THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 290, NO. 36, pp. 22155–22162, September 4, 2015

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

Received for publication, May 27, 2015, and in revised form, July 14, 2015 Published, JBC Papers in Press, July 22, 2015, DOI 10.1074/jbc.M115.668145

Guofeng Xu[‡§1], Dan Shang[¶§1], Zuping Zhang[‖§1], Tanner S. Shaw[§], Yali Ran[§], José A. López[**], and Yuandong Peng[§2]

*From the [‡]XinHua Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200092, China, the [¶]Department of Vascular Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China, the [‖]School of Basic Medicine, Central South University, Changsha 410013, China, the **Department of Medicine, Puget Sound Blood Center, Division of Hematology, University of Washington, Seattle, Washington 98195, and the [§]Cardiovascular Research Section, Department of Medicine, Baylor College of Medicine, Houston, Texas 77030*

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 Ibα 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), P I3-kinase (14), and calmodulin (15)), and co-localization with other receptors on the platelet surface (*e.g.* FcRγ (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), P_2X_1 (20), and P_2Y_{12} (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 CHOs. 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

* This work was supported, in whole or in part, by American Heart Association Scientist Development Grant 0635155N (to Y. P.), National Institute of Health Grant HL095676 (to Y. P.), and funds from the Alkek Endowment Fund (to Y. P.) and the Fondren Foundation. The authors declare that they have no conflicts of interest with the contents of this article.
[1] These authors contributed equally to this work.
[2] To whom correspondence should be addressed: Dept. of Medicine, Baylor College of Medicine, One Baylor Plaza, Alkek N1317.06, Houston, TX 77030. Tel.: 713-798-8639; Fax: 713-798-3415; E-mail: Yuandong@bcm.edu.

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

vWf, tyrosine phosphorylation, and GP Ib-IX complex-initiated thrombus formation.[4] 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 of Trf, a known non-GEMs associating transmembrane protein (28) to examine whether any of them play a role in mediating the localization of the GP Ib-IX complex to the GEMs.

Experimental Procedures

Swapping the TMDs of GP Ibβ and GP IX with That of Trf and Deletion of the CTs of GP Ibβ and GP IX—All constructs containing Ibβ and IX have been previously described (24). The TMDs and the palmitoylation sites of both GP Ibβ (^{123}WGALAAQLALLGLGLLHALLLVLLLC$^{148\text{-palm}}$, 26 amino acids, β_{TC}) and GP IX (^{136}VALVAVAALGLALLAGLLC$^{154\text{-palm}}$, 19 amino acids, IX_{TC}) were replaced with the inverted and depalmitoylated human Trf receptor 1 TMD (^{88}YGLYGIMFGILFFVIVAITGY**A**^{67}IS^{65}, 24 amino acids (accession no. P02786). The underlined/bold alanine is mutated from cysteine (28). Based on the Trf TMD DNA sequence, the following four overlapping PCR primers were designed to replace the TMDs of GP Ibβ and GP IX. The underlined sequences are the Trf TMD coding or complementary sequences: sequence 1, ATAGCCCAAGTAGCCAATCATAAATCCAATCAAGAAAAAGACGATCACAGCAATAGTCCCATAGGCGATACTGCAGAGCGGGCCGGGAG (GP Ibβ overlapping forward primer); sequence 2, AGTATCGCCTATGGGACTATTGCTGTGATCGTCTTTTTCTTGATTGGATTTATGATTGGCTACTTGGGCTATCGCCTGCGGAGG (GP Ibβ overlapping reverse primer); sequence 3, ATAGCCCAAGTAGCCAATCATAAATCCAATCAAGAAAAAGACGATCACAGCAATAGTCCCATAGGCGATACTGTCCCACAAGACCC (IX overlapping forward primer); and sequence 4, AGTATCGCCTATGGGACTATTGCTGTGATCGTCTTTTTCTTGATTGGATTTATGATTGGCTACTTGGGCTATGCCACCACAGAGGC (IX overlapping reverse primer). Whenever overlapping PCR was performed, there were always two rounds of amplification involved. The first reaction was comprised of two PCRs with 1) a GP Ibβ or GP IX cDNA forward primer (5′ prime with 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′ prime 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 a 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 (β_{del+C}) and GP IX (IX_{del+c}), 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 through sequencing.

