Residues within a lipid-associated segment of the PECAM-1 cytoplasmic domain are susceptible to inducible, sequential phosphorylation

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Immunoreceptor tyrosine-based inhibitory motif (ITIM)–containing receptors inhibit cellular responsiveness to immunoreceptor tyrosine-based activation motif (ITAM)–linked receptors. Although tyrosine phosphorylation is central to the initiation of both inhibitory ITIM and stimulatory ITAM signaling, the events that regulate receptor phosphorylation are incompletely understood. Previous studies have shown that ITAM tyrosines engage in structure-inducing interactions with the plasma membrane that must be relieved for phosphorylation to occur. Whether ITIM phosphorylation is similarly regulated and the mechanisms responsible for release from plasma membrane interactions to enable phosphorylation, however, have not been defined. PECAM-1 is a dual ITIM-containing receptor that inhibits ITAM-dependent responses in hematopoietic cells. We found that the PECAM-1 cytoplasmic domain is unstructured in an aqueous environment but adopts an α-helical conformation within a localized region on interaction with lipid vesicles that mimic the plasma membrane. The lipid-interacting segment contains the C-terminal ITIM tyrosine and a serine residue that undergo activation-dependent phosphorylation. The N-terminal ITIM is excluded from the lipid-interacting segment, and its phosphorylation is secondary to phosphorylation of the membrane-interacting C-terminal ITIM. On the basis of these findings, we propose a novel model for regulation of inhibitory signaling by ITIM-containing receptors that relies on reversible plasma membrane interactions and sequential ITIM phosphorylation. (Blood. 2011;117(22):6012-6023)

Introduction

Immunoreceptor tyrosine-based inhibitory motif (ITIM)–containing receptors oppose cellular activation by receptors that are coupled to immunoreceptor tyrosine based-activation motif (ITAM)–containing subunits.1 The inhibitory function of ITIM-containing receptors depends on Src homology (SH) 2 domain–dependent binding of tyrosine or inositol phosphatases to phosphorylated ITIMs within the cytoplasmic domain and subsequent dephosphorylation of signal transduction pathway components that are required for ITAM-dependent cellular activation. Src family kinases initiate signaling by both ITIM- and ITAM-containing receptors; however, the mechanisms that control access of ITIM or ITAM sequences to active Src family kinases are not well understood. Recent studies have revealed that the ITAMs of T-cell receptor–associated signaling subunits, which are intrinsically unstructured in an aqueous environment, exhibit increased α-helical content and decreased phosphorylation on interaction with detergent or phospholipid vesicles that mimic the plasma membrane.2-4 These findings suggested that signal transduction by ITAM-containing receptors is regulated by reversible membrane association. The extent to which this principle applies to ITIM-containing receptors has not been determined.

The ITIM-containing receptor family encompasses a large number of different Ig-domain–containing and C-type lectin receptors that are expressed on an array of cells of hematopoietic origin.1 Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1) is a member of the Ig-ITIM subfamily of ITIM-containing receptors5 that is expressed on all endothelial cells and on most hematopoietic cells, including platelets, mast cells, lymphocytes, and monocytes.6 PECAM-1 is a 130-kDa type I transmembrane glycoprotein that contains 6 extracellular Ig homology domains, a single-pass transmembrane region, and a long cytoplasmic domain that contains 2 ITIMs surrounding tyrosine (Y) residues that are found at positions 663 and 686 within the mature form of human PECAM-1, the phosphorylation of which supports recruitment and activation of Src homology 2 domain–containing protein tyrosine phosphatases, including SHP-2 and, to a lesser extent, SHP-1.7 Formation of PECAM-1/SHP-2 complexes is associated with inhibition of platelet, mast cell, and lymphocyte activation via ITAM-coupled receptors.7 As with other ITIM-containing receptors, Src family kinases are involved in PECAM-1 tyrosine phosphorylation7; however, the mechanisms that control phosphorylation of the PECAM-1 ITIMs are not known.

In the present study, we determined the structure of the ITIM-containing PECAM-1 cytoplasmic domain, in the presence and absence of detergent micelles to mimic the plasma membrane. We found that the PECAM-1 cytoplasmic domain was intrinsically unstructured in aqueous solution but that a localized region of it adopted α-helical conformation on interaction with a plasma membrane mimic. The membrane-interacting portion of the PECAM-1 cytoplasmic domain encompassed the C-terminal ITIM,
which became preferentially phosphorylated relative to the nonmembrane-interacting N-terminal ITIM, as well as a serine residue that was susceptible to inducible phosphorylation. On the basis of these results, we conclude that plasma membrane interactions control access of cytoplasmic ITIM and serine residues to the kinases that phosphorylate them.

