Regulation of von Willebrand Factor Binding to the Platelet Glycoprotein Ib-IX by a Membrane Skeleton-dependent Inside-out Signal*

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The platelet receptor for von Willebrand factor (vWF), glycoprotein Ib-IX (GPIb-IX), mediates initial platelet adhesion and activation. We show here that the receptor function of GPIb-IX is regulated intracellularly via its link to the filament-associated membrane skeleton. Deletion of the filament binding site in GPIbα markedly enhances ristocetin- (or botrocetin)-induced vWF binding and allows GPIb-IX-expressing cells to adhere to immobilized vWF under both static and flow conditions. Cytoskeleton D (CD) that depolymerizes actin also enhances vWF binding to wild type GPIb-IX. Thus, vWF binding to GPIb-IX is negatively regulated by the filament-associated membrane skeleton. In contrast to native vWF, binding of the isolated recombinant vWF A1 domain to wild type and filament binding-deficient mutants of GPIb-IX is comparable, suggesting that the membrane skeleton-associated GPIb-IX is in a state that prevents access to the A1 domain in macromolecular vWF. In platelets, there is a balance of membrane skeleton-associated and free forms of GPIb-IX. Treatment of platelets with CD increases the free form and enhances vWF binding. CD also reverses the inhibitory effects of prostaglandin E1 on vWF binding to GPIb-IX. Thus, GPIb-IX-dependent platelet adhesion is doubly controlled by vWF conformation and a membrane skeleton-dependent inside-out signal.

Platelet adhesion plays a critical role in thrombosis and hemostasis. Under the influence of shear forces created by blood flow, initial platelet adhesion is dependent on the interaction between a platelet receptor for von Willebrand factor (vWF), the glycoprotein Ib-IX complex (GPIb-IX), and matrix-bound vWF (1–5). GPIb-IX consists of three subunits: GPIbα, GPIbβ, and GPIX. GPIX. GPIb-IX is associated with glycoprotein V. The N-terminal domain of GPIbα contains binding sites for vWF and α-thrombin (for reviews, see Refs. 6 and 7). The cytoplasmic domain of GPIbα contains a binding site 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) (6, 8). An intracellular signaling molecule, 14-3-3, is associated with GPIb-IX (10), and a phosphorylation-dependent binding site for 14-3-3 is located at the C terminus of GPIbα (11, 12), distinct from the binding site for filamin, which is located between residues 536–568 (13).

In the normal circulation, GPIb-IX does not interact with soluble vWF. At sites of vascular injury, the interaction between platelet GPIb-IX and vWF occurs when GPIb-IX is exposed to subendothelium-bound vWF. Interaction of vWF with the subendothelium is thought to induce a conformational change in vWF (14) and thus allow GPIb-IX binding (for reviews, see Refs. 6, 7, and 15). In vitro, the change in vWF can be mimicked by surface immobilization of vWF (16), or by the binding of vWF modulators such as ristocetin and botrocetin (17–22). In this study, we found that vWF binding to GPIb-IX is also regulated by changes in the cytoskeletal association of GPIb-IX. GPIb-IX molecules that are associated with the filament-linked membrane skeleton are in a “resting” state that inhibits vWF binding function. Dissociation of GPIb-IX from the membrane skeleton or disruption of the membrane skeleton activates vWF binding to GPIb-IX by increasing the accessibility of the binding site in GPIbα. Thus, GPIb-IX-dependent platelet adhesion and activation is likely to be dynamically controlled by a 2-fold regulatory mechanism: exposure of vWF bound to the subendothelial matrix and a membrane skeleton-dependent inside-out signal.

EXPERIMENTAL PROCEDURES

Reagents—Botrocetin and monoclonal antibodies AK2 and WM23 against GPIbα were kindly provided by Dr. Michael Berndt (Baker Medical Institute, Melbourne, Australia) (19). Human vWF was either kindly provided by Dr. Michael Berndt or purified from cryoprecipitates using the method described previously (23). Recombinant A1 domain fragment of vWF has been described previously (22). The monoclonal antibody against GPIbα, LJ-P3 (24), and monoclonal antibody against A1 domain of vWF, NMC-4 (25), have been previously described. Monoclonal antibodies, SS29 against vWF (26) and S22 against GPIbα (27), were generous gifts from Dr. Changgeng Ruan (Suzhou Medical College, Suzhou, China); CDNA clones encoding wild type GPIbα, GPIbβ, and GPIX were kindly provided by Dr. Jose Lopez (28–30).

