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Specific heteromeric association of four transmembrane peptides derived from platelet glycoprotein Ib-IX complex

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Summary

As the receptor on platelet surface for von Willebrand factor, glycoprotein (GP) Ib-IX complex is critically involved in hemostasis and thrombosis. How the complex is assembled from GP Ib α , GP Ib β and GP IX subunits, all of which are type I transmembrane proteins, is not entirely clear. Genetic and mutational analyses have identified the transmembrane (TM) domains of these subunits as active participants in complex assembly. In this study, peptides containing the transmembrane domain of each subunit have been produced and their interaction with one another characterized. Only the Ib β TM sequence, not Ib α and IX counterparts, can form homo-oligomers in SDS electrophoresis and TOXCAT assays. Following up on our earlier observation that a Ib β -Ib α -Ib β peptide complex ($\alpha\beta_2$) linked through native juxtamembrane disulfide bonds could be produced from isolated Ib α and Ib β TM peptides in detergent micelles, here we show that addition of the IX TM peptide facilitates formation of the native $\alpha\beta_2$ complex, reproducing the same effect by the IX subunit in cells expressing GP Ib-IX complex. Specific fluorescence resonance energy transfer was observed between donor-labeled $\alpha\beta_2$ peptide complex and acceptor-conjugated IX TM peptide in micelles. Finally, the mutation D135K in the IX TM peptide could hamper both the $\alpha\beta_2$ complex formation and the energy transfer, consistent with its reported effect in the full-length complex. Overall, our results have demonstrated directly the native-like heteromeric interaction among the isolated Ib α , Ib β and IX TM peptides, which provides support for the four-helical bundle model of the TM domains in GP Ib-IX complex and paves the way for further structural analysis. The methods developed in this study may also be applicable to other studies of heteromeric interaction among multiple TM helices.

Keywords

GP Ib-IX complex; transmembrane domain interaction; complex assembly; thiol-disulfide exchange; fluorescence resonance energy transfer

Introduction

Glycoprotein (GP) Ib-IX-V complex (CD42) is a membrane protein complex expressed primarily on the platelet surface and serves as a receptor for many proteins involved in hemostasis and thrombosis, of which von Willebrand factor (VWF) is the most prominent¹.

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Through its interaction with VWF immobilized at the damaged blood vessel wall, GP Ib-IX-V complex mediates the initial tethering and rolling of circulating platelets to the injury site². Ligation of VWF to the complex sends an activating signal into the platelet, which helps to activate platelet integrin $\alpha_{IIb}\beta_3$ and induce platelet aggregation³⁻⁵. Conversely, binding of the GP Ib-IX-V complex to VWF can be modulated by certain intracellular signals through the cytoplasmic domains of several subunits in the complex^{6; 7}. However, how the GP Ib-IX-V complex mediates the signals across the membrane, in both directions, is not clear, partly due to the lack of understanding of assembly and organization of this complex.

The GP Ib-IX-V complex is composed of four different type I transmembrane proteins: GP Iba, GP Ib β , GP IX and GP V⁸⁻¹⁰. With the VWF-binding site in its extracellular domain¹¹, GP Iba is linked through membrane-proximal disulfide bonds to two Ib β subunits¹². The disulfide-linked Ib β -Iba-Ib β complex, also known as GP Ib, interacts noncovalently but tightly with GP IX⁹. The resulting GP Ib-IX complex is associated more loosely to GP V with an apparent 2:1 stoichiometry¹⁰. Since removing GP V did not impact the VWF-binding ability of the platelet¹³, research efforts have concentrated on the structure and function of the GP Ib-IX complex.

Efficient expression of the GP Ib-IX complex in the plasma membrane of transfected mammalian cells requires all of its three subunits¹⁴, which accurately reflects a symptom of the Bernard-Soulier Syndrome, a rare hereditary bleeding disorder that can be caused by mutations in either Iba, Ib β or IX subunit¹⁵. Proper assembly of the GP Ib-IX complex in the endoplasmic reticulum appears to be a prerequisite for its stability, trafficking and ultimately efficient expression in the plasma membrane¹⁶. Although the molecular mechanism remains to be elucidated, the good correlation between the complex expression level on the cell surface and the extent of its assembly in the cell has provided a useful venue to probe organization of the GP Ib-IX complex by mutational analysis. The interaction between Ib β and IX subunits was thereby found to involve the N-terminal cysteine-rich region in the Ib β extracellular domain¹⁷. The transmembrane (TM) domains were recently identified as critical participants in assembly of the GP Ib-IX complex¹⁸. In particular, we showed that replacing the IX TM domain with poly-LeuAla sequence not only lowered the expression level of GP Iba, but also hampered formation of native disulfide bonds between GP Iba and GP Ib β ¹⁹. Based on these studies, a four-helical bundle model has been proposed for the TM domains of the GP Ib-IX complex (Fig. 1).

