Characterization of Phosphotyrosine Binding Motifs in the Cytoplasmic Domain of Platelet/Endothelial Cell Adhesion Molecule-1 (PECAM-1) That Are Required for the Cellular Association and Activation of the Protein-tyrosine Phosphatase, SHP-2*

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Recent studies have shown that the Src homology-2 (SH2) domain-containing protein-tyrosine phosphatase, SHP-2, associates with the cytoplasmic domain of PECAM-1 as it becomes tyrosine-phosphorylated during platelet aggregation: a process that can be mimicked in part by small synthetic phosphopeptides corresponding to the cytoplasmic domain of PECAM-1 encompassing tyrosine residues Tyr-663 or Tyr-686. To further examine the molecular requirements for PECAM-1/SHP-2 Interactions, we generated human embryonic kidney (HEK)-293 cell lines that stably expressed mutant forms of PECAM-1 harboring tyrosine to phenylalanine (Tyr → Phe) mutations in the cytoplasmic domain. Y663F and Y686F forms of PECAM-1 were tyrosine-phosphorylated to a somewhat lesser extent than wild-type PECAM-1, and a doubly substituted Y663,686F form of PECAM-1 failed to become tyrosine-phosphorylated, suggesting that the PECAM-1 cytoplasmic domain tyrosine residues 596, 636 and 701 do not serve as substrates for cellular kinases. Interestingly, SHP-2 binding was lost when either Tyr-663 or Tyr-686 were changed to phenylalanine, indicating that both residues are required for SHP-2/PECAM-1 association. Although PECAM-1 phosphopeptides NSDVQPY663TEVQV and DTETVPY686SEVRK stimulated the catalytic activity of the phosphatase to a similar extent, surface plasmon resonance studies revealed that the Tyr-663-containing peptide had approximately 10-fold higher affinity for SHP-2 than did the Tyr-686 peptide. Finally, peptido-precipitation analysis showed that the NH2-terminal SH2 domain of SHP-2 reacted preferentially with the Tyr-663 PECAM-1 phosphopeptide, while the Tyr-686 phosphopeptide associated only with the COOH-terminal SH2 domain of the phosphatase. Together, these data provide a molecular model for PECAM-1/SHP-2 interactions that may shed light on the downstream events that follow PECAM-1-mediated interactions of vascular cells.

Phosphorylation of proteins on tyrosine residues provides a key cellular control mechanism for intracellular signaling processes that regulate cell growth, proliferation, adhesion, differentiation, and metabolism (1, 2). The level of tyrosine phosphorylation of cellular proteins is controlled by the coordinated actions of protein-tyrosine kinases and protein-tyrosine phosphatases. Signal transmission by tyrosine phosphorylation is mediated by the binding of sequence-specific Src homology-2 (SH2)1 domains present on cytosolic signaling molecules to phosphotyrosine (Tyr(P)) sites on activated receptors (3–7). These highly conserved protein modules play an important role in mediating protein-protein interactions and can regulate many facets of the signaling process (8, 9). The association of SH2-containing proteins with Tyr(P)-sites on activated receptors can elicit biochemical changes within the cell, including regulating catalytic activity (10), directing subcellular localization (11), and enhancing tyrosine phosphorylation (12) to potentiate downstream signaling events.

Platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) is a 130-kDa member of the Ig gene superfAMILY expressed on the surface of leucocytes and platelets and is also enriched at the junctions of endothelial cells (13, 14). PECAM-1 is a multifunctional receptor consisting of six extracellular Ig homology domains, a transmembrane and cytoplasmic domain (for reviews, see Refs. 15 and 16). Its amino-terminal Ig homology domains 1 and 2 mediate homophilic cell-cell adhesion (17, 18) during the process of leukocyte transendothelial migration (19–21). Evidence is also emerging that PECAM-1 may be involved in cell migration (22), endothelial cell tube formation (23), and angiogenesis (24).

