The Transmembrane Domains of β and IX Subunits Mediate the Localization of the Platelet Glycoprotein Ib-IX Complex to the Glycosphingolipid-enriched Membrane Domain

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Background: Localization of the GP Ib-IX complex to the lipid domain is mediated by the β and IX subunits.

Results: Mutations in β/IX TMDs inhibit GP Ib-IX localization to the lipid domain.

Conclusion: Localization of the GP Ib-IX complex to the lipid domain is mediated by β/IX TMDs.

Significance: The β/IX TMDs may be a novel therapeutic target.

We have previously reported that the structural elements of the GP Ib-IX complex required for its localization to glycosphingolipid-enriched membranes (GEMs) reside in the Ibβ and IX subunits. To identify them, we generated a series of cell lines expressing mutant GP Ibβ and GP IX where 1) the cytoplasmic tails (CTs) of either or both GP Ibβ and IX are truncated, and 2) the transmembrane domains (TMDs) of GP Ibβ and GP IX were swapped with the TMD of a non-GEMs associating molecule, human transferrin receptor. Sucrose density fractionation analysis showed that the removal of either or both of the CTs from GP Ibβ and GP IX does not alter GP Ibα-GEMs association when compared with the wild type. In contrast, swapping of the TMDs of either GP Ibβ or GP IX with that of transferrin receptor results in a significant loss (−50%) of GP Ib from the low density GEMs fractions, with the largest effect seen in the dual TMD-replaced cells (>80% loss) when compared with the wild type cells (100% of GP Ibα present in the GEMs fractions). Under high shear flow, the TMD-swapped cells adhere poorly to a von Willebrand factor-immobilized surface to a much lesser extent than the previously reported disulfide linkage dysfunctional GP Ibα-expressing cells. Thus, our data demonstrate that the bundle of GP Ibβ and GP IX TMDs instead of their individual CTs is the structural element that mediates the β/IX complex localization to the membrane GEMs, which through the α/β disulfide linkage brings GP Ibα into the GEMs.

The function of the GP Ib-IX complex can be regulated by various mechanisms, including conformational changes in each individual subunit (1–7), extracellular intersubunit interaction (6, 8–11), oligomerization to increase the avidity and affinity to its ligand (12), intracellular association with signaling molecules (e.g. 14–3-3ζ (13), P13-kinase (14), and calmodulin (15)), and co-localization with other receptors on the platelet surface (e.g. FcγR (16) and FcγRIIA (17, 18)). One of the features of cell membranes is the existence of specialized glycosphingolipid-enriched membranes (GEMs),3 to which not only a number of receptors, in platelets, such as GP Ib-IX complex (19), FcγRIIA (19), P13-kinase (20), and P2Y12 (21), but also various kinases, phosphatases, and adaptor proteins (22, 23) are localized. Therefore, one would expect that upon ligand-receptor interaction, the receptor-associated GEMs can act as a platform or carrier to support a transmembrane signal, as well as the interplay of different signals elicited by various GEM-associated receptor-ligand interactions, helping them bind in an efficient, controlled, and synergistic manner. Furthermore, a number of investigations have shown that the single bond between the GP Ib-IX complex and vWF cannot be maintained under high shear force imposed on either platelets or the GP Ib-IX-expressing CHO. In addition, vWF is a large multimeric molecule possessing multiple sites that can interact with GP Ibα; therefore, GP Ib-IX complexes could be clustering on vWF, which allows for resistance to the shear force through the anchorage of the GP Ib-IX complex to the platelet cytoskeleton (3–5). Along this line, a GEM-based localization to concentrate larger numbers of GP Ibα molecules should facilitate such multivalent binding.

It is known that all of the GP Ib-IX subunits (GP Ibα, GP Ibβ, and GP IX) are type I transmembrane proteins that reside in the GEMs. Lack of or dysfunction in this association caused by a disruption of the GEMs structure (e.g. MβCD treatment (19)) or introduction of a loss of association mutations to GP Ibα (24) eliminates or inhibits the GP Ib-IX complex interaction with

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2 The abbreviations used are: GEM, glycosphingolipid-enriched membrane; CT, cytoplasmic tail; TMD, transmembrane domain; vWF, von Willebrand factor; Trf, human transferrin receptor.