Although mutational analyses have provided valuable insight into organization of the GP Ib-IX complex, the underlying structural basis remains unclear. This is partly due to the lack of demonstration of direct interactions between isolated extracellular or TM domains of the complex. In this paper, we show that two methods, thiol-disulfide exchange and fluorescence resonance energy transfer, have been adapted to detect the heteromeric interaction of the Ib-IX-derived TM peptides in detergent micelles, thus providing direct evidence to support the four-helical bundle model for the TM domains of the GP Ib-IX complex. The methods adapted for this study may also be applied to other heteromeric TM-TM interactions.

Results

The TM domain of GP Ib β , not those of GP Iba and GP IX, can oligomerize

Before assessing their ability to interact with one another, we analyzed the self-associating ability of each TM domain in the GP Ib-IX complex using the TOXCAT assay. In the TOXCAT assay, a ToxR-TM-MBP chimeric protein that contains the TM domain of interest is expressed in the inner membrane of *Escherichia coli*²⁰. Dimerization of the TM sequence brings together the neighboring ToxR domains in the cytoplasm, which induce expression of chloramphenicol acetyl transferase (CAT) in the cell. Thus, the CAT activity becomes a good indicator for the

extent of TM self-association in the membrane. As shown in Figure 2, the ToxR-TM-MBP constructs containing the respective TM sequences of GP Ib α , GP Ib β and GP IX were expressed in *E. coli* at comparable levels as GpA-WT and GpA-G83I, the control constructs containing the wild type and mutated TM domain of glycophorin A. MalE complementation tests (Fig. 2C) and protease digestion of spheroplasts (data not shown) confirmed that all the chimeric constructs were correctly inserted into the inner membrane of *E. coli*. Comparison of CAT activities indicated that the Ib β TM domain dimerizes to the extent approaching that of the strongly dimerizing GpA-WT (Fig. 2B). In contrast, neither Ib α nor IX TM domain showed significant dimerization in the assay.

We next assessed the ability of the TM domains in the GP Ib-IX complex to interact with one another in SDS. Three TM peptides, each of which contains the TM domain of a subunit and its flanking sequences, were synthesized and named after the respective subunit. While the peptide Ib α -TM contained a portion of the Ib α cytoplasmic domain, the relatively short cytoplasmic domains of GP Ib β and GP IX were entirely included in Ib β -TM and IX-TM, respectively (Table 1). During electrophoresis in SDS, Ib β -TM could migrate mostly as a dimer or sometimes as higher-order oligomers at higher concentrations (Fig. 3). The bands of both Ib α -TM and IX-TM corresponded to monomers at all peptide concentrations tested, consistent with the TOXCAT results. Mixing Ib α -TM, Ib β -TM and IX-TM prior to electrophoresis failed to produce any additional protein bands in the gel (data not shown), indicating that SDS could not support the heteromeric interaction of these TM peptides.

The IX-TM peptide facilitated formation of disulfide bonds between Ib α -TM and Ib β -TM

We had shown earlier that Ib α -TM and Ib β -TM, mixed in dodecylphosphocholine (DPC) micelles and incubated at room temperature in a buffer containing 1 mM reduced glutathione and 1 mM oxidized glutathione to facilitate thiol-disulfide exchange, could spontaneously form a (Ib β -TM)-(Ib α -TM)-(Ib β -TM) peptide complex ($\alpha\beta_2$) with the same inter-molecular disulfide bonds observed in full-length GP Ib-IX complex¹². Since formation of a disulfide bond in a thiol-disulfide exchange reaction is thermodynamically coupled to the extent of noncovalent association of TM peptides²¹, preferential formation of native disulfide bonds between Ib α -TM and Ib β -TM demonstrated the affinity between the two peptides. At the same time, mixing Ib α -TM and Ib β -TM also generated products containing non-native disulfide bonds (Fig. 4), which was consistent with an earlier observation that CHO cells transfected with full-length GP Ib α and GP Ib β , but not GP IX, produced Ib α -containing complexes with non-native disulfide bonds¹⁹. Since GP IX assists in formation of native GP Ib in transfected CHO cells, we reasoned that, if the interaction among the Ib-IX TM domains drives the assembly process, the peptide IX-TM should facilitate formation of native disulfide bonds between Ib α -TM and Ib β -TM in a thiol-disulfide exchange reaction.