TABLE 1
Chinese hamster ovary cell lines used in this study

Cell line	Characteristics
$\alpha_{WT}\beta_{WT}IX_{WT}$	Wild type
$\alpha_{WT}\beta_{WT}IX_{del+c}$ $\alpha_{WT}\beta_{del+c}IX_{WT}$ $\alpha_{WT}\beta_{del+c}IX_{del+c}$	CT truncated and depalmitoylated
$\alpha_{WT}\beta_{WT}IX_{TC}$ $\alpha_{WT}\beta_{TC}IX_{WT}$ $\alpha_{WT}\beta_{TC}IX_{TC}$	Swapping with Trf TMD

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 SZ2 (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 normalized then normalized to the wild type cells. 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 (2×10^6) were spun down at 3,000

[4] G. Xu, D. Shang, Z. Zhang, T. S. Shaw, Y. Ran, J. A. López, and Y. Peng, manuscript in preparation.

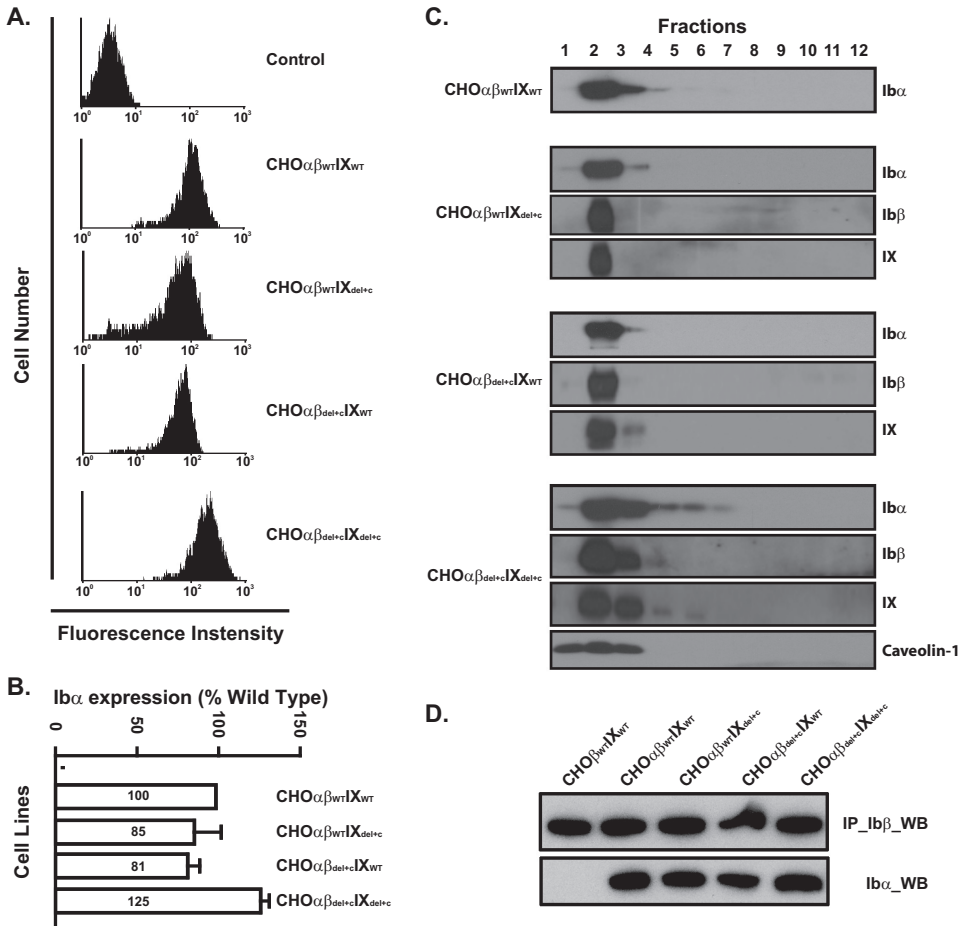

FIGURE 1. **Cytoplasmic tails of GP Ibβ and GP IX are not essential for GP Ibα localization to the GEMs.** *A*, CT-truncated and intracellular membrane cysteine-removed GP Ibβ and GP IX were co-transfected with wild type GP Ibα into CHO cells. Stable cell lines were generated by sorting with a GP Ibα specific antibody. The surface expressed GP Ibα was then incubated with a phycoerythrin-labeled anti-GP Ibα antibody for flow cytometry analysis. *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*, CHO cells expressing the GP Ib-IX complex were lysed with 1% Brij35. Lysates were subjected to sucrose gradient density centrifugation. Equal volume aliquots of each fraction (total 12 fractions) were analyzed by reducing SDS-PAGE and blotted with anti-GP Ibα specific antibody. These blots represent three independent experiments. The GEM-associating molecules are all found within the first four low density fractions. *D*, Brij 35-lysed cells were immunoprecipitated using the anti-GP IX mAb SZ1 antibody. Precipitated proteins were analyzed by reducing gradient SDS-PAGE followed by Western blot analysis with an anti-GP Ibα mAb (WM23).

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/cm^2. 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 CTs 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 (β_{del+c}), GP IX (IX_{del+c}), or both ($\beta_{del+c}IX_{del+c}$). 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

