The Transmembrane Domain of Glycoprotein Ibβ Is Critical to Efficient Expression of Glycoprotein Ib-IX Complex in the Plasma Membrane*

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Lack of expression of glycoprotein (GP) Ib-IX-V complex in platelets often results from mutations in its three subunits: GP Ibα, GP Ibβ, or GP IX. The requirement of all three subunits in the efficient surface expression of the receptor complex has been reproduced in Chinese hamster ovary cells. Here, we probed the role of the transmembrane domains in expression of the GP Ib-IX complex and potential interactions between these domains. Replacing the transmembrane domains of either GP Ibα or GP Ibβ, but not that of GP IX, with unrelated sequences markedly diminished surface expression of the GP Ib-IX complex in transiently transfected Chinese hamster ovary cells. Replacement of the Ibβ transmembrane domain produced the largest effect. Furthermore, several single-site mutations in the Ibβ transmembrane domain were found to significantly decrease overall expression as well as surface expression of GP Ibα, probably by perturbing the interaction between the Ibα and Ibβ transmembrane domains and in turn reducing the stability of GP Ibα in the cell. Mutations S503V and S503L in the Ibα transmembrane domain partly reversed the expression-decreasing effect of mutation H139L, but not the others, in the Ibβ transmembrane domain, suggesting a specific interaction between these two polar residues. Together, our results have demonstrated the importance of the Ibβ transmembrane domain, through its interaction with the Ibα counterpart, to the proper assembly and efficient surface expression of the GP Ib-IX complex.

Platelet glycoprotein (GP)2 Ib-IX-V complex mediates the initial tethering and rolling of platelets on von Willebrand factor (vWF) localized at the vascular injury site (1, 2). Upon ligation with the A1 domain of vWF, the GP Ib-IX-V complex transmits inward a signal that leads to activation of integrin (13, 14). Although the GP Ib-IX-V complex is widely considered to comprise GP Ibα, GP Ibβ, GP IX, and GP V with a 2:2:2:1 stoichiometry (5–7), it is not clear how these subunits assemble into a functional receptor complex in the membrane, partly because intersubunit interactions are largely unknown.

Abnormally low expression of the GP Ib-IX-V complex in human platelets can result from mutations in either GP Ibα, GP Ibβ, or GP IX, but not GP V (8). Such phenomena can be reproduced in transfected Chinese hamster ovary (CHO) cells; missing any of the three subunits leads to no or significantly lowered surface expression of the GP Ib-IX complex (9, 10). In contrast, GP V is not required for efficient expression of the GP Ib-IX complex, although there are mixed reports on whether it enhances the complex expression (11, 12). Furthermore, it has been shown in transfected cells that the GP Ib-IX complex assembles in the endoplasmic reticulum before being transported as a single entity to the plasma membrane. Failure of proper complex assembly results in prompt degradation of GP Ibα in the lysosome and accumulation of nonnative GP IX inside the cell (13, 14).

Although the interdependence among the subunits in the GP Ib-IX complex is well documented, the underlying reasons are not clear. The requirement of all three subunits to efficient surface expression of the complex posits the importance of intersubunit interactions to the proper assembly and therefore stability of the receptor complex. Based on the effects of mutations in GP Ibβ on the expression level of GP IX, the N-terminal cysteine knot region in GP Ibβ was suggested to mediate the interaction with GP IX (15). It helps to explain the dependence of GP IX expression on GP Ibβ. Why the expression of GP Ibα is dependent on GP Ibβ and/or IX nonetheless remains unclear.

The transmembrane (TM) domains of GP Ibα and IX may be important to efficient surface expression of the GP Ib-IX complex. Deletion of either the extracellular or cytoplasmic domain of GP Ibα does not significantly affect complex expression in transfected cells (16, 17). Consistently, the Ibα extracellular domain, but not the combined extracellular and TM domains, can be replaced with counterparts from the interleukin-4 receptor while maintaining reasonable expression level of the receptor complex (18). In addition, a single-site mutation in the IX TM domain was recently identified to cause decreased expression of the GP Ib-IX-V complex in a patient (19). It is not evident, however, whether the Ibβ TM domain is as important as its counterparts in GP Ibα and IX. No mutations within the ...
Ibβ TM domain have been reported to prevent surface expression of the receptor complex, although a nonsense mutation effectively removing the entire TM and cytoplasmic domains of GP Ibβ has (20).

