Transmembrane and Trans-subunit Regulation of Ectodomain Shedding of Platelet Glycoprotein Ibα

Ectodomain shedding of transmembrane proteins may be regulated by their cytoplasmic domains. To date, the effecting cytoplasmic domain and the shed extracellular domain have been in the same polypeptide. In this study, shedding of GPIbα, the ligand-binding subunit of the platelet GPIb-IX complex and a marker for platelet senescence and storage lesion, was assessed in Chinese hamster ovary cells with/without functional GPIbα sheddase ADAM17. Mutagenesis of the GPIb-IX complex, which contains GPIbα, GPIbβ, and GPIX subunits, revealed that the intracellular membrane-proximal calmodulin-binding region of GPIbβ is critical for ADAM17-dependent shedding of GPIbα induced by the calmodulin inhibitor, W7. Perturbing the interaction between GPIbα and GPIbβ subunits further lessened the restraint of GPIbα on GPIbβ shedding. However, contrary to the widely accepted model of calmodulin regulation of ectodomain shedding, the R152E/L153E mutation in the GPIbβ cytoplasmic domain disrupted calmodulin binding to GPIbβ but had little effect on GPIbα shedding. Analysis of induction of GPIbα shedding by membrane-permeable GPIbβ-derived peptides implicated the association of GPIbβ with an unidentified intracellular protein in mediating regulation of GPIbα shedding. Overall, these results provide evidence for a novel trans-subunit mechanism for regulating ectodomain shedding.

A large number of transmembrane proteins expressed on the cell surface undergo ectodomain shedding, a process in which the protein is cleaved at a site close to the outer surface of the cell membrane and subsequently its ectodomain released from the cell (1). Because ectodomain shedding plays a vital role in various cellular processes, including cell growth and proliferation, mammalian development, cell migration, and inflammation, its aberration often leads to disease states including cancer (2–5). Ectodomain shedding is in essence an enzyme-driven proteolytic reaction; therefore, in general there are two ways to regulate it. One way is to modulate the activity of the enzyme that is often referred to as sheddase; the other is to modulate the accessibility of the shedding substrate to the sheddase. Most sheddases belong to the ADAM (a disintegrin and metalloproteinase)2 protease family within the metzincin superfamily (1). Although the structure, function, and regulation of ADAMs have been extensively studied (6–8), mechanisms modulating the accessibility of shedding substrates have not been well explored.

In addition to extracellular signals such as ligand binding to the shedding substrate (9, 10), intracellular signals can also induce shedding of a protein without affecting the catalytic activity of the sheddase. The first reported example of this mechanism is the induction of shedding of L-selectin by calmodulin (CaM) inhibitors (11). CaM co-immunoprecipitates with L-selectin from cell lysates and binds directly to the isolated cytoplasmic domain of L-selectin. Treating L-selectin-expressing cells with trifluoperazine, a CaM inhibitor, causes dissociation of CaM from L-selectin and up-regulates shedding of L-selectin (11, 12). CaM inhibitors rapidly trigger shedding of other membrane proteins by a mechanism independent of protein kinase C and calcium influx, both of which can activate ADAMs (13). Furthermore, point mutations in the L-selectin cytoplasmic domain, such as L320E, disrupt CaM association with the cytoplasmic domain, such as L320E, disrupt CaM association with the cytoplasmic domain, and significantly increase L-selectin shedding (11, 12), providing additional evidence for regulation at the level of substrate. Since the report on L-selectin, treatment of CaM inhibitors, such as trifluoperazine and W7 (N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide), has been found to induce shedding of a number of membrane receptors (13–21). These membrane receptors contain a cluster of basic residues in the membrane-proximal region of their cytoplasmic domain, many of which have been shown to associate with CaM. Thus, it appears that CaM association with the cytoplasmic domain of a membrane protein helps to inhibit its shedding. Dissociation of CaM, either by exogenous inhibitors or disrupting mutations in the CaM-binding site, would up-regulate shedding.

