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Immunoreceptor Tyrosine-Based Inhibitory Motif (ITIM)-Mediated Inhibitory Signaling Is Regulated by Sequential Phosphorylation Mediated by Distinct Nonreceptor Tyrosine Kinases: A Case Study Involving PECAM-1

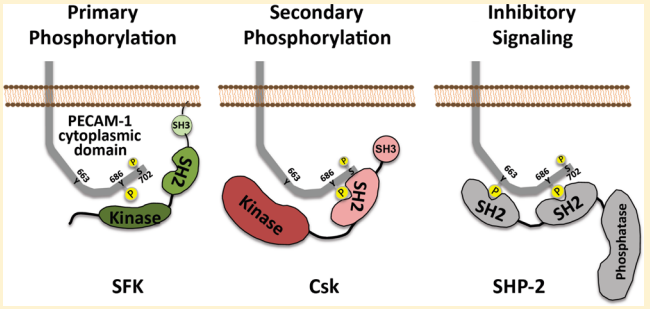
Benjamin E. Tourdot,[†,‡] Michelle K. Brenner,[†] Kathleen C. Keough,[†] Trudy Holyst,[†] Peter J. Newman,[†,‡,§] and Debra K. Newman*[,†,‡,⊥]

[†]Blood Research Institute, BloodCenter of Wisconsin, Milwaukee, Wisconsin 53226, United States

[‡]Department of Pharmacology and Toxicology, [§]Department of Cell Biology, Neurobiology, and Anatomy, and [⊥]Department of Microbiology and Molecular Genetics, Medical College of Wisconsin, Milwaukee, Wisconsin 53226, United States

Supporting Information

ABSTRACT: The activation state of many blood and vascular cells is tightly controlled by a delicate balance between receptors that contain immunoreceptor tyrosine-based activation motifs (ITAMs) and those that contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs). Precisely how the timing of cellular activation by ITAM-coupled receptors is regulated by ITIM-containing receptors is, however, poorly understood. Using platelet endothelial cell adhesion molecule 1 (PECAM-1) as a prototypical ITIM-bearing receptor, we demonstrate that initiation of inhibitory signaling occurs via a novel, sequential process in which Src family kinases phosphorylate the C-terminal ITIM, thereby enabling phosphorylation of the N-terminal ITIM of PECAM-1 by other Src homology 2 domain-containing nonreceptor tyrosine kinases (NRTKs). NRTKs capable of mediating the second phosphorylation event include C-terminal Src kinase (Csk) and Bruton's tyrosine kinase (Btk). Btk and Csk function downstream of phosphatidylinositol 3-kinase (PI3K) activation during ITAM-dependent platelet activation. In ITAM-activated platelets that were treated with a PI3K inhibitor, PECAM-1 was phosphorylated but did not bind the tandem SH2 domain-containing tyrosine phosphatase SHP-2, indicating that it was not phosphorylated on its N-terminal ITIM. Csk bound to and phosphorylated PECAM-1 more efficiently than did Btk and required its SH2 domain to perform these functions. Additionally, the phosphorylation of the N-terminal ITIM of Siglec-9 by Csk is enhanced by the prior phosphorylation of its C-terminal ITIM, providing evidence that the ITIMs of other dual ITIM-containing receptors are also sequentially phosphorylated. On the basis of these findings, we propose that sequential ITIM phosphorylation provides a general mechanism for precise temporal control over the recruitment and activation of tandem SH2 domain-containing tyrosine phosphatases that dampen ITAM-dependent signals.



Multisubunit immunoreceptor tyrosine-based activation motif (ITAM)-coupled receptors are commonly used to activate hematopoietic cells. Such receptors include the T cell receptor (TCR), the B cell receptor (BCR), receptors for the Fc portions of IgE and IgG on mast cells and natural killer cells, respectively, killer cell immunoglobulin-like and lectin-like receptors, and the glycoprotein VI (GPVI) collagen receptor on platelets.[1,2] The ligand binding components of each receptor are coupled to ITAM-containing signaling subunits, including the TCR ζ chain and CD3 subunits, BCR Ig α and β subunits, the FcRγ chain, and DAP-12. In each case, members of three families of nonreceptor tyrosine kinases (NRTK), including Src family kinases (SFK), spleen tyrosine kinases, and Tec family kinases, execute an ordered series of tyrosine phosphorylation events that ultimately result in cell activation.

Two types of inhibitory processes regulate cellular activation by ITAM-coupled receptors. The first of these is mediated by members of the C-terminal Src kinase (Csk) family, which phosphorylate the tyrosine residue at the C-terminus of SFKs, thereby enabling an intramolecular association that suppresses SFK activity.[1,3] The ability of Csk to inhibit ITAM-dependent signal transduction requires binding of its SH2 domain to phosphotyrosine residues in Csk binding proteins (Cbp), which are themselves substrates for phosphorylation by SFKs. SH2 domain-dependent binding of Csk to Cbp has two outcomes that are important for suppression of SFK activity: first, it

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enables recruitment of Csk to sites of SFK activity, and second, it results in a dramatic increase in kinase activity toward its substrates.[3,4]

The second mechanism involved in regulation of cellular activation by ITAM-coupled receptors is mediated by immunoreceptor tyrosine-based inhibitory motif (ITIM)-containing inhibitory receptors. These receptors constitute a large family of molecules that includes immunoglobulin (Ig) superfamily members, sialic acid binding lectin-like molecules (Siglecs), and C-type lectin receptors.[1,5,6] Inhibition of ITAM-mediated signaling by ITIM-containing receptors requires phosphorylation of ITIM tyrosine residues, which enables recruitment and activation of SH2 domain-containing inositol and tyrosine phosphatases that, upon binding, interfere with signaling by ITAM-coupled receptors. Most ITIM-containing inhibitory receptors contain two ITIMs that, upon phosphorylation, bind the tandem SH2 domain-containing tyrosine phosphatases, SHP-1 and SHP-2. SFK activity is required for ITIM tyrosine phosphorylation; however, the precise phosphorylation events for which SFKs are required are not known.[7−9] Furthermore, whether additional enzymes function to control the initiation of inhibitory signaling relative to activating signaling has not been explored.

Platelet endothelial cell adhesion molecule-1 (PECAM-1) is a dual ITIM-containing receptor of the Ig superfamily that is capable of inhibiting ITAM-induced activation of B cells, T cells, and mast cells, but whose best-characterized function is inhibition of GPVI/FcRγ chain-mediated platelet activation.[10] The inhibitory properties of PECAM-1 require phosphorylation of both of its ITIMs, which surround tyrosine residues at positions 663 (VQY$_{663}$TEV) and 686 (TVY$_{686}$SEV) of human PECAM-1, and recruitment of SHP-2 or, less efficiently, SHP-1. The ITIMs of PECAM-1 are not phosphorylated in resting cells but are phosphorylated upon cellular activation in a manner that depends on SFK activity. In platelets, PECAM-1 inhibitory function depends on the SFK Lyn;[11] however, other NRTKs have been shown to mediate PECAM-1 ITIM phosphorylation, including feline sarcoma-related (Fer)/Fujinami poultry sarcoma (Fps) family kinases and Csk.[12−14] The precise phosphorylation events for which these enzymes are responsible are not known. We recently reported that the PECAM-1 ITIMs are phosphorylated sequentially, with phosphorylation of Y$_{663}$ depending on prior phosphorylation of Y$_{686}$.[15] This finding suggests that phosphorylation of dual ITIM-containing receptors proceeds via a two-step process in which the second step requires SH2 domain-dependent interactions of a NRTK with an initially phosphorylated tyrosine residue. In the present study, we explore the roles of different SH2 domain-containing NRTKs known to be involved in ITIM/ITAM signaling in sequential phosphorylation of the PECAM-1 ITIMs. We demonstrate that SFKs efficiently mediate phosphorylation of the C-terminal ITIM but that the N-terminal ITIM is phosphorylated by distinct NRTKs in a manner that requires SH2 domain-dependent interactions with the initially phosphorylated ITIM. We propose that a two-step, two-enzyme mechanism for dual ITIM phosphorylation allows inhibitory signaling to be temporally regulated so as to ensure optimal timing of control over cellular activation.

MATERIALS AND METHODS

Kinases. Recombinant kinases were purchased from commercial vendors. Btk and Lyn were obtained from Invitrogen and Csk from Sigma Aldrich.

Peptide Synthesis. Peptides were synthesized using standard FMOC protocols on an ABI 433 instrument. Phosphotyrosine was coupled as Fmoc-Tyr(PO(OBzl)OH)-OH (EMD Millipore chemicals). The SH2 domain optimal binding peptides were synthesized as amides while the PECAM peptides were synthesized as free acids. The peptide resin was cleaved with 92.5% TFA/2.5% EDT/2.5% TIS/2.5% H_2O and precipitated in cold ethyl ether. The peptides were purified to >90% by RP-HPLC using a Phenomenex Proteo C12 column and a 0.1% TFA/acetonitrile gradient. The mass of final product was verified by MALDI-TOF mass spectrometry analysis.

