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Juxtamembrane basic residues in glycoprotein Ibβ cytoplasmic domain are required for assembly and surface expression of glycoprotein Ib-IX complex

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Abstract

Platelet glycoprotein (GP) Ib-IX complex requires all its three subunits for efficient expression on the cell surface, but the underlying molecular basis is not fully clear. Using transfected Chinese hamster ovary cells as the model system, we demonstrate that juxtamembrane residues 149–154 in the cytoplasmic domain of the GPIbβ subunit is required for assembly and surface expression of the GPIb-IX complex. The complex, or GPIbβ by itself, lacking these residues is retained in the endoplasmic reticulum. Our results thus have illustrated an important role of the GPIbβ cytoplasmic domain in biosynthesis of the GPIb-IX complex.

Structured summary—MINT-6742751, MINT-6742907:

GPIX (uniprotkb:P14770), *GPIbalpha* (uniprotkb:P07359) and *GPIbbeta* (uniprotkb:P13224) *physically interact* (MI:0218) by *fluorescence-activated cell sorting* (MI:0054)

Keywords

GPIb-IX complex; juxtamembrane region; polybasic residue cluster; protein biosynthesis; trafficking

Introduction

Identified primarily as the platelet receptor for von Willebrand factor, glycoprotein (GP)Ibα is also tapped for additional roles in hemostasis [1], inflammation [2], metastasis [3] and platelet genesis [4,5]. For such a multi-talented receptor, the elaborate process by which it is expressed and regulated is not fully understood. GPIbα is expressed in platelets as a part of the GPIb-IX complex; its efficient presentation on the platelet surface depends on the other subunits — GPIbβ and GPIX. Mutations in any subunit of the complex can cause no or low expression of GPIbα, resulting in severe bleeding disorder known as Bernard-Soulier Syndrome (BSS) [6]. The dependence of GPIbα expression on the other subunits has been reproduced in transfected Chinese hamster ovary (CHO) cells [7], making these cells an excellent model to characterize

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expression and regulation of GPIbα. In transfected CHO cells, the GPIb-IX complex assembles in the endoplasmic reticulum (ER) [8]. Through mutational analysis and direct characterization of interactions between the isolated elements, it has become clear that both interactions between GPIbβ and GPIX extracellular domains and among GPIbα, GPIbβ and GPIX transmembrane domains are critical to correct assembly of the GPIb-IX complex [9–11]. The latter interaction may help bring the juxtamembrane Cys residues in GPIbα and GPIbβ into proximity and facilitate formation of inter-subunit disulfide bonds between them [11]. In contrast to the many studies documenting the organization and assembly of the GPIb-IX complex, factors responsible for its prompt trafficking from ER to the plasma membrane are not clear.

Intracellular trafficking involves an elaborate set of cytoplasmic proteins that sort and move the cargo-carrying vesicles. Sequences in the cytoplasmic domain of many receptor proteins have been found to participate in the process, by either sorting the host protein to appropriate transport vesicles or interacting with cargo proteins or both [12,13]. Thus, we reasoned that the cytoplasmic domains of the GPIb-IX complex might play a role in trafficking of the complex. Since deletion of either GPIbα or GPIX cytoplasmic domains did not significantly affect complex expression in the plasma membrane [11,14], we have investigated here the role of the GPIbβ cytoplasmic domain in complex assembly and surface expression of the GPIb-IX complex in transfected CHO cells.

Experimental procedures

Materials

The pDX vector used to express the GPIb-IX complex in CHO cells has been described in earlier studies [7,15]. The CHO K1 cell line was purchased from ATCC. Antibodies against GPIbα (SZ2) and GPIX (sc-7069) were purchased from Beckman Coulter and Santa Cruz Biotechnology, respectively. Anti-HA tag and anti-actin antibodies were purchased from Sigma.

Recombinant DNA cloning

To construct the HA-tagged GPIbβ gene, the gene sequence encoding the HA epitope tag (YPYDVPDYA) was inserted, in multiple steps using a Quikchange mutagenesis kit (Stratagene), into the pDX-GPIbβ vector between those encoding the signal peptide and mature GPIbβ protein. To avoid any spontaneous mutations that may occur during the insertion, the HA-GPIbβ cDNA was extracted from the plasmid by EcoRI digestion and ligated back into a clean pDX vector. Each mutation in the GPIbβ cytoplasmic domain was generated by Quikchange, and the mutant gene fragment was obtained by NotI/SbfI double digestion and ligated back into the pDX-HA-GPIbβ vector. All the gene sequences were confirmed by DNA sequencing (SeqWright, Houston, TX).