2 The abbreviations used are: ADAM, a disintegrin and metalloproteinase; CaM, calmodulin; GP, glycoprotein; DMSO, dimethyl sulfoxide; GC, glycocalcin; Fmoc, 9-fluorenylmethoxycarbonyl; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline.
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The glycoprotein (GP) Ib-IX complex is a platelet receptor for von Willebrand factor and other ligands that play important roles in hemostasis, thrombosis, and inflammation (22). Under physiological conditions ADAM17 cleaves GPIbα, the main ligand-binding subunit of the GPIb-IX complex (Fig. 1A), at a site between membrane-proximal residues Gly^{464} and Val^{465} (23, 24). The soluble shed extracellular domain of GPIbα is a potential biomarker for platelet storage lesion and senescence (23, 24). The soluble shed extracellular domain of GPIbα is a potential biomarker for platelet storage lesion and senescence (23, 24).

Although shedding of GPIbα can be induced by CaM inhibitors (24), GPIbα does not conform to previous observations in that its cytoplasmic domain does not contain a CaM-binding sequence. Instead, the cytoplasmic domain of GPIbβ, another subunit in the GPIb-IX complex that is not shed, contains a membrane-proximal positively charged sequence that interacts with CaM (27). In the GPIb-IX complex GPIbα is associated with two GPIbβ subunits via extracellular membrane-proximal disulfide bonds and noncovalent transmembrane domain interactions (28, 29), we investigated in this study whether CaM association with the cytoplasmic domain of GPIbβ regulates ectodomain shedding of GPIbα in a trans-subunit manner.

**EXPERIMENTAL PROCEDURES**

**Materials**—The vector pDX was used in earlier studies on expression of the GPIb-IX complex in Chinese hamster ovary (CHO) cells (30). The CHO K1 cell line was obtained from ATCC (Manassas, VA). The CHO M2 cell line that lacks functional ADAM17 has been described (31). CaM inhibitor W7 and the broad-spectrum hydroxamic acid-based metalloprotease inhibitor GM6001 were purchased from Calbiochem (La Jolla, CA) and dissolved in 5% dimethyl sulfoxide (DMSO) before use.

**Mutant Constructs of GPIba, GPIbβ, and GPIX**—All cDNA constructs in this study were cloned in the pDX vector for transient transfection to CHO K1 or CHO M2 cells (31). The vectors containing genes encoding Ibα_{G84A/C485S} (GPIbα with both membrane-proximal cysteines, Cys^{484} and Cys^{485}, mutated to serines), Ibα_{pl} (GPIbα with its transmembrane domain changed to polyoleic sequence), HA-Ibβ (GPIbβ with an N-terminal HA epitope tag, YPYDVPDYA), Ibβ_{H139L} (GPIbβ without cytoplasmic residues 161–181), Ibβ_{H141L} (GPIbβ with the H139L mutation in its transmembrane domain), Ibβ_{pl} (GPIbβ with its transmembrane domain changed to polyoleic sequence), and IXpA (GPIX with its cytoplasmic domain changed to polyalanine sequence) have been previously described (28, 32–35).

To obtain the Ibα_{H141L} construct, in which the GPIbα cytoplasmic domain was replaced with the GPIX counterpart, a DNA sequence encoding GPIX residues Ala^{155}–Asp^{161} and a stop codon was appended to that encoding the GPIbα transmembrane domain by consecutive PCR. The resulting gene fragment was then ligated as a BspEI/XbaI fragment into the pDX-Ibα vector as described (33). Glu-scanning mutagenesis in the GPIbβ cytoplasmic domain was carried out using QuikChange (Stratagene, La Jolla, CA). The mutant gene fragment was inserted as a NotI/XmaI fragment into the pDX-HA-Ibβ vector (34). All gene sequences were confirmed by DNA sequencing (SeqWright, Houston, TX).

**Transfected CHO Cells Expressing the GPIb-IX Complex**—The pDX vectors expressing the wild-type or mutant GPIbα, GPIbβ, and GPIX subunits were transiently transfected into CHO K1 or CHO M2 cells using Lipofectamine 2000 (Invitro-
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![Diagram](image)

**FIGURE 2.** W7-induced shedding of GPIbα does not require the GPIbα and GPIX cytoplasmic domains or the membrane-distal region of the GPIbβ cytoplasmic domain. A, illustration of various constructs used in this study. GPIbα, GPIbβ, and IX wild-type GPIb-IX constructs; GPIbα, mutant GPIb in which its cytoplasmic domain was replaced by the GPIX counterpart; GPIbβ, mutant GPIb in which its cytoplasmic domain was replaced with a polyalanine sequence; and GPIbα, mutant GPIb in which cytoplasmic residues following Ala160 were deleted. B, GPIbα shedding in various transfected cells in response to W7 treatment, assessed by release of GC into culture medium and the GPIbα surface expression level on the cell surface. CHO K1 cells transiently expressing the wild-type complex were used as a control. The amount of GC released from the cells was detected by Western blot and the band densities were quantitated as the mean ± S.D. (n = 4).