In Vitro Kinase Assays. The ADP Hunter kit (DiscoveRx Corporation) was used according to manufacturer's specifications. Briefly, recombinant kinases were incubated with synthetic peptides in kinase assay buffer I (50 mM Tris, 1 mM EGTA, 10 mM $MgCl_2$, 0.01% Brij35, 1 mM DTT, 25 μM ATP), and neutralization buffer (provided in kit) for 4 h at 30 °C. Reagents added to each well generate a fluorescence signal that is directly proportional to the amount of ADP produced. The amount of ADP produced during each reaction was quantified against an ADP standard curve (0−25 μM). Fluorescence detected in kinase only wells accounted for ADP generated by nonenzymatic hydrolysis of ATP or baseline autophosphorylation of the kinase and was subtracted as background from peptide-containing wells.

Measurement of ^{32}P-incorporation into biotinylated, synthetic peptides was performed as previously described.[16] All reactions were performed in kinase assay buffer I at 37 °C for 1 h (except as noted) in the presence of 0.1 μCi/μL [γ-^{32}P]ATP (6000 mCi/mmol, Perkin-Elmer). The reaction mixture was placed on ice, and an equal volume of 2 mM unlabeled ATP was added to quench the reaction. The quenched reaction was added to 0.5 mL of PBS containing 50 μL of a 50% slurry of streptavidin-conjugated agarose beads (Pierce) and rotated for 2 h at room temperature. Streptavidin beads were pelleted by centrifugation, washed five times in PBS, and added to 3 mL of scintillation fluid. Radioactivity incorporated into bead-bound substrate peptides was counted on a Wallac 1410 liquid scintilliation counter (Perkin-Elmer).

Phosphorylation of the PECAM-1 Cytoplasmic Domain. Recombinant forms of the PECAM-1 cytoplasmic domain (WT, Y$_{663}$F, and Y$_{686}$F) were generated and purified as previously described.[15] To exclude the possibility of non-ITIM tyrosine phosphorylation, sequences within the constructs that encoded non-ITIM tyrosine residues and that were predicted to serve as potential SFK phosphorylation sites (Y$_{636}$ and Y$_{701}$) were mutated so as to encode phenylalanine residues. Lyn (68 nmol/min activity in 25 μL) was incubated with 1 mM of the PECAM-1 cytoplasmic domain (PECAM-1$_{cyto}$) in kinase assay buffer II (250 μM ATP, 1 mM EGTA, 10 mM $MgCl_2$, 0.01% Brij 35, and 250 μM Na_3VO_4) for 10 min at 30 °C and then heated to inactivate Lyn. Btk, Csk, or Lyn was then added to the reaction mixture along with additional ATP (250 μM). These reactions were carried out for an additional 30 min at 30 °C and stopped by boiling after addition of an equal volume of 2× SDS-PAGE sample reducing buffer; resulting products were separated on a 12% SDS−polyacrylamide gel and stained with Coomassie. Band intensities were measured using a Kodak molecular imaging densitometry system.

Peptido-Precipitation from Platelet Lysate. Platelets were isolated from healthy human volunteers as previously described.[17] The platelets were resuspended in Tyrode's buffer

(137 mM NaCl, 20 mM HEPES, 13.8 mM $NaHCO_3$, 2.5 mM KCl, 0.36 mM NaH_2PO_4, 0.25% BSA, and 0.1% glucose) to a final concentration of 5×10^8 platelets/mL and lysed with an equal volume of 2× lysis buffer (30 mM HEPES, 300 mM NaCl, 20 mM EGTA, 0.2 mM $MgCl_2$, 2% Triton X-100) containing 2× Phosphatase Inhibitor Cocktail Set II, and Protease Inhibitor Cocktail Set I (EMD Millipore). The cytoskeleton was removed from the platelet lysate by centrifugation at 4 °C for 15 min at 14 000*g*. Biotinylated PECAM-1 peptides (Y_{663},F_{686} or Y_{663},pY_{686}) were immobilized on streptavidin sepharose beads (Pierce) and washed twice to remove excess peptide. The peptide−bead complex was then added to the platelet lysate and incubated overnight at 4 °C with constant mixing. The beads were washed 5 times with 1× lysis buffer, resuspended in 2× SDS−PAGE sample buffer and boiled. Proteins eluted from the beads were separated on a 4−20% SDS−PAGE gel and identified by Western blot analysis.

Expression and Purification of GST-Tagged Proteins. Glutathione S-transferase (GST)-conjugated forms of Csk were expressed and purified as described in Yaqub et al.[18] Briefly, full-length Csk was amplified from pDONR223-Csk (A gift from William Hahn and David Root, Addgene plasmid no. 23941) and ligated into the pGEX-KG vector to encode a GST−Csk fusion protein. Point mutations encoding substitutions of lysine for arginine at position 107 ($R_{107}K$) or of cysteine for serine at position 109 ($S_{109}C$), which have previously been shown to abolish phosphotyrosine binding by the Csk SH2 domain,[19,20] were made using a QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies). GST fusion proteins were expressed in BL21 (DE3) cells (Invitrogen) and induced with 1 mM IPTG for 3 h at 37 °C. The bacterial pellet was resuspended in 20 mL of PBS, 1 mM Pefabloc, and 0.1%Triton X-100 per liter of cells and sonicated. After clarification by centrifugation, the lysate was passed over glutathione sepharose 4 Fast Flow (GE Healthcare) and eluted with 50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0. Purified kinases were stored in 20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT, and 50% glycerol at −20 °C.

The plasmid encoding the SH2 domain of Lyn was a generous gift Osamu Miura. The isolated SH2 domain of Btk was amplified from full length Btk in a pDONR223 vector (A gift from William Hahn and David Root, Addgene plasmid no. 23918). Sequences encoding the Lyn and Btk SH2 domains were ligated into the GST-containing vector, pGEX-KG. Expression and purification of the GST-fusion proteins containing the SH2 domains of these enzymes was performed as previously described for the GST-fusion protein containing the SH2 domain of Csk.[26] Purified isolated SH2 domians were stored in 20 mM Tris pH 7.4.

Flow Cytometry Bead-Based Binding Assay. Binding of the isolated SH2 domains of Lyn, Btk and Csk to phosphopeptides corresponding to the C-terminal ITIM of PECAM-1 was assessed using a flow cytometry bead-based binding assay. To assess the ability of the isolated SH2 domains to bind to PECAM-1 phosphopeptides, biotinylated PECAM-1 peptides were incubated with streptavidin-coated polystyrene particles (SVP-60-5, Spherotech) in PBS containing 0.2% BSA (PBS/0.2% BSA) for 45 min at room temperature, and the peptide−bead complex was washed once with PBS/0.2% BSA. Recombinant GST-fusion proteins containing the SH2 domains of Lyn, Btk, or Csk were incubated with the peptide−bead complex for 2 h at room temperature and washed once with PBS/0.2% BSA. An Alexa Fluor-647-conjugated GST-specific antibody was then incubated with the beads and washed once with PBS/0.2% BSA. Beads were analyzed by flow cytometry using an LSR II (Becton Dickinson) high-throughput sampler, and median fluorescence intensities (MFI) were recorded.

To assess the ability of WT, $R_{107}K$, and $S_{109}C$ forms of full-length Csk to bind to a phosphopeptide corresponding to the PECAM-1 C-terminal ITIM, protein G-coated polystyrene particles (PGP-60-5, Spherotech) were incubated with a Csk-specific antibody (SC-286, Santa Cruz) in PBS/0.2% BSA at room temperature for 30 min. The beads were washed twice with PBS/0.2% BSA, after which WT, $R_{107}K$, or $S_{109}C$ forms of GST−Csk (10 μg) were added and incubated 30 min at room temperature. Beads were washed once with 1 M NaCl and twice with PBS/0.2% BSA. Biotinylated PECAM-1 Y_{663},pY_{686} (10 μM) was incubated with Csk-coated beads for 30 min at room temperature and washed once with PBS/0.2% BSA. Alexa Fluor-647-conjugated streptavidin (3 μg/mL; Molecular Probes) was then incubated with the beads for 30 min at room temperature, after which beads were washed once with PBS/0.2% BSA. Flow cytometry was performed as described above, and median fluorescence intensities (MFI) were recorded.