Human PECAM-1 contains five potential tyrosine phosphorylation sites in its cytoplasmic domain (25), which, once phosphorylated, could serve as docking sites for recruitment and physical assembly of cytosolic signaling complexes to potentiate downstream signaling events and evoke changes in biological responses. PECAM-1 has been shown recently to become tyrosine-phosphorylated in response to mechanical stimulation (26), cross-linking with domain-specific monoclonal antibodies (27–32), aggregation of high affinity IgE receptor (33), pervana-

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The abbreviations used are: SH2, Src homology 2; PAGE, polyacrylamide gel electrophoresis; RCM, reduced carboxyamidomethylated and maleylated; PECAM-1, platelet endothelial cell adhesion molecule-1; IRS-1, insulin receptor substrate-1; BIT, brain immunoglobulin-like molecule; pY or TyrP, phosphotyrosine; TAM, tyrosine-based activation motif; N-SH2, NH2-terminal SH2 domain; C-SH2, COOH-terminal SH2 domain; GST, glutathione S-transferase; PDGF, platelet-derived growth factor.
date treatment (34), and integrin αIIbβ3-mediated platelet aggregation (35). Previous studies have demonstrated that tyrosine-phosphorylated PECAM-1 binds the protein-tyrosine phosphatase SHP-2 during the process of integrin αIIbβ3-mediated platelet aggregation in an SH2-dependent manner (35). This association can also be mimicked using synthetic phosphopeptides corresponding to the cytoplasmic domain of PECAM-1 encompassing residues Tyr-663 or Tyr-686 (35). While these phosphopeptide binding studies implicate two discrete regions encompassing residues Tyr-663 and Tyr-686 in the PECAM-1 cytoplasmic domain as targets for SHP-2 association, the binding site(s) on PECAM-1 required for the in vivo cellular association of SHP-2 remain to be determined.

The ubiquitously expressed non-transmembrane protein-tyrosine phosphatase, SHP-2 (also known as SHPTP-2, Syp, PTP1D, PTP2C, and SH-PTP3) contains two tandem amino- and carboxyl-terminal SH2 domains, followed by a catalytic phosphatase domain (36–39). SHP-2 binds protein-tyrosine phosphatase residues of activated growth factor and cytokine receptors, such as platelet-derived growth factor (PDGF), epidermal growth factor, e-kit, interleukin-6, and insulin receptor substrate-1 (IRS-1), in an SH2-dependent manner (40–44). The catalytic activity of SHP-2 is enhanced by its tyrosine phosphorylation (39), and also by occupancy of its amino-terminal SH2 domains. Phosphorylation of its COOH-terminal tyrosine at position 542 of SHP-2 by receptor tyrosine kinases creates a docking site for Grb2, linking Grb2-Sos with SHP-2 to activate the Ras-Raf mitogen-activated protein kinase signaling pathway (45, 46). Grb2, linking Grb2-Sos with SHP-2 to activate the Ras-Raf/mitogen-activated protein kinase signaling pathway, it has been recognized that the cellular association of SHP-2 requires both Tyr-663- and Tyr-686-containing binding sites. The N-SH2 domain of SHP-2 preferentially bound to Tyr-663, while the C-SH2 domain favored Tyr-686 of PECAM-1. These two phosphotyrosine binding motifs, Tyr-663 and Tyr-686, of PECAM-1 stimulated catalytic activation of SHP-2.

**EXPERIMENTAL PROCEDURES**

**Materials**—Phenylmethylsulfonyl fluoride, Triton X-100, bovine serum albumin, leupeptin, dimethyl sulfoxide, isopropyl-β-D-thiogalactoside, ampicillin, and reduced glutathione were purchased from Sigma. Protein G-Sepharose and glutathione- Sepharose 4B were from Pharmacia Biotech Inc. (Uppsala, Sweden). Immunof-P membrane was obtained from Millipore Corp. (Bedford, MA). Sodium dodecyl sulfate, glycine, pre-stained broad SDS-PAGE markers, Tween 20, and TEMED were from Bio-Rad. The enhanced chemiluminescence Western blotting detection kit was obtained from Amersham Life Sciences, Inc. [γ-32P]ATP was obtained from NEI Life Science Products. Small restriction enzyme was purchased from New England Biolabs. Sodium orthovanadate was obtained from LC Laboratories (Woburn, MA).

