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A New Role for Platelet-Endothelial Cell Adhesion Molecule-1 (CD31): Inhibition of TCR-Mediated Signal Transduction

Debra K. Newton-Nash and Peter J. Newman

Platelet-endothelial cell adhesion molecule-1 (PECAM-1) is a 130-kDa transmembrane glycoprotein expressed by endothelial cells, platelets, monocytes, neutrophils, and certain T cell subsets. The PECAM-1 extracellular domain has six Ig-homology domains that share sequence similarity with cellular adhesion molecules. The PECAM-1 cytoplasmic domain contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) that, when appropriately engaged, becomes phosphorylated on tyrosine residues, creating docking sites for nontransmembrane, Src homology 2 domain-bearing protein tyrosine phosphatase (SHP)-1 and SHP-2. The purpose of the present study was to determine whether PECAM-1 inhibits protein tyrosine kinase (PTK)-dependent signal transduction mediated by the immunoreceptor tyrosine-based activation motif-containing TCR. Jurkat cells, which coexpress PECAM-1 and the TCR/CD3 complex, were INDO-1-AM-labeled and then incubated with anti-CD3 mAbs, anti-PECAM-1 mAbs, or both, and goat anti-mouse IgG was used to cross-link surface-bound mAbs. Calcium mobilization induced by CD3 cross-linking was found to be attenuated by coligation of PECAM-1 in a dose-dependent manner. PECAM-1-mediated inhibition of TCR signaling was attributable, at least in part, to inhibition of release of calcium from intracellular stores. These data provide evidence that PECAM-1 can dampen signals transduced by ITAM-containing receptors and support inclusion of PECAM-1 within the family of ITIM-containing inhibitors of PTK-dependent signal transduction. The Journal of Immunology, 1999, 163: 682–688.

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Abbreviations used in this paper: ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; AP, alkaline phosphatase; BCR, B cell receptor for Ag; CB, calcium buffer; GAM, goat anti-mouse IgG; KIR, killer inhibitory receptor; PECAM-1, platelet-endothelial cell adhesion molecule-1; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; SHP, Src homology 2 domain-bearing PTP; SH2, Src homology 2; PerCP, peridinin chlorophyll protein.
to numerous stimuli, including platelet aggregation (13), cross-linking of PECAM-1-specific mAbs (14, 15), mechanical stimulation of endothelial cells (16), engagement of the FcεRI on basophils (17), aggregation of the Ag receptor on T cells (18), and treatment with the FPT inhibitor pervanadate (19). When phosphorylated, these tyrosine residues support the binding of SHP-2 (13, 18, 20–23) and possibly SHP-1 (18, 22, 23). Thus, both structural and biochemical properties of the PECAM-1 cytoplasmic domain suggest that it may belong to the Ig-like, ITIM-containing family of inhibitory receptors.

The pattern of PECAM-1 expression on T lymphocytes also suggests a potential inhibitory function for this molecule. Specifically, the majority of CD4+ (24–27) and half of CD8+ T lymphocytes (24, 25) lose PECAM-1 expression as they make the transition from naive to memory cells. This pattern of expression is consistent with a role for PECAM-1 in dampening the effector function of naive T cells, which are less potent effectors than are memory cells (28). Furthermore, PECAM-1-negative CD4+ T cells provide more effective helper function, in that they respond better to recall Ags, secrete more IL-4, and provide better help for B cell Ig production than do PECAM-1-positive CD4+ T cells (27). Based on these observations, and given the structural and biochemical properties of the PECAM-1 cytoplasmic domain, we hypothesized that PECAM-1 might function as an inhibitory receptor that modulates TCR-mediated signal transduction in T cells. To test this hypothesis, we assessed the impact of PECAM-1 co-ligation on TCR-mediated calcium mobilization in T cells. We found that the PECAM-1 cytoplasmic domain becomes tyrosine phosphorylated in response to cross-linking either the TCR or PECAM-1 itself, with subsequent recruitment of SHP-2. When PECAM-1/SHP-2 was brought into close proximity with the TCR, it resulted in attenuation of TCR-mediated release of calcium from intracellular stores. These data support inclusion of PECAM-1 within the family of ITIM-containing inhibitory receptors.

