Check for updates

Platelet Endothelial Cell Adhesion Molecule-1 Inhibits Platelet Response to Thrombin and von Willebrand Factor by Regulating the Internalization of Glycoprotein Ib via AKT/Glycogen Synthase Kinase-3/Dynamin and Integrin αIIbβ3

Chris I. Jones, Tanya Sage, Leonardo A. Moraes, Sakthivel Vaiyapuri, Umara Hussain, Katherine L. Tucker, Natasha E. Barrett, Jonathan M. Gibbins

Objective—Platelet endothelial cell adhesion molecule-1 (PECAM-1) regulates platelet response to multiple agonists. How this immunoreceptor tyrosine-based inhibitory motif–containing receptor inhibits G protein-coupled receptor–mediated thrombin-induced activation of platelets is unknown.

Approach and Results—Here, we show that the activation of PECAM-1 inhibits fibrinogen binding to integrin αIIbβ3 and P-selectin surface expression in response to thrombin (0.1–3 U/mL) but not thrombin receptor–activating peptides SFLLRN (3×10^{-7}–1×10^{-5} mol/L) and GYPGQV (3×10^{-6}–1×10^{-4} mol/L). We hypothesized a role for PECAM-1 in reducing the tethering of thrombin to glycoprotein Ibα (GPIbα) on the platelet surface. We show that PECAM-1 signaling regulates the binding of fluorescein isothiocyanate–labeled thrombin to the platelet surface and reduces the levels of cell surface GPIbα by promoting its internalization, while concomitantly reducing the binding of platelets to von Willebrand factor under flow in vitro. PECAM-1–mediated internalization of GPIbα was reduced in the presence of both EGTA and cytochalasin D or latrunculin, but not either individually, and was reduced in mice in which tyrosines 747 and 759 of the cytoplasmic tail of β3 integrin were mutated to phenylalanine. Furthermore, PECAM-1 cross-linking led to a significant reduction in the phosphorylation of glycogen synthase kinase-3β Ser[9], but interestingly an increase in glycogen synthase kinase-3α pSer[21]. PECAM-1–mediated internalization of GPIbα was reduced by inhibitors of dynamin (Dynasore) and glycogen synthase kinase-3 (CHIR99021), an effect that was enhanced in the presence of EGTA.

Conclusions—PECAM-1 mediates internalization of GPIbα in platelets through dual AKT/protein kinase B/glycogen synthase kinase-3/dynamin-dependent and αIIbβ3-dependent mechanisms. These findings expand our understanding of how PECAM-1 regulates nonimmunoreceptor signaling pathways and helps to explains how PECAM-1 regulates thrombosis. **(*Arterioscler Thromb Vasc Biol*. 2014;34:1968-1976.)**

Key Words: blood platelets ■ glycoproteins ■ signal transduction ■ thrombin ■ von Willebrand factor

Downloaded from http://ahajournals.org by on May 22, 2024

Platelet endothelial cell adhesion molecule-1 (PECAM-1) is a 130-kDa transmembrane glycoprotein expressed on the platelet surface at between 5000 and 8800 copies per cell.[1–3] It is composed of a 574-amino acid residue extracellular portion organized into 6 immunoglobulin-like homology domains, a 19-amino acid transmembrane domain, and a cytoplasmic domain that contains an immunoreceptor tyrosine-based inhibitory motif (ITIM; (L/V/I/S/T)XYXX(L/V)) and an immunoreceptor tyrosine-based switch motif (TxYxx(V/I)).[4,5] The tyrosine residues (residues 663 and 686) within the ITIM and immunoreceptor tyrosine-based switch motif become tyrosine phosphorylated by Src or Csk family kinases,[6] after homophilic ligation or stimulation of platelets through protease-activated receptor (PAR)-1 and 4, glycoprotein VI (GPVI), or GPIb in a process that is largely, but not wholly, dependent on integrin αIIbβ3–dependent platelet aggregation.[7–10]

The inhibition by PECAM-1 of immunoreceptor signaling downstream of GPVI is well established. Once phosphorylated, these twin ITIM and immunoreceptor tyrosine-based switch motif tyrosine residues support the recruitment of Src-homology 2 domain–containing proteins, including the protein-tyrosine phosphatases SHP-2, and to a lesser extent SHP-1,[11] to which the p85 regulatory subunit of PI3K (phosphatidylinositol 3-kinase) becomes associated destabilizing its association with the LAT (linker for activation of T) cells

Received on: February 13, 2014; final version accepted on: June 10, 2014.

From the Institute for Cardiovascular and Metabolic Research, School of Biological Sciences, University of Reading, Reading, Berkshire, United Kingdom.

The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.114.304097/-/DC1.

Correspondence to Chris Jones, PhD, Institute for Cardiovascular and Metabolic Research, School of Biological Sciences, University of Reading, Harbourne Building, Whiteknights, Reading, Berkshire, RG6 6AS, United Kingdom. E-mail c.i.jones@reading.ac.uk

© 2014 American Heart Association, Inc.

Nonstandard Abbreviations and Acronyms	
GPVI	glycoprotein VI
GSK-3	glycogen synthase kinase-3
ITIM	immunoreceptor tyrosine-based inhibitory motif
PECAM-1	platelet endothelial cell adhesion molecule-1
PMI	PECAM-1–mediated internalization
VWF	von Willebrand factor

and the scaffolding molecule Gab1 (Grb-2–associated binding protein-1).[12] By disturbing the LAT–Gab1–PI3K signaling complex, PECAM-1, therefore, disrupts the recruitment and activation of phospholipase C (PLCγ2),[12] thereby inhibiting immune-like signaling downstream of GPVI and reducing the activation of platelets by collagen, the GPVI-specific ligand cross-linked collagen-related peptide (CRP-XL) and convulxin.[10, 13, 14]

More surprising is the ability of PECAM-1 to reduce platelet activation through G protein-coupled receptor (GPCR)–mediated signaling pathways after stimulation by thrombin or ADP. Although hyper-reactivity to thrombin and ADP has not been detected in PECAM-1–deficient mice,[15] preactivation of PECAM-1 has been shown to reduce fibrinogen binding to platelets, secretion, and calcium mobilization in response to ADP and thrombin,[14] and in a laser injury model of thrombosis in cremaster muscle arterioles, that is dependent on thrombin generation rather than collagen exposure,[16] thrombi formed more rapidly, were more stable, and ≈35% larger in PECAM-1–deficient mice.[17] The mechanism by which PECAM-1 inhibits GPCR-mediated activation of platelets is as yet unclear.

While the inhibitory effect of PECAM-1 on individual pathways is modest, corresponding to 5% to 15% inhibition in platelet function, the physiological importance of PECAM-1 comes from its ability to inhibit multiple activation pathway. The combination of these effects account for a substantial inhibition in thrombus formation.[14,17]

We have previously reported that although PECAM-1 inhibits the activation of platelets by thrombin, it does not inhibit activation stimulated by thrombin receptor agonist peptide (TRAP). Here, we investigate this disparity and show that the activation of PECAM-1 triggers the internalization of GPIb by dual AKT/protein kinase B/glycogen synthase kinase-3 (GSK-3)/dynamin and αIIbβ3-dependent mechanisms, which result in the reduction in platelet binding of, and hence response to, thrombin and reduced platelet binding to von Willebrand factor (VWF).