Cell Lines Expressing Recombinant Proteins—Transfection of cDNA into Chinese hamster ovary (CHO) cells were performed according to the previously described methods using LipofectAMINE (Life Technologies, Inc.) (31). Stably transfected cell lines were selected using selection media containing 0.5 mg/ml G418 and/or 0.2 mg/ml hygromycin and further selected by cell sorting using antibodies recognizing GPIbα. The following cell lines were used: cells expressing wild type GPIb-IX complex (169); cells expressing GPIb-IX mutants with truncated GPIbα cytoplasmic domains at residues 591 (Δ591), 559 (Δ559), and 551...
cells expressing GPIb-IX and/or integrin were selected by cell sorting and monitored by flow cytometry.

Flow Cytometry Analysis of vWF Binding to GPIb-IX-expressing Cells and Platelets—CHO cells expressing wild type and mutant GPIb-IX were detached from the tissue culture plate with 0.5 mM EDTA in PBS and resuspended in PBS containing 1% bovine serum albumin or in phosphate-buffered saline (PBS) solution, pH 7.4. The cells were then reconstituted in PBS containing 1% bovine serum albumin or in PBS solution. Resting washed platelets were treated with various concentrations of cytochalasin D and/or PGE1. Me2SO was used as a control receptor (0.5 or 1 μM, respectively). vWF was measured on images obtained at different positions in the flow path of the chamber, corresponding to selected wall shear rates.

**Regulation of vWF Binding to GPIb-IX by the Filamin-associated Membrane Skeleton—**Wild type and the Δ559 mutant GPIb-IX expressed in CHO cells were allowed to bind soluble vWF in the presence of a vWF modulator, ristocetin, which is known to mimic the effect of subendothelial matrix to induce vWF binding to GPIb-IX. Ristocetin-induced binding of vWF to cells expressing wild type GPIb-IX (1b9 cells) is detectable but very low (Fig. 2A). In contrast, ristocetin-induced vWF binding to Δ559 mutant cells is about 10 times higher than to 1b9 cells (Fig. 2A). The difference in vWF binding to 1b9 cells and Δ559 cells is not due to a difference in the expression levels of GPIb-IX, since expression levels had been adjusted to comparable levels by guest on July 22, 2018http://www.jbc.org/Downloaded from

results

**Results**

GPIb-IX Mutants with Deletions in the Cytoplasmic Domain of GPIbα—To examine the roles of the GPIbα cytoplasmic domain in regulating the receptor function of GPIb-IX, wild type GPIbα or cytoplasmic domain deletion mutants of GPIbα were coexpressed in CHO cells with GPIbβ and GPIIX as previously described (11, 31). The mutants Δ559 and Δ551 lack the C-terminal residues 560–610 and 552–610, respectively, and thus lack the binding sites for filamin and 14-3-3 (11, 32). Both Δ559 (Fig. 1) and Δ551 (not shown) are not associated with the Triton X-100-insoluble membrane skeleton and do not bind 14-3-3 (11). The mutant Δ551 lacks the binding sites for 14-3-3 but retains the filament binding site and has been previously shown to bind to the membrane skeleton but not 14-3-3 (11, 32). The mutant Δ551–570 lacks a critical region in the filament binding site (32) and thus is not associated with the Triton X-100-insoluble membrane skeleton (Fig. 1). This mutant, however, retains a functional 14-3-3 binding site (Fig. 1).

Regulation of vWF Binding to GPIb-IX by the Filamin-associated Membrane Skeleton—Wild type and the Δ559 mutant GPIb-IX expressed in CHO cells were allowed to bind soluble vWF in the presence of a vWF modulator, ristocetin, which is known to mimic the effect of subendothelial matrix to induce vWF binding to GPIb-IX. Ristocetin-induced binding of vWF to cells expressing wild type GPIb-IX (1b9 cells) is detectable but very low (Fig. 2A). In contrast, ristocetin-induced vWF binding to Δ559 mutant cells is about 10 times higher than to 1b9 cells (Fig. 2A). The difference in vWF binding to 1b9 cells and Δ559 cells is not due to a difference in the expression levels of GPIb-IX, since expression levels had been adjusted to comparable levels by cell sorting using anti-GPIbα monoclonal antibody (Fig. 2B). A different truncation mutant, Δ551 (deleting residues 552–610), similarly had an enhanced vWF binding capacity (not shown). These data indicate that the small segment of wild type GPIb-IX binds to vWF, and deletion of the filament and 14-3-3 binding sites of GPIbα enhances vWF binding function of GPIb-IX.