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vWF, tyrosine phosphorylation, and GP Ib-IX complex-initiated thrombus formation. At this point, we only know that GP Ibβ and GP IX possess unidentified structural elements required for the association of the GP Ib-IX complex with GEMs that is independent of the palmitoylation state of GP Ibβ and GP IX. Although the transmembrane domains (TMDs) of these two molecules seem like good candidates because of their location near the membrane lipids (25–28), there is no experimental evidence to support this notion. In addition, previous reports have shown that the association of a transmembrane receptor with GEMs is not predisposed by its TMD, but rather, it is mediated by the specific targeting signals residing in the individual protein itself (29). For instance, conformational changes in the extracellular domain can affect the GEMs targeting of the T-cell adhesion protein CD2 (98); a 60-residue membrane-proximal sequence is reported to mediate the EGF receptor to the GEMs (99); and a membrane-proximal signal in the cytoplasmic tail (CT) of the T-cell co-receptor CD4 is required for association with the GEMs (85).

In this study, because even minor changes in the extracellular portions of GP Ibβ and GP IX may cause nonexpression of the entire GP Ib-IX complex (6, 30), we chose to start our investigation with the roles of the CTs and TMDs of GP Ibβ and GP IX. We sequentially mutated them by either truncation or swapping with the TMD and the palmitoylation sites of both GP Ib and GP IX. Although the transmembrane domains (TMDs) of Ibβ and IX—

\[
\text{AtagCCCAAGTAGCCAATTCATAARTCIIWT} \quad \text{WT}
\]

were first incubated with GP Ibβ and GP IX cDNA forward primer (5′-start codon and restriction site) and the overlapping reverse primer, or 2) the overlapping forward primer and a GP Ibβ or GP IX cDNA reverse primer (3′-start codon with stop codon and restriction site). The resulting two products were purified by agarose gel electrophoresis, mixed in an equal molar ratio, annealed, and extended with high fidelity polymerase (Turbo Pfu; Stratagene) and then used as templates for the second round of reactions where the cDNA forward and reverse primers of the GP Ibβ or GP IX flanked with restriction sites were used to amplify and clone the Trf-TMD-swapped Ibβ and IX cDNAs into the PDX vector (31). To delete both of the CTs and the intramembrane proximal palmitoylation sites of GP Ibβ (βdel+) and GP IX (IXdel+) and Turbo Pfu-driven PCRs were performed with specific primers complementary to the transmembrane sequences where two stop codons were introduced to the original palmitoylation cysteine sites to ensure translational stop. All of the TMD replacements and CT deletions mentioned were verified by sequencing. Generation of CHO Cell Lines Expressing Wild Type and Mutant GP Ib-IX Complex—CHO cells expressing wild type and mutant GP Ib-IX complexes were generated and maintained as previously described (5, 24, 31). GP Ibα-positive cells were first incubated with GP Ibα-specific mouse monoclonal antibody Z2 (Beckman Coulter) and then sorted by anti-mouse IgG conjugated magnetic beads (Invitrogen) (Table 1). The expression levels of GP Ibα in these cells were determined by flow cytometry with a phycoerythrin-labeled anti-GP Ibα antibody (Beckman Coulter), and their mean fluorescence intensities were checked through sequencing. Standard errors were calculated from three independent experiments.

Sucrose Density Gradient Centrifugation—A detailed procedure has been described previously (24). In brief, cells expressing the GP Ib-IX complex were lysed with 1% Brij 35 and then loaded to the top of a sucrose density gradient. After centrifugation at 34,000 rpm in a swinging bucket centrifuge for 18 h at 4 °C, 12 equal fractions were taken from the top of the gradient and analyzed using an SDS-PAGE gel. GP Ibs were then detected by Western blotting with each respective antibody (24). The GEM-associating molecules were all found within the first four low density fractions.

Immunoprecipitation—CHO cells expressing wild type and mutant GP Ib-IX complexes (2 × 10⁶) were spun down at 3,000

