Regulation of Mouse PECAM-1 Tyrosine Phosphorylation by the Src and Csk Families of Protein-tyrosine Kinases*

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PECAM-1 is an adhesion molecule expressed on hematopoietic and endothelial cells. Recently, it was observed that PECAM-1 becomes tyrosine-phosphorylated in response to a variety of physiological stimuli. Furthermore, tyrosine-phosphorylated PECAM-1 was shown to associate with SHP-2, a Src homology 2 (SH2) domain-containing protein-tyrosine phosphatase expressed ubiquitously. In light of the significance of tyrosine protein phosphorylation as a regulatory mechanism, we wished to understand better the nature and impact of the protein-tyrosine kinases (PTKs) mediating PECAM-1 tyrosine phosphorylation. Through reconstitution experiments in COS-1 cells, we determined that mouse PECAM-1 could be tyrosine-phosphorylated by Src-related PTKs and Csk-related PTKs, but not by other kinases such as Syk, Itk, and Pyk2. Using site-directed mutagenesis and peptide phosphorylation studies, we found that these PTKs were efficient at phosphorylating Tyr-686, but not Tyr-663, of PECAM-1. Src-related enzymes also phosphorylated mouse PECAM-1 at one or more yet to be identified sites. In other studies, we demonstrated that phosphorylation of PECAM-1 by Src or Csk family kinases was sufficient to trigger its association with SHP-2. Moreover, it was able to promote binding of PECAM-1 to SHP-1, a SH2-2-related protein-tyrosine phosphatase expressed in hematopoietic cells. Taken together, these findings indicated that the Src and Csk families of kinases are strong candidates for mediating tyrosine phosphorylation of PECAM-1 and triggering its association with SH2 domain-containing phosphatases under physiological circumstances.

Platelet endothelial cell adhesion molecule-1 (PECAM-1; also termed CD31) is a 130-kDa cell surface glycoprotein that belongs to the immunoglobulin (Ig) superfamily (Refs. 1 and 2; reviewed in Refs. 3 and 4). It is abundantly expressed on endothelial cells as well as various hematopoietic cell types, including platelets, myeloid cells, monocytes, mast cells, and defined T-cell subsets. The structure of PECAM-1 is highly conserved across species. It contains an extracellular segment with six Ig-like domains (C2 type), a single hydrophobic transmembrane region, and a cytoplasmic domain of ~120 amino acids. Several isoforms of PECAM-1 having a different cytoplasmic domain are also potentially generated by alternative splicing.

Immunolocalization studies have indicated that PECAM-1 is concentrated at intercellular junctions, suggesting that it may regulate cell-cell interactions (reviewed in Refs. 3 and 4). This notion was further strengthened by the discovery that PECAM-1 is a potent adhesion molecule. Unlike most other adhesion molecules, however, PECAM-1 can participate both in homophilic and heterophilic interactions. In addition to itself (5, 6), it can bind proteoglycans (7, 8), the αβ3 integrin (9, 10), CD38 (Ref. 11), and a yet unidentified ligand on activated T-lymphocytes (12). Interestingly, the participation of PECAM-1 in homotypic versus heterotypic interactions is controlled by its cytoplasmic region (6, 13, 14). A highly conserved tyrosine in this domain, tyrosine 686 (Tyr-686), is important for this regulation (14). The capacity of PECAM-1 to mediate adhesion seems to be involved in a variety of biological processes, including release of leukocytes from the bone marrow, migration of inflammatory cells across endothelia, development of the cardiovascular system, and angiogenesis (reviewed in Refs. 3 and 4).

Further to its participation in cell adhesion, PECAM-1 can operate as a signal transduction molecule. This idea was initially suggested by the observation that PECAM-1 undergoes phosphorylation on tyrosine residues in response to several stimuli. These include mechanical stimulation of endothelial cells (15), platelet aggregation (16), triggering of the high affinity receptor for IgE (FcεRI) on basophils (17), engagement of the antigen receptor on T-cells (18), and treatment with the protein-tyrosine phosphatase (PTP) inhibitors vanadate and pervanadate (19, 20). Whereas the exact impact of tyrosine phosphorylation of PECAM-1 has not been fully elucidated, there is some evidence that it may regulate the adhesive properties of PECAM-1 (Ref. 14).