**Methods**

**NMR spectroscopy**

Generation of the recombinant proteins that encompassed the entire (PECAM594-711) or the C-terminal half (PECAM659-711) of the PECAM-1 cytoplasmic domain is described in supplemental Methods (available on the Blood Web site; see the Supplemental Materials link at the top of the online article). A two-dimensional $^{13}$N,$^{1}$H heteronuclear single quantum coherence (HSQC) titration experiment was performed with a sample that contained $600\mu$M $^{15}$N-labeled PECAM594-711 in nuclear magnetic resonance (NMR) buffer that contained 90% H$_2$O/10% D$_2$O. For dodecylphosphocholine (DPC) dose-response studies, 600nM DPC was added after the first spectrum was collected, and buffer that contained $600\mu$M PECAM659-711 was added for each subsequent titration point. Paramagnetic broadening was measured by acquiring 2-dimensional $^{1}$H-13C HSQC spectra on PECAM594-711 with 600 mM DPC in the presence and absence of 0.5 mM MnCl$_2$. Three-dimensional (3D) heteronuclear backbone experiments and heteronuclear $^{13}$N-1H nuclear Overhauser effect (hetNOE) experiments$^{8}$ were performed as described previously$^9$ with samples that contained 1.25mM $^{13}$C/$^{15}$N-labeled PECAM594-711 or PECAM659-711 with or without 600nM DPC in the same buffer. NMR data were acquired at 25°C on a Bruker 500-MHz spectrometer equipped with a triple-resonance CryoProbe and processed with NMRPipe software.$^{10}$ Initial $^{1}$H, $^{13}$N, and $^{13}$C resonance assignments for PECAM594-711 (+DPC) were obtained from the PINE-NMR server$^{11}$ (version 1.0; http://miranda.nmrfam.wisc.edu/PINE/), with peak lists from 3D HNCO, HN(CA)CO, HNCA, HN(CO)CA, HNCB, and CCONH spectra. Tentative assignments were manually inspected, edited, and completed with XEASY$^{12}$ for 85% of the residues from 629-711. Assignments were transferred to the PECAM594-711 (+DPC) spectra and adjusted manually with the DPC titration data for resonances that had shifted. Backbone assignments were subsequently verified and completed for 90% of the residues in PECAM659-711 in the presence and absence of DPC. The combined chemical shift difference for each residue was calculated as $(0.154*\delta_N + \delta_C)^2/2$, where $\delta_N$ and $\delta_C$ are the $^{13}$N and $^{1}$H chemical shift differences (parts per million [ppm]), respectively, between the two conditions.$^{13}$

**Peptide synthesis**

Peptides encompassing residues 684-711 of PECAM-1 were synthesized with standard Fmoc protocols on an ABI 433 instrument. Phosphorylated serine and tyrosine (pS702 and pY686) residues were coupled as protected amino acids. The peptide resin was cleaved with 92.5% trifluoroacetic acid (TFA), 2.5% ethanedithiol, 2.5% Trisopropylsilane, and 2.5% H$_2$O and precipitated in cold ethyl ether. Peptides were purified to >90% by reversed-phase HPLC with a Phenomenex Proteo C12 column and a 0.1% TFA/acetonitrile gradient. The mass of the final product was verified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry.

**Structure calculations of PECAM659-711**

Side-chain assignments for PECAM594-711 in the presence of DPC were completed manually from 3D HBHACONH, HCHC-TOCSY, $^{13}$C-edited NOEY-HSQC spectra, and $^{13}$C(aromatic)-edited NOEY-HSQC spectra. Backbone $\Phi$ and $\Psi$ dihedral angle constraints were generated from secondary shifts of the $^{1}$H$_N$, $^{1}$H$_C$, $^{13}$C$_B$, $^{13}$C$_N$, and $^{15}$N nuclear shifts with TALOS+.$^{14}$ Distance constraints were obtained from 3D $^{15}$N-edited NOEY-HSQC and $^{13}$C-edited NOEY-HSQC spectra ($\tau_{99} = 80$ ms). Structure calculations were performed with the torsion angle dynamics
Table 1. Statistics for the 20 PECAM-1677-711 conformers

<table>
<thead>
<tr>
<th>Experimental constraints, n</th>
<th>Residues 682-701</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Backbone ( C^\alpha, C^\beta, N )</td>
</tr>
<tr>
<td></td>
<td>Heavy atoms</td>
</tr>
</tbody>
</table>

### Deviations from idealized covalent geometry

| Bond lengths, RMSD, Å    | 0.013 |
| Torsion angle violations, RMSD, degrees | 1.2 |

### Constraint violations

|NOE distance, No. > 0.5 Å* | 0.0 ± 0 |
|NOE distance, RMSD, Å       | 0.028 ± 0.004 |
|Torsion-angle violations, No. > 5 | 0.0 ± 0 |
|Torsion-angle violations, RMSD, degrees | 0.404 ± 0.117 |

### WHAT_CHECK quality indicators

| Z-score | −2.42 ± 0.46 |
| RMS Z-score | 0.67 ± 0.04 |
| Bond angles | 0.66 ± 0.03 |
| Bumps          | 0 ± 0 |
| Lennard-Jones energy, kJ mol⁻¹ | −339 ± 42 |

### Ramachandran statistics, % of all residues

| Most favored | 83.12 ± 4.61 |
| Additionally allowed | 13.44 ± 4.17 |
| Generously allowed   | 2.74 ± 2.87 |
| Disallowed            | 0.68 ± 1.80 |

RMSD indicates root mean square deviation.