When Ib α -TM and Ib β -TM were mixed together with n-dodecyl β -D-maltoside (DM) at a molar ratio of 1:2:200 and incubated for thiol-disulfide exchange, native-like $\alpha\beta_2$ was one of the major products at equilibrium, accounting for $13.5 \pm 0.5\%$ of total Ib β -TM in the reaction (Fig. 4). Adding IX-TM to the mixture, to the same concentration as Ib α -TM, significantly increased production of only $\alpha\beta_2$ at the expense of the other products ($41.2 \pm 4.6\%$ of total Ib β -TM). In contrast, the mutant IX_{D135K}-TM did not increase $\alpha\beta_2$ production to the same extent as the wild type peptide ($21.5 \pm 2.4\%$ of total Ib β -TM). This is consistent with the earlier observation that changing Asp135, a polar residue in the IX TM domain, to Lys markedly hampered formation of the disulfide bonds between GP Ib α and GP Ib β subunits¹⁹.

A key feature of an equilibrated thiol-disulfide exchange reaction is its reversibility²¹. When the disulfide-linked Ib β -TM dimer, instead of the Ib β -TM monomer, was used in the reaction with Ib α -TM and IX-TM, the same increase of $\alpha\beta_2$ production was observed (Fig. 5). Since generation of $\alpha\beta_2$ from the Ib β -TM dimer requires reduction and reformation of disulfide

bonds, this result demonstrated that the exchange reaction, with reversible disulfide bond formation, has reached equilibrium under experimental conditions. We next compared the effect of different detergents, at the same peptide/detergent ratio, on $\alpha\beta_2$ production (Table 2). Consistent with the electrophoretic results, no $\alpha\beta_2$ was observed in SDS; only disulfide-linked $Ib\beta$ -TM dimer and oxidized $Ib\alpha$ -TM with an intramolecular disulfide bond were present (data not shown). DM, with a nonionic head group, supported the largest facilitating effect by IX-TM (Table 3). Smaller IX-TM-induced enhancement on $\alpha\beta_2$ production was observed in DPC and lysomyristoyl phosphatidylcholine (LMPC). Although it was not possible at the present time to delineate contribution of detergents to association of the Ib -IX-derived TM peptides, facilitation of disulfide formation between $Ib\alpha$ -TM and $Ib\beta$ -TM by IX-TM was observed in all nonionic and zwitterionic detergents tested. Overall, our results from the thiol-disulfide exchange assay showed that addition of IX-TM significantly facilitated formation of native-like disulfide bonds between $Ib\alpha$ -TM and $Ib\beta$ -TM in detergent micelles. The simplest explanation for our observation is that IX-TM interacts specifically with one $Ib\alpha$ -TM and two $Ib\beta$ -TM, thereby helping to bring the four Cys residues in latter peptides into proximity for disulfide formation.

FRET between $Ib\alpha$ -TM, $Ib\beta$ -TM and IX-TM peptides in DM micelles

FRET has been widely used to monitor association of TM peptides in detergents and liposomes²²⁻²⁵. Since FRET can be best analyzed and interpreted when only one donor and one acceptor are involved in each transfer, we chose not to label $Ib\beta$ -TM. In this study, Cy3 was used as donor fluorophore to label $Ib\alpha$ -TM, and Cy5 as acceptor to label IX-TM. The Cy3/Cy5 pair was chosen partly for their relatively long Förster radius (50-55 Å)^{25, 26}; the FRET signal was thus largely independent of the details of interaction between TM peptides. The absence of Cys residues in IX-TM afforded us the ability to place a Cys residue at the N-terminal end of the IX TM sequence and to conjugate Cy5 to Cys-IX-TM by the well-established thiol-based method (Table 1). On the other hand, the necessity of keeping native Cys residues in $Ib\alpha$ -TM for disulfide bond formation precluded thiol-based conjugation of Cy3. Instead, a new peptide designated 6H- $Ib\alpha$ -TM (Table 1), which included a hexahistidine motif before the $Ib\alpha$ TM sequence for noncovalent association with a Cy3 derivative of nitrilotriacetic acid chelate of nickel (Cy3-NTA)²⁷, was produced for FRET studies. In addition, most of the $Ib\alpha$ cytoplasmic sequence was not included in 6H- $Ib\alpha$ -TM. Despite these changes, 6H- $Ib\alpha$ -TM retained the ability of forming native disulfide bonds with $Ib\beta$ -TM in a thiol-disulfide exchange reaction, which could be enhanced by IX-TM (Fig. 6).