Transient transfection of CHO cells and characterization of the GPIb-IX complex

GPIbα, GPIbβ and GPIX cDNAs were transiently transfected into CHO K1 cells using Lipofectamine 2000 (Invitrogen) according to protocols published earlier [10,16]. After transfection, surface and cellular expression levels of the GPIb-IX complex and GPIb formation were detected by flow cytometry and Western blot as described [16].

Fluorescence microscopy

Cellular localization of GPIbα and GPIbβ in transiently transfected CHO cells were analyzed by fluorescence microscopy. The cells were re-seeded, one day after transfection, onto the polylysine-coated slide and cultured for additional 24 hr. Cells on the slide were washed three times with ice-cold phosphate buffered saline, fixed in 3.7% paraformaldehyde for 10 min at

room temperature, and permeabilized for 7 min in methanol and 2 min in acetone at −20 °C. The cells were stained with 2 μg/mL anti-GPIbα, anti-HA or anti-calnexin antibodies in phosphate buffered saline containing 0.5% bovine serum albumin for 1 hour at room temperature, followed by Alexa Fluor 488-conjugated donkey anti-mouse IgG or Texas Red-conjugated goat anti-rabbit IgG. After washing to remove unattached antibodies, cells were finally mounted in Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA) and subjected to fluorescence microscopy. Labeled CHO cells were analyzed under an Olympus BX60 system microscope with BX-FLA reflected light fluorescence attachment (Olympus America Inc., Melville, NY, USA) equipped with an UPlanApo oil-immersion lens (100×, numerical aperture 1.35). Alexa Fluor 488 and Texas-Red emission were excited with the UV source and signals were filtered with band-pass 450–480nm and 545–580nm filters, respectively.

Results

In order to detect the GPIbβ level in transfected cells, the HA epitope tag was appended to the N-terminus of mature GPIbβ. Little effect on expression of the GPIb-IX complex or on formation of inter-subunit disulfide bonds between GPIbα and GPIbβ was evident with the HA-tagging of GPIbβ (supplementary Fig. S1). Thus, HA-GPIbβ was treated as the wild type construct throughout this study, and all mutations in the GPIbβ cytoplasmic domain were created on the HA-GPIbβ background. To enable a meaningful comparison of expression levels obtained from different experiments, data normalization was carried out as previously described [10,11].

In the first experiment to explore its importance in complex expression, a series of truncating mutations were introduced to the GPIbβ cytoplasmic domain (Fig. 1A). The truncated GPIbβ constructs were transfected transiently with wild type GPIbα and GPIX into CHO cells, and the expression levels of each subunit in the GPIb-IX complex were measured by flow cytometry and immunoblotting. As shown in Figure 1, while removing residues of the GPIbβ cytoplasmic domain distal to Leu167 (as in $GPIb\beta_{167\Delta}$) had little impact on complex expression compared to the wild type, further removing residues 161–167 (as in $GPIb\beta_{160\Delta}$) caused a small but reproducible decrease in the expression level of the complex. Deletion of the entire GPIbβ cytoplasmic domain abolished the expression of GPIbα and GPIX. Thus, residues 149–167 were necessary for expression and assembly of the complex.

To corroborate results from the truncation study and to further identify critical residues, residues 149–167 of GPIbβ was divided into three segments, each of which was replaced by poly-Ala sequence (Fig. 2). The effect of each Ala-replacement on complex expression and assembly was assessed in the same manner as the truncation constructs. Expression of the GPIb-IX complex in $CHO\alpha\beta_{164–167A}IX$ cells, in which residues 164–167 were replaced by alanines, was comparable to that in wild type CHOαβIX cells. Replacing residues 155–160 with alanines caused a marked decrease in complex expression. Nonetheless, formation of inter-subunit disulfide bonds between GPIbα and GPIbβ, which can be assessed by GPIb (GPIbβ-GPIbα-GPIbβ) formation in a non-reducing SDS gel (Fig. 2D) and used as an indicator for correct complex assembly, was maintained in both cells. In contrast, when the six juxtamembrane residues 149–154 were replaced by alanines, expression of GPIbα and GPIX was mostly abolished. Since the intracellular pool of GPIbβ was the same for wild type and $GPIb\beta_{149–154A}$ (Fig. 2C), it is unlikely that the mutation interfered with GPIbβ synthesis. Moreover, native GPIb formation was disrupted as a result of this mutation, indicating a breakdown of proper assembly of the GPIb-IX complex.