Although the Δ559 mutant lacks both the filament and 14-3-3 binding sites in the cytoplasmic domain of GPIbα, the increase in vWF binding to Δ559 is unlikely to be caused by the lack of...
Regulation of vWF Binding to GPIb-IX

14-3-3 binding site, since cells expressing the mutant Δ591, lacking the GPIbα C-terminal 14-3-3 binding sites (residues 591–610) but retaining the filamin binding site, showed no dramatic increase in vWF binding compared with wild type GPIb-IX (Fig. 3A). Furthermore, cells expressing a GPIbα deletion mutant Δ551–570, lacking the residues required for filamin binding but retaining a functional 14-3-3 binding site, showed a significantly enhanced vWF binding (Fig. 3B). Thus, a lack of the filamin binding site of GPIbα is responsible for the enhanced vWF binding function.

To exclude the possibility that deletion mutation of GPIbα caused a conformational change unrelated to GPIb-IX association with the filamin-linked membrane skeleton actin filaments, cells expressing wild type GPIb-IX were treated with cytochalasin D to disrupt actin filamental structures. This treatment induces a significant increase in vWF binding to GPIb-IX (Fig. 4). Taken together, these data indicate that either disruption of filamin interaction with the cytoplasmic domain of GPIbα or disruption of actin filamental structure enhances vWF binding function of GPIb-IX. Thus, vWF binding to GPIb-IX is negatively regulated by the filamin-linked membrane skeleton.

Binding of Wild Type and Mutant GPIb-IX to Recombinant A1 Domain of vWF—It is known that the GPIb binding site in vWF is located in the A1 domain (36–38). Thus, it is possible that the inability of a majority of the wild type GPIb-IX molecules to bind vWF either results from a lack of A1 domain recognition by the wild type GPIb-IX or from a negative regulation that reduces the accessibility of the A1 domain in vWF macromolecules to the binding sites in GPIb-IX. To differentiate these possibilities, we examined ristocetin-induced (Fig. 5) or botrocetin-induced (not shown) GPIb-IX binding to a small recombinant A1 domain fragment of vWF containing the GPIb-IX binding site (residues 445–733) (22). Similar results were obtained either with ristocetin or botrocetin as an inducer. While the binding of the purified native vWF to wild type GPIb-IX is significantly lower than vWF binding to Δ559 mutant cells, the small recombinant A1 domain fragment of vWF showed comparable binding to wild type GPIb-IX and Δ559 mutant cells (Fig. 5). Thus, association of GPIb-IX with the membrane skeleton negatively regulates the accessibility of the A1 domain in native vWF macromolecules to its binding site in GPIbα.

Deletion of Filamin Binding Sites of GPIbα Enhances GPIb-IX-Mediated Static Cell Adhesion to vWF—The above results show that ristocetin- or botrocetin-induced binding of soluble vWF to GPIb-IX is regulated by the interaction of the membrane skeleton with the cytoplasmic domain of GPIbα. To examine whether ristocetin-induced vWF binding appropriately reflects the function of GPIb-IX to mediate cell adhesion to immobilized vWF, cells expressing wild type GPIb-IX or the mutants were allowed to adhere to vWF-coated microtiter wells at 37 °C for 30 min. As shown in Fig. 6A, cells expressing wild type GPIb-IX adhered to immobilized vWF with significantly higher intensity than cells expressing the Δ559 mutant. Furthermore, cells expressing a GPIbα deletion mutant showing a lack of the filamin binding site of GPIbα, showed a significantly enhanced vWF binding (Fig. 3).

FIG. 2. Effects of GPIbα cytoplasmic domain deletion on vWF binding function of GPIb-IX. A, CHO cells expressing wild type GPIb-IX (1b9) or cells expressing the deletion mutant Δ559 (lacking C-terminal residues 559–610) were solubilized at 22 °C in the presence of 1.25 mg/ml ristocetin and increasing concentrations of purified vWF for 30 min. The bound vWF was detected by flow cytometry analysis of the binding of FITC-labeled monoclonal antibody against human vWF, S229, as described under “Experimental Procedures.” The fluorescence intensity (geomean) of vWF binding was corrected by the ratio of GPIb-IX levels between two cell lines (1b9/Δ559 = 1/1.18) as determined in B. B, cells expressing wild type GPIb-IX (1b9) and the Δ559 mutant were incubated with 20 μg/ml of anti-GPIbα monoclonal antibody S22 and then FITC-labeled goat-anti-mouse IgG to detect surface-expressed GPIb-IX levels.