We report in this paper a study focusing on the TM domains of the GP Ib-IX complex. We found that replacing the Ibβ TM domain with unrelated sequences produced the largest effect, in comparison with TM replacements in GP Ibα and GP IX, on surface expression of the GP Ib-IX complex in transfected CHO cells. We have also identified single-site mutations in the Ibβ TM domain that significantly decrease the complex expression by affecting the stability of GP Ibα, suggesting that these mutations affect the interaction between GP Ibβ and Ibα. In particular, our data suggest an interaction between residue Ser503 in the Ibα TM domain and His139 in the Ibβ TM domain, which helps to explain the requirement of GP Ibβ for efficient expression of GP Ibα.

**EXPERIMENTAL PROCEDURES**

**Materials**—The vector pDX was used in earlier studies on expression of the GP Ib-IX complex in CHO cells (9, 10). The CHO K1 cell line was obtained from ATCC. WM23, an anti-Ibα monoclonal antibody, was kindly provided by Dr. M. Berndt. Other antibodies against individual subunits of the GP Ib-IX complex, including FMC25, SZ1, SZ2, AK2, and Gi27, were purchased from Axxora (San Diego, CA), Beckman Coulter, Chemicon, or Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-actin antibody was purchased from Sigma.

**Domain Replacement and Mutagenesis of TM Domains of the GP Ib-IX Complex**—The mammalian expression vectors pDX containing Ibα, Ibβ, or IX cDNAs (9, 10) were used in this study. To replace the predicted TM domain in each subunit with poly-leucine (pL) or poly-leucine-alanine (pLA) sequences (Fig. 1), unique restriction sites on both sides of the TM domain were established by silent mutations using the QuikChange kit (Stratagene). In pDX-Ibα, the pDX vector containing the Ibα cDNA, a BspEI restriction site was introduced to a site around residue Pro481 (upstream of the TM domain) with primer 5′-AAATGACCCCTTTTCTCCATCCGGCATGCTTGGTTGAC-3′ and its complementary primer. After two endogenous XbaI sites were removed sequentially with poly-leucine (pL) or poly-leucine-alanine (pLA) sequences (Fig. 1), unique restriction sites on both sides of the TM domain in the target cDNA were established by silent mutations using the QuikChange kit (Stratagene) in the target vector. All of the gene sequences were confirmed by DNA sequencing (SeqWright, Houston, TX).

**Transient Transfection of CHO Cells**—Transient transfection of CHO cells with pDX vectors containing GP Ibα, GP Ibβ, and GP IX genes was carried out using Lipofectamine (Invitrogen) as described earlier (10) and according to the manufacturer’s instructions. Briefly, CHO K1 cells were evenly split into 12 wells of the cell culture plate (Corning) and grown in Dulbecco’s modified Eagle’s medium (Invitrogen) and grown in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal bovine serum to 90% confluence prior to transfection. The cells in each well were washed gently, treated with 1 ml of reduced serum medium (Invitrogen), and transfected with a total of 1.5 μg of vectors (0.5 μg of each vector) that had been incubated in suspension with Lipofectamine in reduced serum medium for 20 min at room temperature. When only one or two subunit genes were to be transfected, empty pDX vector was included to keep the total amount of DNA a constant. The transfection lasted for 6 h under standard culture conditions before the medium containing the DNA/Lipofectamine mixture was replaced by 1 ml of Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. The cells were then grown for 2–3 days before being harvested and analyzed for protein expression.