The disulfide-linked ($Ib\beta$ -TM)-(6H- $Ib\alpha$ -TM)-($Ib\beta$ -TM) peptide complex (6H- $\alpha\beta_2$) was subsequently purified and reconstituted in DM micelles. Given the apparent affinity of 3.6 μ M for the association of 6H- $Ib\alpha$ -TM with Cy3-NTA in DM-containing buffers (Fig. 7), we estimated that, in experiments described below (100 nM Cy3-NTA, 1 μ M 6H- $\alpha\beta_2$, 1 mM DM), approximately 25% of Cy3-NTA were bound to 6H- $\alpha\beta_2$. Adding unlabeled IX-TM to the Cy3-NTA/6H- $\alpha\beta_2$ mixture did not alter the Cy3 fluorescence (data not shown). In comparison, adding Cy5-IX-TM to the same mixture resulted in marked quenching of the Cy3 emission, in concurrent with the enhanced Cy5 emission (Fig. 8B). Since the attachment of Cy3-NTA to 6H- $\alpha\beta_2$ requires divalent cations, inclusion of EDTA in the buffer abolished the observed energy transfer between Cy3 and Cy5 (data not shown), indicating that FRET reflected the interaction between 6H- $\alpha\beta_2$ and IX-TM. Moreover, most of the energy transfer was reversed by addition of excess unlabeled IX-TM (Fig. 8B). The mutant Cy5-IX_{D135K}-TM peptide did not quench the Cy3 emission from Cy3-NTA/6H- $\alpha\beta_2$ (Fig. 8C). Overall, these results demonstrated the specificity of association between 6H- $\alpha\beta_2$ and IX-TM in DM micelles.

Discussion

Correct assembly of the GP Ib-IX complex is a prerequisite for its efficient expression on the cell surface. Since mutations that either abolish or sharply reduce surface expression of the complex have been identified in disparate regions of all the subunits^{e.g. 28-33}, the complex assembly must entail elaborate steps involving inter-subunit interactions in the lumen space as well as in the membrane bilayer. Despite the abundance of well-documented genetic evidence, direct detection of the interactions between specific elements of the GP Ib-IX complex, which would enable further structural and mechanistic studies, had not been reported. The Ib α extracellular domain, including the N-terminal ligand-binding domain, can be purified directly from plasma or expressed in a recombinant system^{11; 34}, but there have been no reports on Ib β nor IX extracellular domains. In this study, we focused on the TM domains and have analyzed TM peptides derived from the GP Ib-IX complex. While only the Ib β TM domain, but not those of the Ib α and IX subunits, is capable of self-association, they can interact with one another in detergent micelles. In thiol-disulfide exchange reactions, IX-TM facilitated formation of native-like disulfide bonds between Ib α -TM and two Ib β -TM peptides. Consistent with the observed effect in the full-length complex, IX_{D135K}-TM did not significantly enhance formation of disulfide bonds between Ib α -TM and Ib β -TM. Moreover, specific FRET was observed between Cy3-labeled 6H- $\alpha\beta_2$ complex and Cy5-IX-TM in DM micelles but not between the former and Cy5-IX_{D135K}-TM. Overall, these results have demonstrated a specific heteromeric interaction among the Ib-IX-derived TM peptides.

Understanding the inter-subunit interactions in the GP Ib-IX complex will not only help to elucidate the pathogenesis of Bernard-Soulier Syndrome and other Ib-IX-related diseases, but may also provide insights on the mechanism by which the complex transmits the signal across the platelet membrane. Although only the Ib α subunit in the receptor complex makes the direct contact with its ligand — VWF¹¹, cytoplasmic domains of both Ib α and Ib β subunits are involved in modulation of the ligand-binding activity³⁵⁻³⁷. Interactions between the Ib α and Ib β cytoplasmic domains with intracellular proteins have been under scrutiny³⁸⁻⁴². In this study, we have showed that Ib α -TM, Ib β -TM and IX-TM peptides associate specifically with one another through mostly lateral interaction of the TM domains. It paves the way for further quantitative analysis and structural characterization of the Ib-IX TM peptide complex, which will help us to understand how the changes in the association between the cytoplasmic domains with their binding proteins can be propagated to the TM domains and eventually impact the ligand-binding domain in the Ib α subunit.

Lateral interactions between TM helices are critically involved in folding of multi-span membrane proteins as well as assembly of membrane protein complexes⁴³. A number of methods have been developed to characterize the TM-TM interaction in the context of isolated peptides or fusion proteins^{e.g. 20; 21; 24; 44; 45}. Significant insights on sequence motifs and interaction energetics have been obtained from these studies⁴⁶⁻⁴⁹. However, the majority of studies to date have focused on homo-oligomerization of a single TM helix. For various reasons, reports of direct characterization of heteromeric TM-TM interaction have been scarce, and most published studies focus on the interaction between two TM helices⁵⁰⁻⁵⁴. Taking advantage of the naturally occurring juxtamembrane disulfide bonds, we have successfully detected the heteromeric association of four TM helices in micelles. Moreover, with the two disulfide bonds holding three helices together, the heterotetrameric association between Ib α , Ib β and IX TM peptides can be transformed into a more tractable heterodimeric association. Given the prevalence of juxtamembrane disulfide bonds in many membrane proteins^{e.g. 55-57} and relative ease in placing cysteines at appropriate positions near the target TM domain, our approach described here may be generally applicable for studies of other membrane protein complexes.

