Aggregation of many cell-surface receptors results in tyrosine phosphorylation of numerous proteins. We previously observed the tyrosine phosphorylation of the platelet/endothelial cell adhesion moleculc, PECAM-1 (CD31), after FcεRI stimulation in rat basophilic leukemia RBL-2H3 cells. Here we found that PECAM-1 was also transiently tyrosine-phosphorylated after adherence of these cells to fibronectin. Similarly aggregation of the T cell receptor on Jurkat cells also induced this tyrosine phosphorylation. The protein-tyrosine phosphatase SHP-2 is a widely expressed cytosolic enzyme with two Src homology 2 (SH2) domains. SHP-2, but not the related protein-tyrosine phosphatase SHP-1, associated with PECAM-1. This association of the two proteins correlated with the extent of the tyrosine phosphorylation of PECAM-1. A fusion protein containing the two SH2 domains of SHP-2 precipitated PECAM-1 from cell lysates and also directly bound to phosphorylated SH2 domains of SHP-2 precipitated PECAM-1. In immune precipitate phosphatase assays, there was tyrosine-dephosphorylation of PECAM-1. Therefore, integrin and immune receptor activation results in tyrosine phosphorylation of PECAM-1 and the binding of the protein-tyrosine phosphatase SHP-2, which could regulate receptor-mediated signaling in cells.

Tyrosine phosphorylation and dephosphorylation of molecules regulated by both protein-tyrosine kinases and phosphatases are critical events in signal transduction for cell growth, differentiation, and metabolism. The phosphorylation state of these proteins controls protein-protein association or dissociation thereby propagating and regulating downstream signal transduction. Aggregation of the high affinity receptor for IgE (FcεRI) on basophils or mast cells results in tyrosine phosphorylation of many proteins including the β and γ subunits of FcεRI, Syk, Lyn, phospholipase C-γ, pp125FGR, and other proteins (1–6). Recently, we observed that platelet/endothelial cell adhesion molecule 1 (PECAM-1, also known as CD31) was tyrosine-phosphorylated after receptor aggregation (7). This receptor-induced tyrosine phosphorylation of PECAM-1 was an early event, independent of Ca²⁺ influx or of cell adhesion.

PECAM-1 is a member of the immunoglobulin superfamily of cell adhesion molecules expressed on platelets, endothelial cells, and cells of the myeloid lineage including leucocytes, monocytes, some T cell subsets, and basophils (8, 9). It functions in inter-endothelial cell adhesion, leukocyte-endothelial interactions, and in the transendothelial migration of cells (8, 10). PECAM-1 is a single chain integral membrane glycoprotein containing six extracellular Ig-like homology domains, a single transmembrane region, and a cytoplasmic tail of ~118 amino acids. The cytoplasmic domain has numerous serine, threonine, and tyrosine residues that could potentially become phosphorylated. Receptor activation results in phosphorylation of PECAM-1 on serine and tyrosine residues (7, 11–14). Although the function of PECAM-1 as an adhesion molecule is regulated by its cytoplasmic domain, little is known about the significance of tyrosine phosphorylation of PECAM-1.

The protein-tyrosine phosphatase SHP-2 (previously called SH-PTP2, Syk, PTP1D, PTP2C, and SH-PTP3) is a ubiquitously expressed cytosolic protein that contains two amino-terminal tandem SH2 domains and a carboxyl-terminal catalytic domain (15–17). SHP-2 is the homologue of both the Drosophila casw gene product, Casw and SHP-1 (previously called SH-PTP-1, PTP1C, HCP and SHP). SHP-2 associates with tyrosine-phosphorylated epidermal growth factor receptor, the platelet-derived growth factor receptor, insulin receptor substrate-1, FcεRI, and with the T and B cell receptors (18–20). It becomes tyrosine-phosphorylated upon cell stimulation and may act either as a negative regulator for receptor function or as a positive effector for downstream signaling (21–24).

Previously we observed that the minimal tyrosine phosphorylation of PECAM-1 in nonstimulated cells was dramatically increased after FcεRI aggregation (7). Here we report that PECAM-1 was tyrosine-phosphorylated after either cell adherence or activation of other immune receptors such as the T cell receptor. The protein-tyrosine phosphatase SHP-2 but not SHP-1 was associated with tyrosine-phosphorylated PECAM-1. The tyrosine phosphorylation of PECAM-1 and the recruitment of protein-tyrosine phosphatases may play an important role in regulating signaling from cell-surface receptors.