**SDS-Polyacrylamide Gel Electrophoresis and Western Blot**—Transiently transfected cells were harvested and washed with phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA). Approximately 5 × 10⁵ cells from each transfection were mixed thoroughly with 60 μl of cell lysis buffer (1% Triton X-100, 5 mM CaCl₂, 5 mM N-ethylmaleimide, 58 mM sodium borate, pH 8.0) containing 10% (v/v) protease inhibitor mixture for mammalian tissues (Sigma) and incubated at room temperature for 10 min. Supernatant from the cell lysate was separated from the insolubles by centrifugation at 15,600 × g for 4 min at room temperature. To ensure equal loading, the total protein concentration in each supernatant was also checked using the BCA protein assay kit (Pierce). Supernatant containing ~10 μg of total protein was mixed with standard Tris-glycine SDS sample buffer (pH 6.8), separated in a 4–12% Bis-Tris NuPAGE SDS

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gel (Invitrogen), transferred to a polyvinylidene difluoride membrane, and immunoblotted with appropriate antibodies. Antibody binding was detected by adding peroxidase-conjugated secondary antibodies and chemiluminescence substrates (PerkinElmer). Finally, the membrane was either exposed to BioMax XAR film (Eastman Kodak Co.) or quantitated using a ChemiGenius® Bio-Imaging system (Syngene, Frederick, MD). To quantify the relative intensity of the blotted bands, the density of each Ibα or Ibβ band was measured with automatic background correction, divided by the density of the corresponding actin band, and finally normalized against the value of the wild type bands from αβIX cells.

Flow Cytometry—Surface expression levels of GP Ibα and IX were analyzed by flow cytometry as described recently (21). Briefly, the transfected cells were detached with 0.48 mM EDTA and washed with ice-cold PBS. Resuspended in the PBS/BSA buffer, the cells were incubated with 2 μg/ml primary antibody for 1 h at room temperature, washed twice with PBS/BSA, and stained with fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (Zymed Laboratories Inc.) for 1 h at room temperature. After a final wash with PBS/BSA to remove unbound antibody, the cells were examined in a Beckman-Coulter Epics XL flow cytometer. To quantitate the data, the mean fluorescence value of the entire cell population (10,000 cells) was normalized, with the value of αβIX cells being 100% and that of pDX cells 0%.

RESULTS

Since this study addresses mostly the mutualistic effects on surface expression of the GP Ib-IX complex in transfected CHO cells, it is critical that changes in expression be attributed only to the mutations. To minimize bearings of extrinsic factors such as antibiotic selection pressure on protein synthesis in the cell, transient transfection was employed throughout this study. After transfection, the cells were grown for an additional 2–3 days, and expression levels of GP Ibα as well as GP IX were measured with two methods: Western blot of the transfected cell lysate for overall expression and flow cytometry analysis for surface expression. Furthermore, uniform transfection efficiency in a given experiment was achieved by the transfection of surface expression. The surface expression levels were measured with two methods: Western blot of the transfected cell lysate for overall expression and flow cytometry analysis for surface expression. The surface expression levels of mutant complex were then quantified by the mean fluorescence value of the entire cell population (10,000 cells) and normalized, with the value of αβIX cells being 100% and that of pDX cells 0%. Therefore, the normalized surface expression level can be attributed to only the mutations in the target subunit. It is important to note that although the normalized mean fluorescence value correlates positively with the surface expression level, such correlation is not necessarily a linear one. In other words, a 30% normalized value does not necessarily mean that the number of mutant receptors on the cell surface is 30% of that of wild type.

Replacing the TM Domains of GP Ibα and GP Ibβ, But Not That of GP IX, with an Unrelated Sequence Decreases Complex Expression in CHO Cells—Since a TM domain is physically restricted to the membrane bilayer, it can interact only with other TM domains in a complex. If in the target TM domain there is a sequence motif that mediates TM-TM interaction and contributes to complex assembly and eventual surface expression, then replacing the entire TM domain with an irrelevant sequence, such as the pL or pLA sequence, should remove the motif. Both pL- and pLA-containing peptides form a stable TM α-helix in the lipid bilayer (22). Considered generic and “featureless” TM sequences, they have been used to replace the TM region in various studies (23–25). Given the intrinsic difference between the pL and pLA sequences, at least one of them, if not both, should remove the interaction motif in the target TM domain.