Tyrosine phosphorylation of PECAM-1 prompts its association with intracellular signal transduction molecules. Following platelet aggregation (16) or stimulation of FcεRI on baso-
sophila (18), PECAM-1 associates with SHP-2, a PTP with two Src homology 2 (SH2) domains at its amino terminus. This association was determined to require a pair of tyrosines in the cytoplasmic domain of human PECAM-1 (Tyr-663 and Tyr-686), and the tandem SH2 domains of SHP-2 (Refs. 20 and 21). It is noteworthy that the sequences surrounding Tyr-663 and Tyr-686 are reminiscent of an “immunoreceptor tyrosine-based inhibitory motif” (or ITIM) (22–24). This motif is present in “inhibitory” receptors such as FcγRIIB and the killer inhibitory receptors, and can inhibit cell signaling by recruiting SH2 domain-bearing phosphatases. However, the exact physiological consequences of the interaction of PECAM-1 with SHP-2 remain to be determined.

To better comprehend the role of tyrosine phosphorylation in the function of PECAM-1, it is critical to have a detailed understanding of the protein-tyrosine kinases (PTKs) responsible for this phosphorylation. To this end, we have studied the ability of various classes of cytoplasmic PTKs to induce PECAM-1 tyrosine phosphorylation in COS-1 cells. The results of our studies showed that tyrosine phosphorylation of mouse PECAM-1 could be mediated by the Src family and Csk family of PTKs. Further analyses revealed that both types of kinases were apt at phosphorylating Tyr-666 of PECAM-1, but were unable to cause detectable phosphorylation of Tyr-663. Finally, it was observed that phosphorylation of mouse PECAM-1 by either Src or Csk family kinases was sufficient to induce binding to SHP-2, as well as to the closely related phosphatase SHP-1.

MATERIALS AND METHODS

Cells—COS-1 cells were maintained in a minimal essential medium supplemented with 10% fetal bovine serum, glutamine, and antibiotics.

cDNAs and Constructs—The wild-type mouse pecam-1 cDNA, as well as the tyrosine 666 (Tyr-666)-to-phenylalanine 666 (Phe-666) mutant, were described (14). An additional variant bearing a tyrosine 663 (Tyr-663)-to-phenylalanine 663 (Phe-663) substitution was created by site-directed mutagenesis. The cDNA in which both Tyr-663 and Tyr-666 were replaced by phenylalanines (Y663F/Y686F mutant) was produced by standard recombinant DNA technology, using the Phe-663 and Phe-686 pecam-1 cDNAs as templates. All mutations were confirmed by sequencing (data not shown). Mouse cDNAs coding for the protein-tyrosine kinases Lck (25), Csk (26), Chk (27), Syk (28), and Itk (29) were provided by Drs. David Shalloway (Cornell, Ithaca, NY) and Ashley Dunn (Ludwig Institute, Placid, New York). 4G10 was purchased from Upstate Biotechnology Institute, Lake Placid, New York.

RESULTS

Tyrosine Phosphorylation of Mouse PECAM-1 Can Be Mediated by the Src and Csk Families of Protein-tyrosine Kinases—Earlier reports have documented that PECAM-1 becomes tyrosine-phosphorylated in response to a wide variety of stimuli (11–20). As these stimuli trigger the activation of several classes of tyrosine kinases, it is difficult to infer from these studies which of these kinases is responsible for PECAM-1 tyrosine phosphorylation. As a matter of fact, it is conceivable that more than one of these enzymes carry out PECAM-1 tyrosine phosphorylation in physiological situations. To help identify these PTKs, we wished to dissect the requirements for PECAM-1 tyrosine phosphorylation in a heterologous system. Transient transfections in COS-1 cells were chosen for these analyses, as they have proven to be very useful for exploring the tyrosine phosphorylation of other substrates (35–39).