Characterization of PECAM-1 Immunoprecipitated from Platelet Lysates. Platelets were isolated from healthy human volunteers as previously described.[17] Platelets were resuspended in Tyrode's buffer (7.5×10^8 mL^{-1}) and were supplemented with 1 mM $CaCl_2$ immediately prior to stimulation. Platelets suspensions were stimulated with collagen-related peptide (CRP; 5ug/mL) with stirring in a lumi-aggregometer (Chronolog) in the presence or absence of a PI3K inhibitor (TGX-221; Santa Cruz) to block downstream recruitment and activation of Btk and Csk and of an SHP-2 inhibitor (NSC-87877; Santa Cruz) to block dephosphorylation of singly phosphorylated forms of PECAM-1. Platelets were lysed with 2× lysis buffer for 1 h at 4 °C, after which the cytoskeleton was removed by centrifugation at 4 °C for 15 min at 14 000*g*. PECAM-1 was immunoprecipitated from platelet lysates by overnight incubation at 4 °C with PECAM-1.3-coated protein-G sepharose beads. The beads were washed 5 times with 1× lysis buffer, resuspended in 2× SDS−PAGE sample buffer, and boiled. Proteins eluted from the beads were separated on a 7.5% Bio-Rad TGX gel and analyzed by Western blot using antibodies specific for SHP-2(C-18; Santa Cruz), phosphotyrosine (4G10 platinum; Millipore), and PECAM-1 (PECAM-1.3).

Statistical Analysis. Statistically significant differences were identified by performing a one-way ANOVA followed by a Bonferroni post hoc test using GraphPad Prism 5 software.

RESULTS

Lyn, but not Btk or Csk, Is a PECAM-1 C-Terminal ITIM Kinase. In platelets activated via the GPVI collagen receptor, Lyn is required for PECAM-1 ITIM phosphorylation and SHP-2 binding.[11] However, it is not known whether Lyn phosphorylates both of PECAM-1's two ITIMs or instead mediates initial phosphorylation of one ITIM tyrosine, which is then required for subsequent phosphorylation of the second ITIM tyrosine. To discriminate between these two possibilities, we incubated synthetic peptides corresponding to the N- and C-terminal ITIMs of PECAM-1 with recombinant, purified Lyn. Lyn robustly phosphorylated the Y_{686}-containing C-terminal ITIM peptide but not the Y_{663}-containing N-terminal PECAM-1 ITIM peptide (Figure 1A). These data indicate that

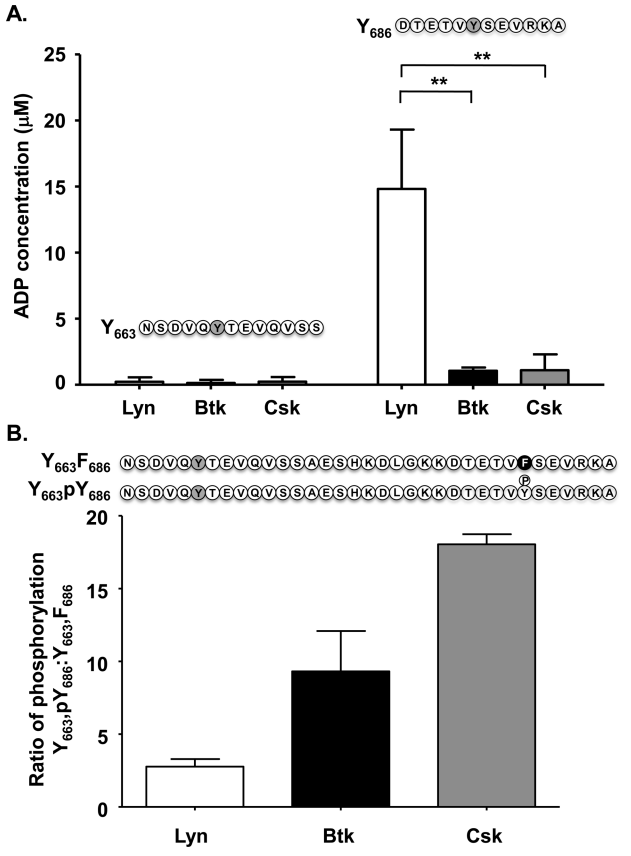

Figure 1. Lyn, but not Btk or Csk, phosphorylates the PECAM-1 C-terminal ITIM, which once phosphorylated enhances phosphorylation of the N-terminal ITIM by Lyn, Btk, and Csk. (A) Synthetic peptides corresponding to the N-terminal (Y_{663}) or C-terminal (Y_{686}) ITIM of PECAM-1 were incubated with Lyn (white bars), Btk (black bars), or Csk (gray bars) for 4 h at 30 °C, after which the amount of ADP generated was determined using an ADP Hunter kit. Results are expressed as the mean ADP concentration ± SD calculated from three independent experiments. Inset: Schematic representation of the sequences of the Y_{663}- or Y_{686}-containing peptides. (B) Synthetic PECAM-1 peptides that span both ITIMs and that contain a tyrosine residue within the N-terminal ITIM (Y_{663}) and either a phenylalanine (F_{686}) or a phosphotyrosine (pY_{686}) within the C-terminal ITIM were incubated with Lyn (white bar), Btk (black bar), or Csk (gray bar), after which ADP generation was measured. The ratio of the amount of ADP generated by each enzyme in the presence of Y_{663},pY_{686} vs Y_{663},F_{686} was determined in three independent experiments. Results are expressed as the mean ratio of phosphorylation ± SD. Inset: Schematic representation of the sequences of the PECAM-1 Y_{663},F_{686} and Y_{663},pY_{686}-containing peptides.

Lyn is capable of mediating initial phosphorylation of the PECAM-1 C-terminal ITIM but that it cannot independently phosphorylate the PECAM-1 N-terminal ITIM.

Since Lyn was unable to phosphorylate the PECAM-1 N-terminal ITIM-containing peptide efficiently, we next sought to determine whether a different NRTK could act as the N-terminal ITIM kinase. The sequence preceding the target tyrosine residue (Y_{663}) in the N-terminal ITIM of PECAM-1 (NSDVQYTEV) conforms well to the motif in the C-terminal tail of SFKs that is targeted for phosphorylation by Csk.[21] Therefore, we evaluated the ability of Csk to phosphorylate the N-terminal ITIM of PECAM-1. In addition, because Btk is closely related to Csk[22] and has a prominent role in late stages of GPVI-mediated platelet activation,[6] we also examined Btk for its ability to phosphorylate PECAM-1 Y_{663}. Interestingly, neither Btk nor Csk efficiently phosphorylated either Y_{663}- or Y_{686}-containing PECAM-1 peptides (Figure 1A), indicating that Btk and Csk are by themselves incapable of mediating either initial phosphorylation of C-terminal Y_{686} or independent phosphorylation of N-terminal Y_{663} of PECAM-1.

Phosphorylation of the PECAM-1 C-Terminal ITIM Enhances Phosphorylation of the N-Terminal ITIM by Btk and Csk. Phosphorylation of multiple sites within a single protein can occur in an interdependent manner, in which phosphorylation at a primary site enhances phosphorylation at a secondary site.[23] In the case of sequential *tyrosine* phosphorylation, the initially phosphorylated tyrosine residue can support SH2 domain-dependent interactions with either the same or a different NRTK, which enhances subsequent phosphorylation by maintaining the NRTK in an active conformation in close proximity to the second phosphorylation site.[23] Previous studies have shown that the SH2 domains of SFKs, Tec family kinases, and Csk are capable of binding to the phosphorylated C-terminal ITIM of PECAM-1.[24] To evaluate the relative abilities of these NRTKs to phosphorylate PECAM-1 N-terminal ITIM Y_{663} in a manner that depends on prior phosphorylation of C-terminal ITIM Y_{686}, representative family members, including Lyn, Btk, and Csk, were incubated with Y_{663}-containing peptides that span both ITIMs and that contain either a phosphotyrosine (pY) or a phenylalanine (F) at position 686. As shown in Figure 1B, Lyn, Btk, and Csk were each able to phosphorylate PECAM-1 Y_{663} in a peptide that contained a phosphotyrosine but not a phenylalanine at position 686; however, Btk and Csk were both much more efficient than was Lyn. Collectively, the results shown in Figure 1 suggest that Lyn is required for PECAM-1 inhibitory function because it mediates initial phosphorylation of the C-terminal ITIM. Subsequent phosphorylation of the N-terminal ITIM, which requires C-terminal ITIM phosphorylation, can be mediated by Lyn, but is more efficiently accomplished by other NRTKs, including Csk and Btk.

