The Cytoplasmic Domain of the Platelet Glycoprotein Ibα Is Phosphorylated at Serine 609*

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The α chain of the platelet von Willebrand factor receptor, glycoprotein (GP) Ib, is not known to be phosphorylated. Here, we report that the cytoplasmic domain of GPIbα is phosphorylated at Ser609; this was detected by immunoblotting with an anti-phosphopeptide antibody, anti-pS609, that specifically recognizes the GPIbα C-terminal sequence S609GLHSL only when Ser609 is phosphorylated. Immunoadsorption with anti-pS609 removed almost all of the GPIbα from platelet lysates, indicating a high proportion of GPIbα phosphorylation. Anti-pS609 inhibited GPIb-IX binding to the intracellular signaling molecule, 14-3-3. Dephosphorylation of GPIb-IX with potato acid phosphatase inhibited anti-pS609 binding and also 14-3-3 binding. A synthetic phosphopeptide corresponding to the GPIbα C-terminal sequence (SIRYGHSL), but not a nonphosphorylated identical peptide, abolished GPIb-IX binding to 14-3-3. Thus, phosphorylation at Ser609 of GPIbα is important for 14-3-3 binding to GPIb-IX. In certain regions of spreading platelets, particularly at the periphery, there was a reduction in GPIbα staining by anti-pS609 as observed under a confocal microscope, indicating that a subpopulation of GPIbα molecules in these regions is dephosphorylated. These data suggest that phosphorylation and dephosphorylation at Ser609 of GPIbα regulates GPIb-IX interaction with 14-3-3 and may play important roles in the process of platelet adhesion and spreading.

Platelet adhesion plays a critical role in thrombosis and hemostasis. Platelets in normal circulation are in a resting, nonadherent state. At sites of vascular injury, platelets adhere to the exposed subendothelial matrix. Under the high shear force of blood flow, platelet adhesion involves multiple steps. Initially, platelets adhere in a reversible manner (1). This process is mediated by the interaction between a platelet receptor for von Willebrand factor (vWF),1 the glycoprotein Ib-IX complex (GPIb-IX), and matrix-bound vWF (1–3). GPIb-IX interaction with vWF mediates signaling leading to activation of integrins that are responsible for platelet spreading and aggregation (1, 4).

GPIb-IX consists of three subunits: GPIba, GPIbb, and GPIc. GPIb-IX is loosely associated with glycoprotein V. The N-terminal domain of GPIbα contains binding sites for vWF and thrombin (for reviews see Refs. 5 and 6). The cytoplasmic domain of GPIbα contains a binding site (residues 536–568 (7)) for filamin (also called actin-binding protein or ABP-280), which links GPIb-IX to cross-linked actin filamental structures underlying the plasma membrane (the membrane skeleton) (8, 9). We found that an intracellular signaling molecule, 14-3-3, is associated with GPIb-IX (10). A binding site for 14-3-3 is located in a 15-amino acid residue serine-rich region (residues 595–610) at the C terminus of GPIbα (29). 14-3-3 binding also involves an additional 14-3-3 binding site in GPIbβ (11, 12).

The 14-3-3 family of highly conserved intracellular proteins interacts with several intracellular serine/threonine kinases and other signaling molecules (13–22) and regulates their functions (15, 23–26). A recognition motif, RXSxFXP, has been identified in c-Raf and several other 14-3-3 ligands, requiring a phosphorylated serine residue (27, 28). Thus, interaction of many intracellular signaling proteins with 14-3-3 is regulated by phosphorylation. We hypothesized previously that serine residues in the 14-3-3 binding site of GPIbα might be important for 14-3-3 binding (29). However, it is not clear whether 14-3-3 binding is regulated by phosphorylation of these serine residues, as 14-3-3 can interact with synthetic nonphosphorylated peptides corresponding to GPIbα cytoplasmic domain (11, 29). Further, GPIbα has been thought to be a nonphosphorylated protein because previous studies failed to show phosphorylation of GPIbα (30). In this study, we re-examined phosphorylation states of GPIbα using a phosphoserine-specific anti-GPIbα antibody. We report here that the cytoplasmic domain of GPIbα is phosphorylated at Ser609 and that phosphorylation at this site is important for 14-3-3 binding to intact platelet GPIb-IX. Furthermore, we show that GPIbα dephosphorylation occurs at the edge of spreading platelets, suggesting that phosphorylation and dephosphorylation of Ser609 in the cytoplasmic domain of GPIbα is involved in regulating GPIb-IX functions during platelet adhesion and spreading.