†The largest NOE violation in the ensemble of structures was 0.30 Å.

‡The largest torsion-angle violation in the ensemble of structures was 2.5°.

*Nonbonded energy was calculated in XPLOR-NIH.

Circular dichroism spectroscopy

Small unilamellar vesicles were prepared with 1,2-dimyristoyl-sn-glycero-3-[phospho-r-i-serine] (DMPS) and/or 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC, Avanti Polar Lipids), which was dried to a film and lyophilized overnight to remove solvent, resuspended in 20mM NaHPO₄ (pH 7), and sonicated at high intensity in a cup horn sonicator (Qsonica). Circular dichroism (CD) measurements (5 scans/measurement) were made with a Jasco J-710 spectropolarimeter at room temperature over a 190- to 250-nm range with a bandwidth of 1 nm and a scanning rate of 50 nm/min. For the data shown in Figure 8A, each sample contained 100μM peptide and a 6nM final concentration of small unilamellar vesicles composed of 100% DMPC, 75% DMPC/25% DMPS, 50% DMPC/50% DMPS, or 100% DMPS. For the data shown in Figures 8B-C, each sample contained 100μM peptide and small unilamellar vesicles composed of 100% DMPS at a final concentration of 0, 1.5, 3, or 6nM. Spectra were corrected for buffer and vesicle effects by subtracting scans obtained with the appropriate concentration of vesicle only. Linear regression analysis was performed with GraphPad Prism 5 software.

In vitro tyrosine phosphorylation of the PECAM-1 cytoplasmic domain and purification of phosphorylated and unphosphorylated species

PECAM594-711Y636,701F was incubated with Src (BIOMOL), Fyn (BIOMOL), or Fer (Cell Signaling) in buffer that contained 0.25M ATP/1mM EGTA/10mM MgCl₂/0.1% Brij-50/0.25M Na₂VO₃ overnight at 30°C. We were unable to perform in vitro kinase assays in the presence of DPC because neither Src nor Fer family kinases were active in the presence of DPC (data not shown). Phosphorylated and unphosphorylated species were separated on a Mono Q column in 10mM Bis-Tris Propane (pH 6.2) that contained 1mM DTT with a gradient of 20-250mM NaCl over 50 minutes at a flow rate of 0.5 mL/min on an Akta FPLC system (GE Healthcare).

Mass spectrometric analysis of PECAM-1594-711 tryptic digests

Peak protein-containing fractions obtained from Mono Q column chromatography were diluted in 0.1% TFA (1:1), desalted, eluted from P10 C18 ZipTips (Millipore) into 80% acetonitrile/0.1% TFA, and dried in a SpeedVac (Thermo Scientific). Samples were resuspended in 25mM NH₄CO₃ (pH 8) and digested with trypsin (62.5 ng) for 1 hour at 37°C. Tryptic digests were eluted from P10 C18 ZipTips with 80% acetonitrile/0.1% TFA, spotted with DHB (2.5-dihydroxybenzoic acid) or CHCA (α-cyano-4-hydroxycinnamic acid) matrix, and dried under vacuum. MALDI-TOF data were acquired with an ABI Voyager-DE Pro mass spectrometer in positive ion, linear mode. Data were analyzed with Data Explorer (Applied Biosystems) and calibrated on known PECAM-1 fragments present in every sample. Observed masses were compared with expected masses generated from in silico digestion of PECAM594-711Y636,701F with Protein Prospector (University of California at San Francisco Mass Spectrometry Facility), which required 5 missed cleavages to ensure the presence of both ITIMs in a single peptide.

Identification of phosphorylated serine residues with mutant forms of recombinant PECAM-1

Human embryonic kidney (HEK) 293T cells were transiently transfected with PECAM-1–encoding plasmids with Superfect (Qiagen), washed with serum- and phosphate-free DMEM, and exposed to32P-orthophosphate (0.2 mCi/mL) with or without 1μM okadaic acid (OA) for 4 hours. Cells were lysed in buffer that contained 20mM Tris/150mM NaCl/1% Triton X-100/1mM EDTA/10mM NaF/20mM β-glycerophosphate, serine phosphatase inhibitors (Calbiochem), and complete protease inhibitor cocktail tablets (Roche). PECAM-1 were immunoprecipitated from precleared cell lysates with PECAM-I, and immunoprecipitates were resolved by SDS-PAGE. Parallel gels were fixed, dried, and used to expose X-OMAT film with a Lightning Plus LK screen overnight at −80°C or subjected to Western blot analysis with rabbit polyclonal anti-human PECAM-1 antibodies to quantify PECAM-1 levels.