FIG. 1. Deletion mutants of GPIb-IX. A, a schematic depicting various cytoplasmic domain deletion mutants of GPIbα. B, CHO cells expressing wild type GPIb-IX (1b9). Cells expressing the deletion mutants Δ559 or Δ551–570 were solubilized as described under “Experimental Procedures.” The Triton X-100-soluble (S) and insoluble (I) proteins were separated by ultracentrifugation at 100,000 × g and then immunoblotted with a monoclonal anti-GPIbα antibody, WM23. Note that wild type GPIb-IX is associated with Triton X-100-insoluble fraction, but the Δ559 and Δ551–570 were Triton X-100-soluble. C, mutant Δ551–570 was solubilized and incubated with 14-3-3-conjugated beads as previously described (34). The bead-bound GPIb-IX was detected by immunoblotting with anti-GPIbα monoclonal antibody SZ2. The binding of wild type and other mutants of GPIb-IX to 14-3-3 has been previously described (11).
Deletion of the Filamin Binding Site of GPIba Enables Cell Adhesion to Immobilized vWF.

Adhesion to vWF under Flow—An important function of GPIb-IX is to mediate initial cell adhesion and rolling under flow conditions. Thus, we examined whether deletion of the filamin-binding site of GPIb-a also affects GPIb-IX-mediated cell adhesion under flow. The difference between wild type and mutant GPIb-IX in mediating cell adhesion to immobilized vWF is dramatic under flow conditions (Fig. 6, B and C). Even at a low shear rate of 200 s⁻¹, very few 1b9 cells (wild type) attached or rolled on vWF-coated surfaces. With the increase in shear rate to >500 s⁻¹, almost no 1b9 cells were seen to roll on vWF-coated surfaces. In contrast, when an equal number of Δ559 cells was perfused through the vWF-coated flow chamber, significant cell adhesion and rolling were observed even at a high shear rate of 1500 s⁻¹, suggesting that the mutant GPIb-IX lacking the filamin binding site is able to mediate efficient cell adhesion and rolling on immobilized vWF under high shear rate flow conditions. Thus, GPIb-IX-mediated cell adhesion to vWF under both low and high shear rate conditions is significantly enhanced by disruption of GPIb-IX interaction with the filamin-associated membrane skeleton.

vWF Binding to CHO Cells Coexpressing GPIb-IX and Integrin α₂bβ₃—Although platelets have two major receptors for vWF, GPIb-IX and the integrin α₂bβ₃ (39), it is known that ristocetin-induced vWF binding to platelets is GPIb-IX-dependent and unaffected by anti-integrin antibodies (39). However, binding of vWF to GIb-IX has been shown to induce fibrinogen binding to integrin α₂bβ₃ (31, 40). Also, the GPIba mutant Δ559 enhanced integrin-dependent CHO cell spreading on vWF (31). Thus, to study whether the membrane skeleton regulates vWF binding function of GPIb-IX in platelets, we first examined whether the ristocetin-induced vWF binding to cells expressing wild type GPIb-IX or the filamin-binding-deficient mutant (Δ559) can be influenced by the presence of the integrin α₂bβ₃ in our assay system. To do this, two CHO cells lines coexpressing wild type GPIb-IX (123 cells) and the mutant (Δ559/2b3a) with the integrin α₂bβ₃ were examined for ristocetin-induced vWF binding. Similar to cells expressing GPIb-IX alone, ristocetin-induced vWF binding is significantly enhanced in Δ559/2b3a cells compared with 123 cells (wild type) (Fig. 7). Ristocetin-induced vWF binding to 123 or Δ559/2b3a cells was unaffected by the integrin inhibitor RGDS peptides (Fig. 7). Thus, ristocetin-induced soluble vWF binding either in wild type or mutant GPIb-IX cells is not significantly influenced by the presence of integrin α₂bβ₃. This finding, together with the data obtained with α₂bβ₃-deficient cells (Fig. 2), suggests that the enhanced vWF binding in the cytoplasmic do-
In contrast, anti-GPIb antibody NMC-4. FITC-labeled anti-A1 domain monoclonal antibody, SZ29 as in Fig. 2. The binding of A1 domain was detected using the FTTC-labeled anti-A1 domain monoclonal antibody NMC-4.