the wild type GP Ibβ and GP IX co-transfected stable cell lines ($\alpha\beta_{WT}IX_{WT}$), the CT truncation and depalmitoylation of either GP Ibβ ($\alpha\beta_{del+c}IX_{WT}$) or GP IX ($\alpha\beta_{WT}IX_{del+c}$) reduced GP Ibα expression by ~20%. In contrast, dual truncation and depalmitoylation of GP Ibβ and GP IX ($\alpha\beta_{del+c}IX_{del+c}$) resulted in a higher level of GP Ibα expression (~25%; Fig. 1*B*). 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. 1*C*) (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. 1*D*). In contrast, only GP Ibβ can be detected in the βIX-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 Cys^{68} 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 (Cys^{68} to Ala^{68}). After sorting, we found that the individual swapping of the TMD of either GP Ibβ ($\alpha\beta_{TC}IX_{WT}$) or GP IX ($\alpha\beta_{WT}IX_{TC}$) 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 ($\alpha\beta_{TC}IX_{TC}$), the GP Ibα expression level was ~16% of that in the wild type cells (Fig. 2*B*), suggesting that the TMD bundle formed by β/IX 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 $\alpha\beta_{WT}IX_{TC}$ and 42% in $\alpha\beta_{TC}IX_{WT}$ cells; Fig. 2*D*), when compared with the wild type cells (100% in $\alpha\beta_{WT}IX_{WT}$; Fig. 1*C*). Interestingly, swapping the GP IX TMD had less of an impact than that of the GP Ibβ TMD. Moreover, dual replacement of both TMDs ($\alpha\beta_{TC}IX_{TC}$) partitioned greater than 80% of the GP Ibα into non-GEM fractions (~18% left in the GEM fractions; Fig. 2*D*). Finally, the GP Ib-IX complex is still intact because all of the GP Ib subunits appeared in similar sucrose density fractions (Fig. 2*C*), and the mutant GP Ibβ and GP IX still associate with GP Ibα upon immunoprecipitation with anti-GP IX antibody SZ1 (Fig. 2*E*). 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/cm^2, 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*), β/IX 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. 1*C*). 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 β/IX TMD-swapped GP Ibα to be 85% × 40%:60% × 50% = 34:30. Moreover, consistent with previous reports, the non-GP Ibα-expressing CHO cells (β/IX) do not roll on the vWf-coated surfaces (35), and the previously reported GP Ibα mutation ($\alpha_{W129F}\beta_{WT}IX_{WT}$) causes no change in the rolling velocity (31), indicating that the faster rolling of the β/IX 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 ($\alpha\beta_{TC}IX_{TC}$), we could find fast rolling cells on the immobilized vWf surface only at a shear stress of 2.5 dyn/cm^2. Because the expression level of GP Ibα in these cells is ~16% of that in the wild type cells (Fig. 2*B*), 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 β/IX 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-