Materials and Methods

Cell lines and cell culture

The acute T cell leukemia cell line, Jurkat (29), and the mature human T cell lymphoblast line HUT 78 (30, 31), were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in log phase growth at a density of 1 × 10^6 cells/ml in a humidified, 37°C, 5% CO_2/95% air atmosphere. Jurkat cells were cultured in RPMI (complete) containing 10% FBS and 10 mM HEPES, 2.8 mM KCl, 10 mM MgCl_2, 10 mM D-glucose, and 0.1% BSA (pH 7.2)). Cells were resuspended at a final concentration of 8 × 10^5 cells/ml with the addition of cold RPMI (complete)/10% FBS, and cell suspensions were placed on ice until calcium flux was induced and measured.

Antibodies

Peridinin chlorophyll protein (PerCP)-conjugated mouse anti-human CD3 (IgG1 isotype), FITC-conjugated goat anti-mouse IgG (GAM, PerCP-conjugated IgG1, and normal mouse IgG1 (Becton Dickinson, San Jose, CA) were purchased from Becton Dickinson Immunocytometry Systems. The mouse anti-human PECAM-1 Abs, PECAM-1-1 (IgG2a), -1.2 (IgG1), and -1.3 (IgG1), were prepared in our laboratory, and have been previously described (9). The mouse anti-human PECAM-1 Ab, 4G6 (IgG2b), was generously provided by Dr. Steven Albeda (University of Pennsylvania Medical Center, Philadelphia, PA) (32).

The human CD3ε-specific Ab, UCHT1 (mouse IgG1, κ), containing no azide and low endotoxin, was purchased from Pharmingen (San Diego, CA). Azide-free GAM F(ab')_2 fragments (Accurate Chemical and Scientific, Westbury, NY) or azide-containing GAM F(ab')_2 fragments (Sigma, St. Louis, MO), from which azide was removed by dialysis, were used for cross-linking of cell-surface-associated molecules. HRP-conjugated mouse anti-phosphotyrosine (clone PY20; Zymed Laboratories, San Francisco, CA), murine monoclonal anti-SHP-2 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-human PECAM-1 (SEW32-34; prepared in our laboratory), and HRP-conjugated GAM (Jackson Immunoresearch, West Grove, PA) or alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (Zymed Laboratories) Abs were used for Western blot analysis.

Induction and assessment of PECAM-1 tyrosine phosphorylation and SHP-2 binding

Jurkat cells in log phase growth were pelleted by centrifugation at 300 × g for 10 min at room temperature and resuspended at a final concentration of 10^5 cells/ml in cold RPMI (complete)/10% FBS. Cell suspensions (1 ml) were incubated with no Ab, with the human CD3ε-specific Ab, UCHT1 (1 μg/ml), with a human PECAM-1-specific Ab, or with both anti-CD3ε and anti-PECAM-1 for 30 min on ice. To remove unbound Ab, cell suspensions were washed twice with 5 ml of cold 0.145 M sodium chloride containing 10 mM HEPES, 2.8 mM KCl, 2 mM MgCl_2, 10 mM D-glucose, and 0.1% BSA (calcium buffer (CB)), and 10 mM CaCl_2. Cells were resuspended in the same buffer at a final concentration of 2 × 10^5 cells/ml.

To induce PECAM-1 phosphorylation and SHP-2 binding, cell suspensions were first prewarmed at 37°C for 10 min. GAM was added to achieve a final concentration of 20–100 μg/ml, which was empirically determined in preliminary experiments to yield maximal stimulation. Following incubation for an additional 3 min at 37°C, the reaction was stopped by the addition of an equal volume of cold 2% lysis buffer (0.145 M NaCl, 4% Triton X-100, 0.2 mM MgCl_2, 20 mM HEPES, 20 mM EGTA, 4 mM sodium orthovanadate, 40 μg/ml leupeptin, 2 mM PMSF (pH 7.4)), and lysates were prepared with gentle rocking for 120 min at 4°C. Lysates were also prepared from unstimulated cells, which were allowed to settle to the bottom of a 15-ml conical tube at 37°C and were not subjected to warming, cooling, or centrifugation. Following cell lysis, the Triton-insoluble fraction was removed by centrifugation at 15,000 × g for 15 min at 4°C.