Affinity-purified rabbit antibodies specific for human PECAM-1 phosphorylated on tyrosine 686 (anti-pY686), serine 702 (anti-pS702), or serine 707 (anti-pS707) were commissioned from BioSource International with the following used as immunogens: acetyl-DTETVpY686SEVRKA(C)-amide, acetyl-(C)VESRYPs702RTEGS-amide, and acetyl-SRTEGps707LDGT(C)-amide phosphopeptides, respectively. To verify specificity, HEK 293T cells transfected with plasmids encoding wild-type (WT) or mutant forms of PECAM-1 were cultured in the presence and absence of OA (to characterize phosphoserine-specific antibodies) or pervanadate (to characterize pY686-specific antibodies), lysed, and subjected to Western blot analysis with rabbit polyclonal anti-human PECAM-1 antibodies to quantify PECAM-1 levels.
(7μM) in the presence (activated) or absence (aggregated) of 2mM RGD and 5mM EDTA. Platelets were lysed (1:1) in 100mM Tris/300mM NaCl/2% Triton X-100/0.2% SDS/2% sodium deoxycholate/2mM EDTA/20mM NaF/40mM /H9252-glycerophosphate with tyrosine and serine phosphatase inhibitors (Calbiochem) and protease inhibitors. Lysates were subjected to PECAM-1 immunoprecipitation and Western blot analysis as described above. Research involving human subjects was approved by the BloodCenter of Wisconsin Institutional Review Board, and all human participants gave written informed consent in accordance with the Declaration of Helsinki.

Results

Structure of the PECAM-1 cytoplasmic domain in the presence and absence of DPC micelles

We used an NMR approach to investigate the structural properties of the PECAM-1 cytoplasmic domain. The 2-dimensional 1H-15N HSQC spectrum of PECAM-1594-711 in aqueous solution displayed ~ 89 backbone amide resonances of the 112 expected, but with a very narrow spread of resonance frequencies in both the 15N and 1H dimensions (Figure 1A). This pattern is characteristic of either unfolded or globally dynamic proteins.21 On the basis of these findings, we conclude that the PECAM-1 cytoplasmic domain is intrinsically unstructured in aqueous solution.

Previous studies have reported that a segment of the ITAM-containing T-cell receptor ζ-chain cytoplasmic domain is also unstructured in an aqueous environment but adopts an α-helical structure on interaction with negatively charged phospholipids.2-4,22 To determine whether the ITIM-containing PECAM-1 cytoplasmic domain behaves similarly, NMR spectroscopy was used to detect interactions between PECAM594-711 and DPC micelles, which are used in NMR studies as a membrane mimetic model system.23,24 As shown in Figure 1B, addition of DPC to PECAM594-711 in 100-mM increments up to 600mM (the solubility limit under the experimental conditions) consistently shifted the HSQC signals for at least 16 residues toward lower 15N chemical shift values. Upfield 15N chemical shift changes previously have been correlated with a micelle-induced conformational change from random coil to α-helix on interaction.25,26 To identify the residues involved in DPC binding, we assigned backbone 15N, 1HN, and 13C resonances, as well as 13Cα and 13Cβ resonances, for approximately 80% of the residues from N 629-T711 of PECAM 594-711 in the absence and presence of 600mM DPC. Backbone 1H/15N chemical shift perturbations clustered in the segment from T682 to S702, which indicates that this is the region within PECAM594-711 that interacts with DPC micelles (data not shown). To confirm and complete the resonance assignments, spectra were obtained with the C-terminal portion of the cytoplasmic domain (PECAM659-711) wherein the amount of resonance overlap decreased significantly (Figure 1C). Chemical shift values for PECAM659-711 were identical to those observed for the full-length form (PECAM594-711), which indicates that the structure and DPC interactions of the PECAM-1 cytoplasmic
domain were unaffected by truncation of the unstructured N-terminal region (supplemental Figure 2). Backbone resonances of PECAM594-711 in the presence of DPC were assigned for all residues except S699, H634, K675, K680, and K691. In the absence of DPC, the additional residues E688, V689, and R700 could not be assigned. The chemical shifts were very similar to those of the full-length PECAM-1 cytoplasmic domain (PECAM594-711), both with and without DPC. As with PECAM594-711, analysis of PECAM594-711 1H/15N chemical shifts in the presence versus absence of DPC revealed that a segment from T682 to S702 was significantly perturbed on binding to DPC micelles, which indicates that the structure and DPC interaction were unaffected by truncation of the unstructured N-terminal region (Figure 1D).