**Antibodies**—The murine anti-PECAM-1 monoclonal antibody, PECAM-1.3 (specific for Ig homology domain 1) and the polyclonal anti-PECAM-1 antibody, SEW16, have been described previously (17, 50). The horseradish peroxidase-conjugated monoclonal anti-phosphotyrosine antibody, PY-20, was obtained from Zymed Laboratories Inc. (South San Francisco, CA). A polyclonal antibody directed to the NH2-terminal SH2 domain of SHP-2 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Normal Mouse IgG1 was obtained from Sigma. Fluorescein isothiocyanate-conjugated goat anti-mouse F(ab)2 antibody was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

**Site-directed Mutagenesis of PECAM-1 cDNA**—A full-length cDNA encoding wild-type human PECAM-1 was cloned into plasmid vector pGEM-7zf(+) (50). The Quick-change site-directed mutagenesis kit (Stratagene, La Jolla, CA) along with two single-nucleotide mismatched primers containing the desired mutation was used to construct PECAM-1 cDNAs encoding mutant Tyr→Phe forms of PECAM-1. An oligonucleotide mismatched primer pair (5'-TCAAGACGTGGATGTCAGG-3') and (3'-AGTCTGCAGTGTAATTGCTTCG-5') from base 2197 to 2223 of PECAM-1 was used to construct a Y663F mutant form of PECAM-1. For the Y686F mutant form of PECAM-1, an oligonucleotide mismatched primer pair (5'-ACAGACAGTGGATGTAATTGCTTCG-3') and (3'-TGTCTCTGTGTCACAAGCTCACCAGA-5') from base 2266 to 2292 of PECAM-1 was used. The double Y683,686F mutant form of PECAM-1 was generated by sequential site-directed mutagenesis of using both mismatched primer sets. The mutated forms of the PECAM-1 cDNAs were exchanged into a wild-type plasmid vector pcDNA3.0 (Invitrogen, Carlsbad, CA). cDNAs were subjected to nucleotide sequence analysis to confirm the presence of the desired mutation(s) and to exclude polymerase chain reaction-induced errors.

**Development of Stable PECAM-1-expressing HEK-293 Cell Lines**—Human embryonic kidney (HEK-293) cells obtained from the American Type Culture Collection (CRL 1573) were cultured in minimal essential medium (Life Technologies, Inc.) containing Earle’s salts, Glutamax, and 10% heat-inactivated fetal calf serum at 37 °C in a humidified atmosphere of 5% CO2. HEK-293 cells were grown to 80–90% confluence in 100-mm dishes, incubated with 10 μg of plasmid pcDNA3.0 (Invitrogen, Carlsbad, CA). cDNAs were subjected to nucleotide sequence analysis to confirm the presence of the desired mutation(s) and to exclude polymerase chain reaction-induced errors.

**Stimulation of HEK-293 Cell Lines**—Adherent HEK-293 cell lines expressing either wild-type or Tyr→Phe mutant forms of PECAM-1 were lifted using 0.1% trypsin and 10 mM EDTA, washed in sterile PBS, and resuspended at 6 × 10^6 cells/ml in serum-free medium. Cells were equilibrated at 37 °C for 15 min and then stimulated with a mixture of 2.5 mM H2O2 and 100 μM sodium orthovanadate (pervanadate) at 37 °C for 10 min. The reaction was stopped by the addition of ice-cold PBS. The cells were pelleted by centrifugation at 2,000 rpm for 5 min at room temperature, then washed twice in ice-cold PBS before lysis with 1 ml of Triton lysis 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) for 1 h at 4 °C.

**Immunoprecipitation Analysis**—Following cell lysis, the 15,000 × g Triton-soluble fraction was preclared with 50 μl of a 50% slurry of
Protein G-Sepharose for 30 min at 4 °C and then centrifuged at 4,000 rpm for 5 min. Preceded cell lysates were incubated with either 10 μg/ml normal mouse IgG, or 10 μg/ml PECA-1.3 IgG overnight at 4 °C. Immune complexes were captured with 50 μl of a 50% slurry of Protein G-Sepharose for 1 h at 4 °C, and then washed five times with immunoprecipitation wash buffer (50 mM Tris, pH 7.4, containing 150 mM NaCl, and 2% Triton X-100). Bound proteins were eluted from the beads by boiling for 10 min in 30 μl of SDS reducing buffer, loaded onto a 10% SDS-polyacrylamide gel, then transferred to Immobilon-P membrane (Millipore Corp., Bedford, MA), and analyzed by horseradish peroxidase-conjugated PY-20 or polyclonal antibodies directed to SHP-2 or PECA-1.