To test the possibility whether significantly decreased expression of the GPIb-IX complex in $CHO\alpha\beta_{149–154A}IX$ cells was primarily due to improper complex assembly, wild type or mutant

GPIbβ was transfected alone into CHO cells. In the absence of GPIbα and GPIX, synthesis of GPIbβ was not markedly impacted by changes in the cytoplasmic domain, since intracellular pools of mutant GPIbβ were comparable to wild type, if not higher (Fig. 3A). While surface expression of $GPIb\beta_{155–160A}$ or $GPIb\beta_{164–167A}$ was higher than that of wild type GPIbβ, surface expression of $GPIb\beta_{149–154A}$ was significantly lower (Fig. 3B). Thus, residues 149–154 are required for efficient expression of the GPIb-IX complex in the plasma membrane, for they not only participate in the assembly of the GPIb-IX complex, but also may influence trafficking of the receptor complex.

To further characterize the role of the GPIbβ cytoplasmic domain in protein trafficking to the plasma membrane, distribution of GPIbα and GPIbβ in transiently transfected CHO cells was visualized by immunostaining and fluorescence microscopy. GPIX was not examined due to the lack of an appropriate conformation-insensitive monoclonal antibody [17]. DAPI and anti-calnexin antibody were used to locate the nucleus and the ER, respectively. As shown in Fig. 4A, GPIbα in CHOαβ IX and $CHO\alpha\beta_{160\Delta}IX$ cells was present only at the peripheral part of the cell (i.e. cell surface). As expected, little GPIbα was detected in $CHO\alpha\beta_{148\Delta}IX$ and $CHO\alpha\beta_{149–154A}IX$ cells. The absence of GPIbα in the ER was consistent with an earlier report that incorrectly folded or assembled GPIbα, which presumably may not be suitable for trafficking to the plasma membrane, is promptly degraded in the lysosome [8]. In contrast to GPIbα, whereas little GPIbβ was located on the cell surface of $CHO\alpha\beta_{148\Delta}IX$ and $CHO\alpha\beta_{149–155A}IX$ cells, a large quantity was visualized in the ER (Fig. 4B). These data supported that deletion of the GPIbβ cytoplasmic domain or replacement of the six juxtamembrane residues with poly-Ala did not affect expression of GPIbβ, but abolished the ability of GPIbβ trafficking to the cell surface.

Discussion

GPIbβ has long been recognized as particularly critical to efficient presentation of GPIbα on the cell surface, because partial complexes of GPIbα/GPIbβ and GPIbβ/GPIX, but not GPIbα/GPIX, can be detected on the surface of transfected CHO cells, albeit at a much lower level than that of the full complex [15]. The importance of GPIbβ was attributed to its interaction with both GPIbα and GPIX, which presumably helps to stabilize the GPIb-IX complex. Hosting many BSS-causing mutations, the extracellular domain of GPIbβ binds to that of GPIX [18]. Moreover, the transmembrane domain of GPIbβ interacts with its counterparts from GPIbα and GPIX, substantiating two reported BSS-causing mutations that result in either deletion of the entire transmembrane and cytoplasmic domains of GPIbβ or their replacement [19,20]. In this study, we have demonstrated that juxtamembrane residues 149–154 in the GPIbβ cytoplasmic domain are essential to proper assembly and efficient expression of the GPIb-IX complex, which added another reason for the importance of GPIbβ.

Very little surface expression of the mutant GPIb-IX complex was detected in $CHO\alpha\beta_{149–154A}IX$ cells. Cys148 between the GPIbβ transmembrane and cytoplasmic domains is palmitoylated in the human GPIb-IX complex [21]; but it is not present in the mouse or rat GPIbβ sequence. Although mutating residues 149–154 may adversely affect palmitoylation of Cys148, lack of palmitoylation does not have an impact on complex assembly and expression (Fig. S2). Moreover, incorrect complex assembly caused by omission of GPIbβ juxtamembrane residues may lead to lowered expression on the cell surface, but it cannot account for all the observed effects. CHO cells transiently transfected with only GPIbα and GPIbβ express exclusively non-native GPIb complex, yet the expression level of GPIbα is approximately 30% of that in wild type CHOαβIX cells [10,11], much higher than that in $CHO\alpha\beta_{149–154A}IX$ cells. A plausible explanation for the detrimental effect caused by the mutation of the juxtamembrane residues may be a defect in trafficking to the plasma membrane. Wild type GPIbβ can be

expressed, without GPIbα and GPIX, on the surface of transfected CHO cells. By contrast, $GPIb\beta_{149–154A}$ cannot, providing support for a role of juxtamembrane residues in protein trafficking. Unable to reach the plasma membrane, the incorrectly assembled GPIb-IX complex is likely to suffer the fate of quick degradation.