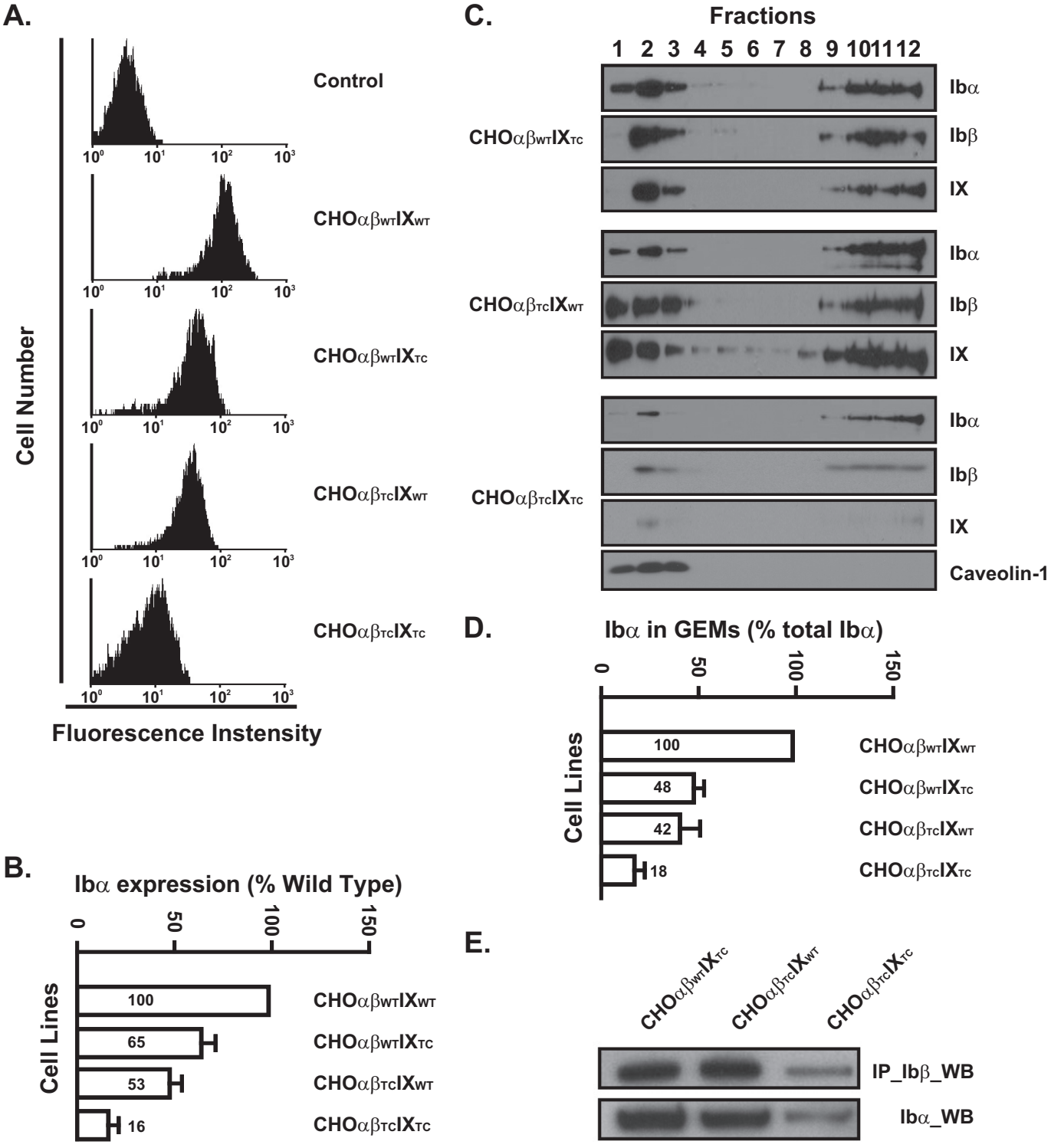

FIGURE 2. **Dissociation of GP Ibα from the GEMs caused by TMD swapping in GP Ibβ and GP IX.** *A*, depalmitoylated GP Ibβ and GP IX with their TMDs replaced with the TMD of human transferrin receptor were co-transfected with wild type GP Ibα to establish stable GP Ibα-expressing CHO cells. *B*, the 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.

plex to the membrane GEMs, 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 Ibβ 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 Ib-IX sub-

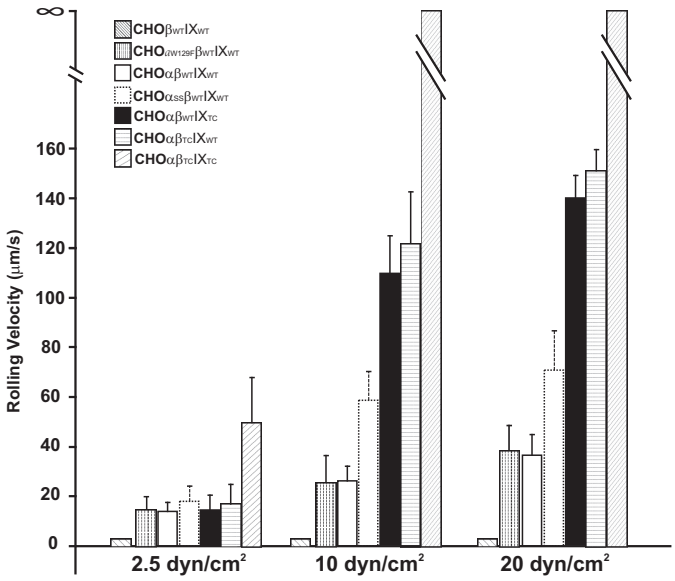

FIGURE 3. **Inhibition of the shear-induced GP Ibα-vWf interaction caused by TMD swapping in GP Ibβ and GP IX.** The cells expressing wild type or mutant GP Ib-IX complexes were perfused over a vWf-immobilized surface in a parallel plate flow chamber at flow rates that generated wall shear stresses of 2.5, 10, or 20 dyn/cm^2. Unlike the disulfide linkage dysfunctional GP Ibα-expressing cells ($\alpha_{SS}\beta_{WT}IX_{WT}$) 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.

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 palmitate modified proteins, a possibility that we have previously ruled out. In our sequential mutagenesis, we first altered the CTs and then the TMDs of GP Ibβ and GP IX. We found that only the changes to the TMDs altered the association with the GEMs, with the largest effect seen in the dual TMD-replaced cells. This observation is particularly interesting because it suggests that the β/IX TMD bundle, as a whole, could be the structural segment for GP Ibα partitioning into the GEMs. Our data implicate a new feature of the regulation of the GP Ib-IX function, *i.e.* the orderly formation of β/IX TMD bundles that produces the most appropriate surface for interaction with the GEM-specific lipids, thereby mediating the GP Ib-IX complex localization to the GEMs.