bovine serum albumin (PBS/BSA), and stained with fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (Invitrogen) for 1 h at room temperature. After a final wash with PBS/BSA to remove unbound antibody, the cells were fixed and examined in a Beckman-Coulter Epics XL flow cytometer. To quantify the GPIbα surface expression level, the mean fluorescence intensity of the entire cell population (10,000 cells) was normalized to the value of cells expressing the wild-type complex set as 100%.

**Ectodomain Shedding of GPIbα from Transfected CHO Cells**—To measure W7-induced shedding of GPIbα, transfected CHO cells (approximately 5 × 10⁶ cells) were washed with PBS containing Ca²⁺ and Mg²⁺ and treated with either 150 μM W7 or 5% DMSO at 37 °C for 3 h. Proteins contained in the PBS supernatant were precipitated with 5% trichloroacetic acid on ice for 1 h. Cells were either assayed for surface expression of GPIbα by flow cytometry, or lysed in lysis buffer (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 5 mM N-ethylmaleimide, 1% Triton X-100) containing a 1:50 dilution of a protease inhibitor mixture for mammalian cells (Sigma). The supernatant from the cell lysate was obtained by centrifugation at 15,600 × g for 5 min at room temperature. To measure constitutive shedding of GPIbα from transiently transfected cells, the culture medium was collected 2 days after transfection, and glicolicin (GC) released into the medium was immunoprecipitated with W23 and protein G-agarose beads (Invitrogen).

In both experiments, glicolicin precipitated from the medium or full-length GPIbα in cell lysates were resolved in an 8% Tris glycine/SDS gel under reducing or non-reducing conditions, transferred to PVDF membranes, and blotted with W23. As a loading control, blots were stained with anti-actin antibody (Sigma). To assess expression of HA-tagged GPIbβ...
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FIGURE 4. Effects of Glu mutations on assembly of the GPIb-IX complex and cellular expression levels of GPIββ and GPIβ. CHO K1 cells were transfected with various GPIb-IX complexes bearing the single Glu or double Glu GPIββ mutation, and the cell lysates were separated in Tris glycine/SDS gels under non-reducing (A) or reducing (B) conditions, transferred to PVDF membrane, and blotted with WM23 (GPIββ), anti-HA (HA-tagged GPIββ), polyclonal anti-GPIX (GPIX), or anti-actin (actin) antibodies. The figure is representative of three independent experiments. The GPIβ and non-GPIβ labels on the right refer to native and non-native GPIβα-containing complexes, respectively (35).

FIGURE 5. Glu mutations do not dissociate GPIββ from GPIβα. Lysates of CHO K1 cells transfected with various GPIb-IX complexes were coimmunoprecipitated (IP) with monoclonal anti-HA antibody and protein G-agarose beads. The samples were separated in a Tris glycine/SDS gel under reducing conditions, transferred to PVDF membrane, and blotted with anti-GPIβα antibody WM23. The figure is representative of two independent experiments.

and GPIX, cell lysates were resolved in 12% Tris glycine/SDS gels, transferred to PVDF membranes, and blotted with anti-HA antibody (Sigma) and polyclonal anti-GPIX antibody (Santa Cruz Biotechnology, Santa Cruz, CA), respectively. Antibody binding was detected using peroxidase-conjugated secondary antibodies and chemiluminescence substrates (PerkinElmer Life Sciences). Finally, the membrane was exposed to Kodak BioMax X-AR film or directly visualized and quantitated using a GeneGnome HR imaging system (Syngene BioImaging, Frederick, MD). The exposure time was adjusted to avoid saturation and to ensure exposure in the linear range. The relative intensity of the blotted bands was quantitated with background correction, and normalized against the intensity of the corresponding actin band.

The GC:GPIβα ratio for the wild-type and mutant GPIb-IX complex, an indicator of the extent of GPIβα shedding, is calculated as the ratio of the level of GC measured by Western blot to the surface expression level of GPIβα quantitated by flow cytometry. The ratios were then normalized to that of the wild-type complex in the same experiment set as 1.0.