Initial Phosphorylation of the PECAM-1 Cytoplasmic Domain by Lyn Enables Its Subsequent Phosphorylation by Btk or Csk. To determine whether observations made using synthetic peptides extend to the intact PECAM-1 cytoplasmic domain, a recombinant form of the PECAM-1 cytoplasmic domain (PECAM-1_{cyto}) was incubated sequentially with Lyn, followed by additional Lyn, Btk, or Csk. Mono- and diphosphorylated forms of PECAM-1_{cyto} generated in the *in vitro* kinase reaction were separated from one another and from unphosphorylated PECAM-1_{cyto} by SDS−PAGE as previously described.[15] As shown in Figure 2A, Lyn, but not Btk or Csk, effectively converted PECAM-1_{cyto} from its unphosphorylated form to a species that was phosphorylated at a single site, as reflected by a slight upward shift in the apparent molecular weight of PECAM-1_{cyto}. Preincubation of PECAM-1_{cyto} with Lyn enabled Btk and Csk to phosphorylate PECAM-1_{cyto} at a second site, as reflected in an even greater upward shift in the apparent molecular weight of the diphosphorylated species (Figure 2A). Quantitatively, addition of Btk or Csk to singly phosphorylated PECAM-1_{cyto} resulted in a significant increase in the amount of dually phosphorylated PECAM-1_{cyto} (Figure 2B). In contrast, addition of Lyn to singly phosphorylated PECAM-1_{cyto} resulted in only a slight, and not significant, increase in the amount of dually phosphorylated PECAM-1_{cyto}. These data demonstrate that Lyn mediates only initial phosphorylation of C-terminal ITIM Y_{686}, which then supports

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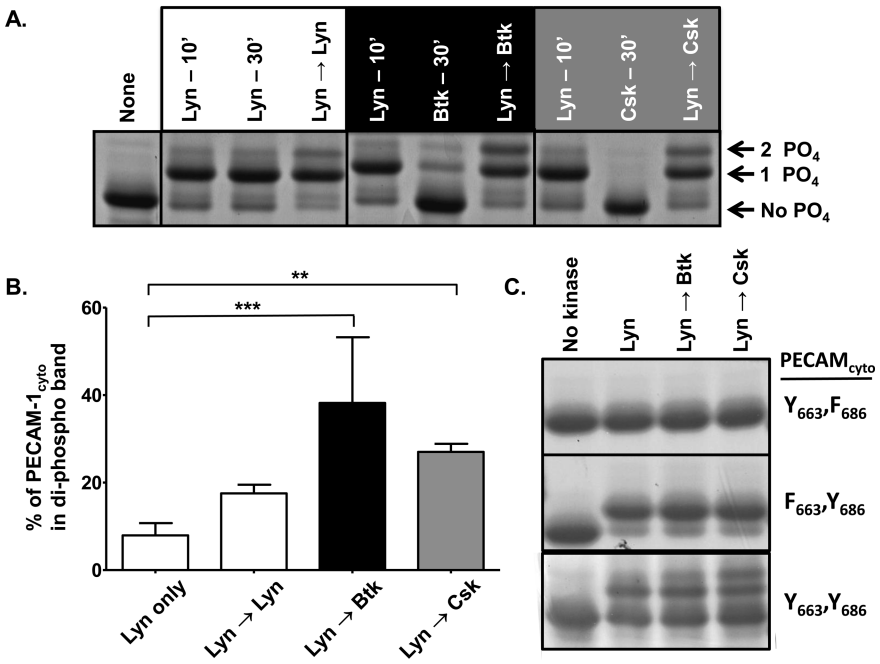

Figure 2. Initial phosphorylation of PECAM-1 by Lyn enables subsequent phosphorylation by Btk and Csk. Purified, recombinant PECAM-1 cytoplasmic domain (PECAM-1_{cyto}) was incubated with no kinase (None) or subjected to a 10 minute incubation with Lyn alone (Lyn 10′), a 30 minute incubation with Lyn (Lyn−30′), Btk (Btk−30′), or Csk (Csk−30′), or a 10 minute incubation with Lyn followed by heat-inactivation of Lyn (60 °C, 20 min), and an additional 30 minute incubation with Lyn (Lyn → Lyn), Btk (Lyn → Btk), or Csk (Lyn → Csk). Phosphorylated PECAM-1_{cyto} species were separated by SDS−PAGE and stained with Coomassie blue. (A) Mono- and di-phosphorylated PECAM-1_{cyto} species migrated at a higher apparent molecular weight than non-phosphorylated PECAM-1_{cyto} with the di-phosphorylated species having a higher apparent molecular weight than the mono-phosphorylated species. The image shown is representative of four independent experiments. (B) Band intensities were quantified using a Kodak Molecular Imaging Densitometry System. Total band intensities (non-phospho + mono-phospho + di-phospho band intensities) were normalized to 100% for each lane. Results are expressed as the mean % of PECAM-1_{cyto} in the di-phosphorylated band relative to the total band intensity ± S.D. calculated from four independent experiments. (**$p < 0.01$, ***$p < 0.001$) (C) PECAM-1_{cyto} with ITIM tyrosine residues mutated to phenylalanine (Y_{663},F_{686}; F_{663},Y_{686}) as well as WT PECAM-1_{cyto} (Y_{663},Y_{686}) were incubated with Lyn alone or with Lyn followed by an additional incubation with either Btk or Csk. As described above, the phosphorylated PECAM-1_{cyto} species generated during these reactions were separated by SDS−PAGE and stained with Coomassie blue. Data shown are representative of three independent experiments.

recruitment of Btk, Csk, or both to carry out subsequent phosphorylation of N-terminal ITIM Y_{663}.

To confirm that phosphorylation of the C-terminal ITIM accounts for the first shift in apparent molecular weight of PECAM-1_{cyto}, we generated a mutant form of PECAM-1_{cyto} in which the C-terminal ITIM tyrosine was substituted with phenylalanine (Y_{663},F_{686}) and evaluated its ability to undergo a mobility shift on SDS−PAGE gels after exposure to conditions that support tyrosine phosphorylation. In the mutant Y_{663},Y_{686}F form of PECAM-1_{cyto}, the single and double shifts in apparent molecular weight that were observed upon incubation of wild-type PECAM-1_{cyto} (Y_{663},Y_{686}) with Lyn or with Lyn plus Btk or Csk were ablated (Figure 2C). These data highlight the importance of Y_{686} as the initiator of PECAM-1 phosphorylation and confirm that neither Y_{663} nor any other residue in the PECAM-1 cytoplasmic domain serves as a substrate for Lyn, Btk, or Csk. As expected, substitution of the N-terminal ITIM tyrosine with phenylalanine (F_{663},Y_{686}) ablated the second but not the first shift in apparent molecular weight of PECAM-1_{cyto} (Figure 2C). On the basis of these findings, we conclude that Lyn phosphorylates only the C-terminal ITIM (Y_{686}) of PECAM-1 and that this phosphorylation event is required for subsequent phosphorylation of the N-terminal ITIM (Y_{663}) by Btk or Csk.

Evidence for Sequential Phosphorylation of PECAM-1 in Platelets Activated via the ITAM-Coupled GPVI Collagen Receptor. The GPVI collagen receptor on platelets is coupled to the ITAM-containing Fc receptor γ chain (FcRγ).[1,2] Platelet activation in response to binding of collagen to the GPVI/FcRγ complex involves an ordered series of events that includes activation of Lyn, recruitment and activation of PI3K, and subsequent recruitment and activation of Btk.[6,25] Fibrinogen binding to activated platelets results in platelet aggregation, which is required for phosphorylation of paxillin family members that serve as Csk binding proteins that recruit Csk to the membrane to participate in inhibitory signaling.[26] Platelets stimulated via the GPVI/FcRγ complex in the presence of a PI3K inhibitor would therefore be expected to engage in SFK-mediated but not Btk- or Csk-mediated signaling events. Our model predicts that, under these conditions of platelet activation, Lyn would be able to phosphorylate the PECAM-1 C-terminal ITIM but subsequent phosphorylation of the N-terminal ITIM by Btk or Csk would be precluded. As shown in Figure 3, PECAM-1 immunoprecipitated from platelets stimulated with collagen-related peptide (CRP), a GPVI/FcRγ-specific ligand, in the presence of a PI3K inhibitor (TGX-221) was tyrosine phosphorylated but was unable to coimmunoprecipitate SHP-2. Since phosphorylation of both ITIMs is required for SHP-2 binding,[27] this finding suggests that PECAM-1 is phosphorylated on *only* one ITIM under these conditions. These data are consistent with a sequential two-enzyme model of ITIM phosphorylation in which Lyn phosphorylates the C-terminal ITIM of PECAM-1 (Y_{686}) after which Btk or Csk, both of which are activated

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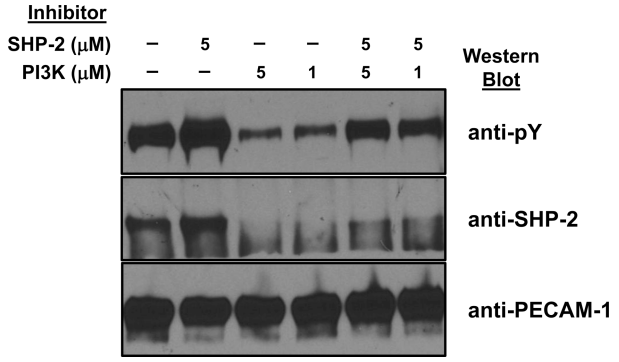

Figure 3. PECAM-1 is phosphorylated on its C-terminal ITIM but cannot bind SHP-2 in platelets activated in the presence of a PI3K inhibitor. Human platelets were activated with collagen-related peptide (CRP) in the presence and absence of a SHP-2 inhibitor, a PI3K inhibitor, or both and then lysed as described in Materials and Methods. PECAM-1 was immunoprecipitated (IP) from platelet lysates. PECAM-1 IPs were probed by Western blot analysis using antibodies specific for phosphotyrosine (anti-pY), coprecipitating SHP-2 (anti-SHP-2) and PECAM-1 antigen (anti-PECAM-1). The data shown are representative of three independent experiments.

downstream of PI3K, phosphorylates the N-terminal ITIM (Y_{663}) and enables recruitment of SHP-2.