Materials and Methods

Materials

E. coli BL21 and DH5 α competent cells were purchased from Stratagene (La Jolla, CA). The expression vector pGEX-4T-3, thrombin, Cy5-maleimide and Cy3 bifunctional dye were purchased from Amersham Biosciences (Piscataway, NJ); DPC and LMPC were from Avanti Polar Lipids (Alabaster, AL); 1,10-phenanthroline, N-lauroylsarcosine, DM, acetyl-CoA, 5,5'-dithiobis-(2-nitrobenzoic acid) were from Sigma (St Louis, MO). Cy3-NTA was synthesized as described²⁷. Expression vectors pccKAN, pccGpA-WT, and pccGpA-G83I, along with *E. coli* strain MM39²⁰, were provided by Dr. Engelman.

Expression and purification of TM peptides

The pHex vector for expression of the His-GST-TM fusion protein has been described¹². Gene fragments encoding residues S473-R542 of human GP Ib α (CTAGAAGGATCCTCCAGAAATGACCCTTTTCTCC and GTCGACTCGAGTCACCTCTGCAGCTCCAGGTG), L121-S181 of human GP Ib β (CTAGAAGGATCCCTCTGCTGGGGG and GTCGACTCGAGTCAGGACTCGTCGG), and V132-D161 of human GP IX (CTAGAAGGATCCGTGCGCCCGGGGGTCGTCGACTCGAGTCAATCCAGGGCCTC TG) were amplified by PCR from the respective cDNAs using forward and reverse primers listed in parentheses above. Each fragment was inserted into the pHex vector as a BamHI-XhoI fragment, and eventually expressed in *E. coli* BL21 cells. The gene encoding 6H-Ib α -TM peptide was generated from the Ib α -TM gene through 3 successive mutagenesis steps using the following primers and their respective complementary primers: CTGGGATCCTCCAGACACCATCACTTTCTCCACCCCGAC, CCTCCAGACACCATCACCATCACCACCCGACTTTTGC, and GTTGGGCATGTGAAATGACAGGCCCTGGACTCTGGC. A cysteine codon was inserted into the IX-TM gene in a similar manner using primer CCGCGTGGATCCTGCGTGCGCCCGGGGGTC and its complementary primer.

Overexpression of the His-GST-TM fusion proteins and their purification followed published protocols¹². Briefly, the fusion protein was separated from water-soluble cytosolic proteins and membrane fractions, solubilized in 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.2 % N-lauroylsarcosine and 5 mM β -mercaptoethanol, and purified by Ni-affinity chromatography. Eluted by the buffer containing 200mM imidazole, the fusion protein was cleaved by thrombin (10-20 units/mg of protein) at room temperature overnight. The generated TM peptides were separated from the His-GST fragment by preparative reverse-phase HPLC and stored at -80 °C as lyophilized powder. Omission of reducing agents from purification of the His-GST-Ib β -TM fusion protein produced a significant portion of disulfide-link Ib β -Ib β TM peptide dimer in the end. The purity and identity of TM peptides was confirmed by SDS-PAGE, analytical HPLC and mass spectrometry (Fig. S1)¹².

SDS-PAGE

Purified TM peptides were dissolved and, if necessary, mixed to desired ratio in ethanol. After ethanol was evaporated under a stream of nitrogen, the peptides were dissolved in the LDS sample buffer with reducing agent (Invitrogen) and boiled at 100 °C for 10min. Then the samples were immediately loaded onto a precast 4-12% Bis-Tris SDS polyacrylamid gel (Invitrogen) that ran in the MES-containing running buffer at 4 °C.

TOXCAT assay

Gene fragments encoding TM domain residues P488-V504 of GP Ib α , G124-L143 of GP Ib β , V136-L153 of GP IX were amplified by PCR from the respective cDNAs, and inserted respectively into the pccKAN plasmid²⁰. The resulting pccKAN-based plasmids were transformed to *E.coli* MM39 cells. The topology of the ToxR-TM-MBP fusion protein expressed in MM39 cells was checked with the maltose complementation and spheroplast tests^{20; 58}. Expression of the fusion protein was measured by Western blot using an anti-MBP monoclonal antibody (Sigma). The activity of CAT expressed in MM39 cells was measured as described⁵⁹.