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Activation of PECAM-1 Inhibits the Binding of Thrombin to Human Platelets and Their Subsequent Activation

Consistent with our previous work, activating PECAM-1 by cross-linking antibodies that recognize the sixth immunoglobulin domain of PECAM-1 (AB468), resulting in PECAM-1 phosphorylation,[12–14] led to a small (5%–15%) but significant inhibition in platelet fibrinogen binding and P-selectin exposure in response to thrombin across a range of concentration (P<0.0001 and P=0.001, respectively, 2-way ANOVA; Figure 1A). Again confirming our previous findings, PECAM-1 cross-linking did not inhibit platelet response to either PAR-1– or PAR-4–activating peptides (Figure 1A). The inhibition of thrombin activation of platelets without inhibiting PAR signaling suggests a role for PECAM-1 in modulating the binding of thrombin to platelets. Consistent with this cross-linking, PECAM-1 led to a significant reduction in the binding to fluorescein isothiocyanate–labeled thrombin and a concomitant reduction of platelet activation measured by the surface exposure of P-selectin (Figure 1B and Figure I in the online-only Data Supplement).

This reduction of thrombin binding was specific to the activation of PECAM-1. To control for the potential steric inhibition caused by the binding and cross-linking of the PECAM-1 antibody, we repeated these experiments using a different PECAM-1 antibody (WM59) that binds PECAM-1 but prevents its activation, thereby providing a control for any steric effects. No reduction in thrombin binding was seen when platelets were incubated with antibodies against the first or second immunoglobulin domain of PECAM-1 (WM59; which prevent homophilic ligation and the activation of PECAM-1[18]) and the cross-linking antibody (Figure 1C). Similarly, the binding and cross-linking of antibodies against GPIbα, GPVI, PAR-1, and PAR-4 had no effect on the binding of thrombin to the platelet surface (Figure 1C).

PECAM-1 Cross-Linking Reduces the Levels of GPIbα at the Cell Surface

GPIbα as part of the GPIb/V/IX complex plays an integral role in thrombin activation. It is a high-affinity receptor for thrombin[19] and acts as a cofactor for PAR cleavage.[20] Furthermore, PECAM-1 plays a role in GPIb signaling, becoming tyrosine phosphorylated on VWF binding to GPIbα, and PECAM-1–deficient mice show enhanced aggregation in response to VWF.[9] We hypothesized that the reduction in thrombin binding to platelets may be attributable to reduced surface expression of GPIbα after PECAM-1 activation. PECAM-1 cross-linking resulted in a significant reduction in the binding of antibodies against GPIbα and a reduction in antibodies against GPIbβ (Figure 2A and Figure II in the online-only Data Supplement). Although the level of GPIbα expressed on the surface of resting platelets varied among subjects, the reduction in GPIbα after PECAM-1 cross-linking occurred in all subjects. The action of PECAM-1 seemed to be specific for GPIb and did not cause a reduction in the binding of antibodies against PAR-1 and PAR-4, or GPVI (Figure 2A).

To confirm that this reduction was attributable to PECAM-1, PECAM-1 on the surface of wild-type mouse platelets was cross-linked using antibodies that recognize the sixth immunoglobulin domain of mouse PECAM-1 (M-185) and as with human blood resulted in a significant decrease in GPIbα on the platelet surface. No such decrease was seen in PECAM-1–deficient mouse platelets (Figure 2B). Again to control for