Preparation and Expression of GST Fusion Proteins Containing the SH2 Domains of SHP-2—300-base pair segments encoding amino acid residues 1–105 (the NH2-terminal SH2 domain) or 112–213 (the COOH-terminal SH2 domain) of SHP-2 were generated by polymerase chain reaction amplification of SHP-2 cDNA cloned into pGEX-2T (kindly provided by Dr. Benjamin Neel, Beth Israel Hospital, Boston, MA). Following amplification, the cDNA fragments were gel-purified and ligated into Salmon-digested pGEX-4T-2 (Pharmacia Biotech Inc.). The resulting bacterial expression constructs, termed pGEX-4T-2-N-SH2 and pGEX-4T-2-C-SH2, respectively, were used to transform Escherichia coli BL21 D3 cells (Novagen Inc., Madison, WI). Full-length SHP-2 and individual NH2- and COOH-terminal SH2 domain GST fusions were then expressed by inducing log-phase 1-liter cultures with 0.2 mM isopropyl-β-D-thiogalactoside, and isolated using glutathione-Sepharose beads as described previously (51).

Peptide-presentation Analysis—Biotinylated PECA-1 phosphopeptides were prepared as described previously (35). These PECA-1 cytoplasmic domain peptides (Ytr5PO4)10 (10 μg/ml) were incubated with 2 μg of recombinant GST alone, a GST fusion protein encompassing the amino- and carboxyl-terminal SH2 domains of SHP-2, termed GST-N-SH2-C-SH2-SHP-2, or GST-N-SH2-SHP-2, or GST-C-SH2-SHP-2 in 1 ml of Triton lysis buffer, overnight at 4 °C with constant mixing. Fifty μl of Ultralink Plus neutravidin-agarose beads (Pierce) were then added to the peptide-protein mixture and incubated for an additional 1 h at 4 °C. The beads were washed five times in immunoprecipitation buffer and centrifuged at 4,000 rpm for 5 min. Bound proteins were eluted by boiling in SDS reducing buffer and resolved by 10% SDS-PAGE.

Surface Plasmon Resonance Measurements—BIAcore sensor chip SA (BIAcore AB, Uppsala, Sweden), consisting of pre-immobilized streptavidin on a carboxymethylated dextran matrix, was conditioned with 1 mM NaCl in 50 mM NaOH according to manufacturer’s instructions. All experiments were carried out in HBS buffer (0.01 M Hepes, pH 7.4, containing 150 mM NaCl, 1 mM EDTA, and 0.005% w/v Tween-20) with a flow rate of 5 μl/min and a constant temperature of 25 °C. For each set of experiments, 30 μl of biotinylated PECA-1 phosphorylated and non-phosphorylated peptides (100 μg/ml) were injected onto the chip. Specificity of the tyrosine-phosphorylated PECA-1 peptides was demonstrated by the binding of an monoclonal anti-phosphotyrosine antibody, or peroxidase-conjugated PY-20 or polyclonal antibodies directed to SHP-2. Previous studies have demonstrated that the NH2-terminal SH2 domain of SHP-2 binds directly to tyrosine-phosphorylated PECA-1/SHP2 Interactions

RESULTS

Two phosphotyrosine binding motifs in the PECA-1 cytoplasmic domain are required for the cellular association of SHP-2. Previous studies have demonstrated that tyrosine-phosphorylated peptides encompassing residues 658–668 and 681–691 of PECA-1 bind the protein-tyrosine phosphatase, SHP-2, and that SHP-2 binds tyrosine-phosphorylated PECA-1 in aggregating human platelets (35). To determine the binding site(s) on PECA-1 required for the cellular association of SHP-2, we prepared PECA-1 cDNA constructs containing Tyr → Phe mutations at positions 663 and 686 and stably expressed them in HEK-293 cells. In preliminary experiments (not shown), we confirmed that the HEK-293 cells do not express endogenous PECA-1, but synthesize abundant amounts of SHP-2. As shown in Fig. 1, the introduction of either a single or double Tyr → Phe mutation at positions 663 and 686 did not affect cellular trafficking or expression of PECA-1 on the cell surface.

