuspended at 1 x 109 platelets/ml in 138 mM NaCl, 2.7 mM KCl, 5 mM glucose, and 50 mM HEPES, pH 7.4, and incubated at 37°C unstirred with 0.5 mM [32P]orthophosphate for 3 h. Phosphorylated platelet pellets were then sedimented by centrifugation and resuspended in gel filtration buffer (137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 1 mm CaCl2, 3.3 mM Na3VO4, and 20 mM HEPES, pH 7.4 with 0.1% (w/v) glucose and 0.1% (w/v) bovine serum albumin). Following the addition of 500 μl of a 2 x radioligand immunoprecipitation assay buffer containing 1 mM sodium orthovanadate, 32P-labeled platelet proteins were immuno-

Pepitide Aggregation/Aggregation Studies—Washed platelets were activated by the addition of 7 μM TRAP in the absence of stirring. In some instances, 2 μM, 1 μM, and 100 μM fibrinogen aggregation was induced by adding 7 μM TRAP to stirred platelets (1000 rpm) in the presence of 2 mM CaCl2, 1 mM MgCl2, and 100 μM/ml fibrinogen for 3–10 min at 37°C in a four-channel automated platelet analyzer (BioData Corp., Horsham, PA). In some experiments, platelets were preincubated for 5 min at 37°C with 0.5 mM RGDS peptide prior to activation by TRAP.

Peptide Aggregation—Platelet extracts subjected to either acti-
vation or aggregation were lysed by the addition of 0.5 ml of Triton lysis buffer (2% Triton X-100, 10 mM EDTA, 15 mM HEPES, 145 mM NaCl, 0.1 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 2 mM sodium orthovanadate, pH 7.4), and the lysate was slowly rocked for 1 h at 4°C. Triton-soluble and -insoluble (cytoskeletal) fractions were separated by centrifugation at 15,000 x g for 5 min at 4°C as described previously (13). In some experiments, samples were lysed by the addition of an equal volume of ice-cold 2 x radioligand immunoprecipitation assay buffer (final concentration = 20 mM Tris, pH 7.4, 1 mM EDTA, 150 mM NaCl, 0.1% (w/v) sodium deoxycholate, 1 mM Na3VO4, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1% (v/v) Triton X-100).
subjected to autoradiography to detect comigrating $^{32}$P-labeled amino acids (17).

RESULTS

PECAM-1 Becomes Tyrosine-phosphorylated during Platelet Aggregation—Previous studies have shown that the cytoplasmic domain of PECAM-1 becomes phosphorylated on serine residues following activation of platelets with either thrombin or phorbol ester (13). To examine whether one or more of the five tyrosine residues within the PECAM-1 cytoplasmic domain might also become phosphorylated under certain physiological conditions, we compared the phosphorylation state of PECAM-1 in human platelets that were 1) resting; 2) activated with TRAP without stirring (i.e. nonaggregated); or 3) TRAP-activated, stirred, and fully aggregated. Following detergent lysis in the presence of the tyrosine phosphatase inhibitor vanadate (see “Experimental Procedures”), the phosphorylation state of PECAM-1 was evaluated both immunologically and biochemically. As shown in Fig. 1A, PECAM-1 was $^{32}$P-labeled in both resting and activated platelets (lanes 1 and 2), but incorporation of $[^{32}P]$orthophosphate into PECAM-1 was greatest under conditions where aggregation had been allowed to occur (lane 3). To determine which residues within the cytoplasmic domain of PECAM-1 had become phosphorylated under each of these three conditions, the three bands shown in the top row of Fig. 1A were excised from the gel and acid-hydrolyzed, and phosphoamino acids were analyzed by two-dimensional electrophoresis. As shown in Fig. 1B, PECAM-1 derived from resting platelets was slightly phosphorylated on serine (panel 1), and the level of serine phosphorylation increased 2–3-fold following platelet activation (panel 2), as reported previously (13). No labeling of tyrosine residues was observed in either resting or activated cells (panels 1 and 2), even when the film was overexposed (data not shown). Interestingly, whereas $^{32}$P had been incorporated solely on serine residues in PECAM-1 derived from resting or activated, nonaggregated platelets, phosphorytrosine was readily detectable in PECAM-1 derived from activated, aggregated platelets. These findings were confirmed immunologically in immunoblot analysis employing the anti-phosphotyrosine-specific monoclonal antibody PY-20 (Fig. 1C, lanes 1–3). Interestingly, the addition of RGD peptide before TRAP stimulation blocked tyrosine phosphorylation of PECAM-1 (lane 4), suggesting that this event required prior integrin-mediated platelet aggregation. Together, these data indicate that PECAM-1 initially becomes phosphorylated on one or more serine residues upon platelet activation, followed by tyrosine phosphorylation sometime during the platelet aggregation process.