Downloaded from http://ahajournals.org by on May 22, 2024

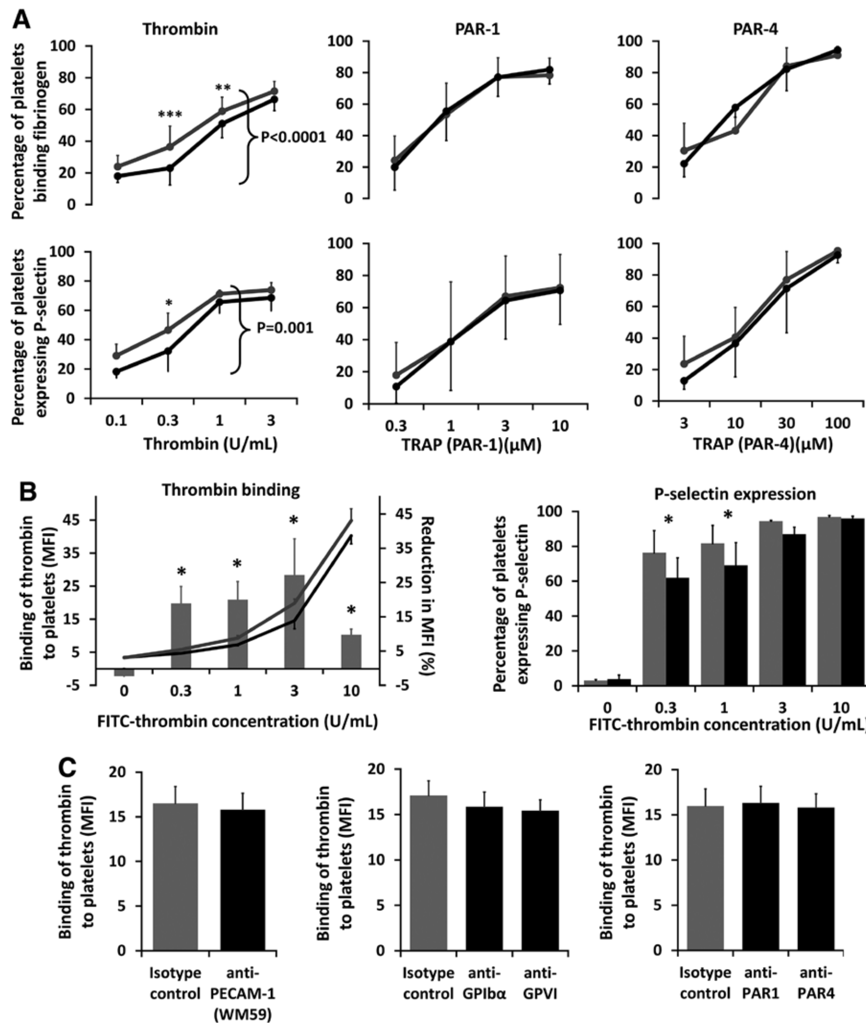

Figure 1. Activation of platelet endothelial cell adhesion molecule-1 (PECAM-1) inhibits the binding of thrombin to human platelets and their subsequent activation. **A**, Fibrinogen binding (**top row**) and P-selectin expression (**bottom row**) to human platelets in response to thrombin-, protease-activated receptor (PAR)-1–, or PAR-4–activating peptides in the presence (black) or absence (gray) of PECAM-1 (AB468) cross-linking and activation were measured by flow cytometry (n=5). *P* values compare the difference between the 2 curves over the entire concentration range using 2-way ANOVA with Bonferroni post-test analysis of the difference at individual concentrations. **B**, To ascertain whether reduced platelet response to thrombin was attributable to reduced thrombin binding, the reduction in the binding of fluorescein isothiocyanate (FITC)–labeled thrombin to human platelets (**left**) and the resultant surface exposure of P-selectin (**right**) were measured after PECAM-1 cross-linking. The binding of FITC-thrombin to platelets was measured by flow cytometry and is presented as the median fluorescent intensity (MFI; left-hand axis, presence [black line] or absence [gray line] of PECAM-1 [AB468] cross-linking) and the percentage reduction in MFI in PECAM-1 cross-linked samples compared with the isotype control (right-hand axis; n=5). **C**, To check that this reduction in thrombin binding was specific to the activation of PECAM-1 and not the binding of antibodies to the platelet surface, the binding of FITC-labeled thrombin to human platelets in the presence of cross-linked isotype control, PECAM-1 (WM59—inhibitory), glycoprotein Ibα (GPIbα), GPVI, PAR-1, or PAR-4 antibodies was measured (n=3). In all cases, *$P<0.05$, **$P\leq0.01$, ***$P<0.001$. TRAP indicates thrombin receptor agonist peptide.

Downloaded from http://ahajournals.org by on May 22, 2024

the effects of steric inhibitions caused by the binding and cross-linking of the PECAM-1 antibody, we repeated these experiments using a different PECAM-1 antibody (WM59) that binds PECAM-1 but prevents its activation. No reduction in GPIbα was seen when platelets were incubated with antibodies against the first or second immunoglobulin domain of PECAM-1 (WM59) and the cross-linking antibody (Figure 2C).

PECAM-1 Activation Inhibits Platelet Binding to VWF

Given these findings, we hypothesized that PECAM-1 cross-linking should also reduce platelet adhesion to VWF. The number of platelets in whole blood binding to VWF (100 μg/mL) under arterial flow conditions was indeed reduced after PECAM-1 cross-linking (Figure 2D–2G). This supports the role of PECAM-1 in regulating surface GPIbα and is in line with previous reports showing enhanced aggregation in response to VWF in PECAM-1–deficient mice.[9]

PECAM-1–Mediated Reduction of Platelet Surface GPIbα Is Attributable to Internalization and Not Cleavage

We sought to determine whether the PECAM-1–mediated reduction in the surface expression of GPIbα was attributable to internalization or cleavage of the receptor. Previous reports

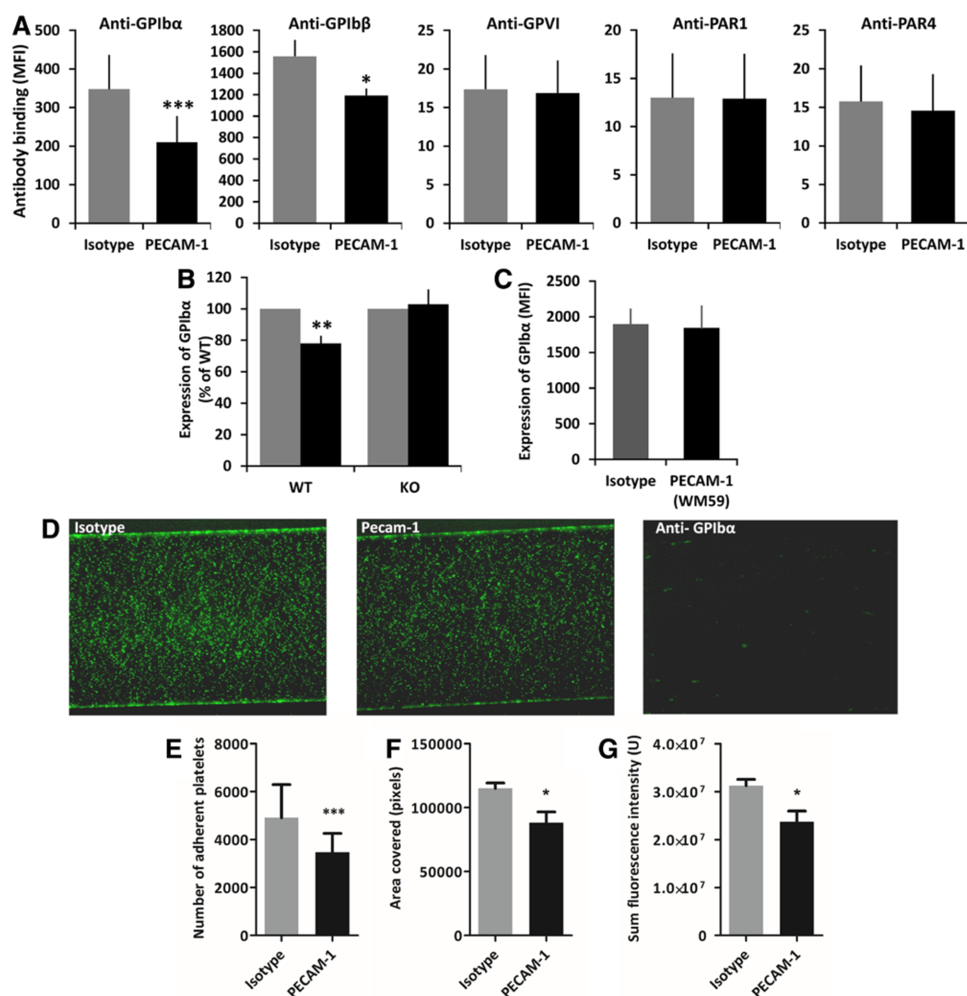

Figure 2. Activation of platelet endothelial cell adhesion molecule-1 (PECAM-1) reduces the surface expression of glycoprotein Ibα (GPIbα) and reduces platelet binding to von Willebrand factor (vWF). **A**, Human platelet surface expression of GPIbα, GPIbβ, GPVI, protease-activated receptor (PAR)-1, PAR-4 in the presence (black) or absence (gray) of PECAM-1 (AB468) cross-linking was measured by flow cytometry (n=12 for anti-GPIbα and n=3 for all other experiments). **B**, To confirm that this was PECAM-1 specific, the expression of GPIbα on the surface of mouse platelets from wild-type (WT; n=4) or PECAM-1 knockout (KO; n=6) mice after the cross-linking of isotype control (gray) or PECAM-1 stimulatory antibodies (black) was measured. **C**, To check that this reduction in the surface of GPIbα was specific to the activation of PECAM-1 and not the binding of antibodies to the platelet surface, human platelet surface expression of GPIbα was measured in the presence of cross-linked isotype control (gray), PECAM-1 (WM59—inhibitory; black; n=4). The difference in median fluorescent intensity (MFI) between **A** and **C** is because they were performed on different flow cytometers, an FACSCalibur and an Accuri C6, respectively. **D** to **G**, Given the importance of GPIbα in platelet tethering to VWF, we assessed the impact of PECAM-1 cross-linking on the binding of platelets to VWF under flow. **D**, Representative images showing human platelets binding to VWF (100 μg/mL) under flow conditions after the cross-linking of isotype control or PECAM-1 stimulatory antibodies, or, as a positive control, antibodies that block the binding of GPIb to VWF (Haematologic Technologies Inc). The number of human platelets binding to VWF (100 μg/mL; **E**), the area covered by (**F**), and the sum fluorescence intensity of (**G**) these platelets after the cross-linking of isotype control (gray) or PECAM-1 (black) stimulatory antibodies (n=3). **P*<0.05, ***P*≤0.01, ****P*<0.001.