EXPERIMENTAL PROCEDURES

Reagents—Anti-peptide antibody anti-Iβc, recognizing the C-terminal domain of GPIbα, was described previously (29). Monoclonal antibody WM23 against GPIbα and purified vWF were kindly provided by Dr. Michael Berndt (Baker Medical Research Institute, Melbourne, Australia) (31). Monoclonal antibody SZ2 against GPIbα was kindly provided by Dr. Changgeng Ruan (Suzhou Medical College, Suzhou, China) (32). Recombinant 14-3-3ζ protein and 14-3-3-conjugated Sepharose beads were prepared as described previously (29, 33). Peptides and phosphopeptides were synthesized by the Protein Research Laboratory, University of Illinois at Chicago and purified by high performance liquid chromatography, and their molecular mass was verified by electron-spray mass spectrometry.

Anti-peptide antibody against the Phosphopeptide Corresponding to the C Terminus of GPIbα—A phosphopeptide (CSGHGSL) corresponding to a C-terminal 5-residue sequence of GPIbα (plus an N-terminal cysteine for conjugation) was conjugated to keyhole limpet hemocyanin (Sigma) as described previously (34). An anti-peptide antibody was raised by im-

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1 The abbreviations used are: vWF, von Willebrand factor; GP, glycoprotein; GPIb-IX, glycoprotein Ib-IX complex; PAGÉ, polyacrylamide gel electrophoresis; MBP, maltose-binding protein; PAP, potato acid phosphatase; pS, phosphoserine.
FIG. 1. Specific binding of anti-pS609 antibody to Ser<sup>609</sup>-phosphorylated peptides corresponding to GPIba C-terminal sequences. A, synthetic phosphopeptide CSGHpsL or nonphosphorylated identical peptide CSGHSL was coated onto microtiter plates. Anti-pS609 antiserum or preimmune serum (negative result not shown) was incubated in the microtiter wells for 2 h at 22 °C. After further incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG, bound antibody was determined by measuring A<sub>490</sub> nm as described under “Experimental Procedures.” B, synthetic phosphopeptide SIRYSGHpsL with phosphorylated Ser<sup>609</sup>, an identical nonphosphorylated peptide (SIRYSGHSL), or an identical peptide with phosphorylation at Ser<sup>609</sup> but not Ser<sup>606</sup> (SIRYpSGHSL) were coated onto the microtiter well, and binding of the anti-pS609 serum to each of these peptides was measured as described in A. Note that anti-pS609 reacts only with the Ser<sup>609</sup>-phosphorylated peptide. C, microtiter plates were coated with synthetic phosphopeptides SIRYSGHpsL, an identical nonphosphorylated peptide SIRYSGHSL, a phosphopeptide SIRYpGSHSL (phosphorylation at Ser<sup>606</sup>), or a negative control peptide corresponding to GPIbβ C-terminal 14-amino acid sequence (IbβC). The microtiter wells were incubated with an antiserum, anti-IbαC, directed against the nonphosphorylated GPIbα C-terminal sequence. The comparable amounts of binding of this antibody to various GPIbα peptides indicates that comparable amounts of these peptides were coated on the microtiter wells. Shown in the figure are the results from three samples (mean ± S.D.).
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Fig. 2. Phosphorylation-dependent binding of anti-pS609 to the platelet GPIbα. A, washed platelets were directly solubilized into SDS-sample buffer, analyzed by SDS-PAGE, and then immunoblotted with a control preimmune serum (Control) or anti-pS609 serum. B, platelets were solubilized in Triton X-100-containing buffer as described under "Experimental Procedures." GPIb-IX were immunoprecipitated with control IgG or an anti-GPIbα monoclonal antibody, SZ2 (SZ-2). Control IgG and SZ-2 immunoprecipitates as well as platelet lysates were immunoblotted with anti-pS609. C, the lysates were treated with (PAP) or without (No PAP) 6 units/ml potato acid phosphatase as described under "Experimental Procedures" and then analyzed by SDS-PAGE and immunoblotting with anti-pS609 or anti-IbC (reactive with both phosphorylated and nonphosphorylated GPIbα C-terminal sequence). Note that potato acid phosphatase treatment inhibited anti-pS609 binding to GPIbα but did not affect the binding of anti-IbC.