Interaction of the Protein-tyrosine Phosphatase SHP-2 with Tyrosine-phosphorylated Peptides Derived from the Cytoplasmic Domain of PECAM-1—The cytoplasmic domain of PECAM-1 is structurally and functionally complex, being encoded by eight distinct exons (exons 9–16) that can be alternatively spliced to yield different PECAM-1 isoforms (18–20). Regions of the cytoplasmic domain encoded by exons 9, 11, and 13–15 each contain a tyrosine residue that could have become phosphorylated during platelet aggregation. To determine whether any of these might be involved in targeting one or more cytoskeletal or cytosolic signaling molecules to the cytoplasmic domain of PECAM-1 in activated, aggregated platelets, we synthesized a matched series of biotinylated 11-amino acid peptides corresponding to a specific region of the PECAM-1 cytoplasmic domain, each, with the exception of PECAM-(594–604) (residue 594 is the first amino acid of the cytoplasmic domain), containing the potential tyrosine phosphorylation site in the center, with and without phosphate (Fig. 2A). These biotinylated peptides were mixed with detergent lysates of

resting human platelets, and bound proteins were recovered using streptavidin-agarose beads. Immunoblot analysis using a series of antibodies specific for signaling molecules known to be present in human platelets identified SHP-2, a 70-kDa protein-tyrosine phosphatase, bound to PECAM-1-(658–668) and PECAM-1-(681–691) (regions encoded by exons 13 and 14 of the PECAM-1 gene, respectively), but only when the peptides themselves were tyrosine-phosphorylated at Tyr$^{663}$ and Tyr$^{686}$ (Fig. 2B). Interaction of SHP-2 with each of the two biotinylated peptides was specific, as formation of the complex could be competitively inhibited by a 20–50-fold molar excess of nonbiotinylated PECAM-1 phosphopeptide (Fig. 2, C and D).

SH2 Domains of SHP-2 Mediate Binding to PECAM-1-(658–668) and PECAM-1-(681–691)—SHP-2 is one of several intracellular cytosolic protein-tyrosine phosphatases that are able to
localize to specific sites within the cell and has been shown to interact with a number of activated growth factor receptors via its SH2 domains located at the amino terminus of the molecule (depicted schematically in Fig. 3A). To examine whether the SH2 domains of SHP-2 were responsible for associating with the two biotinylated PECAM-1 phosphopeptides identified above, the tyrosine-phosphorylated or nonphosphorylated forms were incubated with a GST fusion protein containing both the amino- and carboxyl-terminal SH2 domains of SHP-2, and complexes were captured with streptavidin-agarose beads. As shown in Fig. 3 (B and C, left panels), nonphosphorylated peptides failed to associate with either GST alone (lanes 1–3) or the GST-N-SH2-C-SH2 fusion protein (lanes 4–6). In contrast, phosphopeptides encompassing Tyr<sup>663</sup> and Tyr<sup>686</sup> bound avidly to the GST fusion protein containing the two SH2 domains of SHP-2 (Fig. 3, B and C, right panels, lanes 4–6). This interaction was specific, as the phosphopeptides did not interact with the GST protein alone (right panels, lanes 1–3). These data demonstrate that the interaction of SHP-2 with these two PECAM-1 cytoplasmic domain phosphopeptides is directly mediated by one or both of the amino-terminal SH2 domains of the phosphatase.

**PECAM-2 Binds PECAM-1 in Activated, Aggregated Human Platelets**—Although PECAM-1 becomes phosphorylated on tyrosine residues during platelet aggregation (Fig. 1), and SHP-2 binds to synthetic phosphopeptides corresponding to two small discrete regions within the cytoplasmic domain of PECAM-1 (Fig. 2), the ability of SHP-2 to associate with cellular PECAM-1 in an activated human platelet remained to be demonstrated. To address this issue, PECAM-1 was immunoprecipitated from detergent lysates of platelets that had been (a) treated with buffer (resting), (b) TRAP-activated but not aggreg-
Fig. 3. In vitro binding of PECAM phosphopeptides with the SH2 domains of SHP-2. A, shown are the domain structures of SHP-2 (I) and a GST fusion construct containing only the SH2 domains of SHP-2 (II). B and C, 10 μg of the indicated PECAM-1 cytoplasmic domain peptide were incubated with GST alone (lanes 1–3) or with a GST fusion protein containing both SH2 domains of SHP-2 (lanes 4–6) at final concentrations of 0.5 (lanes 1 and 4), 1.0 (lanes 2 and 5), and 2.0 (lanes 3 and 6) μg/mL. The resulting complexes were precipitated by streptavidin-agarose, resolved by 12.5% SDS-PAGE, and immunoblotted using an anti-SHP-2 polyclonal antibody. Note that the SH2 domains of SHP-2 interacted only with the tyrosine-phosphorylated forms of the PECAM-1-(658–668) and PECAM-1-(681–691) peptides.