Downloaded from http://ahajournals.org by on May 22, 2024

have demonstrated the cleavage of GPIbα by ADAM17, generating a ≈95-kDa fragment and a ≈45-kDa N-terminal fragment.[21] Using a purified cobra metalloproteinase, Nk (10 μg/mL)[22] as a positive control, we identified both the ≈130-kDa intact GPIbα and the ≈95-kDa and ≈45-kDa fragments by Western blotting, using antibodies against the C-terminal and N-terminal of GPIbα respectively (Figure 3A). Neither of these cleaved forms of GPIbα was observed in lysates of resting, CRP-XL–stimulated, or PECAM-1–stimulated platelets, indicating that no detectable cleavage of GPIbα occurred (Figure 3A). Furthermore, there was no evidence of any GPIbα cleavage products in these samples. Although there is a smear in the lane of the PECAM-1–stimulated platelets, this appears at the same height when probing with both the N-terminal and C-terminal antibodies and is present in the antibody control lanes. We therefore conclude that this smear is not attributable to cleavage of GPIbα.

To further test whether GPIbα was cleaved or internalized, we measured the binding of GPIbα antibodies to nonpermeablized and permeabilized platelets. As in all previous experiments, the binding of GPIbα antibodies to nonpermeablized platelets (ie, binding solely to GPIbα expressed on the surface of platelets) decreased after PECAM-1 stimulation. There was, however, no reduction in binding of GPIbα antibodies to permeabilized platelets (ie, when the GPIb antibody could access both external and internal pools of GPIb) indicating that there

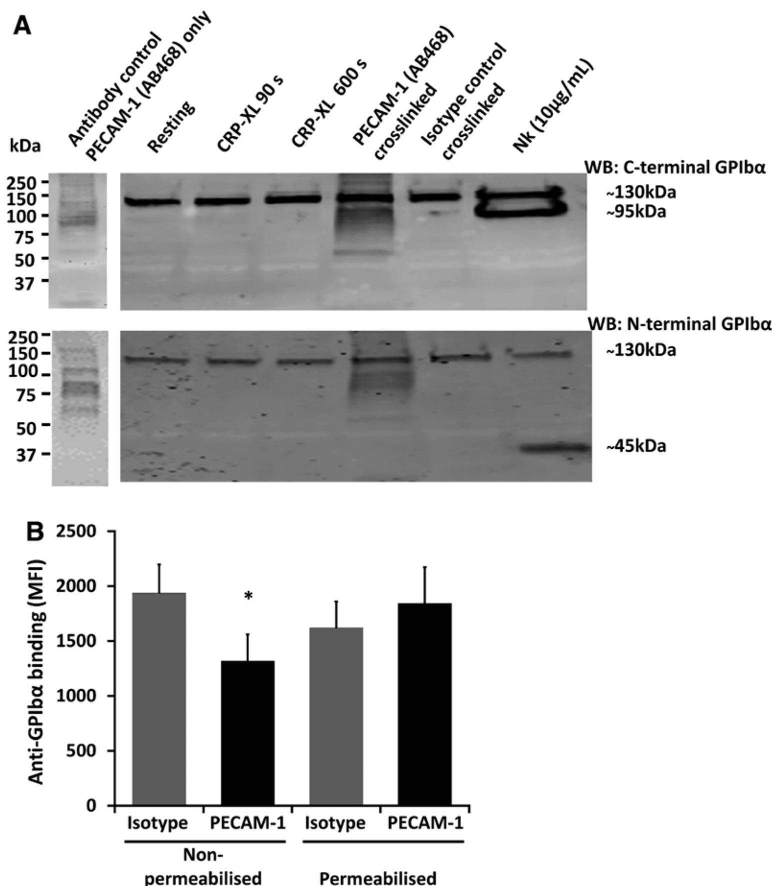

Figure 3. Glycoprotein Ibα (GPIbα) is internalized and not cleaved after platelet endothelial cell adhesion molecule-1 (PECAM-1) activation. **A**, To determine whether GPIbα is cleaved after PECAM-1 activation, human platelets that had been stimulated for 90 or 600 seconds with CRP-XL (1μg/mL), or undergone PECAM-1 activation via antibody cross-linking, or been exposed to purified cobra metalloproteinase, Nk (10 μg/mL), were probed by Western blotting (WB) using antibodies that recognize either the C- or the N-terminal domain of GPIbα. Cleavage products were clearly visible in samples treated with Nk but not CRP-XL– or PECAM-1–stimulated platelets. The smear present in the PECAM-1–stimulated samples appears at the same height when probing with both the N-terminal and C-terminal antibodies and appears when the antibodies are run alone in the absence of platelets. We therefore conclude that it is the result of protein in the stimulating antibodies rather than a result of cleavage. **B**, To identify the internalization of GPIbα after PECAM-1 activation, the binding of GPIbα antibodies to nonpermeablized human platelets (ie, binding to GPIbα expressed on the surface of platelets) or to permeabilized platelets (ie, binding to all GPIbα, internal and surface expressed) after PECAM-1 cross-linking was measured by flow cytometry (n=3). $^{*}P<0.05$.

Downloaded from http://ahajournals.org by on May 22, 2024

was no loss of GPIbα from the platelets (Figure 3B). Taken together, these 2 pieces of evidence suggest that PECAM-1–mediated reduction of platelet surface GPIbα is attributable to internalization and not cleavage.

PECAM-1 Internalization of GPIbα Is Dependent on Cytoskeletal Rearrangement and Exogenous Calcium Ions

The internalization of GPIbα is well known after platelet activation,[23] and PECAM-1 has, in other cells types, been shown to be involved in non–clathrin-mediated endocytosis.[24] To elucidate the mechanism by which PECAM-1–mediated internalization (PMI) of GPIbα occurs in platelets, the reduction in surface expression of GPIbα was measured after PECAM-1 activation in the presence of EGTA to chelate extracellular calcium ions and cytochalasin D or latrunculin and to prevent actin polymerization. Individually none of the compounds significantly inhibited PMI of GPIbα. A combination of EGTA and either cytochalasin D or latrunculin did, however, completely abrogate the effect of PECAM-1 activation on the surface expression of GPIbα (Figure 4A). By contrast, GPIbα internalization after stimulation of GPVI by CRP-XL was not inhibited by EGTA alone but was completely inhibited by either cytochalasin D or latrunculin (Figure 4B). This indicates that although GPVI-mediated internalization of GPIbα is entirely dependent on cytoskeletal rearrangement, the activation of PECAM-1 initiates 2 separate mechanisms, one dependent on cytoskeleton and the other dependent on exogenous divalent cations, both of which are capable of modulating the surface exposure of GPIbα independently.