A mouse pecam-1 cDNA was transiently transfected in COS-1 cells, in the absence or presence of PTKs known to regulate hemopoietic and/or endothelial cell functions. After 60 h, cells were lysed in Nonidet P-40 containing buffer, and

2. F. Gervais and A. Veillette, unpublished data.

3. A. Veillette and N. Beaucournu, unpublished data.

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PECAM-1 was immunoprecipitated using anti-PECAM-1 mAb MEC 13.3. Following several washes, immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis and immunoblotted with anti-phosphotyrosine mAb 4G10 (Fig. 1, A and B, top panel). The abundance of PECAM-1 in the immunoprecipitates was also verified by stripping the immunoblot and reprobing with a polyclonal rabbit anti-PECAM-1 serum (bottom panel). In the absence of exogenous PTKs (Fig. 1A, lane 8), mouse PECAM-1 was not detectably tyrosine-phosphorylated in COS-1 cells. However, upon expression of the Src-related kinase p56\(^{1/c}\) (lane 9), p56\(^{50/3}\) (lane 12), p53\(^{30/3}\) (lane 13), and p60\(^{-c-src}\) (lane 14), there was a prominent increase in its phosphotyrosine content. Introduction of p50\(^{ck}\) (lane 10), which belongs to a separate enzyme family (reviewed in Ref. 40), also induced tyrosine phosphorylation of PECAM-1. By contrast though, Syk (lane 11), a kinase playing an essential role in numerous hemopoietic cell types (reviewed in Ref. 41), was incapable of stimulating PECAM-1 tyrosine phosphorylation. The differential effects of these PTKs on PECAM-1 tyrosine phosphorylation were not caused by variations in the levels of PECAM-1 expression, as indicated by the immunoblot with anti-PECAM-1 antibodies (bottom panel). Moreover, there were no tyrosine-phosphorylated proteins in anti-PECAM-1 immunoprecipitates obtained from cells not transfected with the pcam-1 cDNA (lanes 1–7), showing that the immunoreactive products detected were clearly PECAM-1. It is noteworthy, however, that PECAM-1 frequently migrated as a doublet in these assays, most likely as a result of being differentially glycosylated in its extracellular domain. Tyrosine phosphorylation of PECAM-1 could also be documented when only the cell surface-associated PECAM-1 was immunoprecipitated from transfected COS-1 cells (data not shown).

In another experiment (Fig. 1B), we tested the ability of Chk, a Csk-related enzyme selectively expressed in hemopoietic cells and brain (27, 42–44), to induce tyrosine phosphorylation of PECAM-1. As was the case for Lck (lane 3) and Csk (lane 4), p52\(^{ck}\) (lane 5) was also efficient at provoking tyrosine phosphorylation of PECAM-1. By comparison, unrelated protein-tyrosine kinases such as Lck (lane 6), Ref. 45) and Pyk2 (data not shown; Ref. 46) were unable to induce this phosphorylation.

The Src and Csk Families Are Efficient at Phosphorylating Tyrosine 686 of Mouse PECAM-1—Next, we wished to determine which tyrosine residue(s) in mouse PECAM-1 was phosphorylated by the Src and Csk families of PTKs. To this end, we first tested the ability of mutating the two postulated sites of PECAM-1 tyrosine phosphorylation, Tyr-663 and Tyr-686, on the phosphorylation of PECAM-1 in COS-1 cells (Fig. 2A). Cells were transfected with a pcam-1 cDNA in which either Tyr-663 or Tyr-686 was replaced by phenylalanine, in the presence of either a representative Src family kinase (p56\(^{1/c}\) or p50\(^{ck}\)). Anti-phosphotyrosine immunoblotting of anti-PECAM-1 immunoprecipitates showed that Phe-663 PECAM-1 (lanes 5 and 6) was as extensively tyrosine-phosphorylated by Lck and Csk as wild-type PECAM-1 (lanes 2 and 3). In fact, the phosphotyrosine content of Phe-663 PECAM-1 (lanes 5 and 6) was slightly greater (~2-fold) than that of wild-type molecules (lanes 2 and 3). While the exact basis for this phenomenon is not known, it is possible that Phe-663 PECAM-1 interacted less efficiently with endogenous SH2 domain-containing phosphatases expressed in COS-1 cells (see Fig. 8), thereby allowing more stable phosphorylation of other tyrosines in its cytoplasmic domain. This experiment also revealed that Phe-686 PECAM-1 was efficiently phosphorylated by p56\(^{1/c}\) (lane 8). However, this mutant failed to be detectably phosphorylated by Csk (lane 9).