Like many single-span membrane receptors, the juxtamembrane region of GPIbβ cytoplasmic domain is enriched in basic residues, which maintain the correct topology of the GPIb-IX complex via the positive-inside rule [22]. The juxtamembrane residues may also mediate trafficking of the host receptor complex. A recent study has shown that phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 4,5-disphosphate lipids could target proteins with polybasic clusters to the plasma membrane [23]. Thus it is conceivable that negatively-charged phosphotidylinositides or other lipids may bind to the juxtamembrane basic residues in GPIbβ and play a role in GPIbβ-mediated trafficking. Whether phosphotidylinositides bind the GPIbβ cytoplasmic domain and whether these lipids could target GPIbβ and by extension the GPIb-IX complex to the plasma membrane require further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

GP, glycoprotein; CHO, Chinese hamster ovary; ER, endoplasmic reticulum..

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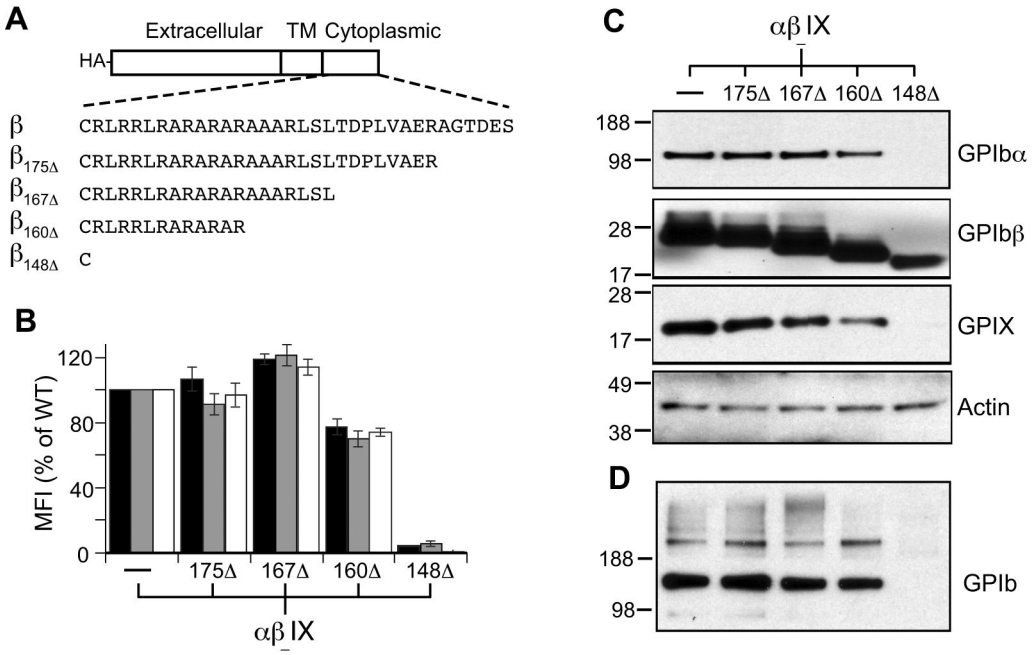

FIGURE 1. Sequential truncation of the GPIbβ cytoplasmic domain

A, sequences of cytoplasmic domains in wild type GPIbβ(β) and truncation constructs. *B*, surface expression levels of GPIbα (black column), GPIbβ (grey) and GPIX (white) in transiently transfected CHO cells measured by flow cytometry. The levels were quantified as relative mean fluorescence intensity (MFI), and normalized with CHOαβIX cells being 100% and cells transfected with sham vector 0%. The data are presented as mean ± S.D. (n=3). *C*, overall expression of individual subunits of the GPIb-IX complex in transfected cells. Cell lysates were resolved in SDS-PAGE under reducing conditions and eventually immunoblotted with antibodies against GPIbα (SZ2), GPIbβ (anti-HA), GPIX (polyclonal antibody), and actin. The identity of each cell lysate was noted on the top. *D*, GPIb formation, as an indicator of complex assembly, in various transfected cells. The lysates were resolved in SDS-PAGE under non-reducing conditions and immunoblotted for GPIbα.