Chelating extracellular calcium inhibits the activation of integrin αIIbβ3, the activation of which is known to be enhanced by PECAM-1.[15,25] To establish whether integrin αIIbβ3 is involved in PMI of GPIbα, we used knock-in mice (DiYF mice) in which tyrosines 747 and 759 of the cytoplasmic tail of the β3 integrin were mutated to phenylalanine, preventing tyrosine phosphorylation and selectively impairing outside-in αIIbβ3 signaling.[26] Internalization of GPIbα after PECAM-1 cross-linking was reduced in heterozygous mice and abolished in knock-in mice (Figure 4C). In corroboration of our findings in humans, internalization of GPIbα after stimulation with CRP-XL was unaltered in DiYF mice (Figure 4D). There was no significant difference among wild-type, heterozygous, or homozygous knock-in mice in the surface expression of either αIIbβ3 or GPVI (Figure 4E and 4F).

PMI of GPIbα Occurs in an AKT/GSK-3/Dynamin-Dependent Manner

PECAM-1 clustering has been reported to induce dynamin-2–dependent endocytosis in endothelial cells,[24, 27] whereas in platelets, dynamin has been shown to be critical to the internalization of both $P2Y_1$ and $P2Y_{12}$.[28] To investigate the role of dynamin in PMI of GPIbα, platelets were incubated with Dynasore (100 μmol/L) before antibody-mediated PECAM-1

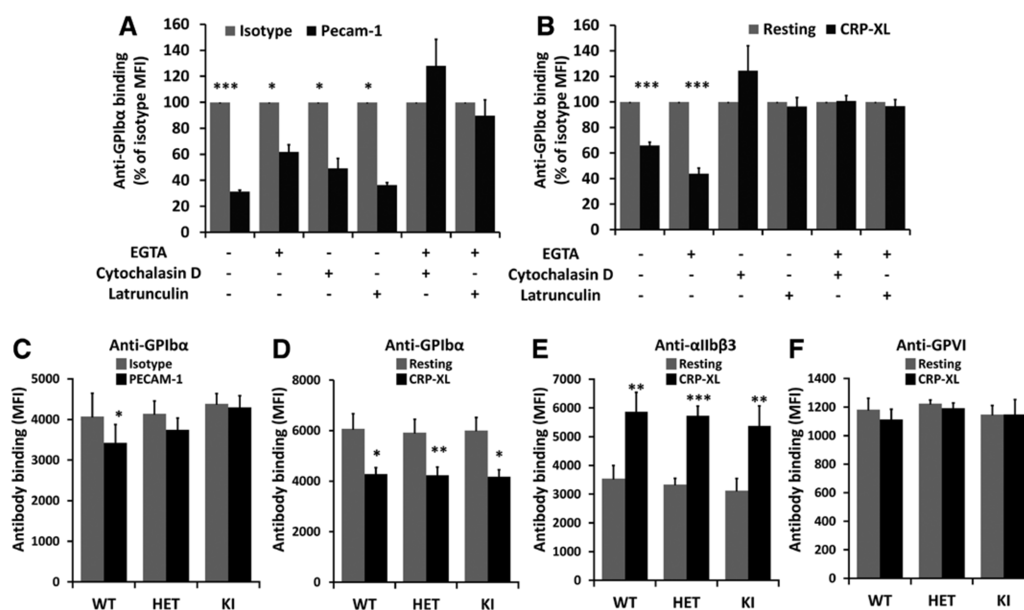

Figure 4. Actin polymerization and αIIbβ3 are required for platelet endothelial cell adhesion molecule-1 (PECAM-1)–mediated internalization of glycoprotein Ibα (GPIbα). Surface expression of GPIbα on human platelets after (**A**) PECAM-1 cross-linking or (**B**) stimulation with CRP-XL in the presence of EGTA (2 mmol/L), cytochalasin D (50 μmol/L), or latrunculin (8.5 μmol/L) was measured by flow cytometry (n=5). To test the involvement of αIIbβ3 in PECAM-1–mediated internalization of GPIbα, platelets from DiYF mice, in which tyrosines 747 and 759 of the cytoplasmic tail of the β3 integrin were mutated to phenylalanine, underwent (**C**) PECAM-1 cross-linking or (**D**) CRP-XL stimulation followed by measurement of surface GPIbα. The surface expression of (**E**) αIIbβ3 and (**F**) GPVI was also measured in these mice and showed no significant difference between the genotypes in resting or CRP-XL–stimulated conditions (wild type [WT]: n=5; heterozygous (Het): n=9; knock in [KI]: n=5). Asterisks indicate significant difference from isotype control or resting platelets in all cases. *$P<0.05$, **$P\leq0.01$, ***$P<0.001$. MFI indicates median fluorescent intensity.

Downloaded from http://ahajournals.org by on May 22, 2024

cross-linking. PMI of GPIbα was reduced in the presence of Dynasore and almost abolished in the presence of Dynasore and EGTA (Figure 5A). In part, the action of dynamin in endocytosis is regulated by the dephosphorylation (by calcineurin[29,30]) and rephosphorylation (by cdk5 and GSK-3[31,32]) of 2 serine residues in the C-terminal proline-rich domain of dynamin. Although dephosphorylation of dynamin is required for clathrin-mediated endocytosis in neurons, non–clathrin-mediated bulk endocytosis (of the type previously shown to result from PECAM-1 activation in endothelial cells[24]) requires both dephosphorylation and rephosphorylation.[32, 33] We have previously shown that PECAM-1 cross-linking resulted in reduced phosphorylation of AKT at Ser[473].[12] This should lead to a reduction in the phosphorylation of GSK-3, which would in turn regulate its kinase activity. After stimulation of platelets with CRP-XL, the phosphorylation of GSK-3β Ser[9] increased compared with the level in unstimulated cells; however, after PECAM-1 cross-linking, the phosphorylation of GSK-3β Ser[9] decreased (Figure 5B and 5C). Interestingly, the same pattern was not repeated for GSK-3α. Ser[21] phosphorylation of GSK-3α did not increase significantly in response to CRP-XL but did increase significantly after PECAM-1 cross-linking (Figure 5B and 5C). To confirm the role of GSK-3 in PMI, we used the highly selective inhibitor of both GSK-3 isoforms, CHIR99021, which inhibited the internalization of GPIbα after the cross-linking of PECAM-1 (Figure 5D). Together, these data suggest that the internalization of GPIbα after PECAM-1 stimulation occurs in a non–clathrin-, AKT/GSK-3/dynamin-dependent manner which may be similar to bulk endocytosis reported in endothelial or neuronal cells.