Thiol-disulfide exchange assay

Purified TM peptides were dissolved in ethanol and mixed with detergents in a glass vial to various peptide-to-detergent molar ratios between 1:50 and 1:1000 and the final individual peptide concentration in the reaction was 25 μ M. For different detergent experiments, the peptide-to detergent molar ratio of 1:200 was used as a constant. The mixture was dried by Argon stream to a thin film, and placed under vacuum overnight. The dried peptide/detergent mixture was hydrated in 40 μ L reaction buffer (100 mM Tris-HCl, pH 8.0, 100 mM KCl, 2 mM EDTA, 1 mM oxidized glutathione, 1 mM reduced glutathione) and incubated at room temperature for 16 h to allow the thiol-disulfide exchange reaction to reach equilibrium. To test the reversibility of disulfide formation in the thiol-disulfide exchange assay, the reaction was quenched at various time point by adding 6.7 μ L 0.5 M HCl and 33.3 μ L organic HPLC solvent. The reaction products were analyzed by reverse-phase HPLC in a diphenyl analytical column as described¹². The assignment of the each peak was assisted by mass determination and the product concentration of each peak was calculated by integration of the corresponding HPLC peak area as the percentage of the total Ib β -TM peak area derived from the adding 2.5 mM freshly prepared dithiothreitol (DTT).

Conjugation of Cy5 to the Cys-IX-TM peptide

Approximate 1 mg of Cys-IX-TM peptide was dissolved in 900 μ L hexafluoroisopropanol, before 100 μ L 100mM Tris-HCl, pH7.4 was slowly added. Cy5-maleimide, at 3 $\times$ excess, was dissolved in 50 μ L dimethylformamide and immediately added to the peptide solution to start conjugation at room temperature. The extent of conjugation was monitored by analytical HPLC (Fig. S2). Upon completion, reduced glutathione in excess was added to prevent further unwanted reaction. The Cy5-conjugated Cys-IX-TM (Cy5-IX-TM, ϵ_{650} = 250000) was further purified by HPLC and its purity confirmed by analytical HPLC, SDS-PAGE, and mass spectrometry (Fig. S2).

Preparation and purification of the disulfide-linked 6H- $\alpha\beta_2$ peptide complex

The disulfide-linked 6H- $\alpha\beta_2$ peptide complex was produced using essentially the same procedure as the thiol-disulfide exchange assay (6H-Ib α -TM:Ib β -TM:IX-TM:detergent, 1:2:1:500), albeit on a larger scale. At the end of the reaction, 1 mM CuSO₄ and 2 mM 1,10-phenanthroline were added to eliminate the (6H-Ib α -TM)-SS-(Ib β -TM) side product. Subsequently, the 6H- $\alpha\beta_2$ complex was purified by HPLC using a diphenyl semi-preparative column (Grace Vydac). The purity of 6H- $\alpha\beta_2$ (ϵ_{280} = 23490) was confirmed by SDS-PAGE, analytical HPLC, and mass spectrometry (Fig. S3).

Fluorescence anisotropy measurement

Fluorescence anisotropy experiments were performed on a PTI QuantaMaster fluorimeter equipped with polarizers (Photon Technology International, Birmingham, NJ). During the measurements, 2-10 μ L aliquots of 100 μ M TM peptide in 100 mM DM was titrated into 1 mL 100 nM Cy3-NTA. Both peptide/DM and dye were prepared in the same buffer (100mM

Tris-HCl, pH 8.0, 100mM KCl, 1mM DTT). Excitation wavelength was set to 520 nm, with both slit widths at 8 nm. Fluorescence anisotropy (A) at any given concentration of TM peptide was calculated using the following equations:

$$A = (I_{VV} - GI_{VH}) / (I_{VV} + 2GI_{VH}), G = I_{HV} / I_{HH}$$

where I is the fluorescence emission at 570 nm with its first subscript letter denoting the position (vertical or horizontal) of the excitation polarizer and the second subscript letter that of the emission polarizer, and G is the gating correction factor. The titration data were plotted as $(A - A_0)/A_0$ vs. the concentration of TM peptide, where A_0 is the fluorescence anisotropy of Cy3-NTA in the absence of any TM peptides. The equilibrium dissociation constant, K_d , was obtained by fitting the titration plot to a hyperbolic binding equation.