The Roles of SH2 Domain-Dependent Interactions in pY_{686}-Dependent Phosphorylation of Y_{663} by Btk and Csk. Models for sequential tyrosine phosphorylation predict that an initially phosphorylated tyrosine residue serves as a docking site to recruit a secondary kinase, which then phosphorylates the secondary site.[23] Such a model requires that the initially phosphorylated tyrosine be contiguous with the secondary target tyrosine. To examine whether Btk and Csk act in this manner on the two ITIMs of PECAM-1, we provided these enzymes with Y_{663} either on the same peptide as pY_{686} (Y_{663},pY_{686}) or on a separate peptide (pY_{686} + Y_{663}). As shown in Figure 4, pY_{686} markedly enhanced phosphorylation of Y_{663} by Btk and Csk only when in the same peptide as Y_{663}. These data demonstrate that pY_{686} acts in cis to serve as a docking site that brings Csk and Btk into close proximity to Y_{663} and enable its phosphorylation.

A second prediction of sequential phosphorylation models is that the SH2 domain of the secondary kinase binds to the primary phosphorylation site. In a previous SH2 domain profiling study, the SH2 domains of both Csk and Tec were shown to bind to PECAM-1 pY_{686}-containing peptides; the Lyn SH2 domain also bound, albeit weakly, to PECAM-1 pY_{686}-containing peptides in this study.[24] To confirm these findings relative to Lyn and Csk and to extend them to the Tec family kinase Btk, we evaluated the abilities of full-length enzymes and their isolated SH2 domains to bind to PECAM-1 pY_{686}-containing phosphopeptides. In concordance with previous findings, we found that the isolated SH2 domain of Lyn bound to pY_{686}; however, the SH2 domain of Csk bound approximately twice as efficiently as that of Lyn (Figure 5A). In contrast to its family member, Tec, however, the Btk SH2 domain bound poorly to PECAM-1 pY_{686}, if at all (Figure 5A). Consistent with these findings, we found that PECAM-1 pY_{686}-containing phosphopeptides were capable of pulling down full-length Csk and Lyn but not Btk from platelet lysates (Figure 5B). These findings indicate that Csk and Lyn, but not Btk, bind to PECAM-1 pY_{686} via their SH2 domains.

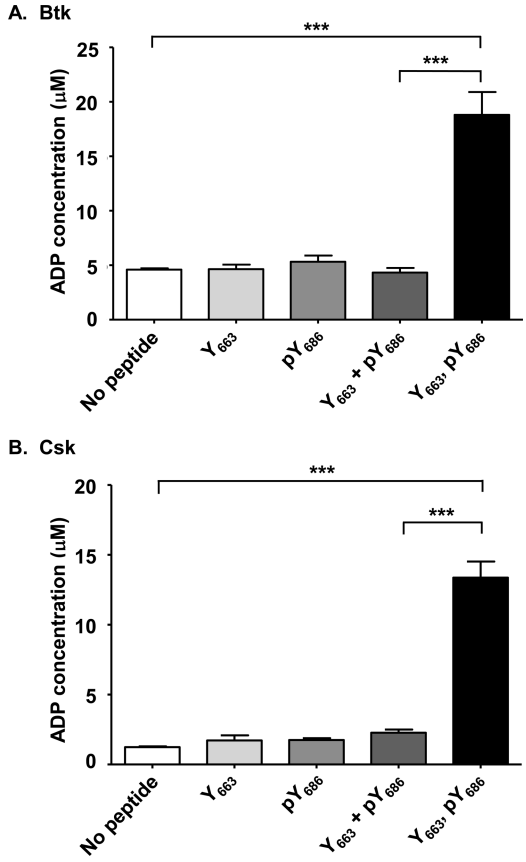

Figure 4. ITIM linkage in a single polypeptide is required for pY_{686}-dependent phosphorylation of Y_{663} by Btk and Csk. Synthetic peptides corresponding to the N-terminal ITIM (Y_{663}) and the phosphorylated C-terminal ITIM (Y_{686}) of PECAM-1 were incubated with (A) Btk or (B) Csk either alone or in combination with each other as separate peptides (Y_{663} + pY_{686}) or as parts of a single peptide corresponding to the portion of the PECAM-1 cytoplasmic domain that encompasses both ITIMs (Y_{663},pY_{686}). Reactions were carried out over 4 h at 30 °C, after which ADP generation was measured. Results are expressed as the mean ADP concentration ± SD calculated from three independent experiments. (***$p < 0.001$).

The finding that the Csk SH2 domain binds directly to PECAM-1 pY_{686} but that the Btk SH2 domain does not raises the question of the role that the SH2 domains of these enzymes play in their abilities to phosphorylate PECAM-1 Y_{663} in a pY_{686}-dependent manner. To address this question, we evaluated the abilities of phosphopeptides known to bind optimally to the SH2 domains of Btk or Csk, referred to as SH2 domain optimal binding peptides (SH2 OBP), as well as a short PECAM-1 pY_{686}-containing peptide, to inhibit phosphorylation of the long PECAM-1 Y_{663},pY_{686} substrate peptide. The Btk SH2 OBP (GDGpYEEISPLLL) corresponds to a sequence that binds to the Btk SH2 domain with high affinity,[28] while the Csk SH2 OBP (DKDHLpYSTVCK) corresponds to a sequence within the paxillin family member, Hic5, that binds with high affinity to the Csk SH2 domain.[29] As shown in Figure 6, phosphorylation of Y_{663},pY_{686} by either Btk (Figure 6A) or Csk (Figure 6B) was significantly inhibited by addition of an excess of the appropriate OBP or of the pY686-containing competitor peptide. None of the competitor peptides inhibited phosphorylation of poly(Glu_4-Tyr), a substrate that does not require SH2 domain-dependent interactions for its phosphorylation, by Btk (Figure 6C) or Csk (Figure 6D), indicating that they do

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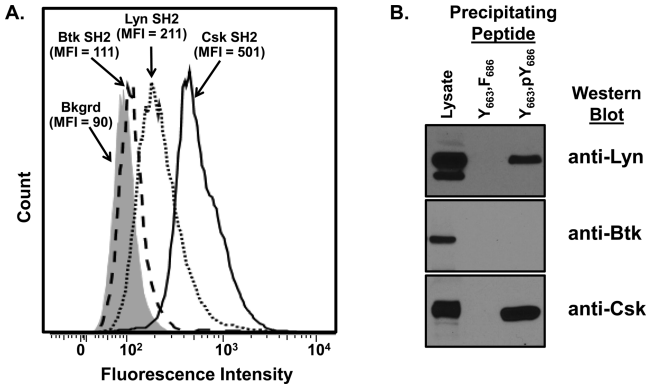

Figure 5. Csk and Lyn, but not Btk, interact with the C-terminal ITIM of PECAM-1 via an SH2 domain-dependent interaction. (A) Polystyrene streptavidin beads coated with the PECAM-1 $Y_{663}pY_{686}$ peptide were incubated with GST fusion proteins containing the SH2 domain of Btk (dashed line), Lyn (dotted line), or Csk (solid line), washed, and stained with an Alexa 647-conjugated GST-specific antibody. The relative ability of these SH2 domains to bind $Y_{663}pY_{686}$ was measured by flow cytometry and reported as median fluorescence intensity (MFI). The negative control (Bkgrd; gray histogram) reflects the MFI observed upon binding of the SH2 domain of Btk to beads not coated with peptide; Csk and Lyn SH2 domains exhibited similar levels of background binding. Results shown are representative of three independent experiments. (B) Lysates of resting human platelets were incubated with streptavidin beads coated with PECAM-1 $Y_{663}F_{686}$- or $Y_{663}pY_{686}$-containing peptides. Whole platelet lysates and peptido-precipitates were separated by SDS−PAGE and probed by Western blot analysis using antibodies specific for Lyn (anti-Lyn), Btk (anti-Btk), and Csk (anti-Csk). The data shown are representative of three independent experiments.

not directly inhibit kinase activity. Interestingly, both the PECAM-1 pY_{686}-containing peptide and the Csk SH2 OBP significantly *enhanced* phosphorylation of poly(Glu_4-Tyr) by Csk (Figure 6D), which is consistent with the known ability of ligation of the Csk SH2 domain to enhance its kinase activity.[3] In contrast, the PECAM-1 pY_{686}-containing peptide also significantly enhanced phosphorylation of Poly (Glu_4-Tyr) by Btk, whereas the Btk SH2 OBP did not (Figure 6C). These findings are consistent with a model in which PECAM-1 pY_{686} provides a docking site for the SH2 domain of Csk, which both tethers the enzyme to the PECAM-1 cytoplasmic domain and stimulates enzymatic activity so as to ensure efficient phosphorylation of the N-terminal ITIM. In contrast, PECAM-1 pY_{686} serves only an activating, and not a tethering, role for Btk, with activation appearing to occur via an SH2 domain independent mechanism.