We next examined the cellular association of SHP-2 with the various Tyr → Phe PECA-1 mutants. PECA-1-expressing HEK-293 cell lines were treated with pervanadate, a protein-tyrosine phosphatase inhibitor, that has been shown previously to elicit proximal signal transduction events, including tyrosine phosphorylation. As shown in Fig. 2, in unstimulated HEK-293 cell lines, a small degree of constitutive tyrosine phosphorylation of PECA-1 was observed in the PY-20 immunoblot (Fig. 2, left middle panel) of PECA-1.3 immunoprecipitates from wild-type, Y663F, and Y686F mutants. In contrast, in pervanadate-treated cell lines, prominent tyrosine-phosphorylation of PECA-1 was observed in the PY-20 immunoblot (Fig. 2, right middle panel) of PECA-1.3 immunoprecipitates from wild-type, and to a slightly lesser extent, Y663 and Y686F mutants. Examination of the double Y663,686F mutant revealed complete loss of tyrosine-phosphorylated PECA-1 in the PY-20 immunoblot (Fig. 2, right middle panel), despite the presence of abundant amounts of PECA-1 in the PECA-1.3 immunoprecipitates (Fig. 2, right upper panel). Interestingly, there was evidence of three potential, unidentified PECA-1-associated phosphoproteins with molecular masses of 90–95, 60–70, and 40–50 kDa in the PY-20 immunoblot of PECA-1.3 immunoprecipitates from pervanadate-treated HEK-293 cells expressing wild-type PECA-1.

Once we had confirmed the presence of tyrosine-phosphorylated PECA-1 in the various HEK-293 cell lines, we next stripped the immunoblot and reprobed with a specific polyclonal antibody directed to SHP-2. As shown in the lower panel of Fig. 2, in pervanadate-treated HEK-293 cell expressing wild-type PECA-1, the presence of SHP-2 could be easily detected in the PECA-1.3 immunoprecipitate. In contrast, SHP-2 failed to co-precipitate with either the Y663F, Y686F, or Y663,686F mutant forms of PECA-1. These data suggest that both phosphotyrosine binding motifs in the cytoplasmic domain of PECA-1 are required for mediating the cellular association of SHP-2.

The NH2-terminal SH2 domain of SHP-2 binds directly to tyrosine-phosphorylated PECA-1 (658–668) peptide, while the COOH-terminal SH2 domain of SHP-2 binds directly to tyrosine-phosphorylated PECA-1 (681–691) peptide. Previous studies have shown that SHP-2 associates with tyrosine-phosphorylated PECA-1, in an SH2-dependent manner. The orientation of the NH2 domains of SHP-2 interacting with tyrosine-phosphorylated PECA-1, however, is unknown. To
address this issue, we prepared recombinant GST fusion proteins expressing individual NH₂- and COOH-terminal SH2 domains of SHP-2 and incubated them with biotinylated peptides corresponding to PECAM-(658–668) and -(681–691). As shown in Fig. 3, nonphosphorylated peptides failed to associate with GST alone or with the GST-N-SH2-C-SH2, GST-N-SH2 and GST-C-SH2 fusion proteins. In contrast, the PECAM-1 phosphopeptide encompassing Tyr-663 bound avidly to GST fusion proteins containing both SH2 domains or the single NH₂-terminal SH2 domain of SHP-2, while the PECAM-1 phosphopeptide encompassing Tyr-686 bound avidly to the GST fusion proteins containing both SH2 domains or the single COOH-terminal SH2 domain of SHP-2. These data demonstrate that the NH₂-terminal SH2 domain of SHP-2 binds via the phosphotyrosine binding motif of PECAM-1 encompassing Tyr-663, while the COOH-terminal SH2 domain of SHP-2 binds via the phosphotyrosine binding motif of PECAM-1 encompassing Tyr-686.
binds via the phosphotyrosine binding motif encompassing Tyr-686.