microtiter plates (Fig. 1C). These data indicate that anti-pS609 specifically binds to GPIbα C-terminal sequences only when Ser<sup>609</sup> is phosphorylated (Fig. 1).

To examine whether Ser<sup>609</sup> of the platelet GPIbα is phosphorylated, washed resting platelets were solubilized directly into SDS-containing sample buffer and immunoblotted with anti-pS609. Fig. 2A shows that anti-pS609 specifically reacted with a band with its molecular weight identical to that of GPIbα. To verify that anti-pS609 indeed reacted with GPIbα, platelets were solubilized and GPIbα was immunoprecipitated with a monoclonal antibody, SZ2, directed against GPIbα. The immunoprecipitates were then immunoblotted with anti-pS609. Fig. 2B shows that anti-pS609 indeed reacted with immunoprecipitated GPIbα. As the binding of anti-pS609 requires phosphorylation at Ser<sup>609</sup>, these results indicate that the Ser<sup>609</sup> in the cytoplasmic domain of GPIbα is phosphorylated. To further verify that the reactivity of anti-pS609 with GPIbα requires phosphorylation, platelets were solubilized and treated with PAP to dephosphorylate proteins. As shown in Fig. 2C, treatment of platelet lysates with PAP dramatically inhibited the binding of anti-pS609 to GPIbα. Inhibition in anti-pS609 binding did not result from the loss of GPIbα because such treatment did not affect the recognition of GPIbα by the antibody (anti-IbC) that reacts with both the phosphorylated and nonphosphorylated GPIbα C-terminal sequence. Thus, binding of anti-pS609 indeed requires phosphorylation of GPIbα. Taken together, the above results indicate that Ser<sup>609</sup> in the cytoplasmic domain of platelet GPIbα is phosphorylated in resting platelets.

Stoichiometry of Ser<sup>609</sup> Phosphorylation—To examine the stoichiometry of Ser<sup>609</sup> phosphorylation, washed platelets were solubilized and lysates were immunoprecipitated with anti-pS609 to deplete the GPIbα population containing phosphorylated Ser<sup>609</sup>. The GPIbα that remained in platelet lysates was then detected by immunoblotting with the antibody (anti-IbC). Fig. 3A shows that preabsorption by anti-pS609, but not by preimmune serum, removed most of the GPIbα molecules (>95%) from platelet lysates. In contrast, anti-pS609 failed to remove GPIbα from the PAP-dephosphorylated platelet lysates (Fig. 3B). Thus, the majority of the GPIbα molecules in Triton X-100-soluble platelet lysates are phosphorylated at Ser<sup>609</sup>. As a population of GPIb-IX is associated with the Triton X-100-insoluble cytoskeleton of platelets, we also examined whether phosphorylated GPIbα is present in the Triton X-100-insoluble fractions corresponding to the cytoskeleton (precipitated by centrifugation at 15,000 × g) and the membrane skeleton (precipitated at 100,000 × g) using the method reported by Fox (9).

