doi:10.1016/j.jmb.2008.07.037 J. Mol. Biol. (2008) 382, 448–457

JMB

Available online at www.sciencedirect.com

ScienceDirect

ELSEVIER

Specific Heteromeric Association of Four Transmembrane Peptides Derived from Platelet Glycoprotein Ib–IX Complex

Shi-Zhong Luo and Renhao Li*

Center for Membrane Biology, Department of Biochemistry and Molecular Biology, The University of Texas Health Science Center at Houston, Houston, TX 77030, USA

Received 30 March 2008; received in revised form 8 June 2008; accepted 13 July 2008
Available online
22 July 2008

As the receptor on the 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 Ibα, GP Ibβ 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 assembly of the complex. In this study, peptides containing the transmembrane domain of each subunit have been produced and their interaction with one another characterized. Only the Ibβ TM sequence, but not the Ibα and IX counterparts, can form homo-oligomers in SDS-PAGE and TOXCAT assays. Following up on our earlier observation that a Ibβ–Ibα–Ibβ peptide complex ($\alpha\beta_2$) linked through native juxtamembrane disulfide bonds could be produced from isolated Ibα and Ibβ TM peptides in detergent micelles, we show here 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 the 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 formation of the $\alpha\beta_2$ complex 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 Ibα, Ibβ and IX TM peptides, which provides support for the four-helix bundle model of the TM domains in the GP Ib–IX complex and paves the way for further structural analysis. The methods developed in this study may 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

Edited by J. Bowie

Introduction

The 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 prominent.[1] Through its interaction with VWF immobilized at the damaged blood vessel wall, the GP Ib–IX–V complex mediates the initial tethering and rolling of circulating platelets to the injury site.[2] 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 aggregation.[3–5] Conversely, binding of the GP Ib–IX–V

*Corresponding author. E-mail address: renhao.li@uth.tmc.edu.

Abbreviations used: 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 glycophorin A; LMPC, 1-myristoyl-2-hydroxy-*sn*-glycero-3-phosphocholine; TM, transmembrane; VWF, von Willebrand factor.

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 Ibα, GP Ibβ, GP IX, and GP V.[8–10] With the VWF-binding site in its extracellular domain,[11] GP Ibα is linked through membrane-proximal disulfide bonds to two Ibβ subunits.[12] The disulfide-linked Ibβ–Ibα–Ibβ complex, also known as GP Ib, interacts noncovalently but tightly with GP IX.[9] The resulting GP Ib–IX complex is associated more loosely to GP V with an apparent 2:1 stoichiometry.[10] Since removing GP V did not impact the VWF-binding ability of the platelet,[13] 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 subunits,[14] which accurately reflects a symptom of the Bernard-Soulier syndrome, a rare hereditary bleeding disorder that can be caused by mutations in either Ibα, Ibβ or IX subunit.[15] 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 membrane.[16] 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 Ibβ and IX subunits was found to involve the N-terminal cysteine-rich region in the Ibβ extracellular domain.[17] The transmembrane (TM) domains

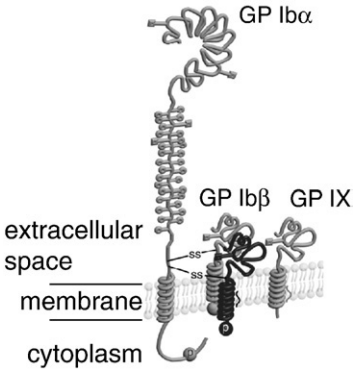

Fig. 1. The organization of the platelet GP Ib–IX complex. The complex consists of one Ibα subunit, two Ibβ sununits, and one IX subunit, all of which are type I transmembrane proteins. Two juxtamembrane disulfide bonds link the Ibα and Ibβ subunits. The extracellular domains of all subunits contain leucine-rich repeats. A parallel four-helix bundle model has been proposed for TM domains of the GP Ib–IX complex.[19] This illustration is adapted from earlier publications.[2,12]

were recently identified as critical participants in assembly of the GP Ib–IX complex.[18] In particular, we showed that replacing the IX TM domain with a poly-LeuAla sequence lowered the expression level of GP Ibα, and hampered formation of native disulfide bonds between GP Ibα and GP Ibβ.[19] On the basis of these studies, a four-helix 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. Here, 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-helix bundle model for the TM domains of the GP Ib–IX complex. The methods adapted for this study may be applied to other heteromeric TM–TM interactions.

Results

The TM domain of GP Ibβ, but not those of GP Ibα 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, CAT activity is a good indicator for the extent of TM self-association in the membrane. As shown in Fig. 2, the ToxR-TM-MBP constructs containing the respective TM sequences of GP Ibα, GP Ibβ and GP IX were expressed in *E. coli* at levels comparable with those of 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 inserted correctly into the inner membrane of *E. coli*. Comparison of CAT activities indicated that the Ibβ TM domain dimerizes to an extent approaching that of the strongly dimerizing GpA-WT (Fig. 2b). In contrast, neither Ibα 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,