To obtain direct evidence that the SH2 domain of Csk is required for its ability to phosphorylate Y_{663} in a pY_{686}-dependent manner, we expressed wild-type (WT) and mutant forms of recombinant active Csk in which SH2 domain substitutions ($S_{109}C$ and $R_{107}K$) that interfere with phosphotyrosine binding ability were introduced.[19,20] We found that these substitutions either ablated ($S_{109}C$) or dramatically reduced ($R_{107}K$) Csk binding to PECAM-1 pY_{686}-containing phosphopeptides (Figure 7A). These substitutions had, however, either no effect ($S_{109}C$) or enhanced by 4-fold ($R_{107}K$) Csk-mediated phosphorylation of a non-SH2 domain-dependent substrate (poly(Glu_4-Tyr); data not shown). As shown in Figure 7B, WT Csk robustly phosphorylated PECAM-1 Y_{663} in a pY_{686}-dependent manner. In contrast, the $R_{107}K$ substitution abolished and the $S_{109}C$ substitution dramatically reduced Csk-mediated pY_{686}-dependent phosphorylation of Y_{663}. These findings strongly support a role for SH2 domain-dependent binding to PECAM-1 pY_{686} in Csk-mediated phosphorylation of Y_{663}.

Siglec-9 ITIMs Undergo Sequential Phosphorylation by Csk. The data presented thus far indicate that phosphorylation of the PECAM-1 ITIMs occurs via a two-step, two-enzyme process. More specifically, our findings provide evidence that the first phosphorylation event, that of phosphorylation of the C-terminal ITIM (pY_{686}), is mediated by a SFK and that the enzyme most likely to carry out the second phosphorylation event, that of phosphorylation of the N-terminal ITIM, is Csk, which binds to and is activated by PECAM-1 pY_{686}. Interestingly, the PECAM-1 N-terminal ITIM (NSDVQYTEV) conforms perfectly to the consensus sequence that is known to be targeted by Csk,[3] in that it possesses an acidic residue at the −3 position and glutamine residue at the −1 position relative to the target tyrosine residue. Among the dual ITIM-containing inhibitory receptors, Siglecs 7 (EREI-QYAPL) and 9 (EGELQYASL) possess N-terminal ITIMs that conform perfectly to consensus sequences for phosphorylation by Csk.[30] We therefore sought to determine the extent to which our two-step model for sequential ITIM phosphorylation extends to other dual ITIM-containing inhibitory receptors by investigating the potential for Csk to sequentially phosphorylate the ITIMs of Siglec-9. Csk was incubated with two peptides, both of which span the two ITIMs of Siglec-9 and contain the N-terminal tyrosine residue (Y_{416}) but that differ from one another in that they contain either a phosphotyrosine (pY) or a phenylalanine (F) at the position of the C-terminal ITIM tyrosine residue (position 439). As shown in Figure 8, Csk was able to phosphorylate Siglec-9 Y_{416} in a peptide that contained a phosphotyrosine but not a phenylalanine at position 439. These results demonstrate that N-terminal ITIM of Siglec-9 can serve as a substrate for phosphorylation by Csk but only when the C-terminal ITIM of Siglec-9 is already phosphorylated, which supports a sequential mechanism for phosphorylation of this receptor.

■ DISCUSSION

ITAM-coupled receptors are utilized by virtually every type of hematopoietic cell to effect cellular activation.[1,5,8] It is important that ITAM-mediated cellular activation be properly regulated to avoid pathological conditions such as autoimmunity, hypersensitivity, tumor progression, and occlusive thrombus formation. Two major mechanisms that regulate ITAM-coupled receptors include inhibition of SFK activity by Csk[1,3] and dephosphorylation of ITAM-coupled receptor signal transduction pathway components by tandem SH2 domain-containing tyrosine phosphatases recruited to dual ITIM-containing inhibitory receptors.[1] ITAM-dependent activation pathways and both Csk- and ITIM-mediated inhibitory pathways are initiated through SFK-mediated phosphorylation of tyrosine residues in ITAMs, Csk binding proteins, and ITIMs, respectively, which has led to the supposition that all of these pathways are engaged simultaneously and that the magnitude of the cellular response is determined by the balance that is struck between activating and inhibitory signals.[1] In the present study, we describe a novel mechanism through which the timing of activation and inhibition can be controlled. Specifically, we use PECAM-1 and the signal transduction pathway that it regulates in platelets as a model system to characterize the mechanism by which cells exert precise control

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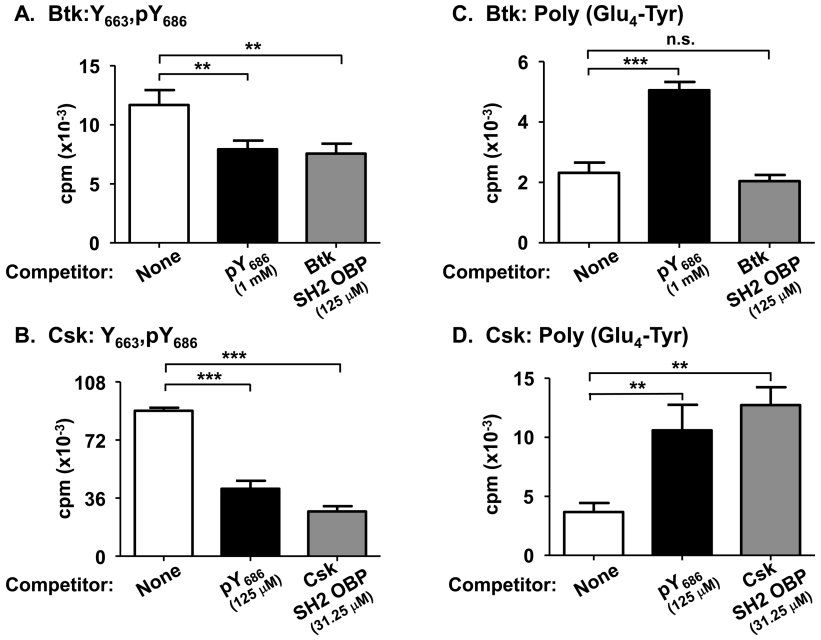

Figure 6. Btk and Csk utilize their SH2 domains to direct them to phosphorylate Y_{663} in a pY_{686}-dependent manner. (A and B) A biotinylated PECAM-1 peptide substrate (Y_{663},pY_{686}; 10 μM) or (C and D) a biotinylated Poly (Glu_4-Tyr) peptide substrate (10 μM) was incubated with 48 nM Btk (A and C) or Csk (B and D) in the presence and absence of unbiotinylated competitor peptides. Dose response studies (not shown) were performed to determine the concentrations of competitor peptides that inhibited phosphorylation of PECAM-1 Y_{663},pY_{686} by 50%. For the purposes of comparison, the concentrations of competitor peptides that decreased activity toward PECAM-1 Y_{663},pY_{686} by approximately 50% are shown. Competitor peptides corresponded to the phosphorylated C-terminal ITIM (pY_{686}) of PECAM-1, an SH2 domain optimal binding peptide (OBP) for either Btk (GDGpYEEISPLLL; Btk SH2 OBP) or Csk (DKDHLpYSTVCK; Csk SH2 OBP). After incubation for one hour at 37 °C in the presence of [γ^{32}P]ATP, biotinylated peptides were captured onto streptavidin beads and washed extensively. ^{32}P incorporation into captured peptides was quantified by scintillation counting of washed beads. Results are expressed as mean counts per minute (cpm) ± S.D. calculated from triplicate reactions with background subtracted. (**p < 0.01, ***p < 0.001, n.s. = not significant).