Surface Plasmon Resonance Studies of Recombinant N-SH2-C-SH2-SHP-2 Association with PECAM-1 Phosphopeptides—To determine the kinetics of the binding of the SH2 domains of SHP-2 with the cytoplasmic domain of PECAM-1, two 11-amino acid biotinylated tyrosine-phosphorylated PECAM-1 peptides encompassing residues 663 and 686 were immobilized onto a streptavidin-dextran-coated BIAcore sensor chip. Preliminary experiments demonstrated that a specific anti-phosphotyrosine monoclonal antibody bound to the tyrosine-phosphorylated, but not the nonphosphorylated versions of each PECAM-1 peptide, establishing the specificity of the interaction (data not shown). To determine the dissociation rate constants of the SH2 domains of SHP-2, varying amounts of the recombinant N-SH2-C-SH2-SHP-2 fusion protein lacking the GST portion were injected over the immobilized PECAM-1 phosphopeptides and the interaction followed continuously by surface plasmon resonance. The overlay surface plasmon resonance plots of the raw data for the recombinant N-SH2-C-SH2-SHP-2 fusion protein interactions with tyrosine-phosphorylated PECAM-1 peptides encompassing Tyr-663 (A) and Tyr-686 (C) are shown in Fig. 4. In addition, these results were analyzed by Scatchard plots derived from the data shown in B and D. Note that the peptide containing Tyr-663 exhibits approximately 10-fold higher affinity for the SH2 domains of SHP-2 than does the Tyr-686-containing peptide. RU, resonance units; \( R_{eq} \), response at equilibrium in resonance units.

### Table I

| Binding protein         | Immobilized phosphopeptide | Sequence         | \( K_D \) nM   |
|-------------------------|-----------------------------|------------------|---------------|
| rN-SH2-C-SH2-SHP-2      | PECAM-(658–668) Tyr(P)-663 | NSDVQPITEVQV     | 3.47          |
|                         |                             |                  | 4.50          |
|                         |                             |                  | 3.20          |
|                         |                             |                  | 3.47 ± 0.5 (mean ± S.D.) |
| rN-SH2-C-SH2-SHP-2      | PECAM-(681–691) Tyr(P)-686 | DTETVPYSEVRK     | 32.47         |
|                         |                             |                  | 59.52         |
|                         |                             |                  | 32.89         |
|                         |                             |                  | 59.20         |
|                         |                             |                  | 48 ± 15 (mean ± S.D) |

Fig. 4. Determination of the binding affinity of the SH2 domains of SHP-2 for tyrosine-phosphorylated regions of the PECAM-1 cytoplasmic domain. A recombinant protein consisting of the two tandem SH2 domains of SHP-2 was incubated at concentrations ranging from 0.8 to 2.5 \( \mu M \), with immobilized biotinylated PECAM-1-(658–668) Tyr(P)-663 (A) or PECAM-1-(681–691) Tyr(P)-686 (C). The resulting surface plasmon resonance sensorgrams are shown. Scatchard plots derived from these data are shown in B and D. Note that the peptide containing Tyr-663 exhibits approximately 10-fold higher affinity for the SH2 domains of SHP-2 than does the Tyr-686-containing peptide. RU, resonance units; \( R_{eq} \), response at equilibrium in resonance units.
DISCUSSION

We have demonstrated previously that tyrosine-phosphorylated peptides encompassing residues 658–668 and 681–691 of the cytoplasmic domain of PECAM-1 bind SHP-2 and that SHP-2 binds tyrosine-phosphorylated PECAM-1 during platelet aggregation (35). While these in vitro studies suggested that tyrosine phosphorylation of amino acid residues 663 and 686 within the PECAM-1 cytoplasmic domain is required for the association of SHP-2 with PECAM-1, we needed to examine mutant forms of PECAM-1 by replacing these tyrosine residues with phenylalanine to define the precise binding site(s) on PECAM-1 for SHP-2 in vitro. Our results indicate that phosphorylation of both Tyr-663 and Tyr-686 is required for the cellular association of SHP-2 with PECAM-1 (Fig. 2). Interest-
gingly, mutation of both tyrosine phosphorylation sites, 663 and 686, resulted in a dramatic loss of tyrosine phosphorylation of PECAM-1 (Fig. 2). There are several possible explanations for this observation. First, these two amino acid residues are primary sites for tyrosine phosphorylation of PECAM-1, while the other three tyrosine residues do not contribute. Second, mutation of these sites may result in the loss of direct or indirect association of SH2-containing signaling molecules such as protein-tyrosine kinases, which may be responsible for phosphorylating the other three tyrosine residues in the cytoplasmic domain of PECAM-1.