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 ($\alpha\beta_{del+c}IX_{WT}$). 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 ($\alpha\beta_{del+c}IX_{del+c}$). 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 (β_{del}) 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 ($^{148\text{-palm}}$CRLRRLRARARARAAAR^{164}) 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 $\alpha\beta_{WT}IX_{del+c}$ cells. On the other hand, mutant GP Ibβ in $\alpha\beta_{del+c}IX_{WT}$ cells can restore to a level ~81% of the wild type cells (Fig. 1*B*) when compared with the CT-removed but palmitoylation intact GP Ibβ (β_{del}) (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 $\alpha\beta_{del+c}IX_{del+c}$ 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 ($\alpha\beta_{TC}IX_{WT}$ and $\alpha\beta_{WT}IX_{TC}$) 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, β_{pL} and IX_{pL}) or polyleucine-alanine (pLA, β_{pLA} and IX_{pLA}) 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 ($\alpha\beta_{TC}IX_{WT}$ and $\alpha\beta_{WT}IX_{TC}$) have comparable levels of GP Ibα found in the GEMs to the disulfide linkage dysfunctional GP Ibα-expressing cells ($\alpha_{SS}\beta_{WT}IX_{WT}$), 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 ($\alpha_{SS}\beta_{WT}IX_{WT}$, 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/cm^2 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 Ibβ and GP IX may have the potential to become a therapeutic approach to specifically and potently interfere with the shear-induced GP Ib-IX-vWf interaction.

Author Contributions—G. X., D. S., Z. Z., and Y. P. designed the study and wrote the manuscript. G. X., D. S., and Z. Z. designed, performed and analyzed the experiments shown in the table and figures. Y. R. helped with the cell culture. T. S. S. helped with manuscript editing. J. A. L. provided critical reagents. All authors have approved the final version of the manuscript.