CaM Pulldown Assay—Cell lysates were incubated with 20 μg of GST-CaM (27) at 4 °C for 1 h, and then incubated with 50 μl of glutathione-Sepharose 4B beads (GE Healthcare) at room temperature for 1 h. Beads were washed with Tris-buffered saline and bound HA-tagged GPIββ was eluted with SDS sample buffer, resolved using a 5–20% Tris glycine/SDS gel under reducing conditions, transferred to PVDF membrane, and blotted with anti-HA antibody.

RESULTS

The human GPIb-IX complex is primarily expressed in anucleate platelets that are not amenable to mutagenesis studies. CHO cells, which do not express endogenous GPIb-IX, have been used in studies of ectodomain shedding (37, 38). As in platelets, GPIβα is found in the plasma membrane of transfected CHO K1 cells, and its efficient expression requires cotransfection of GPIbα and GPIX (30, 34). To assess shedding of GPIβα in CHO cells, we monitored the amount of soluble extracellular domain of GPIβα, also known as GC (39), released into the culture medium by immunoblotting and measured concurrently the level of GPIβα remaining on the cell surface by flow cytometry. As in platelets (24), GPIβα is constitutively shed from stably or transiently transfected CHO cells expressing the GPIb-IX complex (referred in this study as Ibo/IX/IX cells). Shedding can be further up-regulated by W7 in a dose-dependent manner (Fig. 1, B and C, supplemental Fig. S1). In addition, both basal and W7-induced GPIβα shedding from CHO cells is abrogated by GM6001, a broad-spectrum metalloprotease inhibitor. Finally, the complete lack of free GC in the culture medium of transfected CHO M2 cells that lack functional ADAM17 (31), despite expression of a high level of GPIβα, clearly indicates that shedding of GPIβα in transfected CHO cells is catalyzed by ADAM17, as also true for platelets (Fig. 1D). Thus, transiently transected

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CHO cells are a suitable model system for studies of GPIbα shedding.

Identification of Intracellular Elements in GPIbβ That Regulate Shedding of GPIbα—Because W7 treatment induces ectodomain shedding by presumably dissociating CaM from the shedding substrate, we reasoned that the element responding to W7-induced shedding of GPIbα should reside in cytoplasmic domains of the GPIb-IX complex. Moreover, genetic alteration of the cytoplasmic domains should abolish W7-induced shedding of GPIbα and/or enhance constitutive shedding of GPIbα. Initially, whole domain replacement was carried out to locate the cytoplasmic sequence critical for W7-induced shedding of GPIbα. As illustrated in Fig. 2A, the cytoplasmic domain of GPIbα was replaced by the GPIX counterpart, not known to associate with any intracellular proteins or play a role in GPIb-IX assembly (Ibβ160Δ). The cytoplasmic domain of GPIX was replaced by a polyalanine sequence (IX pL). Because the membrane-proximal region of the GPIbβ cytoplasmic domain is absolutely required for surface expression of the GPIb-IX complex (34), only the membrane-distal region that contains residues 161–181 was truncated in this study (Ibβ160L). To characterize the effect of these changes on GPIbα shedding, each mutant construct was cotransfected with the other two wild-type subunits into CHO K1 cells (e.g. Ibβ1X with wild-type GPIbβ and GPIX, designated as Ibβ1X/IbββpL/IX in Fig. 2B). The transfected cells were treated with 150 μM W7 or solvent (5% DMSO) for 2 h in PBS containing Ca2+ and Mg2+ at 37 °C, after which the extent of W7-induced GPIbα shedding was monitored.