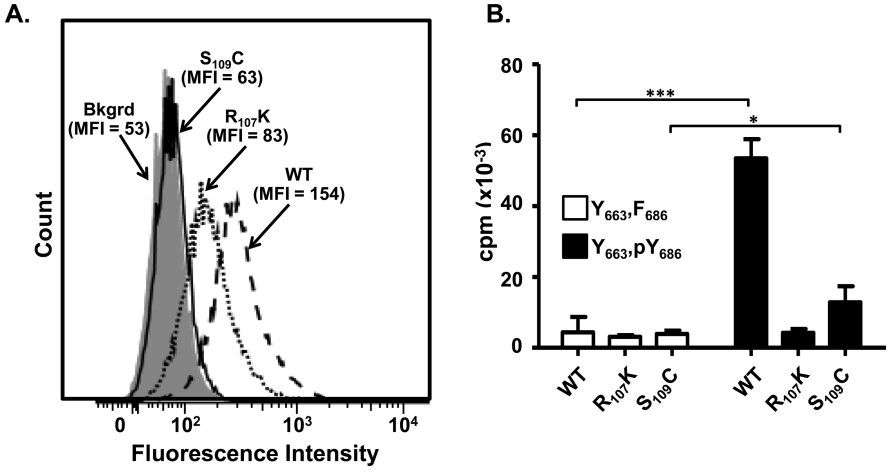

Figure 7. Csk requires the phosphotyrosine binding ability of its SH2 domain to effectively phosphorylate the N-terminal ITIM of PECAM-1 in the presence of the phosphorylated C-terminal ITIM. (A) Protein G-containing polystyrene beads coated with a Csk-specific antibody were used to capture wild-type (WT; dashed line), R_{107}K (dotted line), or S_{109}C (solid line) forms of recombinant Csk, after which beads were washed, incubated with biotinylated PECAM-1 Y_{663},pY_{686} peptides, and stained with an Alexa 647-conjugated streptavidin. The relative ability of WT and mutant forms of Csk to bind PECAM-1 Y_{663},pY_{686}-containing peptides was measured by flow cytometry and reported as median fluorescence intensity (MFI). The negative control (Bkgrd; gray histogram) reflects the MFI observed in the absence of Csk. Results are representative of two independent experiments. (B) A biotinylated PECAM-1 peptide substrate (Y_{663},pY_{686} or Y_{663},F_{686}; 50 μM) was incubated with wild-type (WT) or SH2 domain mutant (R_{107}K or S_{109}C) forms of GST-conjugated Csk for 1 h at 37 °C in the presence of [γ^{32}P]ATP. ^{32}P incorporation into captured peptides was quantified by scintillation counting of washed beads. Results are expressed as the mean counts per minute (cpm) ± SD calculated from triplicate reactions with background (no peptide substrate) subtracted. The data shown are representative of two independent experiments (*p < 0.05, ***p < 0.001).

over the timing of initiation of inhibitory signaling; namely, that dual ITIM phosphorylation takes place in a two-step process (Figure 9) in which a SFK phosphorylates one ITIM, after which the second ITIM is phosphorylated by a different NRTK. Enzymes that efficiently carry out the second phosphorylation event include Csk and Btk, which are involved only in very late

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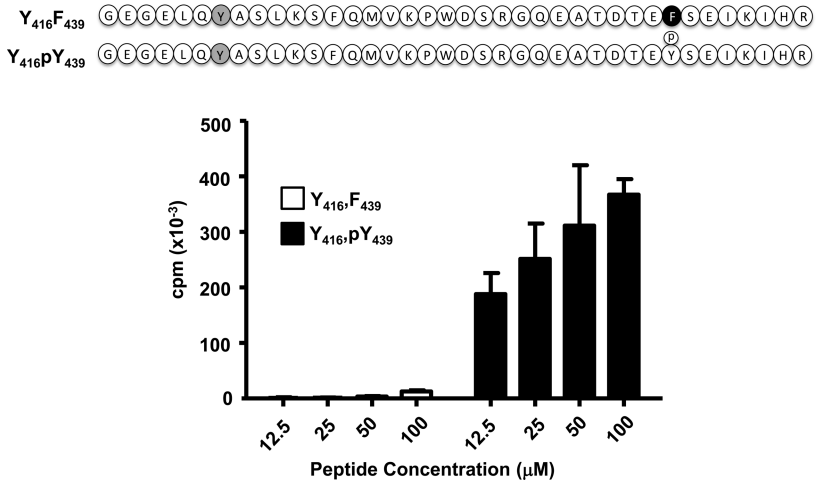

Figure 8. Prior phosphorylation of the C-terminal ITIM of Siglec 9 enhances phosphorylation of the N-terminal ITIM by Csk. Increasing concentrations of a biotinylated peptide substrate corresponding to the sequence of Siglec-9 that spans its two ITIMs (Y_{416},pY_{439} or Y_{416},F_{439}) were incubated with Csk (48 nM) for 1 h at 37 °C in the presence of [γ^{32}P]ATP. ^{32}P incorporation into captured peptides was quantified by scintillation counting of washed beads. Results are expressed as mean counts per minute (cpm) ± SD calculated from triplicate reactions with background (no peptide substrate) subtracted. The data shown are representative of two independent experiments. ^{32}P incorporation into Y_{416},pY_{439} peptides was significantly greater than that incorporated into Y_{416},F_{439} peptides (***$p < 0.001$) at each concentration tested. Inset: Schematic representation of the sequences of the Siglec-9 Y_{416},pY_{439}- or Y_{416},F_{439}-containing peptides.

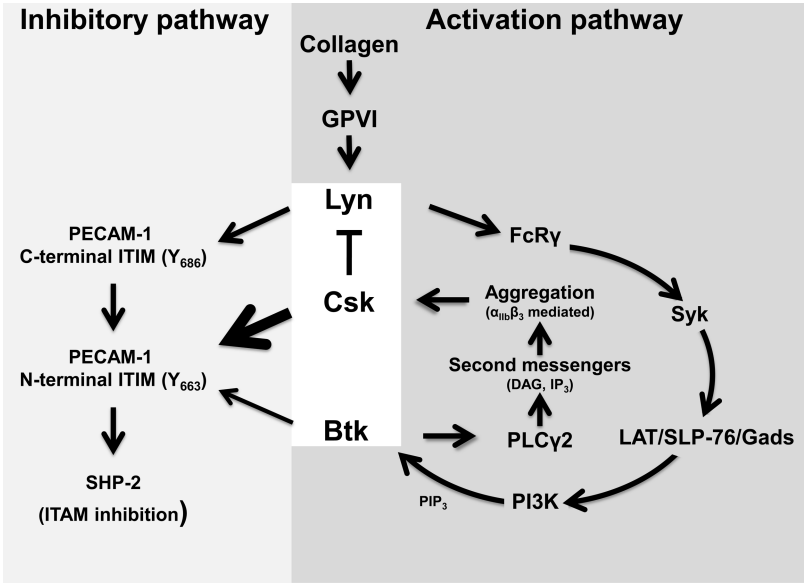

Figure 9. Schematic describing a novel mechanism for control of the timing of ITIM-mediated inhibition vs ITAM-dependent activation. Following binding of collagen to GPVI, the SFK Lyn initiates both inhibitory and activating signaling pathways by phosphorylating the C-terminal ITIM (Y_{686}) of PECAM-1 and the ITAMs of the GPVI-associated FcRγ chain. Phosphorylation of PECAM-1 Y_{686} (pY_{686}) is insufficient for inhibitory function, which requires that both ITIMs be phosphorylated. Phosphorylation of the FcRγ ITAMs enables recruitment of Syk, phosphorylation of the LAT/Gads/SLP-76 signalosome, activation of PI3K, and production of PIP_3, which support recruitment of PLCγ2 and Btk to the plasma membrane. Btk phosphorylates PLCγ2, which generates the second messengers DAG and IP_3 that are required for $\alpha_{IIb}\beta_3$-dependent platelet aggregation. In aggregating platelets, Csk is recruited to the plasma membrane where it deactivates SFKs involved in GPVI signaling. Once recruited, Btk and Csk can phosphorylate pY_{686}-containing PECAM-1 on Y_{663}. Dual ITIM phosphorylation of PECAM-1 allows recruitment and activation of SHP-2, which is required for PECAM-1-mediated inhibitory function. The requirement for NRTKs recruited only very late in the process of ITAM-dependent activation to phosphorylate the second ITIM of a dual ITIM-containing inhibitory receptor provides a novel mechanism by which ITIM-mediated inhibitory function can be delayed relative to ITAM-mediated activation. Abbreviations: Btk, Bruton's tyrosine kinase; Csk, C-terminal Src kinase; DAG, diacylglycerol; FcRγ, Fc receptor γ-chain; Gads, GRB2-related adapter protein 2; GPVI, glycoprotein VI; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; IP_3, inositol (1,4,5)-trisphosphate; LAT, linker for activation of T cells; Lyn, Lck/Yes-related novel protein tyrosine kinase; NRTKs, nonreceptor tyrosine kinase; PECAM-1, platelet endothelial cell adhesion molecule 1; PI3K, phosphatidylinositol 3-kinase; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PLCγ2, phospholipase C γ2 isoform; SLP-76, Src homology 2 (SH2) domain-containing leukocyte protein of 76 kDa; SFK, Src family kinase; SHP-2, SH2 domain-containing protein tyrosine phosphatase 2; Syk, Spleen tyrosine kinase.

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stages of ITAM-dependent platelet activation. Together, the findings that SFKs mediate only the first step of dual ITIM phosphorylation and that other NRTKs recruited only very late during ITAM-dependent cellular activation mediate the second step provide new insights into the mechanism by which ITIM-mediated inhibition can be delayed relative to ITAM-mediated activation so as to ensure proper timing of control over the cellular activation process.