Discussion

Two of the enduring questions surrounding the role of PECAM-1 in regulating platelet response are how this ITIM-containing molecule inhibits nonimmunoreceptor signaling and how a molecule with what seems to be a moderate impact on individual signaling pathways can have such a profound effect on thrombus formation in vitro and in vivo.[14,17] By investigating the disparity between the action of PECAM-1 on platelet response to thrombin and TRAP, we have identified a PECAM-1–mediated receptor internalization pathway mediated via dual AKT/GSK-3/dynamin- and αIIbβ3-dependent mechanisms that result in the internalization of GPIbα, a reduction in thrombin binding to, and activation of, platelets, and reduced platelet binding to VWF. The physiological impact of PMI of GPIbα on the binding of platelets to vessel-bound VWF is uncertain because it is unlikely that PECAM-1 is activated before platelets bind to VWF and adhere to the site of vessel damage. This novel role for PECAM-1 in platelets does, however, explain how PECAM-1 inhibits GPCR-mediated activation of platelets by thrombin and why it has such a potent impact on thrombosis. By simultaneously reducing thrombin stimulation through PMI of GPIb, and GPVI signaling through its action as an ITIM-containing receptor, PECAM-1 directly regulates 2 of the major pathways by which platelets become activated and propagate thrombus formation.

PMI of GPIbα seems to occur via a distinct mechanism that is separate from the internalization of GPIbα that follows GPVI stimulation. This was seen in the internalization of GPIbα in response to CRP-XL in PECAM-1 knockout

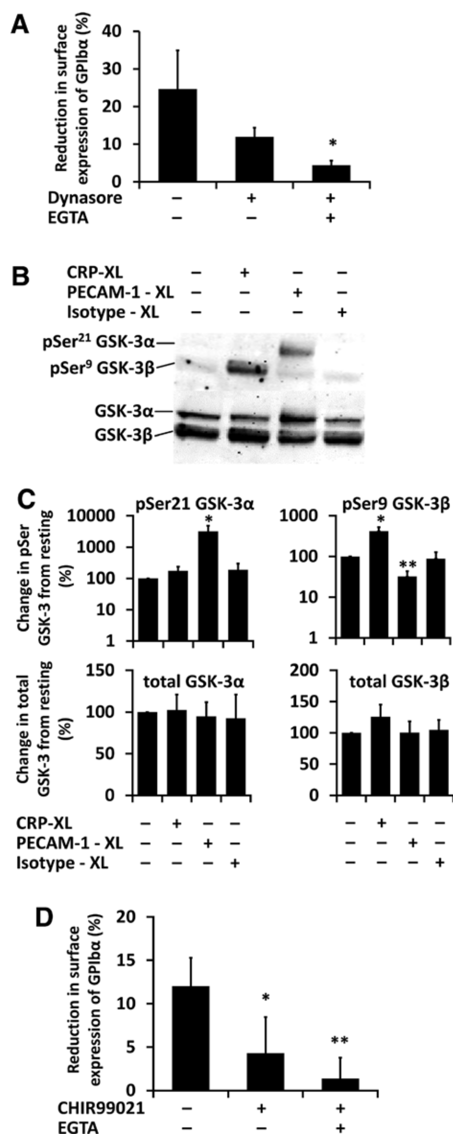

Figure 5. Platelet endothelial cell adhesion molecule-1 (PECAM-1)–mediated internalization of glycoprotein Ibα (GPIbα) is dependent on AKT/protein kinase B/glycogen synthase kinase-3 (GSK-3)/dynamin signaling. **A**, The reduction in the expression of GPIbα on the platelet surface resulting from PECAM-1 cross-linking was measured in the absence or presence of Dynasore (100 μmol/L) or Dynasore and EGTA together (n=8). **B**, A representative image showing phosphorylation of GSK-3α Ser^{21} and GSK-3β Ser^{9}, and total GSK-3α and -3β after platelet stimulation by CRP-XL (1 μg/mL) or PECAM-1 cross-linking. **C**, Quantification of Western blots showing phosphorylation of GSK-3α Ser^{21} and GSK-3β Ser^{9} and total GSK-3α and -3β after platelet stimulation by CRP-XL (1 μg/mL) or PECAM-1 cross-linking (n=3). **D**, The reduction in the expression of GPIbα on the platelet surface resulting from PECAM-1 cross-linking was measured in the absence or presence of the GSK-3 inhibitor CHIR99021 (3 μmol/L) or CHIR99021 (3 μmol/L) and EGTA (2 mmol/L) together (n=3). **P*<0.05, ***P*≤0.01, ****P*<0.001.

mice and the ability of cytochalasin D and latrunculin alone to inhibit CRP-XL–mediated internalization of GPIbα, but not PMI. It is likely, however, that in vivo these 2 mechanisms work in concert, particularly because PECAM-1 becomes phosphorylated after GPVI stimulation and because PECAM-1 and GPVI have opposing effects on AKT and hence GSK-3 phosphorylation. Why such opposing signaling events result in such similar outcomes is not yet known. The importance of PMI during thrombus formation may in part come from the activation of PECAM-1 by homophilic ligation, which only occurs during close platelet–platelet contact. Under these circumstances, stimulation of receptor internalization or endocytosis by PECAM-1 at the site of contact may be a mechanism for recycling receptors that are not occupied or bound into focal adhesion complexes. Trafficking these receptors away from the site of contact to areas of the platelet that are not in contact with other cells provides an attractive mechanism by which to maximize the efficiency of platelet response to multiple simultaneous stimuli and orchestrate thrombus growth.

The mechanism by which integrin β3 regulates PMI is as yet unknown. Its effect, however, seems to be critical. One explanation for this perhaps lies with filamin. Filamin binds to GPIbα in resting platelets anchoring it to the cytoskeleton,[34] helping to maintain the structure and integrity of the plasma membrane after platelet tethering to VWF under high shear.[35] On stimulation, filamin becomes detached from the cytoplasmic tail GPIbα, allowing its translocation.[36] By contrast, filamin binds to αIIbβ3 after platelet activation. One tempting hypothesis is that PECAM-1 plays a role, either directly or indirectly, in the decoupling of filamin from GPIbα and its subsequent binding to integrin β3. We have as yet been unable to confirm or refute this hypothesis, but it will be the focus of future work.

Of the 2 isoforms of GSK-3, it is the β-form that is thought to be predominant in platelets.[37] Proteomic analysis has, however, identified that the copy number per platelet for each isoform is similar, 1300 for GSK-3β and 1000 for GSK-3α.[38] Both isoforms are constitutively active in resting platelets but are inhibited by phosphorylation of Ser^{21} (GSK-3α) or Ser^{9} (GSK-3β) by protein kinase c or AKT on platelet stimulation.[39] It is thought that GSK-3 acts as a negative regulator of platelets; GSK-3 inhibitors increase platelet responses, GSK-3β$^{+/-}$ mice display increased aggregation and thrombus formation, and platelets from mice in which GSK-3α Ser^{21} and GSK-3β Ser^{9} have been mutated to Ala show reduced response.[37,39] The reduction on GSK-3β pSer^{9} after PECAM-1 cross-linking fits with this understanding of platelet GSK-3. PECAM-1, which has a well-established inhibitory effect on platelet signaling, reduces AKT phosphorylation and therefore reduces the phosphorylation and inhibition of GSK-3β, thereby enhancing its inhibitory effect, some of which may be mediated through dynamin and translocation of surface receptors, as we have shown to be the case with GPIb. The increase in GSK-3α phosphorylation after PECAM-1 cross-linking does not, however, fit with our current understanding. Part of the reason for this is that all previous work on GSK-3 in platelets has focused either on GSK-3β alone, as in the case of the GSK-3β$^{+/-}$ mice, or has assumed that both isoforms work in a similar way and have therefore inhibited both isoforms. In the case of dual inhibition, the effect of GSK-3α may be masked by GSK-3β making any interpretation of the physiological relevance of GSK-3α impossible. Although there is 85% similarity in the amino acid sequence between the isoforms and 98% homology in the kinase domain,[40] there is growing evidence

Downloaded from http://ahajournals.org by on May 22, 2024

that the 2 isoforms of GSK-3 have distinct roles.[41–43] Why the activation of PECAM-1 has such contrasting effects on the 2 GSK-3 isoforms and what significance GSK-3α phosphorylation has in PECAM-1 signaling are as yet unknown.