Consistent with previous observations (34, 40, 41), some, but not all, alterations in cytoplasmic domains caused a moderate decrease in expression of the GPIb-IX complex in transiently transfected cells (Fig. 2B). Because the level of GC released into the culture medium depends on both shedding efficiency and the abundance of GPIbα as shedding substrate, the difference in GPIbα surface expression level precluded the direct comparison of GC levels between cell lines as an accurate assessment of the mutational effect on GPIbα shedding efficiency. Instead, a relative ratio of the level of shedding product versus the level of remaining shedding substrate on the cell surface, the GC:GPIbα ratio, was normalized against the corresponding ratio observed in cells expressing the wild-type GPIb-IX complex (Ibβ1X/Ibββ/IX), and used to denote the extent of GPIbα shedding. The inclusion of only the GPIbα surface expression level, but not its overall expression level, in measurement of GPIbα shedding makes irrelevant the possibility that a mutated GPIbβ cytoplasmic domain may alter the distribution of GPIbα between the cell surface and internal compartments. Comparison of GC:GPIbα ratios for cells transiently expressing wild-type and mutant complexes (Fig. 2B) indicated that although replacement of GPIbα or GPIX cytoplasmic domains, or truncation of the membrane-distal region of the GPIbβ cytoplasmic domain, affected surface expression of GPIbα, none of the mutations abolished W7-induced shedding of GPIbα nor did they significantly alter its extent. Thus, it appears that the membrane-proximal region of the GPIbβ cytoplasmic domain, which contains the CaM-binding site and is the only cytoplasmic sequence in...
the GPIb-IX complex that had not been changed in our study, may be critical for mediating W7-induced shedding of GPIbα.

Because replacing the membrane-proximal region of the GPIbβ cytoplasmic domain with a polyalanine sequence abolished surface expression of GPIbα (34), thus making it impossible to monitor its shedding, site-specific mutagenesis was performed to probe the role of this region in the regulation of GPIbα shedding. More specifically, GPIbβ residues 149–154 were mutated to Glu, one or two residues at a time. The mutant GPIbβ was transiently cotransfected with wild-type GPIbα and GPIX in CHO K1 cells, to enable evaluation of constitutive shedding of GPIbα (Fig. 3). In all cases, GPIbα shedding was mediated by ADAM17, because no GC was generated from transfected CHO M2 cells lacking functional ADAM17 and surface GPIbα levels were relatively higher than those in transfected CHO K1 cells (supplemental Fig. S2). Some mutations, such as Ibβ_E149 to which GPIbα residue Arg149 was mutated to Glu, caused a significant decrease in surface expression of GPIbα (Fig. 3B). Nonetheless, in most cells sufficient GPIbα was expressed to allow characterization of GPIbα shedding. All single Glu mutant complexes exhibited GC:GPIbα ratios indistinguishable from wild-type. In contrast, double Glu mutant complexes exhibited different GC:GPIbα ratios. Although the GC:GPIbα ratio of cells expressing the GPIbβ_R152E/L153E mutant complex (Ibβ/Ibβ_R152E/L153E/IX) was the same as that of wild-type, ratios of cells expressing Ibβ/Ibβ_R149E/L150E/IX, Ibβ/Ibβ_R149E/L150E/L151E/IX, or Ibβ/Ibβ_R152E/L153E/IX complexes were significantly higher than wild-type (Fig. 3B). These cells had little GPIbα on the surface, likely due to a combination of decreased GPIbα expression and increased shedding activity. Together, these results show that the membrane-proximal region of the GPIbβ cytoplasmic domain is important for regulation of GPIbα shedding.