**EXPERIMENTAL PROCEDURES**

**Materials and Antibodies—**Glutathione-Sepharose 4B was purchased from Pharmacia Biotech, Inc. MOPS was obtained from Fluka (Ronkonkoma, NY). All other materials not indicated under “Experimental Procedures” were described previously (7, 25, 26).

Polyclonal rabbit anti-SHP-2 (N-16 and C-18), polyclonal goat anti-PECAM-1, and monoclonal anti-GST or anti-CD3 (21-L5) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal anti-SHP-1 and anti-SHP-2 antibodies were obtained from Transduction Laboratories (Lexington, KY). The monoclonal anti-
PECAM-1 antibody (R23) and anti-FcRIIa antibody (BC4) were purified as described previously (7, 27). Polyclonal rabbit anti-human PECAM-1 and anti-rat PECAM-1 antibodies were kindly provided by Dr. Kim Piotrowski (Blood Center of Southwestern Wisconsin, Milwaukee, WI). For immunoprecipitations, the R23 anti-PECAM-1 antibody was covalently coupled to cyanogen bromide-activated Sepharose 4B beads at a concentration of 2 mg/ml. All other antibodies have been described previously (27, 28).

Cell Stimulation and Immunoprecipitation—RBL-2H3 cells were maintained as monolayer cultures, and FcεRI-mediated stimulation of these cells was as described previously (7, 29). Other stimulants used were 0.5 M calcium ionophore A23187 and 40 mM phorbol myristate 13-acetate (PMA). In some experiments, cells were treated with pervanadate formed by mixing 0.4 mM sodium orthovanadate with 1 mM H2O2. The stimulated cells (107) were then rinsed twice with phosphate-buffered saline and lysed with 1 mL of ice-cold Triton X-100, 10 mM Tris, pH 7.5, 100 mM NaCl, 50 mM Na3VO4, 1 mM Na2VO3, 2 mM phenylmethanesulfonil fluoride, 21 μg/mL aprotinin. The Jurkat T cell line (clone E6–1) was obtained from the ATCC (Rockville, MD) and maintained in suspension culture in RPMI 1640 medium. For T cell receptor stimulation, 2 × 107 cells were washed twice with ice-cold RPMI 1640 medium containing 0.1% BSA and 10 mM Tris–HCl, pH 7.5. Cells were then incubated on ice with 1 μg/mL monoclonal anti-CD3 antibody for 30 min followed by 10 μg/mL Fab1 fragments of goat anti-mouse IgG for 3 min at 37 °C. In some experiments the cells were treated with pervanadate as described above.

Post-nuclear supernatants were prepared by centrifuging the cell lysates at 16,000 × g for 30 min and then precleared with Sepharose 4B beads. Immunoprecipitation was with 20 μg of the anti-PECAM-1 antibody R23 coupled to Sepharose 4B beads, 4 μg of rabbit anti-SHP-2, or 10 μg of rabbit anti-PECAM-1 antibodies prebound to protein A-Sepharose 4B beads at 4 °C for 90 min. The beads were then washed five times with lysis buffer, and proteins were eluted from the beads by boiling in SDS-PAGE sample buffer.

Expression of GST Fusion Proteins and Precipitation of Proteins with GST Fusion Proteins—The plasmids containing the two SH2 domains of SHP-2, the full length of SHP-1 and SHP-2 as GST fusion proteins were kindly provided by Dr. Gen-Sheng Feng (Indiana University) and Dr. Benjamin G. Neel (Beth Israel Hospital, Boston). The proteins were expressed in Escherichia coli, affinity purified on glutathione-Sepharose beads, and characterized as recommended by the manufacturer. Precipitation with the 100 pmol of the GST fusion protein was as described previously (20).

Cell Adhesion Assays—This was as described previously (7, 30) with the modification that bacterial culture type Petri plates (100-mm diameter) were used after coating with either 15 μL of 10 μg/mL fibronectin (Calbiochem) or 15 μL of phosphate-buffered saline. RBL-2H3 cells at 5 × 105 cells/ml in Eagle’s modified essential medium containing 10 mM HEPES, pH 7.4, were added to each plate, and the plates were incubated at 37 °C for the indicated times. Although the cells did not adhere to the BSA-coated dishes, more than 90% of the cells attached to fibronectin-coated plates by 30 min. The nonadherent cells from the BSA-coated wells were recovered by centrifugation at 200 × g for 5 min at 4 °C. The cells were solubilized in 1 mL of Triton lysis buffer.