The inhibition of platelet activation by PECAM-1 is well known, but the mechanisms by which this ITIM-containing receptor inhibits platelet response to thrombin were not. Here, we have shown that the activation of PECAM-1 results in the internalization of GPIb by dual GSK-3/dynamin- and αIIbβ3-dependent mechanisms, which result in the reduction in platelet binding of, and hence response to, thrombin and reduced platelet binding to VWF. Furthermore, we have for the first time shown the differential regulation of GSK-3α/β by PECAM-1 in platelets. This novel role for PECAM-1 expands our understanding of how this promiscuous molecule regulates multiple diverse signaling pathways and why it has such a potent impact on thrombosis. It may also suggest a role for PECAM-1 in regulating conditions where the generation of thrombin and the response of platelets to thrombin are central to pathology.

Sources of Funding

This study was supported by British Heart Foundation grants to J.M. Gibbins (RG/05/007, RG/09/011, and FS/07/018).

Disclosures

None.

References

1. Newman PJ. The role of PECAM-1 in vascular cell biology. *Ann N Y Acad Sci*. 1994;714:165–174.
2. Wu XW, Lian EC. Binding properties and inhibition of platelet aggregation by a monoclonal antibody to CD31 (PECAM-1). *Arterioscler Thromb Vasc Biol*. 1997;17:3154–3158.
3. Metzelaar MJ, Korteweg J, Sixma JJ, Nieuwenhuis HK. Biochemical characterization of PECAM-1 (CD31 antigen) on human platelets. *Thromb Haemost*. 1991;66:700–707.
4. Jackson DE, Kupcho KR, Newman PJ. Characterization of phosphotyrosine binding motifs in the cytoplasmic domain of platelet/endothelial cell adhesion molecule-1 (PECAM-1) that are required for the cellular association and activation of the protein-tyrosine phosphatase, SHP-2. *J Biol Chem*. 1997;272:24868–24875.
5. Vivier E, Daëron M. Immunoreceptor tyrosine-based inhibition motifs. *Immunol Today*. 1997;18:286–291.
6. Cao MY, Huber M, Beauchemin N, Famiglietti J, Albelda SM, Veillette A. Regulation of mouse PECAM-1 tyrosine phosphorylation by the Src and Csk families of protein-tyrosine kinases. *J Biol Chem*. 1998;273:15765–15772.
7. Cicmil M, Thomas JM, Sage T, Barry FA, Leduc M, Bon C, Gibbins JM. Collagen, convulxin, and thrombin stimulate aggregation-independent tyrosine phosphorylation of CD31 in platelets. Evidence for the involvement of Src family kinases. *J Biol Chem*. 2000;275:27339–27347.
8. Jackson DE, Ward CM, Wang R, Newman PJ. The protein-tyrosine phosphatase SHP-2 binds platelet/endothelial cell adhesion molecule-1 (PECAM-1) and forms a distinct signaling complex during platelet aggregation. Evidence for a mechanistic link between PECAM-1- and integrin-mediated cellular signaling. *J Biol Chem*. 1997;272:6986–6993.
9. Rathore V, Stapleton MA, Hillery CA, Montgomery RR, Nichols TC, Merricks EP, Newman DK, Newman PJ. PECAM-1 negatively regulates GPIb/V/IX signaling in murine platelets. *Blood*. 2003;102:3658–3664.
10. Jones KL, Hughan SC, Dopheide SM, Farndale RW, Jackson SP, Jackson DE. Platelet endothelial cell adhesion molecule-1 is a negative regulator of platelet-collagen interactions. *Blood*. 2001;98:1456–1463.
11. Hua CT, Gamble JR, Vadas MA, Jackson DE. Recruitment and activation of SHP-1 protein-tyrosine phosphatase by human platelet endothelial cell adhesion molecule-1 (PECAM-1). Identification of immunoreceptor tyrosine-based inhibitory motif-like binding motifs and substrates. *J Biol Chem*. 1998;273:28332–28340.
12. Moraes LA, Barrett NE, Jones CI, Holbrook LM, Spyridon M, Sage T, Newman DK, Gibbins JM. Platelet endothelial cell adhesion molecule-1 regulates collagen-stimulated platelet function by modulating the association of phosphatidylinositol 3-kinase with Grb-2-associated binding protein-1 and linker for activation of T cells. *J Thromb Haemost*. 2010;8:2530–2541.
13. Cicmil M, Thomas JM, Leduc M, Bon C, Gibbins JM. Platelet endothelial cell adhesion molecule-1 signaling inhibits the activation of human platelets. *Blood*. 2002;99:137–144.
14. Jones CI, Garner SF, Moraes LA, Kaiser WJ, Rankin A, Ouwehand WH, Goodall AH, Gibbins JM; Bloodomics Consortium. PECAM-1 expression and activity negatively regulate multiple platelet signaling pathways. *FEBS Lett*. 2009;583:3618–3624.
15. Wee JL, Jackson DE. The Ig-ITIM superfamily member PECAM-1 regulates the “outside-in” signaling properties of integrin alpha(IIb)beta3 in platelets. *Blood*. 2005;106:3816–3823.
16. Falati S, Gross P, Merrill-Skoloff G, Furie BC, Furie B. Real-time *in vivo* imaging of platelets, tissue factor and fibrin during arterial thrombus formation in the mouse. *Nat Med*. 2002;8:1175–1181.
17. Falati S, Patil S, Gross PL, Stapleton M, Merrill-Skoloff G, Barrett NE, Pixton KL, Weiler H, Cooley B, Newman DK, Newman PJ, Furie BC, Furie B, Gibbins JM. Platelet PECAM-1 inhibits thrombus formation in vivo. *Blood*. 2006;107:535–541.
18. Sun QH, DeLisser HM, Zukowski MM, Paddock C, Albelda SM, Newman PJ. Individually distinct Ig homology domains in PECAM-1 regulate homophilic binding and modulate receptor affinity. *J Biol Chem*. 1996;271:11090–11098.
19. Buchanan SG, Gay NJ. Structural and functional diversity in the leucine-rich repeat family of proteins. *Prog Biophys Mol Biol*. 1996;65:1–44.
20. De Candia E, Hall SW, Rutella S, Landolfi R, Andrews RK, De Cristofaro R. Binding of thrombin to glycoprotein Ib accelerates the hydrolysis of Par-1 on intact platelets. *J Biol Chem*. 2001;276:4692–4698.
21. Gardiner EE, Karunakaran D, Shen Y, Arthur JF, Andrews RK, Berndt MC. Controlled shedding of platelet glycoprotein (GP)VI and GPIb-IX-V by ADAM family metalloproteinases. *J Thromb Haemost*. 2007;5:1530–1537.
22. Wijeyewickrema LC, Gardiner EE, Shen Y, Berndt MC, Andrews RK. Fractionation of snake venom metalloproteinases by metal ion affinity: a purified cobra metalloproteinase, Nk, from Naja kaouthia binds Ni2+-agarose. *Toxicon*. 2007;50:1064–1072.
23. Han Y, Nurden A, Combrié R, Pasquet JM. Redistribution of glycoprotein Ib within platelets in response to protease-activated receptors 1 and 4: roles of cytoskeleton and calcium. *J Thromb Haemost*. 2003;1:2206–2215.
24. Muro S, Wiewrodt R, Thomas A, Koniaris L, Albelda SM, Muzykantov VR, Koval M. A novel endocytic pathway induced by clustering endothelial ICAM-1 or PECAM-1. *J Cell Sci*. 2003;116(pt 8):1599–1609.
25. Varon D, Jackson DE, Shenkman B, Dardik R, Tamarin I, Savion N, Newman PJ. Platelet/endothelial cell adhesion molecule-1 serves as a costimulatory agonist receptor that modulates integrin-dependent adhesion and aggregation of human platelets. *Blood*. 1998;91:500–507.
26. Law DA, DeGuzman FR, Heiser P, Ministri-Madrid K, Killeen N, Phillips DR. Integrin cytoplasmic tyrosine motif is required for outside-in alphaIIbbeta3 signalling and platelet function. *Nature*. 1999;401:808–811.
27. Garnacho C, Shuvaev V, Thomas A, McKenna L, Sun J, Koval M, Albelda S, Muzykantov V, Muro S. RhoA activation and actin reorganization involved in endothelial CAM-mediated endocytosis of anti-PECAM carriers: critical role for tyrosine 686 in the cytoplasmic tail of PECAM-1. *Blood*. 2008;111:3024–3033.
28. Mundell SJ, Barton JF, Mayo-Martin MB, Hardy AR, Poole AW. Rapid resensitization of purinergic receptor function in human platelets. *J Thromb Haemost*. 2008;6:1393–1404.
29. Liu JP, Sim AT, Robinson PJ. Calcineurin inhibition of dynamin I GTPase activity coupled to nerve terminal depolarization. *Science*. 1994;265:970–973.
30. Marks B, McMahon HT. Calcium triggers calcineurin-dependent synaptic vesicle recycling in mammalian nerve terminals. *Curr Biol*. 1998;8:740–749.
31. Clayton EL, Sue N, Smillie KJ, O’Leary T, Bache N, Cheung G, Cole AR, Wyllie DJ, Sutherland C, Robinson PJ, Cousin MA. Dynamin I phosphorylation by GSK3 controls activity-dependent bulk endocytosis of synaptic vesicles. *Nat Neurosci*. 2010;13:845–851.
32. Tan TC, Valova VA, Malladi CS, Graham ME, Berven LA, Jupp OJ, Hansra G, McClure SJ, Sarcevic B, Boadle RA, Larsen MR, Cousin MA,