Control
in the presence (\(\text{WT}\)) or absence (\(\text{A1}\)) of ristocetin. The binding of vWF was detected using SZ29 as in Fig. 2. The binding of A1 domain was detected using the FTTC-labeled anti-A1 domain monoclonal antibody NMC-4.

Control
in the presence (\(\text{WT}\)) or absence (\(\text{A1}\)) of ristocetin. These cells were also incubated with the recombinant A1 domain of vWF in the presence (\(\text{A1}\)) or absence (\(\text{Control}\)) of ristocetin. The binding of vWF was detected using SZ29 as in Fig. 2. The binding of A1 domain was detected using the FTTC-labeled anti-A1 domain monoclonal antibody NMC-4.

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Deletion of filamin-binding sites enhances GPIb-IX-mediated cell adhesion to vWF under static and flow conditions. CHO cells expressing wild type GPIb-IX (1b9) or GPIb-IX mutants, Δ559 or Δ551–570, lacking the filamin binding site in the cytoplasmic domain of GPIbα were incubated in vWF-coated microtiter wells for 30 min at 37 °C. After three washes, the adherent cells were quantitated by an acid phosphatase assay as described under “Experimental Procedures.” Shown are results from three samples (mean ± S.D.). A, quantitative evaluation of cell adhesion to immobilized vWF in a flow field at the indicated shear rates. Adhesion of CHO cells expressing either wild type GP Ib-IX (1b9) or the mutant Δ559 was evaluated on single frames randomly selected from a real time recording of flowing cells. The image acquisition rate was 30 frames/s. The results shown are the mean ± S.E. of cell counts/mm² from 15 separate frames. At least two experiments were performed for each experimental condition, with reproducible results. C, the four panels provide a representation of selected single frames (area = 65,536 μm²) for each experimental condition. The sharper images obtained with Δ559 cells at 200 s⁻¹ reflect their slower rolling velocity. All adhesion events observed with the GPIb-IX-expressing cells, whether wild type or Δ559, were transient.

previous study by Cunningham et al. (32), wild type GPIb-IX-expressing cells appeared to contain two populations with respect to vWF binding levels, and half of the cells poorly bound vWF. Although the reason for this difference is not clear, it is possible that two different vWF-binding levels of wild type GPIb-IX-expressing cells under the conditions used by Cunningham et al. (32) reflect two different states of GPIb-IX-associated membrane skeleton in different cell populations. Our data are consistent with the finding of Cunningham et al. (32) that nearly all CHO cells expressing filamin binding-deficient GPIb-IX mutants showed high levels of vWF binding. Our data are also consistent with the finding of Cranmer et al. (43) that CHO cells expressing wild type GPIb-IX poorly adhered to human vWF. Cranmer et al. (43) reported that adhesion and rolling of wild type GPIb-IX-expressing CHO cells on bovine vWF were similar to those of cells expressing a filamin binding-deficient GPIb-IX mutant under shear rates up to 1500 s⁻¹, and were slower in rolling velocity at 3000 s⁻¹. However, it is known that bovine vWF is different from human vWF in that it can bind to human platelet GPIb-IX without requiring any vWF modulation (such as immobilization or ristocetin) (44, 45). This difference indicates that binding of bovine vWF to human platelets is not regulated by the physiological regulatory mechanisms in human. Thus, the data obtained using bovine vWF, although useful in characterizing ligand binding function of GPIb-IX, cannot be applied to elucidate the regulatory mechanism of vWF-GPIb-IX interaction in humans.

Platelets have two major receptors for vWF, GPIb-IX and integrin αIIbβ3 (39). It has been shown that vWF binding to GPIb-IX activates integrin αIIbβ3 and facilitates integrin-dependent cell spreading on vWF (31, 40, 46). Here we show that the levels of ristocetin-induced soluble vWF binding to platelets and CHO cells expressing both integrin and GPIb-IX were not significantly influenced by the integrin αIIbβ3 (Figs. 7 and 8). This result is consistent with the published findings that ristocetin-induced vWF to platelets is completely inhibited by anti-GPIb antibodies but unaffected by anti-integrin antibodies (39). Since vWF molecules are very large multimers that are likely to hinder the access of other vWF molecules to nearby membrane surface, and a single vWF molecule contains multiple distinct binding sites for GPIb-IX and integrin αIIbβ3, these results suggest a possibility that vWF binding to GPIb-IX preferably stimulates the interaction between the integrin and vWF already bound to GPIb-IX, resulting in no significant increase in the total amounts of bound vWF. Thus, disruption of GPIb-IX-association with the membrane skeleton enhances vWF binding by up-regulating ligand binding function of GPIb-IX but not that of integrins.