PTPase, protein-tyrosine phosphatase; Stds, standards; Y(P), phosphotyrosine.

Fig. 4. Interaction of SHP-2 with full-length platelet PECAM-1. Platelets (1 × 10⁹/mL) were incubated at 37°C and stimulated with the following agonists in the presence of 2 mM CaCl₂, 1 mM MgCl₂, and 100 μg/mL fibrinogen: 1) buffer (stirred); 2) 7 μM TRAP for 10 min, without stirring; 3) 7 μM TRAP for 5 min (stirred and aggregated); 4) 7 μM TRAP (stirred) in the presence of 0.5 mM RGDW peptide; 5) 10 μg/mL normal mouse (NM) F(ab)₂ fragments for 30 min (stirred); and 6) 10 μg/mL PECAM-1.2 F(ab)₂, fragments for 30 min (stirred). Following detergent lysis, immunoprecipitations (IP) were performed using either normal mouse IgG₁ (left panel) or PECAM-1.3 (right panel). Bound proteins were resolved by 12.5% SDS-PAGE and analyzed by immunoblotting using anti-SHP-2. The relative intensities of the SHP-2 band were determined densitometrically using an AMBIS scanner and are indicated at the bottom of the gel. Note that the association of SHP-2 with PECAM-1 was increased slightly when platelets were activated by TRAP or when PECAM-1 on the cell surface was cross-linked using the monoclonal antibody PECAM-1.2. PECAM-1/SHP-2 interaction increased approximately 9-fold in fully aggregated platelets.

DISCUSSION

There is increasing evidence to suggest that, following interaction with the extracellular matrix or with other cells, cell surface adhesion receptors may be capable of transmitting signals across the plasma membrane. For example, cross-linking of certain integrins with antibodies or adhesion of cells to specific integrin ligands can stimulate tyrosine phosphorylation of multiple cytosolic components, increase cytoplasmic pH and ionized calcium, initiate phosphoinositide synthesis, and modify patterns of gene expression (22–24). Integrins do not contain intrinsic kinase or phosphatase activity within their cytoplasmic domains, nor do they harbor SH2 or SH3 domains that could serve to recruit signaling molecules following ligand binding. Studies suggest, rather, that outside-in signaling events may be brokered by the accumulation and assembly of specific cytoskeletal (25) and signaling (26–28) molecules at sites of integrin clustering. The molecular basis underlying at least some of these interactions has recently been suggested by the work of Law et al. (29), who showed that the β₃-subunit of the integrin α₅β₃ becomes tyrosine-phosphorylated in response to thrombin-induced platelet aggregation, creating potential docking sites for the SH2-containing adapter proteins Grb2 and Shc.

This mode of signal transduction may represent a general paradigm that is used by PECAM-1 as well. Although the cytoplasmic domain of PECAM-1 lacks demonstrated catalytic
SHP-2 (human platelets specifically with full-length PECAM-1 derived from TRAP-aggregated GST fusion protein containing the SH2 domains of SHP-2 interacted whereas the recombinant GST protein failed to bind PECAM-1, the factor receptor (34–36), the epidermal growth factor receptor (PDGFRb), the latter of which has been shown to bind SHP-2 following receptor autophosphorylation (37, 38).