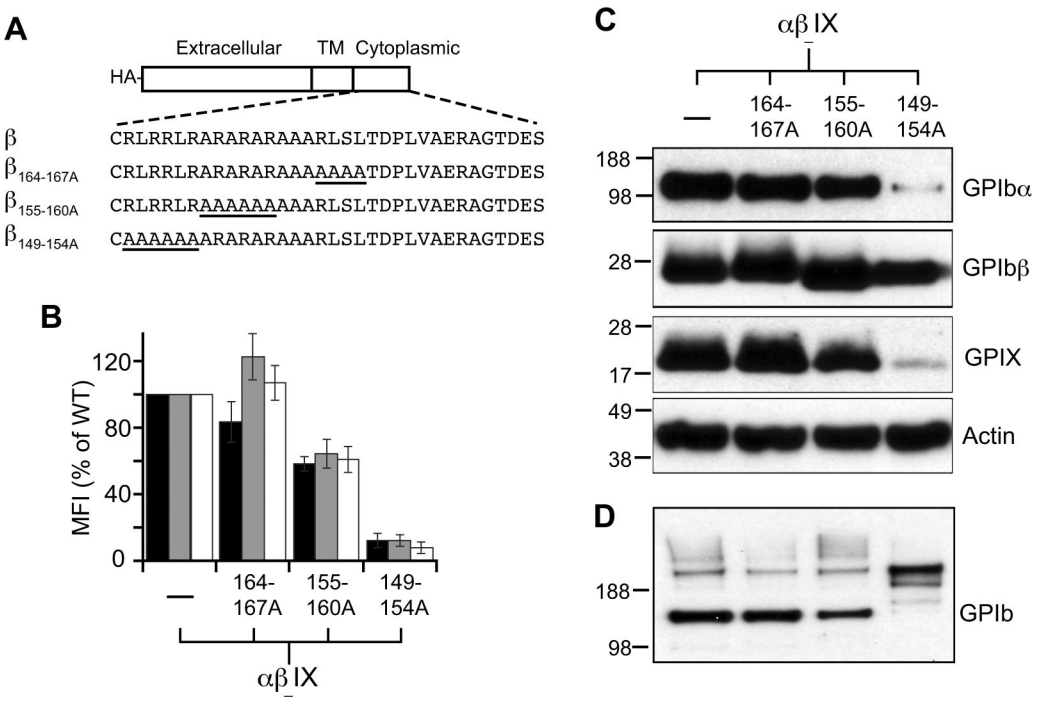

FIGURE 2. Juxtamembrane residues in the GPIbβ cytoplasmic domain are required for complex surface expression

A, sequences of cytoplasmic domains in the wild type and mutant GPIbβ constructs. In each sequence, mutated residues are underlined. Characterization of surface expression (*B*), overall expression levels (*C*) of individual subunits, as well as GPIb formation (*D*), in transfected CHO cells were carried out as outlined in the legend of Figure 1.