PECAM-1.1-coated beads were prepared by incubating PECAM-1.1 Ab (2 μg/ml) with protein A-Sepharose beads for 2 h at 4°C with gentle rocking. Ab-coated beads were washed three times with lysis buffer to remove unbound Ab. Cell lysates were incubated with 100 μl of a 50% slurry of PECAM-1.1-coated beads with gentle rocking overnight at 4°C. Immunoprecipitation was washed three times with immunoprecipitation buffer (50 mM Tris (pH 7.4), containing 150 mM NaCl, 2 mM NaVO_4, 1% Triton X-100, and 1 mM PMSF). Pelleted beads were boiled in 60 μl of 2× SDS-sample reducing buffer. Material eluted from the beads was separated on a 7.5% SDS-polyacrylamide gel and transferred to an Immobilon-P membrane, which was subjected to Western blot analysis for the presence of PECAM-1, phosphotyrosine residues, and SHP-2.

Calcium mobilization assays

All procedures for measurement of intracellular calcium concentrations by fluorescence spectrofluorometry were performed in the dark. Jurkat cells (10^5 cells/ml) were incubated for 30 min at 37°C in RPMI (complete) containing 10% FBS and 10 μM INDO-1-AM (Molecular Probes, Eugene, OR). Cells were then adjusted to a final concentration of 8 × 10^5 cells/ml with the addition of cold RPMI (complete)/10%FBS, and cell suspensions were placed on ice. Cell suspensions (0.5 ml) containing 4 × 10^5 cells/ml were incubated at 4°C for 30 min in the presence of Abs at the indicated final concentrations. To remove unbound Abs, Ab-bound cells were washed three times at 4°C with 3 ml of CB (0.145 mM NaCl, 10 mM HEPES, 2.8 mM KCl, ZmM MgCl_2, 10 mM D-glucose, 0.1% BSA (pH 7.2)). Cells were resuspended at a final concentration of 2 × 10^5 cells/ml in cold CB containing either 10 mM CaCl_2 or 0.1 mM EGTA and maintained on ice until calcium flux was induced and measured.

Immediately before induction of calcium mobilization, cell suspensions were placed in a 37°C water bath for 2 min. Prelabeled cell suspensions were transferred to a cuvette equipped with a magnetic stir bar and transferred to the sample chamber, maintained at 37°C, of an SLM 8100 spectrophotometer (SLM-AMINCO, Urbana, IL). To cross-link surface-bound Abs, GAM was added to achieve a final concentration of 20 μg/ml, which was determined in preliminary experiments to induce maximal stimulation. Fluorescence of stirring cell suspensions was excited at 350 nm and emission of fluorescence at wavelengths of 405 and 490 nm was recorded every 3 s over a period ranging from 3 to 10 min in length. Results are presented as plots of the ratio of fluorescence detected at 405, relative to 490, nm as a function of time.

Results

Previous studies have shown that cross-linking the TCR leads to tyrosine phosphorylation of the cytoplasmic domain of PECAM-1, creating a docking site for the protein tyrosine phosphatase, SHP-2.
We sought to more thoroughly examine the conditions required for, and the downstream consequences of, PECAM-1 tyrosine phosphorylation and PTP binding in T cells. Since T cell expression of PECAM-1 is restricted to certain T cell subsets and maturation states (24), several T cell lines were evaluated for co-expression of TCR and PECAM-1. Consistent with previously reported observations (25, 33), the acute T cell leukemia line, Jurkat, expressed both TCR and PECAM-1 (Fig. 1). The HUT78 cell line expressed TCR, but was PECAM-1-negative by both flow cytometric and immunoblot analyses (data not shown). Therefore, Jurkat T lymphocytes were chosen for further studies.