References

1. Shen, Y., Cranmer, S. L., Aprico, A., Whisstock, J. C., Jackson, S. P., Berndt, M. C., and Andrews, R. K. (2006) Leucine-rich repeats 2–4 (Leu^{60}–Glu^{128}) of platelet glycoprotein Ibα regulate shear-dependent cell adhesion to von Willebrand factor. *J. Biol. Chem.* **281,** 26419–26423
2. Chen, Z., Lou, J., Zhu, C., and Schulten, K. (2008) Flow-induced structural transition in the β-switch region of glycoprotein Ib. *Biophys. J.* **95,** 1303–1313
3. Lou, J., and Zhu, C. (2008) Flow induces loop-to-β-hairpin transition on the β-switch of platelet glycoprotein Ibα. *Proc. Natl. Acad. Sci. U.S.A.* **105,** 13847–13852
4. van der Wal, D. E., Verhoef, S., Schutgens, R. E., Peters, M., Wu, Y., and Akkerman, J. W. (2010) Role of glycoprotein Ibα mobility in platelet function. *Thromb. Haemost.* **103,** 1033–1043
5. Peng, Y., Shrimpton, C. N., Dong, J. F., and López, J. A. (2005) Gain of von Willebrand factor-binding function by mutagenesis of a species-conserved residue within the leucine-rich repeat region of platelet glycoprotein Ibα. *Blood* **106,** 1982–1987
6. Li, R., and Emsley, J. (2013) The organizing principle of the platelet glycoprotein Ib-IX-V complex. *J. Thromb. Haemost.* **11,** 605–614
7. Zhang, W., Deng, W., Zhou, L., Xu, Y., Yang, W., Liang, X., Wang, Y., Kulman, J. D., Zhang, X. F., and Li, R. (2015) Identification of a juxtamembrane mechanosensitive domain in the platelet mechanosensor glycoprotein Ib-IX complex. *Blood* **125,** 562–569
8. McEwan, P. A., Yang, W., Carr, K. H., Mo, X., Zheng, X., Li, R., and Emsley, J. (2011) Quaternary organization of GPIb-IX complex and insights into Bernard-Soulier syndrome revealed by the structures of GPIbβ and a GPIbβ/GPIX chimera. *Blood* **118,** 5292–5301
9. Mo, X., Nguyen, N. X., Mu, F. T., Yang, W., Luo, S. Z., Fan, H., Andrews, R. K., Berndt, M. C., and Li, R. (2010) Transmembrane and trans-subunit regulation of ectodomain shedding of platelet glycoprotein Ibα. *J. Biol. Chem.* **285,** 32096–32104
10. Mo, X., Nguyen, N. X., McEwan, P. A., Zheng, X., López, J. A., Emsley, J., and Li, R. (2009) Binding of platelet glycoprotein Ibβ through the convex surface of leucine-rich repeats domain of glycoprotein IX. *J. Thromb. Haemost.* **7,** 1533–1540
11. Zhou, L., Yang, W., and Li, R. (2014) Analysis of inter-subunit contacts reveals the structural malleability of extracellular domains in platelet glycoprotein Ib-IX complex. *J. Thromb. Haemost.* **12,** 82–89
12. Kasirer-Friede, A., Ware, J., Leng, L., Marchese, P., Ruggeri, Z. M., and Shattil, S. J. (2002) Lateral clustering of platelet GP Ib-IX complexes leads to up-regulation of the adhesive function of integrin αIIbβ3. *J. Biol. Chem.* **277,** 11949–11956