The inability of individual mutations at Tyr-663 and Tyr-686 to abolish Lck-mediated tyrosine phosphorylation of mouse PECAM-1 raised the possibility that both sites were phosphorylated by the Src family kinase. To evaluate this prospect, a pcam-1 cDNA in which both tyrosines were replaced by phenylalanines was created. When expressed in COS-1 cells (Fig. 2B), we found that Y663F/Y686F PECAM-1 was still tyrosine phosphorylated in the company of p56\(^{1/c}\) (lane 5). Such a finding implied that phosphorylation at one or more additional tyrosines on PECAM-1 was induced by Lck. In this assay, we also noted that the phosphotyrosine content of Phe-663 PECAM-1 (lane 4) in the presence of p56\(^{1/c}\) was reduced ~3-fold by comparison to that of wild-type PECAM-1 (lane 2). While not seen in the experiment depicted in Fig. 2A, this effect was occasionally observed in other assays (data not shown). It suggested that Lck may be able to phosphorylate Tyr-686 (see below).

To address more directly the ability of Lck and Csk to phosphorylate Tyr-663 and Tyr-686, in vitro peptide phosphorylation assays were conducted. Peptides corresponding to Tyr-663 (PQNMDVEY663TEVEVSS) and Tyr-686 (TRATETVY686SE- IRKWD) were synthesized, and their ability to act as substrates for Lck and Csk was tested in immune complex kinase reactions (Fig. 3). After immunoprecipitation from transiently transfected COS-1 cells, Lck and Csk were incubated for 15 min at room temperature in the presence of the PECAM-1 peptides and radioactive ATP. The phosphorylated products were resolved in Tricine gels and detected by autoradiography (Fig. 3A). The results of this experiment showed that the Tyr-686 peptide was well phosphorylated by Lck (lane 3) and Csk (lane 6).
In contrast, the Tyr-663 peptide was not detectably phosphorylated by either kinase (lanes 1 and 2). In another assay (Fig. 3), the ability of the Tyr-686 peptide to be phosphorylated by Lck and Csk was compared with that of a peptide derived from the \( \zeta \) subunit of the T-cell antigen receptor, a model substrate for Src family kinases (40). This analysis indicated that Lck (lane 5) and Csk (lane 6) phosphorylated the Tyr-686 peptide nearly as efficiently as Lck could phosphorylate the \( \zeta \)-based peptide (lane 8). No phosphorylated products other than the PTKs themselves were detected when the reactions were conducted in the absence of peptides (lanes 1–3), or when immunoprecipitations with anti-Csk antibodies were carried out from mock-transfected COS-1 cells (lanes 1, 4, and 7).

PECAM-1 Can Associate with SHP-2, as Well as with the Related Protein-tyrosine Phosphatase SHP-1, in Transiently Transfected COS-1 Cells—We also wanted to evaluate whether phosphorylation of mouse PECAM-1 by Src or Csk family kinases was sufficient to allow recruitment of SH2 domain-containing protein-tyrosine phosphatases (reviewed in Ref. 47). Thus, COS-1 cells were transfected with a cDNA encoding wild-type PECAM-1 plus Lck or Csk, in the presence of a cDNA coding for SHP-2 (Fig. 4A). Since SHP-2 was shown to be able to dephosphorylate PECAM-1 (Ref. 18), a catalytically inactive version (cysteine 459 to serine 459 (C459S) SHP-2) was used for these studies. After transfection, cell lysates were immunoprecipitated with an antiserum directed against the carboxy-terminal segment of SHP-2, and the existence of associated PECAM-1 molecules was revealed by immunoblotting with either anti-PECAM-1 (top panel) or anti-phosphotyrosine (middle panel) antibodies.

In agreement with other reports (16, 18, 20, 21), significant amounts of PECAM-1 were observed to co-immunoprecipitate with SHP-2 (Fig. 4A, top and middle panels, lanes 4–6). We were surprised, however, to find that the association between these two molecules occurred in the absence of co-expression of a PTK (lane 4). In agreement with this observation, PECAM-1 was detectably tyrosine-phosphorylated in cells containing the inactive SHP-2, even if no PTK had been co-transfected (middle panel, lane 4). These findings raised the possibility that expression of the catalytically inactive SHP-2 enhanced tyrosine phosphorylation of PECAM-1. To help substantiate this notion, the level of tyrosine phosphorylation of PECAM-1 in the presence of C459S SHP-2 (Fig. 4B, lane 2) was compared with that of PECAM-1 expressed alone (lane 1). This analysis showed that expression of the inactive SHP-2 (lane 2) was indeed capable of augmenting tyrosine phosphorylation of PECAM-1.