To optimize the conditions required for PECAM-1 tyrosine phosphorylation and PTP binding in T cells, murine mAbs specific for CD3, PECAM-1, or CD3 and PECAM-1 were bound to the surface of Jurkat T cells and then cross-linked by addition of F(ab′)2 fragments of a polyclonal goat Ab specific for murine IgG heavy and light chains (GAM). Cells to which no primary Abs were bound served as negative controls. As shown in Fig. 2, a slight increase in PECAM-1 tyrosine phosphorylation was observed in cells stimulated by CD3 cross-linking, relative to the no Ab control. However, cross-linking of PECAM-1 itself induced a higher level of PECAM-1 tyrosine phosphorylation, which was further enhanced upon coligation of PECAM-1 with CD3. Tyrosine phosphorylation of PECAM-1 resulted in the association of SHP-2, and the amount of phosphatase coprecipitated correlated with the degree of PECAM-1 tyrosine phosphorylation (Fig. 2). We also observed a small amount of the related PTP, SHP-1, co-precipitating with PECAM-1, but could not rule out that its visualization was due to Ab cross-reactivity with SHP-2 (data not shown). Therefore, we conclude that the major PTP associated with PECAM-1 in Jurkat cells is SHP-2. However, cross-linking of PECAM-1 itself induced a higher level of PECAM-1 tyrosine phosphorylation, which was further enhanced upon coligation of PECAM-1 with CD3. Tyrosine phosphorylation of PECAM-1 resulted in the association of SHP-2, and the amount of phosphatase coprecipitated correlated with the degree of PECAM-1 tyrosine phosphorylation (Fig. 2).

Previous studies have shown that coligation of ITIM-bearing inhibitory receptors with ITAM-bearing stimulatory receptors results in attenuation or blunting of cellular activation responses, including the rate and/or extent of calcium mobilization (34–39), IP3 generation (38), and cell-mediated cytotoxic responses (38, 39). To determine whether PECAM-1/SHP-2 might be able to modulate TCR-mediated signal transduction, we examined the effect of PECAM-1 coligation with the TCR in INDO-1AM-loaded T cells. Jurkat T lymphocytes (10^7) were treated with no Ab (lane 1), 1 μg/ml of murine anti-human CD3ε (lane 2), 50 μg/ml of murine Ab specific for human PECAM-1 (PECAM-1.3; lane 3), or both anti-CD3ε and PECAM-1.3 (lane 4) at 4°C. After prewarming for 10 min at 37°C, receptor cross-linking was induced by addition of 20 (data not shown) or 100 μg/ml of GAM F(ab′)2. After a further incubation for 3 min, cells were lysed in Triton X-100, and PECAM-1 immunoprecipitates were prepared and analyzed by anti-phosphotyrosine (PY20; top panel), anti-SHP-2 (second panel), and anti-PECAM-1 (bottom panel) immunoblots.

FIGURE 1. Jurkat T lymphocytes coexpress CD3 and PECAM-1. Cell surface staining of the acute lymphocytic leukemia T cell line, Jurkat, was evaluated by flow cytometry. Negative controls included cells stained with normal mouse IgG1 (NMiG1) followed by FITC-conjugated GAM (GAM-FITC) and then with PerCP-conjugated normal mouse IgG1 (IgG1-PerCP) (A). To evaluate cell surface expression of PECAM-1 and CD3, cells were stained with the murine Ab specific for human PECAM-1, PECAM-1.3 (anti-PECAM-1), followed with GAM-FITC and then with PerCP-conjugated mouse anti-human CD3 (CD3-PerCP) (B).