To assess changes in picosecond-nanosecond dynamics of PECAM594-711, we measured heteronuclear 1H-15N NOE values in the presence and absence of DPC. As expected for a dynamically disordered protein, NOE values were exclusively negative for PECAM594-711 in the absence of DPC. In the presence of DPC micelles, significant increases in the NOE values were observed for several residues between T682 and R703 of PECAM594-711, which denotes an increase in local order (Figure 1F). The 13Cα and 13Cβ chemical shifts are highly sensitive to the presence of secondary structure, and the 3-residue averaged secondary shifts (ΔCα-ΔCβ) computed for PECAM594-711 in the presence of DPC were significantly positive (> 0.5 ppm) for residues E688-R700 (Figure 1F). Consistent with our secondary shift analysis, TALOS +14 analysis of 1Hα, 13Cα, 13Cβ, 13Cγ, and 15N shifts predicted that residues D687-Y701 of PECAM594-711 would form an α-helix in the presence of DPC (data not shown). No other regular secondary structure elements were predicted, and no secondary structures were predicted in the absence of DPC.

To confirm the predicted membrane-associated conformation, we solved the NMR structure of PECAM594-711 in the presence of DPC. The first 18 residues were dynamically disordered and were omitted in the final structure refinement (Table 1). As predicted by our TALOS + results, the only regular structure was a helical region that spanned residues T682-Y701 (Figure 2A). Chemical shift and NOE data were consistent with a slight bend in the helix at Y693-P694. The helix is amphiphilic in nature, with hydrophobic side chains on the opposite face (Figure 2B). The helix is not strictly amphiphilic, however, because the basic residue, R700, is found on the hydrophobic face. It is therefore likely that binding to the plasma membrane is mediated by both hydrophobic interactions with the phospholipid tails and electrostatic interactions between the basic amino acid side chains and the negatively charged phosphatidylserine and phosphatidylinositol head groups. To more directly demonstrate contact between this region of the PECAM-1 cytoplasmic domain and plasma membrane mimetics, we evaluated selective paramagnetic broadening of aromatic side-chain NMR signals. The data shown in Figure 2C reflect the lower accessibility for side chains of tyrosine residues Y686 and Y701, which are in the membrane-associated helix, relative to that of the histidine residue (H623) that is away from the helix and exposed to solvent and the paramagnetic effect of Mn2+. These data provide independent evidence for membrane association of the PECAM-1 cytoplasmic domain.

Sequential phosphorylation of PECAM-1 ITIMs

Phosphorylation of the ITIM tyrosine residues at positions 663 and 686 enables PECAM-1 to mediate inhibitory functions by recruit-
in Table 2) or from either peak 1 (Figure 4B; “Peak 1” in Table 2) or peak 2 (Figure 4C; “Peak 2” in Table 2) species of phosphorylated PECAM594-711Y636,701F. Tryptic fragments that contain both Y663 and Y686 (peptides F and G) had masses consistent with absence of phosphate groups when derived from unphosphorylated PECAM594-711Y636,701F (Figure 4A; “UN” in Table 2), addition of 1 phosphate group when derived from peak 1 of phosphorylated PECAM594-711Y636,701F (Figure 4B, “Peak 1” in Table 2), and addition of 2 phosphate groups when derived from peak 2 of phosphorylated PECAM594-711Y636,701F (Figure 4C; “Peak 2” in Table 2). These results demonstrate that peak 1 corresponds to a monophosphorylated species, whereas peak 2 corresponds to a diphosphorylated species of PECAM594-711Y636,701F. The tryptic fragment that contained only Y686 (peptide B) had a mass consistent with addition of a phosphate group when derived from either the monophosphorylated (Figure 4B; “Peak 1” in Table 2) or the diphosphorylated (Figure 4C; “Peak 2” in Table 2) species of PECAM594-711Y636,701F, which suggests that Y686 can be phosphorylated independently of phosphorylation of Y663. In contrast, tryptic fragments that contained only Y663 (peptides C and D) had masses consistent with the addition of a phosphate group when they were derived from the diphosphorylated species of PECAM594-711Y636,701F (Figure 4C; “Peak 2” in Table 2) but not when derived from the monophosphorylated species of PECAM594-711Y636,701F (Figure 4B; “Peak 1” in Table 2). These results implicate a sequential mechanism for PECAM-1 ITIM phosphorylation, in which phosphorylation of Y663 is dependent on prior phosphorylation of Y686.

### Identification of PECAM-1 serine phosphorylation sites

PECAM-1 is both constitutively and inducibly phosphorylated on serine residues; however, the sites of serine phosphorylation within the PECAM-1 cytoplasmic domain are not known. To determine which of the 12 serine residues within the cytoplasmic domain of human PECAM-1 are capable of being phosphorylated, we assessed the extent of 32P incorporation into WT or mutant forms of PECAM-1 that contained either all 12 serine residues (12S), alanine substitutions for all 12 serine residues (12S A), PECAM-1 constructs encoded phenylalanine substitutions at positions 663 and 686 (Y663,686F) to minimize