We also wanted to contrast the effect of C459S SHP-2 on PECAM-1 tyrosine phosphorylation with that of wild-type SHP-2 (Fig. 5). Thus, COS-1 cells expressing PECAM-1 in the presence of Lck or Csk, and the phosphorylated peptides are indicated on the left. Exposures: 8 min.
either wild-type or C459S SHP-2. As was the case in the absence of Lck or Csk (Fig. 4B), expression of C459S SHP-2 increased the extent of tyrosine phosphorylation of PECAM-1 in cells containing Lck (first panel, compare lanes 2 and 4) or, especially, Csk (compare lanes 5 and 7). By contrast, wild-type SHP-2 had either no effect (lane 3) or decreased (lane 6) the phosphotyrosine content of PECAM-1. As a corollary to these results, we found that the degree of co-immunoprecipitation of PECAM-1 was greater for the phosphatase-inactive SHP-2 (second panel, lanes 4 and 7) than for wild-type SHP-2 (lanes 3 and 6). These results implied that the ability to detect an increase in PECAM-1 tyrosine phosphorylation in the presence of SHP-2 required suppression of its phosphatase activity.

While the precise basis of this phenomenon remains to be determined, a similar effect of the phosphatase-inactive SHP-2 was noted on SHPS-1, another immunoreceptor tyrosine-based inhibitory motif-containing receptor (47). As reported for other SH2 domain-containing molecules (48, 49), we believe that it is likely that the tandem SH2 domains of SHP-2 protected PECAM-1 from the action of endogenous PTPs, thus causing an increase in PECAM-1 tyrosine phosphorylation. While the results of the experiments of Figs. 4 and 5 demonstrated that the association of PECAM-1 with SHP-2 could be reconstituted in COS-1 cells, it should be pointed out, however, that the impact of Src and Csk family kinases on this binding could not be conclusively assessed. The results of an alternative approach will be presented later.

SHP-1 is an SH2 domain-containing PTP that belongs to the same family as SHP-2 (reviewed in Ref. 50). Unlike SHP-2, which is expressed ubiquitously, SHP-1 accumulates mostly in hemopoietic cells and, to a lesser extent, epithelial cells. Given the similarities between these two PTPs, we wanted to determine whether SHP-1 also had the capacity to bind SHP-1. Thus, a similar approach was taken to ascertain its binding to SHP-1. However, in cells containing either Lck (lane 2) or Csk (lane 3), PECAM-1 was found to co-immunoprecipitate with SHP-1.

Either Src or Csk Family Kinases Are Sufficient to Induce

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5 A. Veillette, unpublished results.
PECAM-1 Binding to the Tandem SH2 Domains of SHP-2 and SHP-1—These last findings demonstrated that phosphorylation of mouse PECAM-1 by Src or Csk family kinases was sufficient to allow binding of SHP-1. However, as stated above, we were unable to determine whether this effect also applied to SHP-2, as expression of C459S SHP-2 independently increased PECAM-1 tyrosine phosphorylation in COS-1 cells. To circumvent this caveat, the ability of PECAM-1 to associate with the SH2 domains of SHP-2 and SHP-1 was examined in in vitro binding assays (Fig. 7). PECAM-1 was first expressed in COS-1 cells, either alone or in combination with Lck or Csk. Cell lysates were then incubated with GST fusion proteins encompassing the tandem SH2 domains of SHP-2 or SHP-1, immobilized on agarose-glutathione beads. After extensive washing, the PECAM-1 molecules captured with these SH2 domains were detected by immunoblotting with anti-PECAM-1 antibodies.