FIGURE 2. Effect of receptor cross-linking on PECAM-1 tyrosine phosphorylation and PTP binding in T cells. Jurkat T lymphocytes (10^7) were treated with no Ab (lane 1), 1 μg/ml of murine anti-human CD3ε (lane 2), 50 μg/ml of murine Ab specific for human PECAM-1 (PECAM-1.3; lane 3), or both anti-CD3ε and PECAM-1.3 (lane 4) at 4°C. After prewarming for 10 min at 37°C, receptor cross-linking was induced by addition of 20 (data not shown) or 100 μg/ml of GAM F(ab′)2. After a further incubation for 3 min, cells were lysed in Triton X-100, and PECAM-1 immunoprecipitates were prepared and analyzed by anti-phosphotyrosine (PY20; top panel), anti-SHP-2 (second panel), and anti-PECAM-1 (bottom panel) immunoblots.
mobilization or inhibit TCR-mediated calcium mobilization in Jurkat T lymphocytes. A dose of anti-CD3ε (50 ng/ml) that induced measurable but suboptimal increases in intracellular calcium concentrations only when cross-linked with GAM, and an optimal concentration of GAM (20–100 μg/ml), were identified in preliminary dose response studies (data not shown). A suboptimal concentration of anti-CD3ε was used to enable detection of either an enhancing or an inhibitory effect of PECAM-1 on TCR-induced calcium mobilization. Furthermore, we chose conditions that required GAM cross-linking so that we could control whether or not PECAM-1 was colocalized with the TCR from the time of initiation of T cell activation. As shown in Fig. 3, cross-linking surface-bound anti-CD3ε mAb, PECAM-1.3, failed to induce calcium mobilization, even though PECAM-1 became tyrosine phosphorylated and bound SHP-2 under these conditions (Fig. 2). Evaluation of three other PECAM-1-specific mAbs revealed that none by themselves induced calcium mobilization in Jurkat T lymphocytes (Table I). In contrast, cross-linking of CD3ε induced strong and rapid biphasic calcium mobilization characterized by a transient, initial rise in intracellular calcium, followed by a lower but more sustained elevation of intracellular calcium levels (Fig. 3). Importantly, coligation of PECAM-1/SHP-2 with the TCR attenuated anti-CD3ε-mediated calcium mobilization (Fig. 3), an effect that was proportional to the amount of PECAM-1/SHP-2 brought into the TCR complex (Fig. 4). The ability of PECAM-1-specific Abs to inhibit TCR-mediated calcium flux also appeared to be influenced by the Ig homology domain-specificity of the anti-PECAM-1 mAb. Thus, two different Ig domain 6-specific mAbs (PECAM-1.2 and 4G6), and one specific for domain 1 (PECAM-1.3), effected inhibition of TCR signaling, whereas PECAM-1.1, which is specific for PECAM-1 Ig domain 5, failed to attenuate the early rise in intracellular calcium induced by CD3 cross-linking (Table I), most likely due to the orientation with which PECAM-1.1 interacts with cell surface PECAM-1 (see Discussion). The early rise in intracellular calcium reflects both release of calcium from intracellular stores and influx of calcium across the plasma membrane, whereas the later, sustained elevation of intracellular calcium levels is attributable to influx of calcium across the plasma membrane (40–42). PECAM-1 coligation appeared to inhibit the initial rise in intracellular calcium, whereas the later, sustained elevation of intracellular calcium induced by TCR cross-linking was largely unaffected by PECAM-1 coligation (Fig. 3). From these results, we predicted that PECAM-1/SHP-2 acts by inhibiting calcium release from intracellular stores, but not influx of calcium from extracellular sources. To test this prediction, we assessed the inhibitory effect of PECAM-1/TCR coligation in the absence vs the presence of extracellular calcium. As shown in Fig. 5, the presence of extracellular EGTA did not affect the inhibitory activity of PECAM-1 on TCR-mediated calcium mobilization. Rather, the effect of PECAM-1 inhibition was to attenuate TCR-induced release of calcium from intracellular stores.