The defined crystal structure of the tandem SH2 domains of SHP-2 has suggested that the orientation and spacing between the phosphotyrosine residues of an activated receptor is critical for the binding of one or both SH2 domains and stimulation of enzymatic activation (53). Tyrosines 663 and 686 in the PECAM-1 cytoplasmic domain are spaced 23 amino acids apart, which is similar to known colinear docking sites characteristic of tyrosine-based activation motifs (TAM), described for ZAP-70 and a brain immunoglobulin-like molecule (54, 55). The phosphotyrosine binding motif encompassing Tyr-663 in the PECAM-1 cytoplasmic domain most closely correlates with Tyr-1009 in the carboxyl-terminal region of the β-subunit of PDGF. Previous studies have shown that the Tyr-1009-containing motif alone is able to associate with the N-and C-SH2 domains of SHP-2 with equivalent affinity (43, 56). These observations prompted us to examine the orientational relationship between the SH2 domains of SHP-2 with the two phosphotyrosine binding motifs in the PECAM-1 cytoplasmic domain. To address this question, we generated individual recombinant NH2- and COOH-terminal SH2 domains of SHP-2 in E. coli and examined their ability to associate with nonphosphorylated and tyrosine-phosphorylated forms of 658–668 and 681–691 PECAM-1 peptides. Our results showed that the N-SH2 and C-SH2 domains of SHP-2 have distinct differences of selectivity in recognition of phosphotyrosine sequences in PECAM-1. Tyr(P)-663 is the binding site for the N-SH2 of SHP-2 on PECAM-1, while Tyr(P)-686 is the binding site for the C-SH2 of SHP-2 on PECAM-1 in vitro (Fig. 3). However, the association mediated by the NH2-terminal SH2 domain of SHP-2 with Tyr(P)-663 of PECAM-1 appears to be more efficient. This finding has also been previously observed with a number of other SH2-associated receptors such as the β-subunit of PDGF and epidermal growth factor (42, 57).

Direct determination by surface plasmon resonance of the affinity of the tandem N-SH2-C-SH2 domains of SHP-2 for PECAM-1 Tyr(P) peptides 663 and 686 revealed that PECAM-1 pY-663 (pY663TEV) binds with a $K_a$ of 4 nM, consistent with a high affinity ligand for the tandem SH2 domains of SHP-2, while a 10-fold loss in affinity ($K_a$ of 46 nM) was observed with PECAM-1 pY-686 (pY686SEV) (Fig. 4). These results are in agreement with the predicted consensus sequence required for SH2-binding, a Val residue at −2 position, a phosphotyrosine residue, a β-branched residue at +1 (Thr/Val/Ile), and a hydrophobic residue at +3 position (Val/Leu/Ile), where Tyr-663 binding motif fulfills all requirements, while Tyr-686 binding motif only partially conforms (6, 58, 59).

A recent crystallographic study showed that the tandem SH2 domains of SHP-2 in complex with two phosphotyrosyl peptides corresponding to the Tyr-1009 binding site in PDGF revealed a fixed orientation of the domains in widely spaced and antiparallel orientations (53). Mutational analyses of tyrosine residues at positions 1009 and 1021 of the PDGF receptor have demonstrated that the cellular association of SHP-2 with ligand-stimulated PDGF requires only Tyr-1009, but not Tyr-1021 to create a binding site (60). In contrast, when IRS-1 is

FIG. 5. SHP-2 catalytic activity is stimulated by PECAM-1 Tyr-663 and Tyr-686 phosphopeptides. A, activation of SHP-2 catalytic activity by PECAM-1 cytoplasmic domain tyrosine-phosphorylated peptides was determined by incubating 2 μg/ml recombinant SHP-2 in the presence or absence of the indicated PECAM-1 Tyr(P) peptides at 150 μM final concentration and then measuring the release of 32P counts in 5 min from the artificial substrate, [32P]pY-RCM-lysozyme. Note that PECAM-1 Tyr-663 and Tyr-686 phosphopeptides stimulate the catalytic activity of SHP-2, while the other three PECAM-1 tyrosine-phosphorylated peptides were without effect. Values shown are the mean ± S.D. of three independent experiments. B, dose-dependent profile of activation of SHP-2 by PECAM-1 Tyr-663 and Tyr-686 phosphopeptides. SHP-2 catalytic activity was assayed using [32P]pY-RCM-lysozyme in the presence of increasing concentrations of phosphorylated PECAM-1-(658–668) Tyr(P)-663 (open circles), unphosphorylated PECAM-1-(658–668) (closed circles), phosphorylated PECAM-1-(681–691) Tyr(P)-686 (open triangles), or unphosphorylated PECAM-1-(681–691) (closed triangles). Values shown are the mean ± S.D. of three independent experiments.