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**Table 2. MALDI-TOF analysis of peptides derived from a limited tryptic digest of Fer-phosphorylated PECAM594-711Y636,701F**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Potential phospho-serines</th>
<th>Expected monoisotopic masses</th>
<th>Observed monoisotopic masses</th>
<th>Phosphorylated</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Peak 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Peak 2</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>None</td>
<td>1297.6</td>
<td>N/A</td>
<td>1296.1</td>
<td>NAMKPINDEK</td>
</tr>
<tr>
<td>B</td>
<td>660(1)-690(1) Y663</td>
<td>1326.7</td>
<td>1406.6</td>
<td>1406.5</td>
<td>KDTETYYEQV</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1407.0</td>
<td>SEVR(K)</td>
</tr>
<tr>
<td>C</td>
<td>Y663</td>
<td>4023.0</td>
<td>4022.5</td>
<td>4013.1</td>
<td>N. KEPLNSDVQYQEQTEQVASSAESKDLGK</td>
</tr>
<tr>
<td>D</td>
<td>664-680</td>
<td>4151.1</td>
<td>4231.0</td>
<td>4231.0</td>
<td>N. KEPLNSDVQYQEQTEQVASSAESKDLGK</td>
</tr>
<tr>
<td>E</td>
<td>605-643 (F636)</td>
<td>4408.1</td>
<td>4408.6</td>
<td>4408.6</td>
<td>AKOMPVSMPRPAPLNSNKEKMDSPNPMEASHFGHNDVR</td>
</tr>
<tr>
<td>F</td>
<td>Y663, Y686</td>
<td>5330.6</td>
<td>5490.5</td>
<td>5490.9</td>
<td>N. KEPLNSDVQYQEQTEQVASSAESKDLGKDTETYYEQV</td>
</tr>
<tr>
<td>G</td>
<td>Y663</td>
<td>5458.7</td>
<td>5538.6</td>
<td>5538.5</td>
<td>N. KEPLNSDVQYQEQTEQVASSAESKDLGKDTETYYEQV</td>
</tr>
</tbody>
</table>

UN indicates unphosphorylated; and N/A, not applicable.

Bold type denotes peptides containing phosphate groups, with a single asterisk (*) representing addition of a single phosphate moiety and 2 asterisks (**) representing addition of 2 phosphate moieties.
potential incorporation of $^{32}$P into tyrosine residues, and transfectants were cultured with the serine/threonine phosphatase inhibitor, OA, to maximize incorporation of $^{32}$P into serine residues. As shown in Figure 5A, both untreated and OA-treated cells incorporated high levels of $^{32}$P into WT and Y$_{663,686}$F(12S), but not Y$_{663,686}$F(12S→A), forms of PECAM-1. Among the 12 potential sites for serine phosphorylation, PECAM-1 became highly phosphorylated on S$_{702}$ and S$_{707}$, with weak phosphorylation on S$_{673}$.

To examine the conditions under which PECAM-1 becomes phosphorylated at its major site and tyrosine phosphorylation sites, antibodies specific for phosphoserine residues at positions 702 and 707 (anti-pS$_{702}$ and anti-pS$_{707}$, respectively), as well as an antibody specific for a phosphotyrosine at position 686 (anti-pY$_{686}$), within PECAM-1 were developed. To verify the specificity of the antibodies, WT and mutant forms of human PECAM-1 were immunoprecipitated from lysates of transiently transfected HEK 293T cells with the human PECAM-1-specific monoclonal antibody PECAM-1.3, separated by SDS-PAGE, and analyzed by Western blot analysis with anti-pS$_{702}$, anti-pS$_{707}$, and anti-pY$_{686}$. As shown in Figure 5B, recognition of PECAM-1 by anti-pS$_{702}$ and anti-pS$_{707}$ depended on the presence of a serine residue at position 702 or 707, respectively, and both reactivities were enhanced by (but did not require) treatment of HEK 293T cell transfectants with OA. Similarly, recognition of PECAM-1 by anti-pY$_{686}$ required the presence of a tyrosine residue at position 686 and coincided with PECAM-1 tyrosine phosphorylation, as measured by Western blot analysis with the phosphotyrosine-specific antibody PY20 (Figure 5C). The finding that PY20 recognized the Y$_{663}$F form of PECAM-1 better than the Y$_{686}$F form is consistent with a sequential mechanism for PECAM-1 ITIM phosphorylation, in which Y$_{686}$ is phosphorylated more readily than Y$_{663}$, which is phosphorylated only poorly in the absence of Y$_{686}$. Treatment of PECAM-1 immunoprecipitates with alkaline phosphatase to dephosphorylate both serine and tyrosine residues abrogated recognition of PECAM-1 by anti-pS$_{702}$- and anti-pS$_{707}$-specific IgGs (data not shown). Together, these results demonstrate that antibodies raised against pS$_{702}$, pS$_{707}$, and pY$_{686}$-containing PECAM-1 peptides are both sequence specific and phosphorylation dependent.