The filamin-associated membrane skeleton appears to regulate the vWF binding function of GPIb-IX by controlling ligand access to the binding site of the receptor. This conclusion is supported by the observation that while the binding of native macromolecular vWF to wild type GPIb-IX is minimal, binding of the small recombinant A1 domain of vWF to wild type GPIb-IX is comparable with that seen with filamin-binding deficient GPIb-IX mutants (Fig. 5). Also, the difference in vWF binding between wild type and the mutants is unlikely to result from the difference in amounts of GPIb-IX molecules exposed...
treated with Me_2SO (DMSO) or cytochalasin D (CD) and then solubilized in Triton X-100-containing buffer as previously described (9). Platelet lysates were centrifuged at 14,000 g for 3 h. The Triton X-100-insoluble pellets and supernatant were immunoblotted with a monoclonal antibody against GPIbα, SSA. The reaction was detected by peroxidase-conjugated secondary antibody and enhanced chemiluminescence (Amersham Pharmacia Biotech). The intensities of GPIbα bands were scanned and quantitated using NIH Image software. Results (mean ± S.D.) from three experiments are shown.

3 Platelet lysates were centrifuged at 14,000 g and the membrane skeleton was isolated in Triton X-100-containing buffer as previously described (9). Platelet lysates were centrifuged at 14,000 g for 4 min and then ultracentrifuged at 100,000 × g for 3 h. The Triton X-100-insoluble pellets and supernatant were immunoblotted with a monoclonal antibody against GPIbα, SSA. The reaction was detected by peroxidase-conjugated secondary antibody and enhanced chemiluminescence (Amersham Pharmacia Biotech). The intensities of GPIbα bands were scanned and quantitated using NIH Image software. Results (mean ± S.D.) from three experiments are shown.

B–D washed resting platelets (1.5 × 10⁹/ml) in modified Tyrode’s solution were incubated with or without cytochalasin D for 5 min. The platelets were then washed, centrifuged at 14,000 × g for 3 min and then ultracentrifuged at 100,000 × g for 3 h. The Triton X-100-insoluble pellets and supernatant were immunoblotted with a monoclonal antibody against GPIbα, SSA. The reaction was detected by peroxidase-conjugated secondary antibody and enhanced chemiluminescence (Amersham Pharmacia Biotech). The intensities of GPIbα bands were scanned and quantitated using NIH Image software. Results (mean ± S.D.) from three experiments are shown.

Effects of cytochalasin D on GPIb-IX-associated membrane skeleton and on vWF binding to GPIb-IX in platelets. A, washed resting platelets (1.5 × 10⁹/ml) in modified Tyrode’s buffer were treated with Me_2SO (DMSO) or cytochalasin D (CD) and then solubilized in Triton X-100-containing buffer as previously described (9). Platelet lysates were centrifuged at 14,000 × g for 4 min and then ultracentrifuged at 100,000 × g for 3 h. The Triton X-100-insoluble pellets and supernatant were immunoblotted with a monoclonal antibody against GPIbα, SSA. The reaction was detected by peroxidase-conjugated secondary antibody and enhanced chemiluminescence (Amersham Pharmacia Biotech). The intensities of GPIbα bands were scanned and quantitated using NIH Image software. Results (mean ± S.D.) from three experiments are shown. B–D, washed resting platelets (5 × 10⁹/ml) in modified Tyrode’s solution were incubated with or without 0.5 μM cytochalasin D (CD) for 5 min. The platelets were then further incubated for 30 min in the presence of ristocetin (1 mg/ml) and RGDS (1 mM) with or without the addition of 10 μg/ml vWF (B). FITC-labeled SZ-29 was used to detect vWF binding. The cytochalasin D-treated platelets were also allowed to bind vWF in the presence of either control IgG (C) or the inhibitory anti-GPIb antibody AR2 (D) in the absence of RGDS. The cytochalasin D-treated platelets were also allowed to bind vWF in the presence of RGDS (D). E, washed resting platelets were treated with or without cytochalasin D for 5 min. These platelets were then further incubated in the presence of PGE1 for 15 min. vWF binding to these platelets was measured as described for B.