Mechanism of GPIbβ Regulation of GPIbα Shedding—We next explored the mechanism underlying regulation of GPIbα shedding by the membrane-proximal region of the GPIbβ cytoplasmic domain. In the wild-type GPIb-IX complex, GPIbα connects with two GPIbβ subunits via disulfide bonds to form the GPIb complex (28). Misassembly of the complex, due to disruption of native inter-subunit interactions, often results in formation of so-called non-GPIb complexes in which GPIbα mostly forms non-native disulfide bonds with GPIbβ (35). GPIb formation, as an indicator for GPIb-IX assembly, can be distinguished from non-GPIb complexes by SDS-PAGE under non-reducing conditions followed by immunoblot for GPIbα (Fig. 4A). Similar to earlier findings (34), many Glu mutations in this study disrupted or perturbed correct assembly of the GPIb-IX complex as evidenced by the lack of, or decrease in native GPIb formation and concurrent formation of non-GPIb complexes (Fig. 4). Consistently, coimmunoprecipitation studies showed that GPIbα formed a complex with each mutant GPIbβ tested (Fig. 5). The extent of complex misassembly correlated well with the mutation-induced decrease in GPIbα expression, but it did not correlate with mutational effects on GPIbα shedding. For example, the R151E mutation hampered formation of native GPIbα–GPIbβ disulfide bonds to a higher degree than R152E, and caused a more pronounced decrease in GPIbα expression. However, both mutations had the same GC:GPIbα ratio as wild-type and little effect on GPIbα shedding (Fig. 3B). Moreover, other mutations known to cause misassembly and decrease surface expression of the GPIb-IX complex, such as the H139L mutation in the GPIbβ transmembrane domain, or a poly-leucine-replaced transmembrane domain in either GPIbβ or GPIbα (33), did not increase the level of GPIbα shedding (Fig. 6). Even when GPIbα was expressed alone in transfected CHO cells, in the absence of GPIbβ and GPIX, it was shed at a rate similar to that of the wild-type GPIb-IX com-
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In the wild-type GPIb-IX complex, GPIbα is associated with GPIbβ and GPIX subunits. A, ectodomain shedding of GPIbα is hampered by the association of an intracellular protein or protein complex (shown in stripes) with the GPIbβ cytoplasmic domain. B, the R152E/L153E double mutation in the membrane-proximal region of the GPIbβ cytoplasmic domain should not disrupt the interaction of GPIbβ with this protein and thus does not up-regulate GPIbα shedding. C, the R149E/L150E/R152E double mutation should disrupt the association and make GPIbβ more susceptible to ectodomain shedding. D, the membrane-permeable pal-WT peptide or E, the pal-152E/153E peptide, added exogenously to the wild-type GPIb-IX complex, competitively dissociates the binding intracellular protein from the GPIbβ cytoplasmic domain, leading to the increase in GPIbα shedding. F, the pal-151E/152E peptide is not able to dissociate with the intracellular protein and thus has little effect on GPIbα shedding.

The membrane-proximal region of the GPIbβ cytoplasmic domain and the shedding cleavage site of GPIbα are connected physically through GPIbα–GPIbβ disulfide bonds and their transmembrane domains. Therefore, we reasoned that the association between GPIbα and GPIbβ through covalent disulfide bonds and noncovalent transmembrane domain interactions (28, 29, 35) should be important in mediating GPIbβ regulation of GPIbα shedding. Because GPIbα containing the C484S/C485S double mutation (GPIbα_{C484S/C485S}) does not form disulfide bonds with GPIbβ but still forms a wild-type-like complex with GPIbβ and GPIX through altered interactions between transmembrane domains (32), we characterized the effects of GPIbβ cytoplasmic mutations on ectodomain shedding of GPIbα_{C484S/C485S} (Fig. 7). Although the C484S/C485S double mutation did not affect constitutive shedding of GPIbα, most of the Glu-containing GPIbβ mutations significantly increased the GC:GPIbα ratio in cells expressing GPIbα_{C484S/C485S} compared with wild-type GPIbα (Fig. 7 and supplemental Fig. S4). The increase was most notable for many single-Glu mutants and GPIbβ_{R152E/L153E}. These results indicate that the GPIbβ cytoplasmic domain may regulate GPIbα shedding through interactions between the two subunits. Both the membrane–proximal region of the GPIbβ cytoplasmic domain and the interactions between GPIbα and GPIbβ may be required for regulation of GPIbα shedding.

The membrane-proximal region of the GPIbβ cytoplasmic domain contains a CaM-binding site (27). To test whether Glu mutations in this region affect association of CaM with GPIbβ, N-terminal HA-tagged wild-type or three representative mutant GPIbβ (HA-Ibβ) were each cotransfected with wild-type GPIbα and GPIX into CHO K1 cells, and HA-Ibβ was pulled down by GST-fused CaM and glutathione-coated beads. Although wild-type HA-Ibβ associated with GST-CaM as expected, a negligible amount of mutant HA-Ibβ construct was pulled down (Fig. 8), indicating a lack of CaM association. This did not correlate with the differential effects of the three mutations on constitutive shedding of GPIbα (Fig. 3B), suggesting that mutation-induced dissociation of CaM from the GPIbβ cytoplasmic domain may not be sufficient to induce shedding of GPIbα.