### Table I. Ability of PECAM-1-specific Abs to induce calcium mobilization or inhibit TCR-mediated calcium mobilization in Jurkat T lymphocytes

<table>
<thead>
<tr>
<th>Anti-PECAM-1 mAb</th>
<th>Ig Domain Specificity</th>
<th>Induction of Ca²⁺ Flux</th>
<th>Inhibition of TCR-Mediated Ca²⁺ Flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>PECAM-1.1</td>
<td>5</td>
<td>No</td>
<td>No*</td>
</tr>
<tr>
<td>PECAM-1.2</td>
<td>6</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>PECAM-1.3</td>
<td>1</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>4G6</td>
<td>6</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

* Levels of PECAM-1 binding to cell surface PECAM-1 and of GAM binding to surface-bound PECAM-1.1, as assessed by flow cytometry, did not differ from those observed with PECAM-1.2 and PECAM-1.3 Abs (data not shown).

**Discussion**

The purpose of the present study was to evaluate the downstream consequences of PECAM-1 tyrosine phosphorylation and binding of PTPs, to determine whether PECAM-1, like other ITIM-containing members of the Ig-superfamily, can function as an inhibitor of PTK-dependent signal transduction. To this end, we evaluated the ability of PECAM-1 to inhibit calcium mobilization induced by TCR cross-linking in Jurkat T lymphocytes. We show in the present study that, in response to PECAM-1 or TCR coligation,
PECAM-1 becomes tyrosine phosphorylated and binds SHP-2 (Fig. 2). Furthermore, we demonstrate that calcium mobilization induced by TCR cross-linking is attenuated by coligation of the TCR with PECAM-1, which specifically affects the early rise, but not the late, sustained elevation of intracellular calcium (Fig. 3). PECAM-1-mediated attenuation of TCR-induced calcium mobilization is dose-dependent (Fig. 4), affects release of calcium from intracellular stores (Fig. 5), and is influenced by the domain-specificity of the cross-linking Ab (Table I). Together, these findings demonstrate that PTPs associated with the PECAM-1 cytoplasmic domain can, upon receptor coligation, be brought into proximity with the TCR and interfere with PTK-dependent signal transduction events in T cells. The importance of the PECAM-1 ITIM for this inhibitory function, the components of the TCR signal transduction pathway that are affected, and the extent to which PECAM-1 inhibition of TCR signal transduction normally occurs during the course of Ag stimulation are currently under active investigation in our laboratory. Nevertheless, these data demonstrate that PECAM-1 is able to attenuate cellular activation stimulated by an ITAM-containing stimulatory receptor, and provide the first evidence that PECAM-1 contains a functional ITIM. The identity of the PTK responsible for phosphorylating tyrosine residues in the PECAM-1 cytoplasmic domain in T cells is not known. We observed a low level of PECAM-1 tyrosine phosphorylation upon cross-linking of the TCR, and higher levels upon cross-linking of PECAM-1 either alone or when coligated with TCR. The PTKs that may be responsible for PECAM-1 tyrosine phosphorylation under these conditions include members of the Src family of PTKs (p59^c-src^, p56^ly-src^) and ZAP-70, all of which can be recruited to the TCR signaling complex and activated following TCR ligation (1). Ample evidence supports a role for members of the Src family of PTKs in PECAM-1 tyrosine phosphorylation. Specifically, Fyn coprecipitates with PECAM-1 (21) and c-Src both coprecipitates with and can phosphorylate the cytoplasmic domain of bovine (16, 21, 43) or murine PECAM-1 (23). In addition, overexpression of Src family members, p56^c-src^, p56^ly-src^, and p53^c-src^ in COS-1 cells resulted in tyrosine phosphorylation of murine PECAM-1 (23). A role for ZAP-70 in PECAM-1 tyrosine phosphorylation is supported by the observation that low levels of PECAM-1 tyrosine phosphorylation in Syk-deficient cells could be reconstituted by the stable transfection of Syk (17), a close homologue of ZAP-70 in non-T cells. However, Cao et al. (23) have recently shown that overexpression of Syk in COS-1 cells is incapable of stimulating PECAM-1 tyrosine phosphorylation. Finally, somewhat surprisingly, members of the Csk family of PTKs are also able to phosphorylate murine PECAM-1 (23). Further studies are required to determine which of the many PTKs capable of phosphorylating PECAM-1 on tyrosine residues actually do so in activated T cells.