### TABLE 1

| Cell line Characteristics | WTIXWT | WTIXdel | WTIXTC | WTIXdelTC | WTIXTCWT |
|--------------------------|--------|---------|--------|-----------|-----------|
| CHO cells expressing wild type and mutant GP Ib-IX complex | Wild type | CT truncated and depalmitoylated | Swapping with Trf TMD | | |
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rpm for 1 min and lysed with 1% Brij 35 for 1 h on ice. After centrifugation at 15,000 × g for 10 min, the supernatants were precleared with 50 μl of protein A/G beads (GE Healthcare Life Sciences) for 30 min at 4°C. After removing the protein A/G beads by centrifugation, the samples were incubated overnight with 1 μg of anti-GP IX monoclonal SZ1 antibody (Beckman Coulter) (32) and then immunoprecipitated with protein A/G beads for 1 h at 4°C. The beads were then pelleted by centrifugation and washed three times, and the bound protein was eluted by boiling in 2× sample buffer containing 2% β-mercaptoethanol. Immunoprecipitated proteins were resolved by 4–12% Novex® Tris-glycine gels (Life Technologies) under reducing conditions and transferred to PVDF for Western blotting and detection of GP Ibα and GP Ibβ.

Flow Chamber Assay—A detailed procedure has been described previously (31). In brief, CHO cells were incubated on immobilized vWF (20 μg/ml) for 1 min in a parallel plate flow chamber and then perfused with TBS, 0.5% BSA (Sigma) at flow rates that generated wall shear stresses of 2.5, 10, or 20 dyn/cm². The experiments were recorded in real time for 1 min, by a high speed digital camera (model Quantix; Photometrics, Tucson, AZ) connected to an inverted stage microscope (Eclipse TE300; Nikon, Garden City, NY). The rolling velocity was defined as the distance a cell traveled during a defined period (μm/s). Each rolling experiment was performed three times, and the error bars were calculated from the mean rolling velocities of 100 cells in five different view fields.

Results

CT Truncation and Depalmitoylation of Either or Both of GP Ibβ and GP IX Do Not Interfere with GP Ibα Localization to the GEMs—To investigate whether the TMDs of GP Ibβ and GP IX play any roles in the localization of GP Ibα to the membrane GEMs, we generated stable CHO cell lines where wild type GP Ibα was co-transfected with either CT-truncated and depalmitoylated GP Ibβ (βdel+c), GP IX (IXdel+c), or both (βdel+c,IXdel+c). To rule out the possibility that the transfection reagents change the nature of the membrane in the cells, we made stable cell lines instead of using a transient expression approach. As shown in Fig. 1 (A and B), when compared with...
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the wild type GP Ibβ and GP IX co-transfected stable cell lines (αβWT1/IXWT1), the CT truncation and depalmitoylation of either GP Ibβ (αβWTdelc/IXWTc) or GP IX (αβWT1/IXWT1) reduced GP Ibα expression by ~20%. In contrast, dual truncation and depalmitoylation of GP Ibβ and GP IX (αβWTdelc/IXWTdelc) resulted in a higher level of GP Ibα expression (~25%; Fig. 1B). Upon extraction with 1% Brij 35, nearly identical distribution patterns of the GP Ib subunits from the sucrose gradient fractionation were seen in both the mutant and wild type cells (24).

All the GP Ib molecules are present in the low density GEM fractions (fractions No. 1–3), as identified by blotting the known GEM-associating caveolin (Fig. 1C) (24). To test whether the mutations affect the formation of the GP Ib-IX complex, we lysed the cells with 1% Brij 35 and incubated the lysates with SZ1, an anti-GP IX specific antibody (32, 33). Upon immunoprecipitation, we observed that all the GP Ib subunits can be detected in the precipitates of our αβIX-expressing cells (Fig. 1D). In contrast, only GP Ibβ can be detected in the βLIX-expressing cells, validating previous findings that GP Ibβ and GP IX are tightly associated (10, 34). Taken together, our data not only clearly demonstrate that the structural segment possessing the GEM-targeting signal does not reside in the CTS of GP Ibβ and GP IX but also further supports our previous finding that the association of the GP Ib-IX complexes with the GEMs is not predisposed by the palmitoylation state of either GP Ibβ or GP IX.