This approach revealed that, in the absence of any transfected PTK (Fig. 7A, lane 1), there was little binding of PECAM-1 to the SH2 domains of SHP-2. However, in the presence of Lck (lane 2) or Csk (lane 3), PECAM-1 clearly became associated with the SH2 SH2 domains. Similar results were obtained with the SHP-1 SH2 domains (Fig. 7B). By contrast, PECAM-1 did not bind to GST alone (Fig. 7C).

Tyrosines 663 and 686 of Mouse PECAM-1 Are Required for Binding to SHP-1 and SHP-2—Previous studies have shown that Tyr-663 and Tyr-686, the only two detectable sites of tyrosine phosphorylation in human PECAM-1, were needed for its interaction with SHP-2 (20). Since our data indicated that additional tyrosine phosphorylation sites potentially existed in mouse PECAM-1 (Fig. 2), we wished to evaluate whether Tyr-663 and Tyr-686 were also central for the association of the murine molecule with SHP-2, or whether other tyrosines could mediate this interaction (Fig. 8). COS-1 cells were transfected with cDNAs for wild-type, Phe-663, Phe-686, or Y663F/Y686F mouse PECAM-1, in the presence of Lck and either C459S SHP-1 (lanes 1–4) or C459S SHP-2 (lanes 5–12). The capacity of PECAM-1 to associate with the two phosphatases was then assessed by immunoblotting of SHP-1 or SHP-2 immunoprecipitates (lanes 1–8) with anti-PECAM-1 antibodies (first panel). This analysis showed that mutation of Tyr-663 (lanes 2 and 6), Tyr-686 (lanes 3 and 7), or both (lanes 4 and 8) dramatically interfered with the capacity of mouse PECAM-1 to co-immunoprecipitate with SHP-1 (lanes 2–4) and SHP-2 (lanes 6–8). While the various PECAM-1 mutants generally failed to bind SHP-1 and SHP-2, it is noteworthy that Phe-663 PECAM-1 was still able to associate weakly with SHP-2 (lane 6). In combination, these findings indicated that the association of mouse PECAM-1 with SHP-1 and SHP-2 observed in our system was fully dependent on Tyr-663 and Tyr-686, and that it could not be rescued by other sites of tyrosine phosphorylation in PECAM-1.

DISCUSSION

Herein, we have attempted to gain further insight into the PTKs mediating tyrosine phosphorylation of PECAM-1 in vivo. By transient expression in COS-1 cells, it was determined that two classes of cytoplasmic PTKs, the Src and Csk families, had the ability to induce tyrosine phosphorylation of mouse PECAM-1. Other kinases such as Syk, Ikk, and Pyk2 were incapable of causing this phosphorylation. Through a combination of site-directed mutagenesis and peptide phosphorylation studies, evidence was provided that Src and Csk family kinases were efficient at phosphorylating Tyr-686 of PECAM-1. By contrast, they seemed incapable of phosphorylating Tyr-663, another highly conserved tyrosine in the cytoplasmic domain of PECAM-1. In addition, Src-related enzymes had the ability to phosphorylate one or more other tyrosines in mouse PECAM-1. Finally, it was observed that members of the Src and Csk families were independently apt at promoting mouse PECAM-1 association with SHP-2, as well as SHP-1, and that this binding was dependent on both Tyr-663 and Tyr-686.

Others previously reported that Src was able to phosphorylate PECAM-1 during in vitro kinase reactions (19, 21). In addition to extending these findings by showing that Src was apt at phosphorylating PECAM-1 in vivo, our results demonstrated that other members of the Src family were also able to negotiate PECAM-1 tyrosine phosphorylation. These included the two isoforms of Lyn, p535yn and p565yn, which are particularly abundant in platelets, endothelial cells, and mast cells, as well as p56c, which is contained in high amounts in T-cells (reviewed in Ref. 40). As these Src-related enzymes are frequently more abundant than Src in PECAM-1-expressing cell types, they may be more likely candidates for effecting PECAM-1 phosphorylation in these cells.