Downloaded from http://ahajournals.org by on May 22, 2024

Robinson PJ. Cdk5 is essential for synaptic vesicle endocytosis. *Nat Cell Biol*. 2003;5:701–710.
33. Clayton EL, Anggono V, Smillie KJ, Chau N, Robinson PJ, Cousin MA. The phospho-dependent dynamin-syndapin interaction triggers activity-dependent bulk endocytosis of synaptic vesicles. *J Neurosci*. 2009;29:7706–7717.
34. Andrews RK, Fox JE. Identification of a region in the cytoplasmic domain of the platelet membrane glycoprotein Ib-IX complex that binds to purified actin-binding protein. *J Biol Chem*. 1992;267:18605–18611.
35. Cranmer SL, Ashworth KJ, Yao Y, Berndt MC, Ruggeri ZM, Andrews RK, Jackson SP. High shear-dependent loss of membrane integrity and defective platelet adhesion following disruption of the GPIbα-filamin interaction. *Blood*. 2011;117:2718–2727.
36. Dyson JM, Munday AD, Kong AM, Huysmans RD, Matzaris M, Layton MJ, Nandurkar HH, Berndt MC, Mitchell CA. SHIP-2 forms a tetrameric complex with filamin, actin, and GPIb-IX-V: localization of SHIP-2 to the activated platelet actin cytoskeleton. *Blood*. 2003;102:940–948.
37. Li D, August S, Woulfe DS. GSK3beta is a negative regulator of platelet function and thrombosis. *Blood*. 2008;111:3522–3530.
38. Burkhart JM, Vaudel M, Gambaryan S, Radau S, Walter U, Martens L, Geiger J, Sickmann A, Zahedi RP. The first comprehensive and quantitative analysis of human platelet protein composition allows the comparative analysis of structural and functional pathways. *Blood*. 2012;120:e73–e82.
39. Moore SF, van den Bosch MT, Hunter RW, Sakamoto K, Poole AW, Hers I. Dual regulation of glycogen synthase kinase 3 (GSK3)α/β by protein kinase C (PKC)α and Akt promotes thrombin-mediated integrin αIIbβ3 activation and granule secretion in platelets. *J Biol Chem*. 2013;288:3918–3928.
40. Woodgett JR. Molecular cloning and expression of glycogen synthase kinase-3/factor A. *EMBO J*. 1990;9:2431–2438.
41. Azoulay-Alfaguter I, Yaffe Y, Licht-Murava A, Urbanska M, Jaworski J, Pietrokovski S, Hirschberg K, Eldar-Finkelman H. Distinct molecular regulation of glycogen synthase kinase-3alpha isozyme controlled by its N-terminal region: functional role in calcium/calpain signaling. *J Biol Chem*. 2011;286:13470–13480.
42. Ciaraldi TP, Nikoulina SE, Bandukwala RA, Carter L, Henry RR. Role of glycogen synthase kinase-3 alpha in insulin action in cultured human skeletal muscle cells. *Endocrinology*. 2007;148:4393–4399.
43. Hoeflich KP, Luo J, Rubie EA, Tsao MS, Jin O, Woodgett JR. Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. *Nature*. 2000;406:86–90.

Significance

We show 2 new facets of platelet endothelial cell adhesion molecule-1 (PECAM-1) biology in platelets: (1) that PECAM-1 mediates the internalization of glycoprotein Ibα leading to a reduction in the activation of platelets by thrombin and a reduction in their binding to von Willebrand factor and (2) that PECAM-1 differentially regulates the phosphorylation of the 2 isoforms of glycogen synthase kinase-3. In so doing, this article answers several outstanding questions concerning the regulation of platelets by PECAM-1: how this immunoreceptor tyrosine-based inhibitory motif–containing molecule inhibits nonimmunoreceptor signaling and importantly, how PECAM-1 has such a profound effect on thrombus formation in vitro and in vivo.

Downloaded from http://ahajournals.org by on May 22, 2024