Tyrosine phosphorylation of PECAM-1 results in the creation of sites with the potential for binding the tandem SH2 domain-containing PTPs, SHP-1 and SHP-2. Our data show that, in T cells, SHP-2 preferentially associates with the tyrosine-phosphorylated cytoplasmic domain of PECAM-1. These results are consistent with those of numerous studies demonstrating coprecipitation of SHP-2 with tyrosine-phosphorylated PECAM-1 (13, 18, 20, 22, 23). Although we occasionally observed, as have others (18, 23), coprecipitation of small amounts of SHP-1 with PECAM-1, SHP-2 appears to bind preferentially, most likely due to the presence of specific amino acid residues surrounding the phosphorylated tyrosines in the PECAM-1 ITIM, with which the SH2 domains of SHP-1 and SHP-2 must interact. The tandem SH2 domains of SHP-1 and SHP-2 are structurally similar, enabling both to recognize the consensus ITIM sequence (44). The PECAM-1 ITIM sequence of VxP^Y_xox_xhxVxP_yox_xhxV (12) is like that of another member of the Ig-like, ITIM-containing family of inhibitory receptors, Ly49, in that it possesses a valine instead of a leucine residue at the +3 position relative to the tyrosine residues in its ITIM (45). The ability of the Ly49 ITIM to activate SHP-1 is reduced relative to that of another inhibitory receptor, p58 KIR, and this difference has been attributed to the substitution of valine for leucine at ITIM position +3 (45). In contrast, the binding of SHP-2 to phosphorylated ITIMs appears not to be affected by the substitution of valine for leucine at position +3, as peptides containing either amino acid at this position were selected equally well from a degenerate peptide library by the SH2 domains of SHP-2.
Thus, the preferential binding of SHP-2, relative to SHP-1, to the PECAM-1 ITIM may similarly be attributable to the existence of valine, as opposed to leucine, residues at the +3 positions of the PECAM-1 ITIM.

We found that PECAM-1 oligomerization resulted in PECAM-1 tyrosine phosphorylation and SHP-2 binding, but not calcium mobilization in Jurkat T cells. Our observations are consistent with the previous observation of PECAM-1 tyrosine phosphorylation in response to binding of the PECAM-1-specific Ab, PECAM-1.2 (15). They are, however, inconsistent with the previous observation that the PECAM-1-specific mAb, 4G6, induces calcium mobilization in human endothelial cells and PECAM-1-transfected REN cells (46). This difference could be due to different signal transduction properties for PECAM-1 in T cells relative to endothelial cells, or to a difference in the assays used for assessment of calcium mobilization. We conclude that PECAM-1 tyrosine phosphorylation and SHP-2 binding, at least in T cells, are not coupled to signal transduction pathways that elevate intracellular calcium. Thus, though a positive signaling function for SHP-2 has been proposed based upon its ability to bind Grb2/SOS and activate the Ras/MAPK signal transduction pathway (47–51), our results support the hypothesis that induction of protein tyrosine phosphorylation and activation of signal transduction pathways that result in calcium mobilization can be independently regulated (52).

Whereas PECAM-1 oligomerization, tyrosine phosphorylation and SHP-2 binding were not sufficient to induce calcium mobilization in T lymphocytes, they did interfere with calcium mobilization induced by TCR cross-linking. Only one of four PECAM-1-specific Abs tested, PECAM-1.1, failed to produce this effect. It is possible that the orientation of PECAM-1.1, when bound to PECAM-1, imposes geometrical constraints on the receptor that interfere with the ability of the GAM cross-linker to either dimerize PECAM-1 or to bring it productively into proximity with Ab-bound CD3ε. All of the other PECAM-1-specific Abs tested, however, supported PECAM-1-mediated inhibition of TCR-induced calcium mobilization.