Immune Complex Phosphatase Assays—These assays were carried out as described previously (31). Briefly, the RBL-2H3 cells (5 × 106) were solubilized with 1 mL of ice-cold Brj lysis buffer (3% Brj 96, 20 mM Tris, pH 7.5, 100 mM NaCl, 1 mM Na2VO3, 2 mM phenylmethanesulfonil fluoride, 21 μg/mL aprotinin). After immunoprecipitation with anti-PECAM-1, the beads were washed twice with ice-cold protein-tyrosine phosphatase assay buffer (80 mM MOPS, 10 mM EDTA, 10 mM dithiorthreitol, pH 7.0) and resuspended with 50 μL of this buffer in the presence or the absence of 1 mM Na2VO3. The beads were then incubated at the indicated temperatures. After the reaction, the beads were either added to 5 × SDS-PAGE sample buffer or washed twice with Brj lysis buffer before the addition of sample buffer. The proteins were eluted from the beads by boiling for 5 min.

Blotting of Proteins—Total cell lysates and precipitated proteins were separated by SDS-PAGE (Novex, San Diego, CA) and then electrotransferred to polyvinylidine difluoride membranes (Immobilon, Millipore Corp., Bedford, MA). Immunoblotting or blotting with GST fusion proteins was as described previously (7, 20).

RESULTS

Association of PECAM-1 with SHP-2—We recently observed that the adhesion molecule PECAM-1 (CD31) became tyrosine-phosphorylated after FcεRI aggregation. Therefore we investigated whether other signaling molecules associate with PECAM-1 after it becomes tyrosine-phosphorylated. For these experiments we utilized the following two cell lines: rat basophilic leukemia RBL-2H3 cells and the Jurkat T cells which are known to express PECAM-1 (7, 32). In preliminary experiments we utilized the following two cell lines: rat basophilic leukemia RBL-2H3 cells and the Jurkat T cells which are known to express PECAM-1 (7, 32). In preliminary experiments we utilized the following two cell lines: rat basophilic leukemia RBL-2H3 cells and the Jurkat T cells which are known to express PECAM-1 (7, 32). In preliminary experiments we utilized the following two cell lines: rat basophilic leukemia RBL-2H3 cells and the Jurkat T cells which are known to express PECAM-1 (7, 32). In preliminary experiments we utilized the following two cell lines: rat basophilic leukemia RBL-2H3 cells and the Jurkat T cells which are known to express PECAM-1 (7, 32). In preliminary experiments we utilized the following two cell lines: rat basophilic leukemia RBL-2H3 cells and the Jurkat T cells which are known to express PECAM-1 (7, 32). In preliminary experiments we utilized the following two cell lines: rat basophilic leukemia RBL-2H3 cells and the Jurkat T cells which are known to express PECAM-1 (7, 32).
protein-tyrosine phosphatase SHP-2 binds to PECAM-1 (CD31), and this binding is increased during cell adhesion. We examined the direct interaction of SHP-2 with PECAM-1 using GST-fusion proteins containing SH2 domains.

**Figure 2.** Correlation in the extent of the tyrosine phosphorylation of PECAM-1 and its association with SHP-2 after FcεRI aggregation. RBL-2H3 cells were stimulated with 0.03 μg/ml monoclonal anti-FcεRI mAb BC4 for the indicated times (A) or with different concentrations of mAb BC4 for 10 min (B). Lysates from 5 × 10⁶ cells were immunoprecipitated with anti-PECAM-1 and analyzed by immunoblotting with anti-phosphotyrosine (Anti-pTyr), anti-SHP-2, and anti-PECAM-1 antibodies.

**Figure 3.** T cell receptor aggregation induced increased tyrosine phosphorylation of PECAM-1 and increased association of SHP-2. Jurkat cells (10⁶) were stimulated with 1 μg/ml monochlonal anti-CD3 antibody for 30 min on ice, transferred to 37 °C, and treated with 10 μg/ml goat anti-mouse IgG for 3 min. Lysates were immunoprecipitated (IP) with anti-PECAM-1 antibody and analyzed by immunoblotting with anti-phosphotyrosine (Anti-pTyr), anti-SHP-2, and anti-PECAM-1 antibodies.

Therefore, the activation of immune receptors results in the tyrosine phosphorylation of PECAM-1 and its interaction with SHP-2.