13. Du, X., Harris, S. J., Tetaz, T. J., Ginsberg, M. H., and Berndt, M. C. (1994) Association of a phospholipase A_2 (14-3-3 protein) with the platelet glycoprotein Ib-IX complex. *J. Biol. Chem.* **269,** 18287–18290
14. Mu, F. T., Cranmer, S. L., Andrews, R. K., and Berndt, M. C. (2010) Functional association of phosphoinositide-3-kinase with platelet glycoprotein Ibα, the major ligand-binding subunit of the glycoprotein Ib-IX-V complex. *J. Thromb. Haemost.* **8,** 324–330
15. Andrews, R. K., Munday, A. D., Mitchell, C. A., and Berndt, M. C. (2001) Interaction of calmodulin with the cytoplasmic domain of the platelet membrane glycoprotein Ib-IX-V complex. *Blood* **98,** 681–687
16. Falati, S., Edmead, C. E., and Poole, A. W. (1999) Glycoprotein Ib-V-IX, a receptor for von Willebrand factor, couples physically and functionally to the Fc receptor γ-chain, fyn, and lyn to activate human platelets. *Blood* **94,** 1648–1656
17. Wu, Y., Suzuki-Inoue, K., Satoh, K., Asazuma, N., Yatomi, Y., Berndt, M. C., and Ozaki, Y. (2001) Role of Fc receptor γ-chain in platelet glycoprotein Ib-mediated signaling. *Blood* **97,** 3836–3845
18. Sullam, P. M., Hyun, W. C., Szöllösi, J., Dong, J. F., Foss, W. M., and López, J. A. (1998) Physical proximity and functional interplay of the glycoprotein Ib-IX-V complex and the Fc receptor FcγRIIA on the platelet plasma membrane. *J. Biol. Chem.* **273,** 5331–5336
19. Shrimpton, C. N., Borthakur, G., Larrucea, S., Cruz, M. A., Dong, J. F., and López, J. A. (2002) Localization of the adhesion receptor glycoprotein Ib-IX-V complex to lipid rafts is required for platelet adhesion and activation. *J. Exp. Med.* **196,** 1057–1066
20. Vial, C., Fung, C. Y., Goodall, A. H., Mahaut-Smith, M. P., and Evans, R. J. (2006) Differential sensitivity of human platelet P2X1 and P2Y1 receptors to disruption of lipid rafts. *Biochem. Biophys. Res. Commun.* **343,** 415–419
21. Quinton, T. M., Kim, S., Jin, J., and Kunapuli, S. P. (2005) Lipid rafts are required in $G\alpha_i$ signaling downstream of the P2Y12 receptor during ADP-mediated platelet activation. *J. Thromb. Haemost.* **3,** 1036–1041
22. Jin, W., Inoue, O., Tamura, N., Suzuki-Inoue, K., Satoh, K., Berndt, M. C., Handa, M., Goto, S., and Ozaki, Y. (2007) A role for glycosphingolipid-enriched microdomains in platelet glycoprotein Ib-mediated platelet activation. *J. Thromb. Haemost.* **5,** 1034–1040
23. Pradhan, S., and Vijayan, K. V. (2013) Lipid rafts contribute to agonist-induced serine/threonine phosphatase activation and platelet aggregation. *J. Thromb. Haemost.* **11,** 1612–1615
24. Geng, H., Xu, G., Ran, Y., López, J. A., and Peng, Y. (2011) Platelet glycoprotein Ib β/IX mediates glycoprotein Ib α localization to membrane lipid domain critical for von Willebrand factor interaction at high shear. *J. Biol. Chem.* **286,** 21315–21323