To test which TM domain in the GP Ib-IX complex is necessary for its efficient surface expression, we replaced the TM domains, one at a time, with the pL and pLA sequences and measured their effects on complex expression in transfected CHO cells. Residues Leu487–Leu510 in GP Ibα except for Pro488 near the N-terminal end of the TM domain were replaced by either the pL or pLA sequence (Fig. 1A). Residues Gly124–Leu147 in GP Ibβ and Val136–Leu153 in GP IX were likewise replaced. The TM-replaced cDNA were then transiently transfected into CHO cells together with cDNA encoding the other two wild type subunits. In this paper, CHO cells are designated by the transfected subunits, with the subscript of each subunit describing the mutation within. Lack of subscript indicates the wild type subunit. For example, αpLβIX cells denote the CHO cells transfected by the Ibα subunit with the pl TM domain along with wild type Ibβ and IX subunits.

The effects of TM replacement mutations on complex expression in the cell were first characterized through immunoblotting of GP Ibα and IX from cell lysates (Fig. 1B). GP Ibα was blotted with two monoclonal antibodies that target different regions; both produced the same results. In comparison with wild type αβIX cells, the Ibα expression levels were notably reduced in αpLβIX and αpLAβIX cells. They were even lower in αβpLIX and αβpLAIX cells. In contrast, no significant change in Ibβ expression was observed in αβIXpL and αβIXpLA cells. GP IX was blotted with a polyclonal antibody, and the IX expression levels in various transfected cells were consistent with those of GP Ibα.

The surface expression levels of GP Ibα and IX in CHO cells containing TM-replacement mutations were measured by flow cytometry and further quantitated with the mean fluorescence
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FIGURE 1. Replacing the TM domain of GP Ibα or GP Ibβ, but not that of GP IX, with an unrelated sequence decreases expression of the GP Ib-IX complex in transfected CHO cells. A, sequences of the TM domains in the wild type or TM-replaced subunits. In each subunit, the TM domain sequence, defined in the GenBank™ annotations and marked by the boxes, was replaced by the pL or pLA sequence. The extracellular and cytoplasmic domains remained unchanged. The name of each construct is listed on the left. α, GP Ibα; β, GP Ibβ; IX, GP IX. B, overall expression of GP Ibα and GP IX in transfected CHO cells as detected by Western blot. Various cell lysates were resolved in a SDS gel under nonreducing conditions and subsequently blotted for GP Ibα (Fig. 3). In αβIX cells, the dominant band corresponded to GP Ibα (i.e. Ibα-Ibβ), revealing the intersubunit disulfide. Little or no GP Ib band was observed in αβIX, αβLAβIX, αβpLIX, and ββpLαIX cells, indicating that formation of the intersubunit disulfide was affected when either the Ibα or Ibβ TM domain was replaced with an unrelated sequence.

It is interesting to note that the Ib band was wild type-like in αβIXαL cells but was notably diminished in αβIXpLα cells. This suggested that replacing the IX TM domain with the pLA sequence, but not the pL sequence, may interfere with the Ibα-Ibβ disulfide formation. Moreover, in some mutant cells in which the Ibα-Ibβ disulfide was largely absent, most of GP Ibα formed intermolecular disulfide(s) with unidentified protein(s) in a complex with an apparent molecular mass larger than 220 kDa (Fig. 3). Its molecular weight is consistent with that of a Ibα homodimer, which was reported in CHO cells expressing only GP Ibα (26).

In short, our results indicated that the Ibα and Ibβ TM domains are important to formation of the membrane-proximal disulfide between the two subunits. Replacing either TM domain with an unrelated sequence may disrupt the interaction between the two domains, resulting in removal of the disulfide and formation of new disulfides involving GP Ibα. The role of the IX TM domain in formation of the Ibα-Ibβ disulfide remained a question, since ambiguous results were obtained when it was replaced with unrelated sequences.

Site-specific Mutations in the Ibβ TM Domain Affect Stability of GP Ibα and the Complex Expression—To guard against the caveat that decrease in the GP Ib-IX complex expression by TM replacement is due to the input of the pL or pLA sequence rather than disruption of the intrinsic property of the native TM domains, we proceeded to identify site-specific mutations within the TM domains that affect surface expression of GP Ib-IX complex. Given its largest effect by TM replacement, we focused on the Ibβ TM domain.