SFK activity has been shown to be required for the phosphorylation of many ITIM-containing receptors.[8,11,12,23−29] By defining in the present studies the precise phosphorylation event for which SFK activity is required in phosphorylation of PECAM-1, we demonstrate that SFK activity is necessary but not sufficient to initiate dual ITIM-containing inhibitory receptor function. Specifically, we found that SFKs phosphorylate only the C-terminal ITIM of PECAM-1, which in turn confers the ability to recruit a second ITIM kinase that phosphorylates the N-terminal ITIM. We chose to establish this principle using the SFK Lyn, since Lyn is known to inhibit ITAM-mediated cellular activation in several cell types and to be required for PECAM-1 phosphorylation and for PECAM-1-mediated inhibitory function in platelets.[11] The ability to phosphorylate only one of the two PECAM-1 ITIMs is, however, not unique to Lyn but extends to other SFKs as well (Supplemental Figure 1, Supporting Information), many of which have been reported to phosphorylate PECAM-1 in cells other than platelets and to be involved in phosphorylation of other ITIM-containing receptors. The extent to which SFK activity is, in general, necessary but not sufficient for dual ITIM-containing inhibitory receptor function remains to be determined.

Our findings identify Lyn, Btk, and Csk as three kinases that are able to phosphorylate PECAM-1 N-terminal ITIM Y_{663} in a manner that depends on prior phosphorylation of C-terminal ITIM Y_{686}. Csk utilizes SH2 domain-dependent interactions with PECAM-1 pY_{686} to support subsequent phosphorylation of Y_{663}, and Lyn appears to share this property with Csk. Thus, our findings suggest a possible physiologically relevant explanation for previously reported interactions of PECAM-1 with SFKs including Lyn.[31] Nevertheless, the observation that the SH2 domain of Lyn domain binds PECAM-1 pY_{686} less strongly than does that of Csk, coupled with the observation that pY_{686}-dependent phosphorylation of Y_{663} by Lyn is less efficient than is that by Csk suggests that, whereas Lyn is indeed able to bind PECAM-1 pY_{686} and subsequently phosphorylate Y_{663}, Csk performs these functions more efficiently than does Lyn.

Csk has long been known to function as a PECAM-1 ITIM kinase.[14] Our findings expand upon this early observation by demonstrating that Csk specifically phosphorylates the PECAM-1 N-terminal ITIM, but only when the C-terminal ITIM has already been phosphorylated by a SFK. Furthermore, we demonstrate that this function of Csk extends to a second dual ITIM-containing inhibitory receptor, specifically Siglec-9. Csk is well-known for its ability to inhibit SFK-dependent signal transduction by phosphorylating the SFK C-terminal tyrosine residue, which enables an intramolecular inhibitory interaction with the SFK SH2 domain.[1,3] An interesting conundrum in SFK biology is that the affinity of the intramolecular interaction between the SFK C-terminal phosphotyrosine residue and its SH2 domain is relatively low, such that a SFK will remain in an active conformation even after its C-terminal tail is phosphorylated if it is bound to a tyrosine phosphorylated protein that has higher affinity for the SFK SH2 domain.[32] Csk binding proteins, which recruit Csk to sites of SFK activity, possess tyrosine-containing sequences that upon phosphorylation bind with high affinity to SFK SH2 domains, making it necessary for Csk binding proteins to be dephosphorylated for Csk-mediated inactivation of SFKs to be effective.[3] Interestingly, SHP-2, which binds with high affinity to dually phosphorylated PECAM-1 and is an important mediator of its inhibitory function,[33−36] has been implicated in dephosphorylation of two Csk binding proteins, specifically Cbp/PAG and paxillin.[37,38] On the basis of these findings, we propose that Csk inhibits SFK-dependent signaling in two steps: first by phosphorylating the C-terminal inhibitory tyrosine residue of the SFK and second by completing phosphorylation of a dual ITIM-containing inhibitory receptor and enabling binding of SHP-2. This proposed model explains how SHP-2 becomes available to dephosphorylate Csk binding proteins so as to enable SFK SH2 domains to *dissociate from activating intermolecular interactions* and instead *engage in inhibitory intramolecular interactions* with the tyrosine phosphorylated SFK C-terminal tail. Studies are underway in our laboratory to determine whether this function extends to other dual ITIM-containing receptors and to the SHP-2 homologue, SHP-1.

We found in the present studies that, like Csk, Btk is capable of phosphorylating the N-terminal ITIM of PECAM-1 in a manner that depends on prior phosphorylation of the C-terminal ITIM. When directly compared, however, Csk performed this function more efficiently than did Btk (Supplemental Figure 2, Supporting Information), which suggests that Csk may be a more physiologically relevant PECAM-1 N-terminal ITIM kinase than is Btk. The mechanism by which the phosphorylated C-terminal ITIM of PECAM-1 enhances phosphorylation of its N-terminal ITIM by Btk appears to differ from that utilized by Csk. Thus, whereas PECAM-1 pY_{686} engages in SH2 domain-dependent interactions with Csk that both dock and activate the enzyme, this tyrosine phosphorylated sequence within PECAM-1 serves only to activate Btk and does support an SH2-dependent interaction with this enzyme. This finding was unexpected, given our observation that a peptide that binds optimally to the Btk SH2 domain inhibits pY_{686}-dependent phosphorylation of Y_{663} and our previously published finding that the SH2 domain of the Btk homologue, Tec, does bind to peptides corresponding to the phosphorylated C-terminal ITIM of PECAM-1.[24] The mechanism used by Btk to phosphorylate the N-terminal ITIM of PECAM-1 may instead involve a remote substrate docking mechanism, which has previously been shown to be used by Tec family kinases to achieve substrate specificity.[39] This possibility remains to be tested. Finally, it is important to note that the primary function of Btk is to phosphorylate and activate PLCγ2 and thereby induce calcium mobilization downstream of ITAM-coupled receptor ligation.[6,25] That this function is important is supported by the observations that mutations in the gene encoding Btk are responsible for X-linked agammaglobulinemia (XLA) in humans and X-linked immunodeficiency in mice[40] and that Btk deficiency results in profound platelet hyporesponsiveness to GPVI specific agonists in both humans and mice.[41,42] It will ultimately be important to determine whether Btk has both stimulatory and inhibitory functions and to evaluate the relative extents to which Btk and Csk are required for PECAM-1-mediated inhibitory functions, both of which issues can be resolved by studying the relative

levels of responsiveness of cells derived from PECAM-1-positive and PECAM-1-deficient mice on a Btk-deficient[40] or conditionally Csk-deficient[43] background.

Studies of sequences within the PECAM-1 cytoplasmic domain that are required for efficient phosphorylation of its N-terminal ITIM by Lyn, Btk, and Csk may also prove useful in attempts to engineer forms of PECAM-1 with enhanced inhibitory activity. Endowment of platelets with a potent dual ITIM-containing inhibitory receptor whose involvement in regulation of the platelet response can be delayed relative to ITAM-mediated activation would be expected to enable efficient control of bleeding with minimal complications due to pathological thrombus formation. Lessons learned from studies of sequential phosphorylation of the PECAM-1 ITIMs may also contribute to a better understanding of how other dual ITIM-containing receptors regulate the biological activities of a variety of hematopoietic cells that impact a large number of important disease processes, including autoimmunity, immediate-type hypersensitivity, and anti-tumor immunity.[44−46]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplemental Figure 1 shows that the ability to phosphorylate only one of the two PECAM-1 ITIMs is not unique to Lyn but extends to other SFKs and Supplemental Figure 2 shows that Csk phosphorylates the N-terminal ITIM of PECAM-1 in a pY686 dependent manner at the same rate but to a greater extent than Btk. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail address: Debra.Newman@bcw.edu. Tel: 414-937-3820. Fax: 414-937-6284.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

BCR, B-cell receptor; BLNK, B-cell linker protein; Btk, Bruton's tyrosine kinase; Cbp, Csk binding proteins; Csk, C-terminal Src kinase; DAG, diacylglycerol; FcRγ, Fc receptor γ-chain; Fer, feline sarcoma-related; Fps, Fujinami poultry sarcoma; Gads, GRB2-related adapter protein 2; GPVI, glycoprotein VI; Ig, immunoglobulin; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; IP3, inositol (1,4,5)-trisphosphate; LAT, linker for activation of T cells; Lyn, Lck/Yes-related novel protein tyrosine kinase; NRTKs, nonreceptor tyrosine kinases; PECAM-1, platelet endothelial cell adhesion molecule 1; PI3K, phosphatidylinositol 3-kinase; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PLCγ2, phospholipase C γ2 isoform; SLP-76, Src homology 2 (SH2) domain-containing leukocyte protein of 76 kDa; SFK, Src family kinase; SHP-2, SH2 domain-containing protein tyrosine phosphatase 2; Siglecs, sialic acid binding lectin-like molecules; Syk, spleen tyrosine kinase; TCR, T cell receptor; XLA, X-linked agammaglobulinemia

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