Fig. 3. Stoichiometry and distribution of GPIbα Ser<sup>609</sup> phosphorylation. A, washed platelets were solubilized, immunoadsorbed with anti-pS609 to remove Ser<sup>609</sup>-phosphorylated GPIbα, and then analyzed by SDS-PAGE and immunoblotting with the monoclonal anti-GPIbα antibody, WM23. B, platelets were first treated with (+PAP) or without (No PAP) PAP and then immunoadsorbed with anti-pS609 or control preimmune serum. Lysates were then analyzed by SDS-PAGE and immunoblotting with anti-GPIbα monoclonal antibody SZ2. C, washed platelets were solubilized as described previously (9). The platelet lysates were centrifuged at 14,000 × g for 5 min (low speed) and the supernatant was again centrifuged at 100,000 × g for 3 h (high speed). The pellets from low speed and high speed centrifugations as well as the final supernatants were solubilized in identical final volumes of SDS-sample buffer and Western blotted with anti-pS609 (phosphorylation-specific) or anti-IbC (reactive with both phosphorylated or nonphosphorylated GPIbα). Note the similar distribution patterns of GPIbα as detected with anti-pS609 or anti-IbC.

Anti-pS609 Inhibits 14-3-3 Binding to Platelet GPIb-IX—To examine whether the phosphoserine-dependent epitope of anti-pS609 is involved in 14-3-3 binding, platelet lysates were preincubated with anti-pS609 and then with 14-3-3-coated Sepharose beads. As a control, platelet lysates were preincubated with preimmune serum from the same rabbit. Preincubation with anti-pS609 but not the control serum inhibited the binding of GPIb-IX to 14-3-3-coated beads, suggesting that the SGP35-36 sequence recognized by anti-pS609 is proximate to the 14-3-3 binding site (Fig. 4).

Phosphorylation at Ser<sup>609</sup> of GPIbα Is Important for the Binding of Platelet GPIb-IX to 14-3-3 Protein—To investigate whether 14-3-3 binding to GPIb-IX is regulated by phosphorylation, the platelet lysates were pretreated with PAP to dephosphorylate proteins. This treatment inhibited the binding of anti-pS609 to GPIbα (Fig. 2B), suggesting that Ser<sup>609</sup> is dephosphorylated. PAP-treated platelet lysates were then allowed to interact with recombinant 14-3-3-ζ-conjugated beads. As shown in Fig. 5, GPIb-IX from platelet lysates bound to 14-3-3-ζ-coated beads, and this binding was dramatically reduced by PAP treatment. Thus, phosphorylation of GPIb-IX is required for high affinity binding between GPIb-IX and 14-3-3-ζ.

To further investigate whether phosphorylation at Ser<sup>609</sup> of GPIbα is important for the interaction between GPIbα C-terminal sequence and 14-3-3-ζ, a nonphosphorylated peptide with a sequence corresponding to the C-terminal region of
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FIG. 4. Inhibition of 14-3-3 binding to GPIb-IX by anti-pS609. Platelet lysates were first incubated with anti-pS609 serum or preimmune negative control serum and then further incubated with control MBP-conjugated beads or 14-3-3 conjugated beads using the method described previously (29). After three washes, the 14-3-3-bound GPIb-IX was detected by Western blot with monochonal antibody WM23.

FIG. 5. Phosphorylation of GPIb-IX regulates 14-3-3 binding. Platelet lysates were first incubated with (+PAP) or without (−PAP) potato acid phosphatase as described under “Experimental Procedures” and then further incubated with 14-3-3- or MBP-conjugated beads. Bead-bound GPIb-IX was detected by Western blot with anti-GPIb monoclonal antibody, WM23. GPIbα in platelet lysates (Lysate) treated with (+PAP) or without PAP (−PAP) were also immunoblotted with WM23 to show that amounts of GPIb-IX were not significantly changed following PAP incubation.