Src-related enzymes are activated in response to engagement of a variety of receptors, including antigen and Fc receptors on hemopoietic cells, tyrosine kinase receptors, G-protein-coupled receptors, and integrins (reviewed in Ref. 40). Hence, it is plausible that stimulation of one or more of these receptors triggers PECAM-1 tyrosine phosphorylation via activation of Src family kinases. Consistent with this idea, it was reported that stimulation of FcεRI, which involves Lyn activation, provoked strong tyrosine phosphorylation of PECAM-1 in a basophil leukemia cell line (17). In a similar way, engagement of the T-cell antigen receptor complex, which triggers activation of the Src-related enzymes Lck and Fyn, yielded a measurable increase in PECAM-1 tyrosine phosphorylation (18).

Our studies revealed that the Csk family of PTKs was also capable of causing tyrosine phosphorylation of mouse PECAM-1 in vivo and in vitro. This result was unexpected, as Csk-related enzymes are presumed to phosphorylate a very limited set of substrates in vivo, represented mostly by the inhibitory carboxyl-terminal tyrosine of Src-related enzymes (reviewed in Ref. 40). Nonetheless, it is unlikely that the phosphorylation of PECAM-1 by Csk family kinases resulted from a loss of their substrate specificity in COS-1 cells, as they were incapable of phosphorylating several other proteins including phospholipase C-γ1, SLP-76, Cbl, HS1, CD5, and ζ (6). Moreover, the ability of Csk-like enzymes to phosphorylate targets other than Src-related enzymes was also suggested by the findings of Bergman et al. (51). These authors showed that overexpression of Csk in HeLa cells led to an increase in tyrosine protein phosphorylation at focal adhesions, which could not be explained by enhanced phosphorylation of Src family kinases. As
Csk overexpression also inhibited spreading of HeLa cells in response to integrin stimulation, it was postulated that Csk modulated cell adhesion by phosphorylating undefined components of focal adhesions. The finding that Csk family kinases have the capacity to phosphorylate an adhesion molecule such as PECAM-1 lends further support to this notion.

Our data indicated that both Src and Csk family kinases could phosphorylate Tyr-686 of PECAM-1. However, neither seemed capable of phosphorylating Tyr-663, the other presumed site of PECAM-1 tyrosine phosphorylation. At first glance, this result may seem different from that reported by Jackson and colleagues (20). This group showed that the phosphotyrosine content of human PECAM-1 in human embryonic kidney cells treated with pervanadate was partially reduced by mutation of either Tyr-686 or Tyr-663. Even though phosphorylation at these sites was not formally demonstrated, this result implied that Tyr-663 became phosphorylated under these conditions. Our inability to detect Tyr-663 phosphorylation may suggest that human embryonic kidney cells expressed PTKs other than Src and Csk family kinases that were capable of phosphorylating Tyr-663. Alternatively, it is possible that human and mouse PECAM-1 have distinct propensities to be phosphorylated at these sites. Our inability to detect Tyr-663 phosphorylation can be mediated by Src and Csk family kinases provides a valuable advance toward this goal.

As Src and Csk family kinases were inefficient at phosphorylating Tyr-663 and as Csk did not detectably phosphorylate any other tyrosine on PECAM-1, phosphate occupancy at Tyr-686 would seem sufficient to trigger the association with SHP-2. This notion is also supported by the earlier observation that a monophosphorylated Tyr-686 peptide could avidly bind the tandem SH2 domains of SHP-2 in vitro (16). Nonetheless, it should be pointed out that unphosphorylated Tyr-663 appeared to play an important role in defining the ligand specificity of PECAM-1 binding. In the L-cell aggregation system, mutation of Tyr-686 to phenylalanine, deletion of this tyrosine residue, or its phosphorylation caused a switch in adhesion from heterophilic to homophilic (6, 13, 14). Possibly, phosphate occupancy at Tyr-686 changes the interactions of the cytoplasmic domain of PECAM-1 with cellular proteins, thereby indirectly impacting on the conformation of its extracellular region or altering the capacity of PECAM-1 to cluster at the cell surface. It is also conceivable that tyrosine phosphorylation is aimed at recruiting cellular effectors and/or regulators of PECAM-1. In the case of SHP-2 and SHP-1, these may trigger the dephosphorylation of other cellular proteins or, alternatively, provide a negative feedback mechanism by dephosphorylating PECAM-1 (Ref. 18). Whereas future studies are needed to dissect these possibilities, the knowledge that PECAM-1 tyrosine phosphorylation can be mediated by Src and Csk family kinases provides a valuable advance toward this goal.

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