Swapping the TMD of Either or Both of GP Ibβ and GP IX with That of Trf Inhibits GP Ibα Localization to the GEMs—To investigate whether the TMDs and/or which TMD of GP Ibβ and GP IX plays a role in partitioning the GP Ib-IX complex into the GEMs, we replaced the TMDs of GP Ibβ and GP IX with that of Trf, a known non-GEMs associating transmembrane protein. Previous studies have shown that upon replacement of the TMD with Trf TMD, IgE receptor function was abolished because of a segregation of the host molecule from the GEMs (28). Because the Trf is a type II transmembrane protein, whereas GP Ibβ and GP IX are type I transmembrane proteins, we inserted an inverted Trf TMD sequence into both, such that the orientation of the Trf TMD across the plasma membrane is the same as its normal orientation in the Trf receptor. Moreover, it was shown that Cys68 can be palmitoylated, another piece of evidence demonstrating that palmitoylation itself does not predispose the host protein to associate with the GEMs. To prevent obscuring the interpretation, we also mutate this cysteine to an alanine (Cys68 to Ala68). After sorting, we found that the individual swapping of the TMD of either GP Ibβ (αβWT1/IXWT1) or GP IX (αβWT1/IXWT1) greatly reduced the expression of GP Ibα to a level ~60% of that in the wild type cells (Fig. 2, A and B). This suggests that the TMDs of both GP Ibβ and GP IX contribute to the expression of GP Ibα. When both of the TMDs were replaced (αβWT1/IXWT1), the GP Ibα expression level was ~16% of that in the wild type cells (Fig. 2B), suggesting that the TMD bundle formed by βLIX harbors the most appropriate conformation and surface nature for the GP Ib-IX complex to be efficiently expressed on the cell surface. Furthermore, when we subjected our cell lines to sucrose density fractionation analysis, we found that individual replacements of the TMD of GP Ibβ and GP IX caused more than 50% of the GP Ibα to translocate to the non-GEM fractions (48% in αβWT1/IXWT1 and 42% in αβWT1/IXWT1 cells; Fig. 2D), when compared with the wild type cells (100% in αβWT1/IXWT1; Fig. 1C). Interestingly, swapping the GP IX TMD had less of an impact than that of the GP Ibβ TMD. Moreover, dual replacement of both TMDs (αβWT1/IXWT1) partitioned greater than 80% of the GP Ib into non-GEM fractions (~18% left in the GEM fractions; Fig. 2D). Finally, the GP Ib-IX complex is still intact because all of the GP Ib subunits appeared in similar sucrose density fractions (Fig. 2C), and the mutant GP Ibβ and GP IX still associate with GP Ibα upon immunoprecipitation with anti-GP IX antibody SZ1 (Fig. 2E). Thus, for the first time our data demonstrate that 1) the TMDs of both GP Ibβ and GP IX contribute to the association of the GP Ib-IX complex with GEMs, and 2) the TMD bundle of GP Ibβ and GP IX is a critical structural segment in the GP Ib-IX complex for GEM association.

Loss of GEM Association Inhibits GP Ib-IX Complex Interaction with vWf under Flow—We have reported that the disulfide linkage dysfunctional GP Ibα expresses at a level ~85% of the wild type GP Ibα, of which ~40% still associates with the CHO GEMs. This reduction greatly increases the rolling velocity of these cells at high shear stresses when compared with wild type cells (~2-fold and ~3-fold increase at 10 and 20 dyn/cm², respectively) (24). To our surprise, however, when we perfused our CHO cells expressing individually Trf-TMD replaced GP Ibβ or GP IX, they rolled at a velocity 4–5-fold faster than the wild type cells (Fig. 3). As shown in Fig. 2 (B and D), βLIX individual swapped cells express GP Ibα at an average level that is ~60% of the wild type GP Ibα, and there were ~50% of the GP Ibα present in the GEMs of these CHO cells when compared with the wild type cells (100%; Fig. 1C). Therefore, we estimated the ratio of the GP Ibα copy number in the GEMs of CHO cells expressing either disulfide linkage deficient GP Ibα or βLIX TMD-swapped GP Ibα to be 85% × 40%:60% × 50% = 34:30. Moreover, consistent with previous reports, the non-GP Ibα-expressing CHO cells (βLIX) do not roll on the vWf-coated surfaces (35), and the previously reported GP Ibα mutation (αβWT1/IXWT1) causes no change in the rolling velocity (31), indicating that the faster rolling of the βLIX individual TMD-swapped cells is specifically due to an impaired vWf binding ability. Thus, our data suggest that at high shear stress, loss of the GEM association and alteration of the transmembrane domains of GP Ibβ and GP IX may synergistically impair the vWf interaction with GP Ibα on these individual TMD-swapped cell surfaces. Unfortunately, when we examined our GP Ibβ/GP IX TMD dual swapped mutant CHO cells (αβWT1/IXWT1), we could find fast rolling cells on the immobilized vWf surface only at a shear stress of 2.5 dyn/cm². Because the expression level of GP Ibα in these cells is ~16% of that in the wild type cells (Fig. 2B), we speculate that the loss of vWf binding could not be solely attributed to the reduction of the GEM association upon dual swapping of the βLIX TMDs. Rather, it might be due to the decreased density of GP Ibα on the cell surface (35).