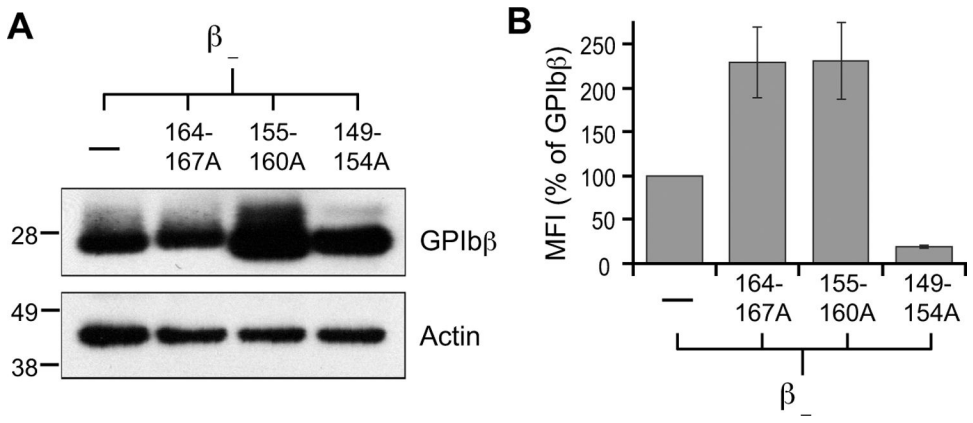

FIGURE 3. Individual expression of GPIbβ in CHO cells

Wild type GPIbβ and Ala-replacement mutants were transfected separately into CHO cells, in the absence of GPIbα and GPIX, and their expression measured by immunoblotting (*A*) and flow cytometry (*B*). The mean fluorescence values were normalized with GPIbβ cells being 100% and cells transfected with empty vector 0%, and presented as mean ± S.D. (n=4).

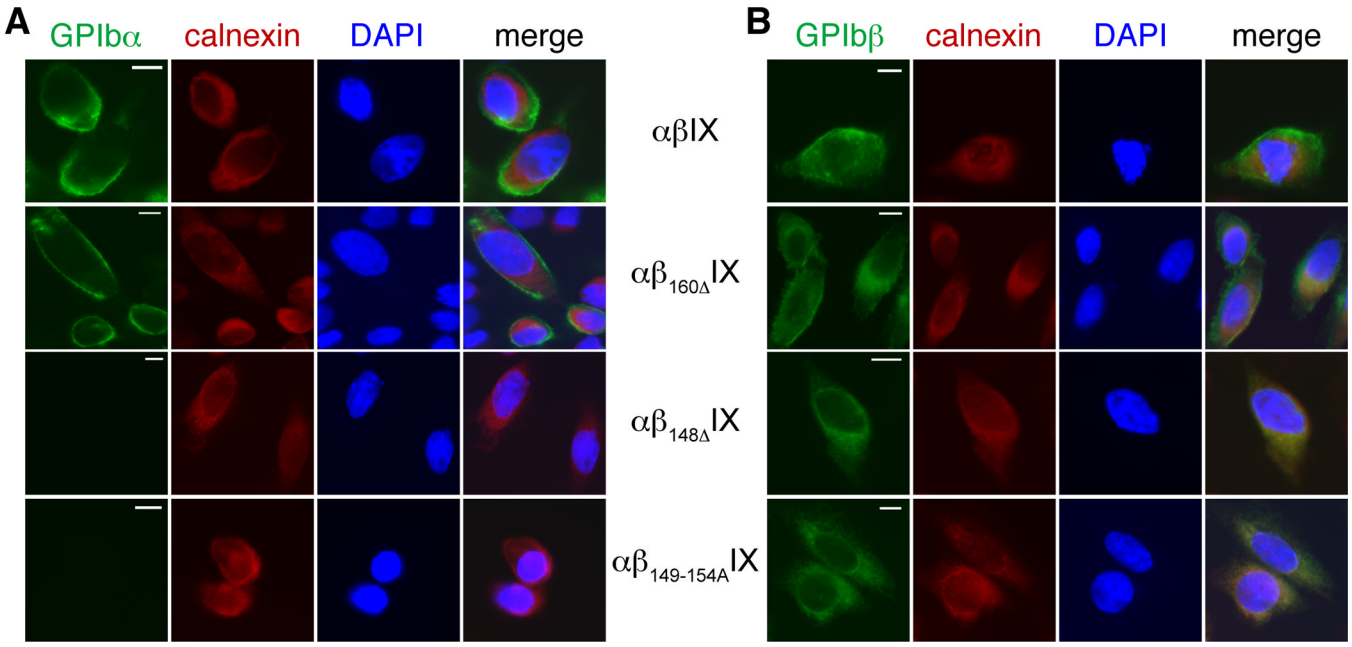

FIGURE 4. Cellular localization of GPIb*α* and GPIbβ in transfected CHO cells

CHO cells expressing the wild type or mutant GPIb-IX complex were detached and immobilized on poly-lysine-coated culture slides. After fixation and permeabilization, the cells were labeled with both anti-GPIbα antibody SZ2 (*A*) or anti-HA(GPIbβ) antibody (*B*) and the ER-marker calnexin. The cell nucleus was stained with DAPI contained in the mounting medium. The microscopic immunofluorescence images were obtained using filters adapted for Alexa Fluor 488 (green, SZ2 and anti-HA antibody), Texas Red (red) or DAPI (blue).