Since the replacement of TM domain with the pL sequence is essentially the replacement of all of the non-Leu residues with Leu residues, certain non-Leu residues should be important to the interaction with GP Ibα and efficient surface expression of the receptor complex. The α-helical structure of a TM domain dictates that its residues interacting with another TM α-helix be located on the same helical interface. To screen quickly for the site-specific mutations, we divided the residues in the Ibβ TM domain into seven “sides,” each of which represents one side of the TM α-helix and was assigned arbitrarily as side a–g (Fig. 4A). All of the non-Leu residues on each side, one at a time, were changed to Leu (Fig. 4B). Each of the seven corresponding mutant subunits, denoted as βpLα to βpLg, was then co-trans-

values (Fig. 2). Partly transfected cells (αβ, αIX, and βIX cells) were included as controls, and they produced GP Ibα or GP IX on the cell surface at a level significantly lower than αβIX cells, consistent with the finding that all three subunits are required for efficient surface expression of the GP Ib-IX complex (9, 10). It is noteworthy that the surface expression level of GP Ibα or GP IX is not the same in these partly transfected cells; αβ cells expressed significantly more GP Ibα than αIX cells, and βIX cells expressed more GP IX than αIX cells.

Of all of the TM replacement mutant cells, αβpLIX and αβpLaIX cells had the lowest surface expression levels of GP Ibα and IX, with ~6% of the mean fluorescence value of αβIX cells. The expression levels in αβpLIX and αβpLaIX cells were slightly higher than αβpLIX cells but remained much lower than αβIX cells. In contrast, surface expression in αβIXpL cells was indistinguishable from the wild type. The expression levels in αβIXpLa cells were significantly lower, with the mean fluorescence value being ~45% of the wild type value.

Overall, surface expression of the Ibα and IX subunits was remarkably consistent with their overall expression in the cell. These results showed that replacing the TM domains of GP Ibα or GP Ibβ, but not that of GP IX, with an unrelated sequence led to a marked decrease in expression of the GP Ib-IX complex, suggesting the importance of the Ibα and Ibβ TM domains in the complex assembly.

TM-replacing Mutations Affect Formation of the Disulfide Bond between GP Ibα and GP Ibβ—The disulfide bond between GP Ibα and Ibβ is located very close to the TM domains. To test whether TM replacement mutations affect formation of this intersubunit disulfide, cell lysates were resolved in a SDS gel under nonreducing conditions and subsequently blotted for GP Ibα (Fig. 3). In αβIX cells, the dominant band corresponded to GP Ibα (i.e. Ibα-Ibβ), revealing the intersubunit disulfide. Little or no GP Ib band was observed in αβIX, αβLAβIX, αβpLIX, and ββpLαIX cells, indicating that formation of the intersubunit disulfide was affected when either the Ibα or Ibβ TM domain was replaced with an unrelated sequence.
Ibβ expression was decreased, the Ibβ expression level, relative to the wild type, was substantially higher than the Ibα level. For instance, the levels of GP Ibα and GP Ibβ in αβpIbIX cells were 15 and 81%, respectively, of those in αβIX cells. This indicated that the Ibα expression was not limited by the decreased expression of GP Ibβ. Rather, the decrease in Ibα expression was probably due to the decrease in Ibα stability as a result of the destabilized interaction between GP Ibα and GP Ibβ.

In contrast to the variations in their overall expression levels, little difference was detected between the surface expression levels of GP Ibα and IX in mutant cells (Fig. 4D), consistent with the earlier finding that the GP Ib-IX complex forms in the endoplasmic reticulum and is transported to the cell surface as a single entity. Furthermore, the surface expression levels in the mutant cells correlate well with the overall expression levels of GP Ibα rather than those of GP Ibβ; the three mutant cells with the lowest overall expression of GP Ibα also produced the least Ib-IX complex on the cell surface. These results suggested that surface expression of the complex was limited by the stability of GP Ibα in the cell.