(>500 μM). The nonphosphorylated versions of PECAM-(658–668) and -(681–691) peptides showed no significant activation of SHP-2 within the concentration range tested.
stimulated by insulin, two tyrosine phosphorylation sites, 1172 and 1222, are necessary to create docking sites for the selective recruitment in vitro of the tandem N- and C-SH2 domains of SHP-2 (52). Furthermore, the simultaneous occupancy of both SH2 domains of SHP-2 by the two tyrosine phosphorylation sites, 1172 and 1222 of IRS-1, resulted in potent stimulation of SHP-2 catalytic activity (61).

Since both SH2 domains of SHP-2 appear to bind two distinct phosphorysine binding motifs in PECAM-1, we speculated that occupancy of one or both SH2 domains would contribute to the catalytic activation of SHP-2. To study the relationship between SH2 domain recognition and allosteric activation, we analyzed five different Tyr(P) peptides comprising potential in vitro phosphorylation sites within the cytoplasmic domain of human PECAM-1 for their ability to stimulate the catalytic activity of full-length SHP-2. Our data showed that SHP-2 was activated by only two of the five tyrosine-containing monophosphopeptides, those encompassing either Tyr-663 or Tyr-686 of PECAM-1 (Fig. 5). Monophosphopeptide occupancy of either SH2 domain of SHP-2 is thought to stimulate catalytic activation by stabilizing an "open" active configuration of the catalytic domain (61). More potent stimulation of catalytic activity of SHP-2 is observed at lower concentrations of biphasrophylated peptides constituting a TAM motif due to the high affinity interaction with both SH2 domains of SHP-2. This finding has been observed for IRS-1 and brain immunoglobulin-like molecule (BIT) (10, 55, 61). PECAM-1 contains a TAM-like binding motif encompassing sequences pY460TEV and pY686SEV, which is similar in consensus sequence to IRS-1 pY1172IDL and pY1222ASI and the two TAM motifs described for BIT, N-TAM pY436ADL and pY460ASI, or C-TAM pY477ADL and pY501ASV. We predict that a PECAM-1 biphosphorylated peptide encompassing the two tyrosine phosphorylation sites, 663 and 686, would also potently enhance the catalytic activation of SHP-2. Due to its length, this has been difficult to test in vitro.

Unlike growth factor receptors such as the β-subunit of PDGF and epidermal growth factor, the association of SHP-2 with tyrosine-phosphorylated PECAM-1 in either 1) pervanadate-treated HEK-293 cells expressing wild-type PECAM-1 or 2) aggregated platelets does not appear to result in tyrosine phosphorylation of SHP-2 itself (data not shown). Therefore, it is unlikely in these settings that SHP-2 serves as an adapter molecule to bind Grb2 and link SHP-2 with the Ras/Raf/mitogen-activated protein kinase pathway. The fact that the binding of SHP-2 with activated PECAM-1 leads to stimulation of its catalytic activity suggests that SHP-2 could exert either positive or negative signaling responses via catalytic dephosphorylation of nearby signaling substrates, such as Src family kinases.

It is well established that PECAM-1 serves as a key participant in adhesion cascades that occur during cellular processes such as leukocyte transendothelial migration, cell migration, cell-cell contact, and junctional localization. PECAM-1 is predominantly distributed on the surface of transmigrating lymphocytes, monocytes, and neutrophils that come into contact with adjacent PECAM-1 molecules highly enriched in the endothelial cell intercellular junctions. In the process of transmigration, homophilic PECAM-1 cell-cell adhesion appears to be sufficient without the need for a chemotactic gradient to induce a signaling process to allow migration of cells into peripheral tissues (19, 20, 62). Engagement of PECAM-1 molecules or integrin-mediated cell adhesion that occurs during leukocyte-endothelial cell interactions could initiate outside-in signal transduction that induces tyrosine phosphorylation of PECAM-1 leading to formation of docking sites that can recruit SH2-containing signaling molecules, such as SHP-2. Activation of downstream signaling events may then initiate inside-out signal transduction leading to modulation of either integrin or PECAM-1 function. Further work will be required to define PECAM-1-mediated cell signaling pathways and the associated cytosolic signaling molecules operational in various cell types. The observation that SHP-2 is associated with PECAM-1 implicates this phosphatase in cellular events that follow interaction of blood and vascular cells.
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