**Cell Adhesion Caused Tyrosine Phosphorylation of PECAM-1**—Although PECAM-1 is an adhesion molecule, its interaction with antibodies does not induce tyrosine phosphorylation of PECAM-1 itself or tyrosine phosphorylation of other cellular proteins (7). Here we investigated whether integrins could induce PECAM-1 tyrosine phosphorylation. When RBL-2H3 cells were added to either BSA or fibronectin-coated dishes, more than 90% of the cells adhered to fibronectin-coated surfaces, but none of them attached to BSA-coated dishes. After 30 min there was an increase in the tyrosine phosphorylation of PECAM-1 in adherent compared with nonadherent cells (Fig. 4A). The cells were still attached at 90 min, but the phosphorylation of PECAM-1 decreased to levels similar to nonadherent cells (data not shown). Although the tyrosine phosphorylation of PECAM-1 due to cell adherence was not as dramatic as that by FcεRI stimulation, there was still increased association of SHP-2 with PECAM-1 after cell adhesion (Fig. 4, A and B). Interestingly, adhesion also increased the tyrosine phosphorylation of SHP-2.

PECAM-1 Was Precipitated by Fusion Proteins Containing SH2 Domains of SHP-2—RBL-2H3 cells were either nonstimulated or stimulated with anti-FcεRI mAb, and the cell lysates were precipitated with the different GST fusion proteins immobilized on glutathione-coupled beads (Fig. 5). PECAM-1 was detected in the precipitates with the SHP-2 but not the SHP-1 fusion proteins from anti-FcεRI-stimulated cells. Although the two SH2 domains expressed separately bound PECAM-1, binding was better when both were expressed in tandem. After maximal tyrosine phosphorylation by pervanadate treatment, there was some precipitation of PECAM-1 by SHP-1 that was, however, still much less than with SHP-2 (data not shown). These data indicate that the association of PECAM-1 and SHP-2 is mediated by the SH2 domains of SHP-2 and requires the tyrosine phosphorylation of PECAM-1.

**Direct Interaction of Fusion Protein Containing the Two SH2 Domains of SHP-2 with Tyrosine-phosphorylated PECAM-1**—Although there was association of PECAM-1 and SHP-2 in immunoprecipitates, it was still possible that this interaction was indirect and mediated by other proteins. Therefore, membrane binding studies were used to study the direct interaction of PECAM-1 with the SHP-2 fusion protein (Fig. 6). PECAM-1 was immunoprecipitated from both nonstimulated and stimulated cells and the proteins separated by SDS-PAGE and blotted with GST-SHP-2. As expected, the GST-SHP-2 fusion protein bound to PECAM-1 only when the lysates were from stimulated cells. These results indicate that the direct interaction of PECAM-1 with SHP-2 can be mediated by the SH2 domains of SHP-2.

**Protein-tyrosine Phosphatase Activity of SHP-2 Coimmunoprecipitated with PECAM-1**—We examined whether the SHP-2 coimmunoprecipitated with PECAM-1 was still enzymatically active and could dephosphorylate PECAM-1 in vitro. PECAM-1 was immunoprecipitated from stimulated RBL-2H3 cells and then subjected to immune complex phosphatase assays (Fig. 7). The extent of the tyrosine phosphorylation of PECAM-1 decreased during the 30 min of in vitro incubation. As previously observed, there was SHP-2 present in these immunoprecipitates. However, if the immunoprecipitates were washed after the in vitro phosphatase assay, there was dissociation of SHP-2 that correlated with the dephosphorylation of PECAM-1. These data strongly suggested that the SHP-2 in the immunoprecipitates still had catalytic activity in vitro and further show that the interaction of these molecules depends on the tyrosine phosphorylation state of PECAM-1.
DISCUSSION

Activation of cells by adherence to fibronectin or by aggregating immune receptors induced the tyrosine phosphorylation of PECAM-1. The FcRI-induced tyrosine phosphorylation of PECAM-1 is an early event, independent of Ca\(^{2+}\) influx or of cell adhesion (7). Receptor aggregation is thought to result in activation of a protein-tyrosine kinase, probably Lyn, which

results in tyrosine phosphorylation of the receptor subunits (33). Syk then binds to the tyrosine-phosphorylated receptor subunits and is activated to propagate downstream signals including the tyrosine phosphorylation of phospholipase C-\(\gamma\) and the rise in intracellular calcium. There is minimal tyrosine phosphorylation of PECAM-1 in Syk-deficient cells (7). By analogy to those results the tyrosine phosphorylation of PECAM-1 in T cells must be downstream of the activation of the protein-tyrosine kinase ZAP-70.