Characterization of constitutive and inducible serine and tyrosine phosphorylation sites in PECAM-1

Our chemical shift perturbation analysis of the PECAM-1 cytoplasmic domain revealed that S$_{702}$ is contained within a region of the PECAM-1 cytoplasmic domain that interacts with lipid, whereas S$_{707}$ is not. Furthermore, localization of Y$_{686}$ within the segment of the PECAM-1 cytoplasmic domain that interacts with lipid correlates with its preferential phosphorylation relative to Y$_{663}$, which is located within a region of the PECAM-1 cytoplasmic domain that does not interact with lipid. We therefore sought to determine
Our studies revealed that a short segment of the PECAM-1 cytoplasmic domain, encompassing residues 682-702, underwent interactions with plasma membrane-mimetic detergent micelles that promoted adoption of localized α-helical structure (shown schematically in Figure 7A). The membrane-interacting segment contains both a serine (S702) and a tyrosine (Y686) residue that were inducibly and sequentially phosphorylated. Collectively, these findings suggest a model in which ordered phosphorylation and plasma membrane dissociation events coordinate sequential phosphorylation of the PECAM-1 cytoplasmic domain (Figure 7B). Thus, in resting cells, residues near the C-terminus of the PECAM-1 cytoplasmic domain engage in structure-inducing membrane interactions that interfere with its phosphorylation (Figure 7Bi). On cellular activation, phosphorylation of S702 alters the overall affinity of the lipid-associating region for the plasma membrane by reducing the local net positive charge at the C-terminal end of the helix (Figure 7Bii). Subsequent dissociation of the PECAM-1 cytoplasmic domain from the plasma membrane renders Y686 accessible for phosphorylation, which then enables subsequent phosphorylation at Y663 (Figure 7Biii).

To test this model, we used CD spectroscopy to evaluate the conformation of a peptide that contained the membrane-interacting region of the PECAM-1 cytoplasmic domain (PECAM-1684-711) in the presence of lipid vesicles that mimicked the composition of the inner face of the plasma membrane and to determine the effect of phosphorylation of S702 and Y686 on conformation-inducing plasma membrane interactions (Figure 8). As shown in Figure 8A, vesicles that contained increasing amounts of the anionic phospholipid DMPS, but not those that contained 100% of the zwitterionic phospholipid DMPC, induced changes in PECAM-1684-711 that were consistent with loss of random coil and gain of α-helical content. This effect was most evident in the ~210- to 230-nm range of the PECAM-1684-711 CD spectrum, which exhibited a shallower minimum at 222 nm and acquisition of a new maximum at ~215 nm in the presence of DMPS-containing vesicles. Importantly, the effects of DMPS-containing vesicles on the shape of the PECAM-1684-711 CD spectrum (Figure 8B) and the minimum observed at 222 nm (Figure 8C) were diminished by the presence of pS702 and abolished by the presence of both pS702 and pY686 in the peptide sequence. These results verify the conclusions that residues within the C-terminal segment of the PECAM-1 cytoplasmic domain adopt α-helical conformation in the presence of negatively charged phospholipids and that phosphorylation of serine and tyrosine residues within this segment induces loss of secondary structure, and they support our proposed model for ordered phosphorylation and plasma membrane dissociation events that coordinate sequential phosphorylation of the PECAM-1 cytoplasmic domain.
Discussion

A central dogma of structural biology is that protein function requires adoption of a particular 3D structure; however, it has recently become apparent that certain classes of functional proteins contain extensive regions of disorder. The family of intrinsically disordered proteins includes signal transducing molecules, which are susceptible to posttranslational modification. The presence of lipids or lipid-like moieties that mimic the inner face of the plasma membrane is one environment but adopt an α-helical conformation in the disordered state and are capable, in certain environments, of undergoing a disorder-to-order transition that interferes with posttranslational modification. We found that the PECAM-1 cytoplasmic domain is intrinsically unstructured in an aqueous environment but that a short segment of it encompasses the C-terminal ITIM engages in membrane interactions similarly regulate signaling by ITIM-containing receptors. We found that the PECAM-1 cytoplasmic domain is intrinsically unstructured in an aqueous environment but that a short segment of it that encompasses the C-terminal ITIM engages in interactions with plasma membrane mimetics that induce adoption of an α-helical conformation (Figures 1-2 and 8; shown schematically in Figure 7). This finding suggests that phosphorylation of the PECAM-1 C-terminal ITIM, like that of TCR-associated ITAM-containing subunits, is constitutively suppressed by plasma membrane interactions in resting cells. Whether adoption of secondary structure is in fact necessary for plasma membrane interactions to control susceptibility of the PECAM-1 C-terminal ITIM to phosphorylation or is simply a function of the composition of the detergent micelles and lipid vesicles that were used for the present studies, as has been suggested for immune receptor ITAMs, remains to be determined.