Activity, it contains numerous potential sites for phosphorylation of serine, threonine, and tyrosine residues (30). We found that PECAM-1, like the integrin b3-subunit, becomes tyrosine-phosphorylated during the platelet aggregation process (Fig. 1). Once “activated” by tyrosine phosphorylation, PECAM-1 associated with the 70-kDa protein-tyrosine phosphatase SHP-2 (Figs. 2 and 4). SHP-2 (also known as SHPTP-2, Syp, PTP1D, PTP2C, and SH-PTP3 (31)) is a ubiquitously expressed protein-tyrosine phosphatase that is composed of two SH2 domains. The catalytic activity of SHP-2 can be regulated both by its phosphorylation of serine, threonine, and tyrosine residues (30). We found that PECAM-1, like the integrin b3-subunit, becomes tyrosine-phosphorylated during the platelet aggregation process (Fig. 1). Once “activated” by tyrosine phosphorylation, PECAM-1 associated with the 70-kDa protein-tyrosine phosphatase SHP-2 (Figs. 2 and 4). SHP-2 (also known as SHPTP-2, Syp, PTP1D, PTP2C, and SH-PTP3 (31)) is a ubiquitously expressed protein-tyrosine phosphatase that is composed of two SH2 domains. The catalytic activity of SHP-2 can be regulated both by its phosphorylation and through direct activation by receptor tyrosine kinases (32–35). Previous studies have shown that the SH2 domains of SHP-2 direct its interaction with the cytoplasmic domains of a number of tyrosine-phosphorylated (i.e. activated) growth factor receptors, including the platelet-derived growth factor receptor (PDGFRb), the latter of which has been shown to bind SHP-2 following receptor autophosphorylation (37, 38). SHP-2 has also been shown to associate through its SH2 domains with the activated platelet-derived growth factor receptor at Tyr(P)1109 (38, 39). The sequence specificity for this interaction has been examined in great detail (40–42), and studies to date indicate that the sequence recognition elements surrounding the Tyr(P) residue that are required for high affinity binding of the N-terminal SH2 domain of SHP-2 include Val at position −2, a b-branched residue (Thr/Val/Ile) at position +1, and a hydrophobic residue (Val/Leu/Ile) at position +3. Of the five tyrosine residues found within the cytoplasmic domain of PECAM-1, only the two encoded by exons 13 and 14, with the sequences VQpY663TEV and TVpY686SEV, fulfill these requirements. Sequence alignment of each of these sequences among various species (Fig. 6) reveals absolute conservation of the four key residues surrounding PECAM-1 Tyr663 that constitute the SHP-2-binding motif. The residues surrounding Tyr686, however, only loosely conform to the consensus sequence, suggesting that the C-terminal site on the PECAM-1 cytoplasmic domain may constitute only a low affinity binding site for SHP-2. It should be pointed out that while we have shown that tyrosine-phosphorylated peptides encompassing residues 658–668 and 681–691 of the PECAM-1 cytoplasmic domain bind SHP-2 (Figs. 2 and 3) and that SHP-2 binds tyrosine-phosphorylated PECAM-1 (Figs. 4 and 5), further studies employing mutant forms of PECAM-1 will be required to determine the binding site on PECAM-1 for SHP-2 in vivo. In this regard, it is notable that deletion of exon 14 results in the loss of the ability of PECAM-1-transfected fibroblasts to bind PECAM-1-negative cells (7, 20, 43), a function that we attribute to loss of PECAM-1-mediated signal transduction (see below). Whether Tyr686 becomes phosphorylated and acts synergistically with Tyr663 to localize SHP-2 to activated PECAM-1 or whether it serves as an independent docking site for the recruitment of additional signaling molecules is the subject of current investigation in our laboratory.

The catalytic activity of SHP-2 can be regulated both by its physical interaction with activated (i.e. tyrosine-phosphorylated) receptor tyrosine kinases (39) as well as by receptor tyrosine kinase-mediated phosphorylation of SHP-2 itself (34, 35, 37). Unlike the platelet-derived growth factor receptor, which can both bind to and phosphorylate SHP-2, PECAM-1 is incapable of phosphorylating SHP-2 at its carboxyl terminus following their interaction. Preliminary studies suggest, however, that PECAM-1 might be able to directly activate SHP-2 by virtue of its binding, as tyrosine-phosphorylated peptides comprising PECAM-1-(668–686) and PECAM-1-(681–691) stimulate the phosphatase activity of SHP-2.2 One scenario whereby PECAM-1 might initiate cellular signaling, therefore, might take advantage of its ability to localize SHP-2 to the inner face of the plasma membrane and activate it, thereby bringing the phosphatase into proximity with nearby phospho-
PECAM-1-mediated Signal Transduction

rlylated signaling molecules. One particularly attractive set of substrates for SHP-2 may be protein-tyrosine kinases, such as pp60 
ene-C, which are constitutively bound to the membrane via post-translationally added fatty acid side chains and which normally exist in an inactive state due to intramolecular interactions with a negative regulatory phosphotyrosine residue (44). Whether PECAM-1/SHP-2 interactions promote cellular signaling by dephosphorylating Src family kinases is not yet known.