25. Perschl, A., Lesley, J., English, N., Hyman, R., and Trowbridge, I. S. (1995) Transmembrane domain of CD44 is required for its detergent insolubility in fibroblasts. *J. Cell Sci.* **108,** 1033–1041
26. Scheiffele, P., Roth, M. G., and Simons, K. (1997) Interaction of influenza virus haemagglutinin with sphingolipid-cholesterol membrane domains via its transmembrane domain. *EMBO J.* **16,** 5501–5508
27. Bock, J., and Gulbins, E. (2003) The transmembranous domain of CD40 determines CD40 partitioning into lipid rafts. *FEBS Lett.* **534,** 169–174
28. Gosse, J. A., Wagenknecht-Wiesner, A., Holowka, D., and Baird, B. (2005) Transmembrane sequences are determinants of immunoreceptor signaling. *J. Immunol.* **175,** 2123–2131
29. Brown, D. A. (2006) Lipid rafts, detergent-resistant membranes, and raft targeting signals. *Physiology* (*Bethesda*) **21,** 430–439
30. López, J. A., Andrews, R. K., Afshar-Kharghan, V., and Berndt, M. C. (1998) Bernard-Soulier syndrome. *Blood* **91,** 4397–4418
31. Peng, Y., Berndt, M. C., Cruz, M. A., and López, J. A. (2004) The α1 helix-β13 strand spanning Leu^{214}–Val^{229} of platelet glycoprotein Ibα facilitates the interaction with von Willebrand factor: evidence from characterization of the epitope of monoclonal antibody AP1. *Blood* **104,** 3971–3978
32. López, J. A., Li, C. Q., Weisman, S., and Chambers, M. (1995) The GP Ib-IX "complex-specific" monoclonal antibody SZ1 binds to a conformation-sensitive epitope on GP IX: implications for the target antigen of quinine/quinidine-dependent autoantibodies. *Blood* **85,** 1254–1258
33. Du, X., Beutler, L., Ruan, C., Castaldi, P. A., and Berndt, M. C. (1987) Glycoprotein Ib and glycoprotein IX are fully complexed in the intact platelet membrane. *Blood* **69,** 1524–1527
34. Berndt, M. C., Gregory, C., Kabral, A., Zola, H., Fournier, D., and Castaldi, P. A. (1985) Purification and preliminary characterization of the glycoprotein Ib complex in the human platelet membrane. *Eur. J. Biochem.* **151,** 637–649
35. Fredrickson, B. J., Dong, J. F., McIntire, L. V., and López, J. A. (1998) Shear-dependent rolling on von Willebrand factor of mammalian cells expressing the platelet glycoprotein Ib-IX-V complex. *Blood* **92,** 3684–3693
36. Mo, X., Luo, S. Z., López, J. A., and Li, R. (2008) Juxtamembrane basic residues in glycoprotein Ibβ cytoplasmic domain are required for assembly and surface expression of glycoprotein Ib-IX complex. *FEBS Lett.* **582,** 3270–3274
37. Brown, D. A., and London, E. (1998) Functions of lipid rafts in biological membranes. *Annu. Rev. Cell Dev. Biol.* **14,** 111–136
38. Mo, X., Lu, N., Padilla, A., López, J. A., and Li, R. (2006) The transmembrane domain of glycoprotein Ibβ is critical to efficient expression of glycoprotein Ib-IX complex in the plasma membrane. *J. Biol. Chem.* **281,** 23050–23059