The characteristics of PECAM-1-mediated inhibition of TCR-induced calcium mobilization differed somewhat from the effects of other Ig-ITIM inhibitory receptors. Specifically, PECAM-1/TCR coligation inhibited release of calcium from intracellular stores only incompletely and failed to block or reduce the late, sustained elevation in intracellular calcium concentration that is normally observed after TCR ligation. This pattern of inhibition differs from that of FcγRIIB, which inhibits B cell receptor (BCR)-induced influx of calcium from extracellular sources, a property that is consistent with the ability of FcγRIIB, upon tyrosine phosphorylation, to recruit the 5′-inositol phosphatase, SHIP (reviewed in Ref. 53). The inhibitory effect of PECAM-1 differed less dramatically from those of inhibitory receptors that, like PECAM-1, recruit protein tyrosine phosphatases to the membrane upon tyrosine phosphorylation. Thus, p58 KIR (34) and PIR-B (35, 36) functioned similarly to PECAM-1 in that they inhibited, respectively, FcεR or BCR-mediated release of calcium from intracellular stores. Unlike PECAM-1, however, the levels of intracellular calcium sustained later in these responses were also reduced upon coligation of p58 KIR with FcεR (34) or upon coligation of the BCR with either PIR-B (35, 36) or CD22 (37).

The absence of an effect of PECAM-1 on the late, sustained elevation of intracellular calcium concentrations may determine the manner in which T cell effector function is inhibited by PECAM-1. The late, sustained calcium signal is important for nuclear localization of NF-AT (54), induction of IL-2 transcription (55), and irreversible commitment of T cells to full activation (56). Thus, the inability of PECAM-1 coligation to reduce the magnitude of the late, sustained calcium signal may influence its ability to affect the magnitude of a T cell effector response. However, stimuli that delay the onset of a rise in intracellular calcium following initial T cell activation, as does PECAM-1, also delay the onset of a T cell proliferative response (57). Thus, we predict that PECAM-1 coligation with the TCR may delay the onset of T cell effector responses, but not affect their ultimate magnitude. That the overall level of inhibition caused by PECAM-1/TCR coligation is incomplete is consistent with the observation that PECAM-1-deficient mice do not display any overt defects in T cell maturation or homing (58). It is possible that an effect of PECAM-1 on T cell responses other than maturation and migration may have been missed in preliminary characterizations of the PECAM-1-deficient mice. However, it is also important to consider that T cell activation is controlled by other inhibitory receptors, including KIRs and CTLA-4. If the function of PECAM-1 in T cells is subtle, so as to delay the onset of, or raise the threshold required for activation, it is possible that the effect of PECAM-1 deficiency may be observable only in the absence of these other inhibitory receptors. Studies of T cell function in PECAM-1-deficient mice that are also heterozygous for CTLA-4 deficiency are currently ongoing in our laboratory to explore this possibility.

Upon interaction with appropriate APC, T cells receive signals through both activating and inhibitory receptors. The relative strength of the signal delivered by each of these two classes of receptors determines whether the degree of activation of the signal transduction pathway reaches a critical threshold required for commitment to activation. In general, the function of inhibitory receptors is to recruit phosphatases that counteract the activity of kinases recruited to activating receptors, thus making it more difficult for a given T cell to reach the critical activation threshold. We show here that PECAM-1, a molecule that is expressed on the surfaces of both endothelial cells and certain T cell subsets, and that mediates homophilic interactions, is capable of interfering with TCR-mediated signal transduction. On the basis of this understanding, we propose that one context in which PECAM-1 exerts its inhibitory function may involve T cell transmigration across endothelial cell barriers, during which process PECAM-1 may serve to prevent T cells from becoming aberrantly activated. Studies of in vitro T cell effector activity, as well as in vivo studies involving comparisons of the immune reactivity of wild-type and PECAM-1−/− mice, are currently ongoing in our laboratory to test the validity of this newly demonstrated function for PECAM-1.

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