GPIbα, S<sup>602</sup>IRYSGHSL<sup>610</sup>, and identical peptides phosphorylated at Ser<sup>609</sup> or Ser<sup>606</sup> were synthesized. Sepharose beads conjugated with recombinant 14-3-3<sub>ξ</sub> were preincubated with these peptides (1 mM) and then allowed to interact with GPIb-IX. As shown in Fig. 6, preincubation with nonphosphorylated or Ser<sup>606</sup>-phosphorylated peptides did not significantly affect the binding of GPIb-IX to 14-3-3<sub>ξ</sub>-conjugated beads. In contrast, preincubation with the Ser<sup>609</sup>-phosphorylated peptide almost completely abolished GPIb-IX binding. Inhibition by the Ser<sup>609</sup>-phosphorylated GPIbα cytoplasmic domain peptide was concentration-dependent, with the half-maximal inhibition at ~50 μM (Fig. 6B). These data suggest that phosphorylation at Ser<sup>609</sup> of GPIbα is required for the high affinity binding of platelet GPIb-IX to 14-3-3<sub>ξ</sub>.

In Situ Distribution of Ser<sup>609</sup>-phosphorylated and Dephosphorylated GPIbα in Spreading Platelets—To examine whether phosphorylation of GPIbα is regulated in intact platelets, freshly washed platelets were allowed to adhere to a vWF- or fibrinogen-coated surface and were then fixed and permeabilized. These platelets were double-stained with anti-pS609 and a monoclonal antibody, WM23, against the extracellular region of GPIbα and were then scanned under a confocal microscope. Platelets adherent on fibrinogen and vWF were similar in spreading Platelets—(A). synthetic peptides (1 mM) corresponding to the GPIbα C-terminal sequence SIRYSGHSL, a Ser<sup>609</sup>-phosphorylated identical peptide, SIRYpGHSL, a Ser<sup>609</sup>-phosphorylated form of the same peptide, SIRYSGHpSL, or a negative control peptide corresponding to the GPIbα C-terminal 14 amino acid residues (IbβC) were incubated with 14-3-3<sub>ξ</sub>-conjugated Sepharose beads (14-3-3) or MBP-conjugated beads (Control) at 4 °C for 1 h. Platelet lysates (150 μl) were then added and incubated for additional 1 h at 4 °C, and after washing, the bead-bound GPIb-IX was detected by immunoblotting with anti-GPIbα antibody, anti-IbαC. B, control MBP-conjugated beads or 14-3-3-conjugated beads were preincubated with increasing concentrations of nonphosphorylated GPIbα C-terminal peptide (SIRYSGHSL) or the Ser<sup>609</sup>-phosphorylated peptide (SIRYSGHpSL) and then allowed to bind to GPIb-IX as described in A. The relative quantity of bead-bound GPIb-IX was estimated by scanning the GPIbα bands and then analyzing them by NIH Image for optical density. Percentages of inhibition by the peptides were calculated by the formula: Inhibition % = [1 − OD (sample)/OD (positive control)] × 100. Shown in the figure are the results (mean ± S.D.) from three experiments.