HSQC spectral analysis in the presence versus absence of DPC micelles revealed that the C-terminal ITIM of PECAM-1 engages in structure-inducing plasma membrane interactions, whereas the N-terminal ITIM of PECAM-1 does not (Figure 1). The reason for the difference in lipid-binding behavior of the 2 PECAM-1 ITIMs is not apparent; however, some interesting possible explanations arise from a comparison of the physicochemical properties of the PECAM-1 ITIMs with those of lipid-interacting and non–lipid-interacting immune receptor ITAMs. It has been proposed that the
lipid-binding strength of different ITAMs correlates with protein net charge and with the presence of clustered basic amino acid residues in the region encompassing the ITAM. The present findings support generalization of this model to encompass ITIMs as well. Thus, comparison of the PECAM-1 ITIMs with immune receptor ITAMs (Table 3) reveals that the lipid-interacting portion of the PECAM-1 cytoplasmic domain, which encompasses the C-terminal ITIM, is similar to lipid-interacting ITAMs in that it has an isoelectric point of approximately 6 or higher and possesses 2 adjacent basic residues in close proximity to the ITIM tyrosine residue, Y686. In contrast, the non–lipid-interacting region of the PECAM-1 cytoplasmic domain, which encompasses the N-terminal ITIM, is similar to non–lipid-interacting ITAMs in that it has an isoelectric point of less than 5 and does not contain a cluster of basic residues near the ITIM tyrosine residue, Y663. The finding that Y663, which like Y686, is susceptible to inducible phosphorylation, does not engage in plasma membrane interactions suggests that its phosphorylation is not directly regulated by plasma membrane interactions. Instead, Y663 was only phosphorylated when Y686 had been phosphorylated first (Table 2; Figures 3-4). Thus, Y686 phosphorylation, which is itself regulated by plasma membrane interactions, may be the event that regulates phosphorylation of Y663 and, ultimately, initiation of PECAM-1–mediated inhibitory signaling. The mechanisms involved in sequential phosphorylation of the PECAM-1 ITIMs are not known and are the subject of continuing investigation in our laboratory.

It has long been known that PECAM-1 is susceptible to both constitutive and inducible phosphorylation on serine residues. However, neither the sites within PECAM-1 that serve as targets for serine phosphorylation nor the functional relevance of this posttranslational modification have been established. The present study is the first to definitively identify S702 and S707 as the major sites of serine phosphorylation within PECAM-1 and to demonstrate that PECAM-1 is constitutively phosphorylated on S707, whereas phosphorylation of S702 is inducible (Figures 5-6). Previous studies have reported that PECAM-1 serine phosphorylation affects its ability to associate with the cytoskeleton in platelets and endothelial cells, albeit positively in the former and negatively in the latter; however, to the extent that they have been studied, PECAM-1 cytoskeletal interactions do not appear to require either S702 or S707. We propose a new role for PECAM-1 serine phosphorylation in regulating its tyrosine phosphorylation. This proposal is predicated on the fact that the inner face of the plasma membrane is enriched in negatively charged phospholipids, including phosphatidylserine and phosphatidylinositol. Electrostatic interactions that involve phosphatidylserine and phosphatidylinositol contribute significantly to membrane binding of several proteins that interact with the inner face of the plasma membrane, including dynamin I, the myristoylated alanine-rich C substrate (MARCKS), and numerous members of the Ras, Rho, Arf, and Rab subfamilies of small guanosine triphosphatases (GTPases). Such electrostatic interactions rely heavily on association of polybasic clusters of positively charged lysine (K) and arginine (R) residues with negatively charged phospholipids. Interestingly, phosphorylation of serine residues in the vicinity of the polybasic regions has been found to disrupt the electrostatic interactions on which membrane interactions depend and to induce dissociation of these molecules from the membrane, with subsequent redistribution to the cytosol. We propose that serine phosphorylation plays a similar role in inducing plasma membrane dissociation of the PECAM-1 cytoplasmic domain so as to enable its tyrosine phosphorylation. Previous studies have demonstrated that inducible PECAM-1 serine phosphorylation precedes
its tyrosine phosphorylation in platelets,36 and in the present study, we have identified S702 as the site at which PECAM-1 is inducibly serine phosphorylated. Results of the structural studies reported herein demonstrate that S702 is located at the extreme C-terminal end of the membrane-interacting portion of the PECAM-1 cytoplasmic domain and that phosphorylation of S702 alters the ability of this segment to adopt secondary structure in the presence of plasma membrane mimetics (Figures 1-2 and 8). Thus, we propose that S702 phosphorylation is predicted to reduce the local net positive charge of the membrane-interacting portion of the PECAM-1 cytoplasmic domain and therefore its overall affinity for the plasma membrane. Subsequent dissociation of the PECAM-1 cytoplasmic domain from the plasma membrane would render the C-terminal ITIM accessible for phosphorylation, which would then enable subsequent phosphorylation of the N-terminal ITIM (shown schematically in Figure 7). The extent to which phosphorylation of PECAM-1 on S702 affects phosphorylation of the PECAM-1 ITIMs and ITIM-dependent inhibitory function is an important area of future investigation.

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Residues within a lipid-associated segment of the PECAM-1 cytoplasmic domain are susceptible to inducible, sequential phosphorylation

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