We next measured the effects of each of the six single-site mutations included in the side a, b, and f side mutations: G124L and A131L from side a, A125L and H139L from side b, and Q129L and G136L from side f. Disparate effects on the overall expression levels of individual subunits were observed, as in the side-scanning mutant cells (Fig. 5A). Furthermore, little difference between surface expression levels of GP Ibα and IX was evident for all of the single-site mutant cells, and the mutational effects on surface expression of the Ib-IX complex correlated well with the Ibα expression in the cell. Overall, whereas A131L had little effect on expression of GP Ibα, the other five mutations markedly decreased the Ibα expression level. The expression level of GP Ibβ in αβIX cells, relative to the wild type level, was sufficiently low that it might limit expression of GP Ibα in the cell. Nonetheless, the expression levels of GP Ibβ in the other mutant cells were relatively higher than that of GP Ibα. Mutation H139L resulted in the lowest expression level of GP Ibα in the cell, with only 18% of the wild type level (Fig. 5A). Consistently, the αβIX cells produced the lowest level of

**FIGURE 2.** Surface expression of the GP Ib-IX complex in TM-replaced mutant cells. CHO cells were transiently co-transfected with vectors containing Ibα, Ibβ, and IX cDNAs. After 48–72 h, the cells were harvested, stained by anti-Ibα conformation-sensitive antibody AK2 or anti-IX antibody FMC25, and analyzed by fluorescence-activated cell sorting analysis. Other antibodies, anti-Ibα SZ2 and anti-IX SZ1, were also used, and similar results were obtained (data not shown). A, overlays of representative histograms from the same transfection experiment. Identities of transfected cells are denoted by the subunits transfected, with the subscript describing the mutation within. B, surface expression levels of GP Ibα (black column) and GP IX (white column), quantified as relative mean fluorescence, in TM-replaced mutant cells. The mean fluorescence values were normalized, with αβIX cells being 100% and cells transfected with empty vectors 0% (not shown). The data are presented as the mean ± S.D. calculated from 4–11 independent experiments. WT, wild type.
GP Ib-IX complex on the cell surface, with a mean fluorescence value being only 40% of the wild type value (Fig. 5B).

In summary, we have identified five single-site mutations in the Ib TM domain, each of which significantly decreased expression of GP Ibα. The mutation H139L, in particular, produced the largest decreasing effect. These results unambiguously demonstrated the importance of the Ib TM domain to efficient surface expression of the GP Ib-IX complex.

S503L and S503V in the Ib TM Domain Reverse the Decreasing Effect on Ibα Expression Caused Only by H139L in the Ib TM Domain—Polar and ionizable amino acids constitute a significant portion of the residues in the hydrophobic TM helices (27, 28). The Ib TM domain contains two polar residues: Gln129 and His139. Changing either residue, in particular the latter, to Leu led to marked decrease in surface expression of the GP Ib-IX complex. Since αβ1139LIX cells produced relatively more GP Ibβ (51% of the wild type level) than GP Ibα (18%), decreased expression of GP Ibα was not due to the lack of GP Ibβ. Rather, it is likely that residue His139 is involved in an interaction with GP Ibα, and the mutation H139L perturbs such interaction and consequently affects the stability of GP Ibα and surface expression of the entire complex. Since polar residues typically interact with other polar residues in the membrane bilayer through hydrogen bonds (29, 30), sequence alignment of the Ibα and Ibβ TM domains identified residue Ser503 in GP Ibα as a potential interacting partner for His139 (Fig. 6, A and B). Its location in the Ibα TM domain matches that of His139, and the side chains of Ser and His could form hydrogen bonds in the membrane bilayer (31).

For polar-polar interactions in the TM domains, replacing a polar residue with an apolar one would result in energetically unfavorable exposure of unpaired side chain of the other polar.

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residue to the hydrophobic environment and therefore greatly destabilize the host membrane protein complex. However, if both polar residues are replaced with apolar ones, the host complex stability may not be affected as much. To probe the possible interaction between Ser503 and His139, we tested initially whether the mutation S503L in the Ib TM domain can reverse the decreasing effect of H139L on the surface expression of the GP Ib-IX complex. We also measured the effect of S503L in other Ibβ single-site mutant cells as well (Fig. 6, C and D).