FRET measurement

The 6H- $\alpha\beta_2$ complex was mixed with DM at a molar ratio of 1:1000; then the mixture was dried by Argon stream to a thin film, and placed under vacuum overnight. The dried 6H- $\alpha\beta_2$ /DM mixture was dissolved in the buffer (100 mM Tris-HCl, 100mM KCl, pH 8.0) to a final 1 μ M peptide concentration as determined by absorption at 280 nm. In each experiment, 3.3 μ L 30 μ M Cy3-NTA and 10 μ L 30 μ M Cy5-IX-TM or IX-TM, all of which were prepared in the same buffer, were added in various combinations to 1 mL 6H- $\alpha\beta_2$ /DM mixture for fluorescence measurement. Each emission spectrum (530-720 nm) was the average of 2 scans using a step size of 1 nm, 1s integration time, and corrected for buffer background. The excitation wavelength was set to 520 nm with both slit widths at 3 nm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CAT, chloramphenicol acetyltransferase
 CHO, Chinese hamster ovary cell
 Cy3-NTA, Cy3 derivative of nitrilotriacetic acid chelate of nickel
 DM, n-dodecyl β -D-maltoside
 DPC, dodecylphosphocholine
 DTT, dithiothreitol
 FRET, fluorescence resonance energy transfer
 GP, glycoprotein
 GpA-WT, wild type transmembrane domain of glycoprotein A
 LMPC, 1-myristoyl-2-hydroxy-*sn*-glycero-3-phosphocholine
 SDS, sodium dodecyl sulfate
 TM, transmembrane
 VWF, von Willebrand factor

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Figure 1. The organization of the platelet GP Ib-IX complex

The complex consists of one Ib α , two Ib β , and one IX subunit, all of which are type I transmembrane proteins. Two juxtamembrane disulfide bonds link Ib α and Ib β subunits. The extracellular domains of all subunits contain leucine-rich repeats. A parallel 4-helical bundle model has been proposed for TM domains of the GP Ib-IX complex¹⁹. This illustration is adapted from earlier publications^{2; 12}.

Figure 2. The Ib β TM domain, not Ib α and IX counterparts, can self-associate in the cell membrane (A) Ib β , Ib α and IX TM sequences analyzed by the TOXCAT assay. (B) The enzymatic activity of CAT induced by self-association of the target TM domain and expressed as the percentage of that induced by GpA-WT. The GpA-WT and GpA-G83I constructs²⁰ were used as positive and negative controls, respectively. Data are shown as mean $\pm$ s.d. (n=3). The lower panel shows the expression levels of chimeric ToxR-TM-MBP proteins probed by Western blot. (C) MaleE complementation to test the topology of chimeric proteins. The pMAL-c2 and pMAL-p2 plasmids that express MBP protein in the cytoplasm and periplasm, respectively, were included as controls.

Figure 3. SDS-PAGE analysis of TM peptides derived from the GP Ib-IX complex
Ib β -TM, but not Ib α -TM and IX-TM, can form oligomers in SDS. Purified peptides were dissolved in the LDS sample buffer, resolved by electrophoresis in a SDS gel, and visualized by Coomassie Blue staining. The amount of peptide loaded in each lane is marked on top and molecular markers (in kDa) are shown in left. The predicted molecular masses of Ib β -TM, Ib α -TM and IX-TM are 6.8, 7.9 and 3.5 kDa, respectively.

Figure 4. IX-TM peptide facilitates formation of disulfide bonds between Iba-TM and Ibβ-TM
 Iba-TM, Ibβ-TM (and IX-TM) were mixed with 5.0 mM DM at a ratio of 1:2:(1):200 and incubated at room temperature for the thiol-disulfide exchange reaction to reach equilibrium. The reaction was quenched by lowering the pH and the products analyzed by reverse-phase analytical HPLC and mass spectrometry. In control reactions (dashed traces), DTT was added to prevent disulfide formation. The dotted line highlights the difference in $\alpha\beta_2$ production between the reactions. Peak 1: (Ibβ-TM)-SS-glutathione; 2: Ibβ-TM; 3: (Ibβ-TM)-SS-(Ibβ-TM); 4: $\alpha\beta_2$, or (Ibβ-TM)-SS-(Iba-TM)-SS-(Ibβ-TM); 5: (Iba-TM)-SS-(Ibβ-TM); 6: oxidized Iba-TM with an intramolecular disulfide; 7: Iba-TM; 8: (Ibβ-TM)-SS-(Iba-TM)-SS-(Iba-TM)-SS-(Ibβ-TM); 9: oxidized Iba-TM dimer with two intermolecular disulfides; 10: IX-TM; 11: IX_{D135K}-TM.