SHP-2 was associated with the tyrosine-phosphorylated PECAM-1. Recent studies suggest a role for the protein-tyrosine phosphatase SHP-2 in several signal transduction pathways (17). SHP-2 associates with the tyrosine-phosphorylated epidermal growth factor or platelet-derived growth factor receptors by its amino-terminal SH2 domain and functions as a negative regulator by dephosphorylating the autophosphorylated receptors (22, 34). However, SHP-2 in some systems becomes tyrosine-phosphorylated and then functions as an adapter protein with positive effects for downstream signaling. For
example, the Grb2-Sos complex binds to the tyrosine-phosphorylated SHP-2 bound to the platelet-derived growth factor receptors and this then activates mitogenic signaling by the Ras pathway (35). In activated RBL-2H3 cells Grb2 was coprecipitated with PECAM-1 and SHP-2 (data not shown). Therefore, the SHP-2 associated with PECAM-1 may function as a phosphatase and also as an adaptor molecule.

The SH2 domains of SHP-2 were critical for its association with PECAM-1. The first of these sites is similar to the recently recognized tyrosine phosphorylation motif YXpXpXpXpX (IV/V)pXpXpXpXpX (16, 19) which is important for down-regulating immune receptor function (34–36). This immunoreceptor tyrosine-based inhibitory motif or ITIM is found on the cytoplasmic domain of several molecules including FcyRIIB in B cells and mast cells, CD22 in B cells, the killer-inhibitory receptors in NK cells, and gp49B1 in mast cells (37, 38). Phosphorylation of the tyrosine in this ITIM recruits SHP-1, SHP-2, and the SH2 domain-containing inositol polyphosphate 5-phosphatase, SHIP, that down-regulates receptor-mediated signal transduction (45, 46). Therefore, the cytoplasmic domain of PECAM-1 has similarities to these negative ITIM sequences and can recruit SHP-2.

The binding of tyrosine-phosphorylated peptides to the SH2 domain of SHP-2 increases its phosphatase activity (47, 48). The enzymatic activity of protein-tyrosine phosphatases is also enhanced by phospholipids (49). Therefore, the recruitment of SHP-2 to the membrane by binding to the tyrosine-phosphorylated PECAM-1 could enhance its phosphatase activity by these two mechanisms. The recruitment of SHP-2 to PECAM-1 would bring it in close proximity to other potential substrates such as FcεRI, the Src family tyrosine kinase Lyn, the protein-tyrosine kinase Syk, the adaptor protein Shc, phospholipase C, and of the SH2 domain of SHP-2 preferentially binds the Tyr(P)-hydrophobic-X-hydrophobic amino acid sequence (37, 38). The residue at pY-2 which is a valine at the site of SHP-2 binding to platelet-derived growth factor receptor may also contribute to binding (39). However, the binding preference of the carboxy-terminal SH2 domain has not been reported. The deduced amino acid sequences of the cytoplasmic domains of PECAM-1 have four (human) or five (bovine and mouse) tyrosine residues. The sequences V\(\text{V}^{177}\)ETYE\(\text{V}\) and T\(\text{V}^{106}\)YSEI\(\text{Y}\) of murine PECAM-1 could be potential sites for binding by the SH2 domains of SHP-2. The first of these sites is similar to the recently recognized tyrosine containing amino acid motif (IV/V)pXpXpXpXpX which is important for down-regulating immune receptor function (40–42). This immunoreceptor tyrosine-based inhibitory motif or ITIM is found on the cytoplasmic domain of several molecules including FcyRIIB in B cells and mast cells, CD22 in B cells, the killer-inhibitory receptors in NK cells, and gp49B1 in mast cells (43, 44). Phosphorylation of the tyrosine in this ITIM recruits SHP-1, SHP-2, and the SH2 domain-containing inositol polyphosphate 5-phosphatase, SHIP, that down-regulates receptor-mediated signal transduction (45, 46). Therefore, the cytoplasmic domain of PECAM-1 has similarities to these negative ITIM sequences and can recruit SHP-2.

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Protein-tyrosine Phosphatase SHP-2 Associates with PECAM-1 (CD31)