on the cell surface as the monoclonal anti-GPIbα antibodies bind to wild type and mutant GPIb-IX at comparable levels (Figs. 2 and 3). Thus, the association of GPIb-IX with the membrane skeleton maintains GPIb-IX in a “resting” state that hinders the access of the A1 domain in the native vWF macromolecules to the ligand binding site of GPIbα. While the structural basis of this inside-out regulation is unclear, it is possible that the association with the membrane skeleton has a conformational effect on the extracellular ligand binding domain of GPIbα. It is also possible that the membrane skeleton association affects the lateral mobility (47) and/or interaction between GPIb-IX molecules (clustering). It is known that vWF binding to GPIb-IX is regulated by conformational changes in vWF induced by immobilization to subendothelial matrix and affected by shear forces (14). Moreover, conformational changes affecting receptor binding may also occur in the A1 domain of vWF and may be mimicked by the action of the functional modulators, botrocetin or ristocetin (22). Our results indicate that vWF-GPIb-IX interaction can be modulated not only by changes in vWF but also by changes in the ligand binding function of GPIb-IX. The latter may be induced by the membrane skeleton-derived signals that control the access of the A1 domain in macromolecular vWF to the binding site in GPIbα. This suggests that even when vWF is activated by immobilization, its interaction with GPIb-IX can still be a regulated event controlled by the platelet membrane skeleton-dependent signals. This 2-fold control mechanism of vWF-GPIb-IX interaction may reflect a need to strictly regulate GPIb-IX-dependent platelet adhesion and activation in circulation in order to prevent thrombosis.

While the mechanism is not clear, the GPIb-IX-associated membrane skeleton is likely to be regulated in platelets. In resting platelets, the association between GPIb-IX and the membrane skeleton is dynamic as resting platelets maintain a balance of membrane skeleton-associated (75%) and dissociated forms of GPIb-IX (25%) (Fig. 8) (9). This is in contrast to CHO cells, where almost all expressed wild type GPIb-IX molecules are associated with the Triton X-100-insoluble actin filaments under similar conditions (Fig. 1) (32). The difference between platelets and CHO cells suggests the possibility that a mechanism in platelets that dynamically regulates GPIb-IX association with the membrane skeleton is absent or inhibited in CHO cells and explains why wild type GPIb-IX in platelets but not in CHO cells is able to mediate cell adhesion to immobilized human vWF without the addition of vWF modulators such as botrocetin. Since we show here that association of GPIb-IX with the membrane skeleton negatively affects vWF binding, it is possible that regulation of the ratio between membrane skeleton-associated and –dissociated forms of GPIb-IX is a potential mechanism for regulating the adhesion function of resting platelets. Consistent with this hypothesis is a previous finding that cytochalasin D abolished the inhibitory effect of PGE1 on ristocetin-induced platelet aggregation (41). We further show here that cytochalasin D reverses the inhibitory effect of PGE1 on ristocetin-induced vWF binding to GPIb-IX in resting platelets (Fig. 8), suggesting that the inhibitory role of PGE1 on ligand binding function of GPIb-IX is affected by the disruption of membrane skeleton actin filaments. However, since PGE1 may affect multiple aspects of platelet signaling, it remains to be investigated whether the effect of PGE1 can be mediated via the membrane skeleton-dependent signals.

The GPIb-IX-associated membrane skeleton is also likely to be regulated during platelet activation. This contention is supported by the well known fact that platelets lose discoid shape during platelet activation. Interestingly, it was reported that vWF binding function of GPIb-IX was inhibited following thrombin-induced platelet activation (48). Thrombin-induced inhibition of vWF binding to GPIb-IX was reversed by cytochalasin D treatment and was not caused by GPIb-IX internalization (48). Since thrombin induces increased incorporation of GPIb-IX into the Triton X-100-insoluble cytoskeleton (49), these data suggest that thrombin may negatively regulate ligand binding function of GPIb-IX via the membrane skeleton-dependent pathway. It is possible that this regulatory mechanism may prevent thrombin-stimulated platelets (if still circulating) from adhering to the vascular wall in places other than the site of original vascular injury. It will be interesting to investigate further how the association of GPIb-IX with the
membrane skeleton and the membrane skeleton organization is regulated in platelets.

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