FIG. 6. A GPIbα C-terminal peptide phosphorylated at Ser<sup>609</sup> inhibits 14-3-3 binding to GPIb-IX. A, synthetic peptides (1 mM) corresponding to the GPIbα C-terminal sequence SIRYSGHSL, a Ser<sup>609</sup>-phosphorylated identical peptide, SIRYpGHSL, a Ser<sup>609</sup>-phosphorylated form of the same peptide, SIRYSGHpSL, or a negative control peptide corresponding to the GPIbα C-terminal 14 amino acid residues (IbβC) were incubated with 14-3-3<sub>ξ</sub>-conjugated Sepharose beads (14-3-3) or MBP-conjugated beads (Control) at 4 °C for 1 h. Platelet lysates (150 μl) were then added and incubated for additional 1 h at 4 °C, and after washing, the bead-bound GPIb-IX was detected by immunoblotting with anti-GPIbα antibody, anti-IbαC. B, control MBP-conjugated beads or 14-3-3-conjugated beads were preincubated with increasing concentrations of nonphosphorylated GPIbα C-terminal peptide (SIRYSGHSL) or the Ser<sup>609</sup>-phosphorylated peptide (SIRYSGHpSL) and then allowed to bind to GPIb-IX as described in A. The relative quantity of bead-bound GPIb-IX was estimated by scanning the GPIbα bands and then analyzing them by NIH Image for optical density. Percentages of inhibition by the peptides were calculated by the formula: Inhibition % = [1 − OD (sample)/OD (positive control)] × 100. Shown in the figure are the results (mean ± S.D.) from three experiments.
phosphorylated Ser606-containing sequence (SIRYpSGH) did not react with GPIb-IX by the anti-phosphopeptide antibody (Fig. 3A); this result is consistent with the previous result of Andrews et al. (11) showing that a nonphosphorylated GPIb-IX C-terminal peptide failed to abolish the binding between 14-3-3 and GPIb-IX. Furthermore, dephosphorylation of GPIb-IX by PAP or preincubation with anti-pS609 inhibited 14-3-3 binding (Figs. 4 and 5). Thus, it is likely that high affinity interaction between the intact platelet GPIb-IX and 14-3-3 requires phosphorylation of Ser609 of GPIbα. It is interesting to note that the 14-3-3 binding site of GPIbα (RYSGHSL) shares similarities with the RXS-PXP-like motifs of other phosphorylated 14-3-3 ligands; they all contain an arginine and a serine at the N-terminal side of the phosphorylated serine (27, 28). Most of the identified RXS-PXP motifs are present in the middle of the protein sequence, and the proline in the motif may possibly form a turn exposing the phosphoserine. Because the 14-3-3 binding site in GPIbα is already exposed at the C terminus, it may not require the presence of a proline residue. However, despite the similarities, there are striking differences between GPIbα and the RXS-PXP-like ligands. The prototype RXS-PXP-like ligand of 14-3-3, c-Raf, requires the helix G region of 14-3-3 (33), and the crystal structure data suggest that phosphoserine in the RXS-PXP motif may interact with residues in the more N-terminal helix C and E region of 14-3-3 (28, 39). In contrast, GPIbα binds to the helix I region of 14-3-3 (33), which forms an amphipathic ligand contact surface (40). Furthermore, synthetic peptides corresponding to C-terminal 15 residues of GPIbα bound to 14-3-3 without requiring phosphorylation (11, 29), and the recombinant GPIbα cytoplasmic domain, which is not phosphorylated at Ser609 (data not shown), also binds to 14-3-3 but with phosphorylation of GPIbα remains to be identified. Several protein kinase inhibitors had no effect on GPIbα phosphorylation, including inhibitors of protein kinase A, protein kinase G, and protein kinase C (data not shown). It is thus possible that these kinases are not involved in GPIbα phosphorylation. However, as the default state of GPIbα appears to be a phosphorylated form, it is also possible that the ineffectiveness of these protein kinase inhibitors is due to the fact that GPIbα is already in a relatively stable phosphorylated state and thus immune to the effects of protein kinase inhibitors.