To test the idea that a GPIbβ cytoplasmic domain-mediated protein binding event is involved in the regulation of GPIbα shedding, three palmitoylated peptides based on the wild-type (pal-WT) or mutant sequences (pal-151E/152E, pal-152E/153E) of the membrane-proximal region of GPIbβ cytoplasmic domain were synthesized. N-terminal palmitoylation facilitates peptide diffusion through the cell membrane into the cytoplasm (42). CHO cells stably expressing the GPIb-IX complex were treated separately with these membrane-permeable peptides, and their ability to induce GPIbα shedding was characterized (Fig. 9A). Although treatment with pal-WT, or pal-152E/153E, induced release of GC into the culture medium and a corresponding decrease of GPIbα on the cell surface in a dose-dependent manner, treatment with pal-151E/152E had little effect. Treatment with palmitoylated peptides did not affect GPIb formation as shown by Western blot under non-reducing conditions (Fig. 9B), thus excluding the possibility that the
treatment may induce GPIbα shedding by dissociating GPIbα from GPIbβ. Instead, the relative effects of these synthetic peptides on GPIbα shedding matched perfectly those of the corresponding Glu mutations in transfected cells, suggesting that these peptides may affect shedding of GPIbα by inducing dissociation of intracellular proteins from the endogenous GPIbβ cytoplasmic domain (Fig. 10).

DISCUSSION

Biological signals are transmitted through cell surface receptors across the cell membrane in both directions. The shedding cleavage site is located near the junction of extracellular and transmembrane domains of a transmembrane protein. Thus modulation of ectodomain shedding by its cytoplasmic domain can be considered as an inside-out signaling process. Previous reports have shown that the effecting cytoplasmic domain and the shedding enzyme interacts with the wild-type GPIbα (11, 13–21). However, our studies showed that the R152E/L153E mutation in GPIbα greatly increased constitutive shedding of GPIbα (Fig. 3). Further, perturbing the noncovalent interaction between GPIbβ and GPIbα further lessened the restraint of the GPIbβ cytoplasmic domain on shedding of GPIbα (Fig. 7). Together, these results demonstrate that the membrane-proximal region of the GPIbβ cytoplasmic domain helps to maintain stable surface expression and inhibits shedding of GPIbα. To the best of our knowledge, this is the first report of regulation of protein ectodomain shedding by the cytoplasmic domain of another polypeptide. This novel trans-subunit aspect of GPIbα shedding regulation will have significant implications for general shedding regulatory mechanisms.

How do cytoplasmic domains regulate ectodomain shedding on the other side of the cell membrane? The underlying molecular and structural basis remains elusive, but the association of the cytoplasmic domain with intracellular proteins clearly plays a role. CaM is known to be associated with the cytoplasmic domain of a number of shed proteins (11, 13–21). However, our study showed that the R152E/L153E double mutation in the GPIbβ cytoplasmic domain disrupts GPIbβ association with CaM but exerts little effect on GPIbα shedding (Fig. 3, 8). This would argue against a critical role for CaM association in directly mediating regulation of GPIbα shedding by the GPIbβ cytoplasmic domain. Our results suggest that an intracellular binding partner other than CaM may be associated with the membrane-proximal region of the GPIbβ cytoplasmic domain. Disruption of the association, either by site-specific mutations or membrane-permeable synthetic peptides based on this GPIbβ sequence may lead to an increase in the accessibility of GPIbα to the sheddase (Fig. 10). Although the identity of this intracellular protein remains to be uncovered, its ability to associate with the wild-type GPIbβ cytoplasmic domain or the R152E/L153E mutant, but not the R151E/R152E or R149E/L150E mutants, may be helpful in its identification.

Induction of ectodomain shedding by membrane-permeable CaM inhibitors is well documented, implicating CaM as a regulatory factor in ectodomain shedding (11–21). However, if CaM association with the GPIbβ cytoplasmic domain is not directly involved in regulating shedding, how could the effect of CaM inhibitors on GPIbα shedding be explained? One possibility is that CaM inhibitors may have other effects in addition to dissociating CaM from GPIbβ in the cell. For instance, W7 was recently shown to insert into the plasma membrane and change the electrostatic surface potential of the membrane (43), which may influence a number of cellular processes and lead to stimulation of ectodomain shedding. Another possibility is that CaM may regulate other intracellular interactions involving the GPIbβ cytoplasmic domain. For example, it was recently shown that CaM and moesin bind to the same intracellular membrane-proximal region of L-selectin, and that both proteins may be involved in L-selectin shedding (44, 45). Additional investigation will be required to explore these possibilities. In this regard, our findings on trans-subunit regulation of GPIbα shedding by GPIbβ are significant because they imply intracellular regulators need not interact directly with the shed protein, but may act via an associated transmembrane protein.

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