Discussion

Even though we have known that the GP Ibβ/GP IX complex possesses the targeting signal for the localization of the com-
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The detailed structural necessity of them remains unknown (24). In this study, we aimed to identify these structural elements. The reason we began our investigation from the CTs and the TMDs of GP Ibα/H9251 and GP IX is because both types of domain have been shown to mediate GEM association in other proteins (25–28). In the case of the GP Ib-IX complex, three features may give the complex the ability to associate with the GEMs: 1) all of the GP Ibα sub-

FIGURE 2. Dissociation of GP Ibα from the GEMs caused by TMD swapping in GP Ibβ and GP IX. A, depalmitoylated GP Ibβ and GP IX with their TMDs replaced with the TMD of human transferrin receptor were co-transfected with wild type GP Ibα to establish stable GP Ibα-expressing CHO cells. B, the expression level of GP Ibα in each cell line is presented as a percentage of expression compared with the wild type GP Ibα (mean fluorescence intensity). Standard errors were calculated from three independent experiments. C, cells were then lysed with 1% Brij 35 followed by a sucrose density gradient fractionation. The localization of GP Ibα and GP Ibβ were determined by Western blotting. D, the GEM association level of GP Ibα in each cell line is presented as the percentage of GEM-associating GP Ibα in respect to the total GP Ibα across all sucrose density fractions. The value was averaged from three independent experiments. E, GP Ibα and GP Ibβ were immunoprecipitated with an anti-GP IX specific antibody, SZ1.
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units are transmembrane proteins with TMDs of different length that can interact with membrane lipids; 2) the intramembrane proximal amino acids in GP Ibβ are positively charged, which possibly can bind to negatively charged GEM-specific lipids; and 3) both GP Ibβ and GP IX are palmitoyl modified proteins, a possibility that we have previously ruled out. In our sequential mutagenesis, we first altered the CTs and modified proteins, a possibility that we have previously ruled for GP Ibα.

The cells expressing wild type or mutant GP Ib-IX complexes were perfused over a vWf-immobilized surface in a parallel plate flow chamber at flow rates that generated wall shear stresses of 2.5, 10, or 20 dyn/cm². Unlike the disulfide linkage dysfunctional GP Ibα-expressing cells (αβssIXWT), that rolled 2–3 times faster than the wild type cells (24), the individual TMD-swapped CHO cells translocated 4–5-fold faster than the wild type cells. However, none of the dual TMD-swapped mutant cells interacted with the immobilized vWf.

It is interesting to note that CT-removed and depalmitoylated GP Ibβ only slightly reduced GP Ibα expression when wild type GP IX is co-transfected (αβdel+IXWT). In comparison, when both GP Ibβ and GP IX have their CT domains removed, and they are depalmitoylated, the expression of GP Ibα is even higher than the wild type cells (αβdel+IXdel+c). These observations are surprising, because they are largely opposite to what has been described previously where the cells transfected with CT-removed but palmitoylation intact GP Ibβ (βdel) were unable to express GP Ibα on their surface (36). The only difference between these two CT-removed GP Ibβ proteins is our mutant GP Ibβ is also depalmitoylated. It is well known that palmitate modifications play a role in the sorting and trafficking of host proteins (37). Even though no function has been attributed to the acylation of GP Ibβ, previous evidence (36) and our data implicate that both the palmitoylation and the intramembrane proximal basic amino acids (14th-palmito-CRLR-LRAAR-RAARaar164) of GP Ibβ are needed to determine the intracellular sorting route of wild type GP Ibβ for successful expression, which then allows GP Ibα to reach the cell surface. The insertion of an acyl group into and the binding of the positively charged amino acids to the inner leaflet of the phospholipid bilayer of the intracellular organelles might be the mechanism. The acyl group and the short CT in the GP IX subunit may also contribute to this, because without it, the GP Ibα expression was also slightly reduced in αβWTIXdel+c cells. On the other hand, mutant GP Ibβ in αβdel+IXWT cells can restore to a level ~81% of the wild type cells (Fig. 1B) when compared with the CT-removed but palmitoylation intact GP Ibβ (βdel) (36). These data suggest that without the governing factors (CT and palmitate modification), GP Ibβ might employ a different sorting route to achieve a relatively successful expression of itself and therein GP Ibα. The choice of such different sorting route may also be facilitated by simultaneous removal of the acyl chain and CT from the GP IX subunit, thereby achieving a higher GP Ibα expression in αβWTIXdel+c cells than in the wild type cells. Further investigations on the sorting and secretory pathways in these cells for the GP Ib-IX complex expression are needed to clarify these possibilities.