SHP-2 has the capacity to be a multifunctional signaling molecule. C-terminal to its phosphatase domain, human SHP-2 contains three tyrosine residues, and the sequence around two of these (Y542TNI and Y580ENV), when tyrosine-phosphorylated, conforms conservatively with the consensus binding site for the SH2 domain of the adapter protein Grb2 (40). In fact, tyrosine phosphorylation of SHP-2 induced by platelet-derived growth factor (45, 46), interleukin-3 and granulocyte-macrophage colony-stimulating factor (47), and steel factor (48) has been shown to result in the direct binding of Grb2 in vivo. Since Grb2 normally exists bound, via its SH3 domains, to the guanine nucleotide-releasing factor Sos (49–56), it has been proposed that SHP-2 functions as an adapter protein, linking the activation of these transmembrane growth factor and cytokine receptors to the Ras signaling pathway in hematopoietic cells. In addition, Welham et al. (47) have found that SHP-2 coprecipitates with the p85 subunit of phosphatidylinositol3'-kinase, although the molecular nature of this interaction is not well understood. Whether SHP-2 integrates signals that derive from PECAM-1 homophilic interactions with either the Ras or phosphatidylinositol 3'-kinase pathway in platelets and other vascular cells remains to be determined.

In resting platelets, a majority of PECAM-1 molecules are either free within the plane of the membrane or associated with the underlying membrane skeleton (13). During platelet aggregation, however, >50% of PECAM-1 receptors become associated with the actin cytoskeleton. In contrast, SHP-2 exists either free in the cytosol or associated with the membrane skeleton, with virtually none becoming attached to the cytoskeleton in activated human platelets (57). In our studies (Fig. 4), we found that SHP-2 become associated with PECAM-1 present in the 15,000 × g supernatant of activated, detergent-solubilized platelets, i.e., those PECAM-1 receptors that were either linked to the membrane skeleton or free within the plane of the membrane. While we have not yet determined the stoichiometry of SHP-2:PECAM-1 in any of these subcellular fractions, it is tempting to speculate that a subpopulation of PECAM-1, perhaps attached to the membrane skeleton of human platelets, serves a unique functional role in the assembly of signaling complexes during the αIIbβ3-mediated platelet aggregation process. Two other nontransmembrane protein-tyrosine phosphatases present in platelets, PTP1B and SHP-1, have been found to be associated with the 15,000 × g detergent-insoluble cytoskeleton during platelet aggregation, where they are thought to participate in the dephosphorylation of tyrosine-phosphorylated proteins that have become associated within the cytoskeleton (57). However, we have found no evidence for their association with the cytoplasmic domain of PECAM-1 to date.

Finally, we predict that bidirectional signaling through PECAM-1 may be of functional importance in other vascular cells. We have shown that PECAM-1 can function downstream from integrin engagement, as tyrosine phosphorylation of PECAM-1 and its subsequent association with SHP-2 are each dependent upon prior integrin-mediated platelet/platelet contact (Figs. 1 and 4). The relationship between PECAM-1- and integrin-mediated signaling, however, appears to be able to work in both directions. The ability of PECAM-1, upon its engagement, to serve as an amplifier of integrin-mediated cell adhesion (8–12) provides one cogent series of examples in which PECAM-1 functions upstream of integrins, serving primarily as an agonist receptor rather than as a cell adhesion molecule per se. “PECAM-1-mediated” binding to the integrin α₅β₅ of lymphocyte-activated killer cells (58) and monocyte-like U937 cells (59) and the interaction of PECAM-1-transfected L-cells with PECAM-1-negative murine L-cell fibroblasts (60, 61) may represent additional examples of adhesive interactions that are enabled by outside-in signal transduction through PECAM-1. This process may be relevant to both inflammation and thrombosis during the process of selectin-mediated leukocyte (62) and platelet (63) rolling on activated endothelium. Homophilic interactions between PECAM-1 located at endothelial cell intercellular junctions and either platelet or leukocyte PECAM-1 might act in concert with selectin-mediated signaling to promote integrin activation and subsequent tight adhesion necessary for transendothelial migration. Whether the anti-inflammatory effects of anti-PECAM-1 antibodies observed in a number of in vitro (4) and in vivo (64, 65) models are due, in part, to inhibition of PECAM-1-mediated outside-in signaling remains to be determined. Further studies aimed at identifying the mechanisms by which PECAM-1/SHP-2 interactions broke these events and the signaling molecules that participate in these complex cellular processes should shed additional light on the role of the cell adhesion and cell signaling molecule PECAM-1 in vascular cell biology.

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