Phosphorylation at Ser609 of GPIbα is important for GPIb-IX interaction with 14-3-3. This conclusion is supported by our finding that the Ser609-phosphorylated GPIbα C-terminal domain peptide (SIRYpSGHSL), but not the identical nonphosphorylated or Ser609-phosphorylated peptides, inhibited GPIb-IX interaction with 14-3-3 in a concentration-dependent manner (Fig. 6), suggesting that interaction between GPIb-IX and 14-3-3 involves a binding site in 14-3-3 that interacts with the Ser609-phosphorylated GPIbα C-terminal sequence. This result is consistent with the previous result of Andrews et al. (11) showing that a nonphosphorylated GPIbα C-terminal peptide failed to abolish the binding between 14-3-3 and GPIb-IX. Furthermore, dephosphorylation of GPIb-IX by PAP or preincubation with anti-pS609 inhibited 14-3-3 binding (Figs. 4 and 5). Thus, it is likely that high affinity interaction between the intact platelet GPIb-IX and 14-3-3 requires phosphorylation of Ser609 of GPIbα. It is interesting to note that the 14-3-3 binding site of GPIbα (RYSGHSL) shares similarities with the RXS-PXP-like motifs of other phosphorylated 14-3-3 ligands; they all contain an arginine and a serine at the N-terminal side of the phosphorylated serine (27, 28). Most of the identified RXS-PXP motifs are present in the middle of the protein sequence, and the proline in the motif may possibly form a turn exposing the phosphoserine. Because the 14-3-3 binding site in GPIbα is already exposed at the C terminus, it may not require the presence of a proline residue. However, despite the similarities, there are striking differences between GPIbα and the RXS-PXP-like ligands. The prototype RXS-PXP-like ligand of 14-3-3, c-Raf, requires the helix G region of 14-3-3 (33), and the crystal structure data suggest that phosphoserine in the RXS-PXP motif may interact with residues in the more N-terminal helix C and E region of 14-3-3 (28, 39). In contrast, GPIbα binds to the helix I region of 14-3-3 (33), which forms an amphipathic ligand contact surface (40). Furthermore, synthetic peptides corresponding to C-terminal 15 residues of GPIbα bound to 14-3-3 without requiring phosphorylation (11, 29), and the recombinant GPIbα cytoplasmic domain, which is not phosphorylated at Ser609 (data not shown), also binds to 14-3-3 but with phosphorylation of GPIbα.
a much lower affinity than GPIb-IX from platelets (33). This suggests that the interaction of 14-3-3 with GPIbα may involve both phosphorylation-dependent and phosphorylation-independent binding mechanisms. However, in intact platelet GPIb-IX, Ser609 phosphorylation is required for the high affinity binding of 14-3-3.

Phosphorylation of the Ser609 of GPIbα is likely to play important roles in GPIb-IX-mediated platelet adhesion and signaling. First, phosphorylation of Ser609 of GPIbα regulates 14-3-3 binding (Figs. 4 and 5), and we have evidence that 14-3-3 binding to GPIb-IX plays an important role in GPIb-IX signaling. Furthermore, our data indicate that a population of GPIbα becomes dephosphorylated at the peripheral of platelets during platelet spreading on vWF or fibrogen (Fig. 7), suggesting that the phosphorylation state of GPIbα can be dynamically regulated and that phosphorylation or dephosphorylation of GPIbα may have a functional role during platelet spreading. Although further studies are required to understand how phosphorylation of GPIbα may play a role in GPIb-IX function, one possibility is that phosphorylation regulates GPIb-IX-associated membrane skeleton organization and thus regulates the movement of GPIb-IX. This possibility is supported by the finding of Dong et al. (41) that a GPIb-IX mutant, lacking the C-terminal 4 amino acid residues including Ser609 is more likely to move laterally on the membrane. However, we show in Fig. 3 that Ser609-phosphorylated GPIbα is distributed in both cytoskeleton and non-cytoskeleton fractions, suggesting that Ser609 phosphorylation does not directly regulate association between GPIb-IX and the membrane skeleton. Consistent with this result, GPIbα association with the membrane skeleton has been shown to be mediated by filamin, which binds to the central region of the GPIbα cytoplasmic domain distinct from the C terminus (7), and mutant GPIb-IX that lacks the C-terminal domain of GPIbα is still associated with filamin and the membrane skeleton (42). However, it is possible that GPIb-IX-associated cytoskeleton organization or movement of GPIb-IX may be indirectly regulated by phosphorylation of GPIbα and 14-3-3 binding via intracellular signaling pathways.

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