Comparison of the α5S03LβIX and αβIX cells revealed that the S503L mutation caused a modest yet consistent decrease in Ibα expression, since the former produced ~58% of GP Ibα in the latter (Fig. 6C). Surface expression of the GP Ib-IX complex was lowered in α5S03LβIX cells (compare Figs. 5B and 6D). Furthermore, S503L caused a similar effect in most cells bearing a single-site mutation in the Ibβ TM domain, reducing the Ibα expression in the cell by 5–20% of the wild type level (compare the Ibα bands in Figs. 5A and 6C). Expression of GP Ibβ was likewise affected, although the degree of reduction did not match that in Ibα expression. Surface expression of the GP Ib-IX complex in these cells was also decreased further by the S503L mutation; the extent of reduction, in terms of the percentage of wild type mean fluorescence value, varied from <5% in α5S03βG124LIX cells to >30% in α5S03βQ129IX cells (compare Figs. 5B and 6D). In contrast, α5S03βH139IX cells produced slightly more GP Ibα but less GP Ibβ than αβH139IX cells (Figs. 5A and 6C). Surface expression of the GP Ib-IX complex in α5S03βH139IX cells was consistently higher than in αβH139IX cells (Figs. 5B and 6D). In other words, the S503L mutation reversed the decreasing effect on complex expression caused by the H139L mutation.

Since the side chains of Ser and Leu are quite different in terms of size, we tested the effect of the mutation S503V. The size of Val resembles that of Ser. Like S503L, S503V reversed the decreasing effect on Ibα expression caused by the H139L mutation but further decreased the surface expression levels of the Ib-IX complex in CHO cells bearing other Ibβ TM mutations (Fig. 6E and F). Comparing to S503L, the reverse effect of S503V on H139L was more prominent. Overall, these results suggest a direct interaction between Ser503 and His139.

**DISCUSSION**

We have demonstrated in this study that the Ibβ TM domain is essential to efficient surface expression of the GP Ib-IX complex in transfected CHO cells. Either whole domain replacement or site-specific mutations in the Ibβ TM domain led to a significant decrease in Ibα expression in the cell and surface expression of the whole complex. The decrease of Ibα expression was not due to the lowered expression of mutant GP Ibβ, since the Ibβ expression levels in the mutant cells, in terms of the percentage of that in wild type αβIX cells, are higher than those of GP Ibα. Furthermore, the extent of decrease in Ibα expression in the cell did not correlate with that in Ibβ. The decrease of Ibα expression caused by the Ibβ TM mutations may be due to the perturbation of the interaction between the Ibα and Ibβ TM domains and therefore the decrease in the stability of GP Ibα in the cell. In particular, our results pointed to a specific interaction between Ser503 in the Ib TM domain and His139 in the Ibβ TM domain, since S503L and S503V reversed the expression-decreasing effect of only H139L and not the other Ibβ mutations.

Interactions among the subunits are obviously important to the stability and function of the GP Ib-IX-V complex. The interactions between GP Ibα and Ibβ, between GP Ibα and IX, and between GP Ibβ and V have been established (10, 11, 32). The direct interaction between GP Ibα and IX remains questionable (10, 33). Because of its interaction with both GP Ibα and IX, GP Ibβ plays a central role in the assembly of the receptor complex (10). GP Ibβ interacts with GP IX through its N-terminal cysteine knot region (15). In comparison, the exact interfacial regions or interacting residues between GP Ibα and GP Ibβ have remained unknown, although an intersubunit disulfide connects the two subunits. The evidence presented in this paper suggested a direct interaction between the Ibα and Ibβ TM domains. Consistently, our recent characterization of the peptides containing the Ibα or Ibβ TM domain showed a direct and specific interaction between the two domains and their flanking regions, and this interaction is directly involved in formation of the Ibα-Ibβ disulfide between the TM peptides. The extracellular domain of GP Ibα, also known as glycocalcin, is readily found in the plasma upon proteolytic cleavage from the GP Ib-IX-V complex on the platelet surface (35), it is unlikely that glycocalcin interacts with the extracellular domains of other subunits in the complex, or at least their interaction is not strong enough to prevent spontaneous dissociation of glycocalcin from the platelet surface. There has been no report on the interaction between the cytoplasmic domains of GP Ibα and GP Ibβ. Therefore, the interaction between the two TM domains may be the major force that mediates association of GP Ibα and GP Ibβ. Such association stabilizes GP Ibα in the endoplasmic reticulum, impeding its intracellular degradation.