Figure 5. Reversibility of disulfide formation in thiol-disulfide exchange reactions
 Ib α -TM and IX-TM were mixed with (A) (Ib β -TM)-SS-(Ib β -TM) or (B) Ib β -TM at beginning of the reaction, and generated the same products after reaching equilibrium. The Ib α -TM/Ib β -TM/IX-TM/DM molar ratio was 1:2:1:200 in both reactions. The distribution of three Ib β -TM-containing products, expressed as the percentage of the total Ib β -TM, is measured at various time points of the reaction. $\square$: $\alpha\beta_2$; $\circ$: Ib β -TM; Δ : (Ib β -TM)-SS-(Ib β -TM).

Figure 6. Thiol-disulfide exchange reactions of 6H-Ib α -TM and Ib β -TM in 5.0 mM DM without (top panel) or with (bottom) IX-TM

The reactions as well as the assignment of HPLC peaks followed the description in the legend of Figure 4. Peak 12: 6H- $\alpha\beta_2$, or (Ib β -TM)-SS-(6H-Ib α -TM)-SS-(Ib β -TM); 13: (6H-Ib α -TM)-SS-(Ib β -TM); 14: oxidized 6H-Ib α -TM with an intramolecular disulfide; 15: 6H-Ib α -TM; 16: (Ib β -TM)-SS-(6H-Ib α -TM)-SS-(6H-Ib α -TM)-SS-(Ib β -TM). The dotted line highlights the difference in 6H- $\alpha\beta_2$ production between the two reactions.

Figure 7. Binding of 6H-Ib α -TM to Cy3-NTA monitored by Cy3 fluorescence anisotropy
 The binding curve was fitted to a hyperbolic binding equation with a binding affinity of $3.6 \pm 0.5 \mu\text{M}$. Note that Ib α -TM induced little change in Cy3 fluorescence anisotropy, indicating that Cy3-NTA specifically bound to the hexahistidine sequence in 6H-Ib α -TM.

Figure 8. Specific FRET between Cy3/6H- $\alpha\beta_2$ complex and Cy5-IX-TM in DM micelles

(A) Illustration of FRET between Cy3-NTA and Cy5 that are associated with the proposed four-helical TM peptide bundle¹⁹. The TM helix in each peptide is shown as a rod, and Cys residues participating in inter-subunit disulfide bonds are marked. Cy3-NTA is noncovalently bound to 6H- $\alpha\beta_2$ and Cy5 is covalently conjugated to IX-TM. (B, C) Fluorescence emission spectra of fluorophore-labeled TM peptides in DM micelles.

Table 1

Sequences of the Ib-IX-derived TM peptides used in this study

Peptide name	primary sequence ^a
Ib α -TM	GSSRNDPFLHPDF CCLPLG FYVLGLFWLLFASVVLILLSSWVGHVKPQALDSGGGAALTATQTTHLELQR
6H-Ib α -TM	GSSRHHHHHPDF CCLPLG FYVLGLFWLLFASVVLILLSSWVGHVK
Ib β -TM ^b	GSGGGL C WGALAAQLALLGLGLLHALLVLLSRLRRLRARARARAAARLSLTDPLVAERAGTDES
IX-TM ^c	GSVRPGVLWDVALVAVAALGLALLAGLLNATTEALD
IX _{D135K} -TM	GSVRPGVLWKVALVAVAALGLALLAGLLNATTEALD
Cys-IX-TM	GSCVRPGVLWDVALVAVAALGLALLAGLLNATTEALD
Cys-IX _{D135K} -TM	GSCVRPGVLWKVALVAVAALGLALLAGLLNATTEALD

^aThe TM sequence is indicated in bold and Cys residues underlined.

^bResidue C148 in the Ib β cytoplasmic domain was changed to Ser (the residue in the rat sequence).

^cResidue C154 in the IX cytoplasmic domain was changed to Asn (the residue in the rat sequence).

Table 2

The effect of detergents on production of the $\alpha\beta_2$ peptide complex in thiol-disulfide exchange reactions. The molar ratio of Ib α -TM, Ib β -TM, IX-TM (if included) and detergent was 1:2:1:200 in all reactions. The amount of $\alpha\beta_2$ is measured through integration of the corresponding HPLC peak and expressed as the percentage of total Ib β -TM in the reaction

Detergent	TM peptides	$\alpha\beta_2$ production (% of Ib β -TM)
DM	Ib α -TM/Ib β -TM	13.5 $\pm$ 0.5
	Ib α -TM/Ib β -TM/IX-TM	41.2 $\pm$ 4.6
DPC	Ib α -TM/Ib β -TM	27.7 $\pm$ 3.4
	Ib α -TM/Ib β -TM/IX-TM	32.8 $\pm$ 2.7
LMPC	Ib α -TM/Ib β -TM	20.5 $\pm$ 0.8
	Ib α -TM/Ib β -TM/IX-TM	29.4 $\pm$ 4.1