One other interesting observation is that both of our individual TMD-swapped cells (αβTCIXWT and αβWTIXTC) achieved appreciable and comparable GP Ibα expression on their cell surfaces (Fig. 2, A and B). In contrast, previous reports have shown that transient expression of mutant GP Ibβ and GP IX whose TMDs are replaced either by polyleucine (pL, βpl and IXpl) or polyleucine-alanine (pLA, βpLa and IXpLa) residues causes nonexpression of GP Ibα only in mutant GP Ibβ cells instead of mutant GP IX cells (38). Therefore, it is possible that 1) the pL or pLA type of TMD may by itself interfere with the expression of GP Ibβ and therein GP Ibα, and/or 2) the Trf TMD in the swapped GP Ibβ has an improved capability of forming stable bonds with the TMDs of GP Ibα and GP IX to maintain a relatively higher expression of GP Ibα on the cell surface. On the other hand, in contrast to the CT removal and depalmitoylation, TMD swapping also altered the GP Ibα-GEM association. Therefore, it is possible that the impaired GEM association may also contribute to the reduction in GP Ibα expression in the TMD-swapped cells.

We further provide evidence showing that the reduction in GP Ibα-GEM association by the altered TMDs of GP Ibβ and GP IX can inhibit GP Ibα interaction with vWf at high shear. Unexpectedly, even though the TMD-swapped cells (αβTCIXWT and αβWTIXTC) have comparable levels of GP Ibα found in the GEMs to the disulfide linkage dysfunctional GP Ibα-expressing cells (αSSβWTIXWT), we found that these cells have significantly reduced capability of resisting the high shear force (4–5 times faster than the wild type cells) when compared with the disulfide linkage dysfunctional GP Ibα-expressing cells (αSSβWTIXWT, 2–3 times faster than the wild type cells) (24). Thus, our data suggested that the faster rolling of the TMD-swapped cells may be caused by both the reduction of GEM
association and the alteration of the β/IX TMDs. For the latter, because it has been shown that 1) the interaction between GP Ibβ and GP IX is primarily mediated by their TMDs (10) and 2) the GP Ibα TMD can also interact with GP Ibβ and GP IX forming a four-helix bundle (6), it is possible that the inter-TMD interactions among α, β, and IX in our TMD-swapped cells are weakened. Upon binding of the GEM-associating GP Ib-IX complex to the immobilized vWF, an impaired clustering of the GP Ib-IX complex inside the GEMs caused by inefficient inter-TMD interactions may also affect the multivalency of the vWF-GP Ibα bonding, thereby contributing to the poor resistance to high shear force and faster movement of the β/IX TMD-swapped cells under flow. Furthermore, we did not find dual TMD-swapped cells adhere to the vWF-immobilized surface at shear stress of 10 dyn/cm² or higher. Nevertheless, because we were unable to increase the GP Ibα expression to a level comparable with that in either individual TMD-swapped cells, we do not intend to attribute the loss of vWF binding solely to the dramatic decrease in the GP Ibα-GEM association in these cells. Based on previous report regarding the effect of GP Ibα density on cell rolling velocity, instead, we speculate that the loss of vWF binding might be primarily due to the low level of GP Ibα expression on the cell surface.

Taken together, our study provides the first evidence that the TMDs of GP Ibβ and GP IX are critical for the GP Ib-IX complex to associate with the GEMs (membrane location). In addition, our data also suggest that β/IX TMDs contribute to the oligomerization of the GP Ib-IX complex (clustering) upon vWF binding, a notion that needs further investigation to ascertain whether that is the case. Nevertheless, considering the significantly increased rolling velocities of the TMD-swapped mutant cells, we speculate that targeting the TMDs of GP Ibα and IX may have the potential to become a therapeutic approach to specifically and potently interfere with the shear-induced GP Ib-IX-vWF interaction.

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