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Although our results pinpoint an interaction between the polar residues in the Ibα/H9251 and Ibβ/H9252 TM domains, the Ser503-His139 interaction is unlikely to be the only one between the two domains. Although replacing either residue with Leu residues caused a decrease in expression of the GP Ib-IX complex, the extent of decrease did not match those caused by TM-replacing mutations in Ib/H9251/H9252pLIX and Ib/H9251/H9252pLAIX cells. This indicated that neither S503L nor H139L mutations caused complete disruption of the receptor complex. Furthermore, the side-scanning mutagenesis study identified three sides of the Ibβ/H9252 TM helix to be critical. The proximity of sides b and f suggested that they might be a critical part of the binding interface to another TM helix. Indeed, all of the four single-site mutations in sides b and f can cause decreased expression of GP Ibα to various degrees, indicating that these residues may also participate in the interaction with GP Ibα. On the other hand, the effect of G124L, the mutation that is not on sides b and f but still causes a significant decrease in the expression of GP Ibα, is puzzling. Finally, the Ibβ TM domain contains a number of Leu residues, the importance of which was not probed by the side-scanning mutations. It is possible that some of the Leu residues participate in the interaction between the Ibα and Ibβ TM domains. Therefore, elucidating the molecular and structural details of the interaction between the Ibα and Ibβ TM domains will require a systematic study that is currently under way.

Replacing the Ibβ TM domain resulted in a more significant effect on complex expression than replacing the Ibα counter-
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part, suggesting additional roles for the Ibβ TM domain in determining complex assembly and expression. One possibility is that the Ibβ TM domain interacts with that of the GP IX, or the IX TM domain can modulate the interaction between GP Ibα and GP Ibβ. Although replacing the IX TM domain with the pL sequence did not affect formation of the Ibα-Ibβ disulfide, replacing it with the pLA sequence did (Fig. 3). Removal of the entire TM and cytoplasmic domains of GP IX abolishes the expression of the GP Ib-IX-V complex in human platelets, suggesting that the IX extracellular domain is not sufficient for efficient surface expression of the complex (36). In addition, a single-site mutation in the IX TM domain has been identified from a patient to suppress the complex surface expression (19). Thus, the intersubunit interactions in the TM domains may not be limited to the one between GP Ibα and GP Ibβ. Our study will provide tools to further explore the organizing principles of the GP Ib-IX-V complex.

The interaction between the Ibα and Ibβ TM domains may also contribute to functional regulation of the GP Ib-IX-V complex. An antibody against the extracellular domain of GP Ibβ can inhibit adhesion of GP Ib-IX-V-transfected cells to vWF under flow (37). Phosphorylation of Ser<sup>166</sup> in the Ibβ cytoplasmic domain mediates its interaction with the cytoplasmic signaling protein 14-3-3ζ and inhibits the vWF binding activity of GP Ibα (38–40). The membrane-proximal region of the Ibβ cytoplasmic domain has been implicated in the thrombin- and ristocetin-induced platelet activation and aggregation (41). Furthermore, mutations in the Ibβ cytoplasmic tail and extrinsic peptides that affect the Ibα-14-3-3ζ interaction can modulate the vWF binding activity (17, 34, 42). It is clear, therefore, that an inside-out modulation signal can originate from the Ibα and/or Ibβ cytoplasmic domain and end with the likely change in the conformation, as well as the ligand-binding ability, of the N-terminal domain of GP Ibα outside the plasma membrane. It is conceivable that the interaction between the Ibα and Ibβ TM domains plays an important role in mediating such a signal across the membrane.

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Note Added on Proof—During the review process, a patient with Bernard-Soulier syndrome was reported (Strassel, C., David, T., Eckly, A., Baas, M.-J., Moog, S., Ravanat, C., Trzeciak, M.-C., Vinciguerra, C., Cazenave, J.-P., Gachet, C., and Lanza, F. (2006) J. Thromb. Haemost. 4, 217–228). This patient was reported to have the new transmembrane and cytoplasmic domains of GP Ibα due to a frame-shifting mutation. This report highlights the importance of the transmembrane and cytoplasmic domains of GP Ibβ in GP Ib expression and function, which is consistent with our conclusion in this study.

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