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Summary \\

\textbf{Background}—The glycoprotein (GP) Ib-IX-V complex, the von Willebrand factor receptor on platelet surface, is critically involved in hemostasis and thrombosis. GPV subunit interacts with GPIb-IX to form the GPIb-IX-V complex, but the underlying molecular basis remains unclear. It was observed earlier that efficient expression of GPV in the plasma membrane requires coexpression of GPIb-IX. \\

\textbf{Objectives and methods}—Hypothesizing that GPIb-IX stabilizes GPV through direct interaction and consequently enhances GPV surface expression, we aim in this study to identify structural elements in the complex that mediate the interaction between GPV and GPIb-IX by analyzing mutational effects on GPV surface expression in transfected Chinese hamster ovary cells. \\

\textbf{Results}—Enhancement of GPV surface expression by GPIb-IX requires transmembrane domains of both GPV and GPIbα, as replacing GPV transmembrane domain with an unrelated poly-leucine-alanine sequence abolished the enhancing effect of GPIb-IX. Additional mutagenesis analysis of the GPV transmembrane helix identified three helical sides containing conserved polar residues as critical to efficient GPV surface expression. Similarly, replacing residues in three sides (Gly495/Ala502/Leu509, Phe491/Trp498/Val505, and Y492/L499/L506) of the GPIbα transmembrane domain to leucines preserved the surface expression level of GPIb-IX but significantly altered that of GPV. \\

\textbf{Conclusions}—Our results demonstrate for the first time the importance of transmembrane domains to efficient surface expression of GPV and suggest that GPV and GPIbα transmembrane domains interact with each other, contributing to assembly of the GPIb-IX-V complex.

\textbf{Keywords} \\
GPIb-IX-V complex; complex assembly; transmembrane domain interaction

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Disclosure of Conflict of Interests \\
The authors state that they have no conflict of interest.
Introduction

Highly expressed in the plasma membrane of platelets, glycoprotein (GP) Ib-IX-V complex plays critical roles in hemostasis and thrombosis. Upon binding to its ligand von Willebrand factor (VWF), the GPIb-IX-V complex initiates platelet adhesion to the injury sites, and subsequently induces platelet activation and aggregation [1–4]. The GPIb-IX-V complex is composed of four single-span transmembrane protein subunits — GPIbα, GPIbβ, GPIX and GPV, with a stoichiometry of 2:4:2:1 [5–7]. GPIbα, GPIbβ and GPIX subunits interact with one another, through interactions among their transmembrane (TM) and extracellular domains, to form the tightly integrated GPIb-IX complex [5, 8–13]. Mutations in any of the three subunits can reduce significantly expression of the GPIb-IX complex in platelets and result in a severe bleeding disorder known as the Bernard–Soulier Syndrome (BSS) [14, 15]. Such requirement of all 3 subunits for the assembly and efficient surface expression of GPIb-IX has been reproduced in transfected Chinese hamster ovary (CHO) cells, making these cells an excellent model to investigate the assembly process of GPIb-IX [16]. For instance, GPIX cannot be expressed alone on the surface of transfected CHO cells. But its expression is significantly enhanced when co-expressed with GPIbβ, because GPIbβ interacts with and presumably stabilizes GPIX [17, 18]. Through mutational analysis in transfected CHO cells, we recently showed that BSS-causing missense mutations in the GPIbβ extracellular domain decrease the GPIX surface expression level by disrupting either folding of their host domain or quaternary association between GPIbβ and GPIX extracellular domains [13]. Similar approach has also been adopted to dissect and identify the interaction among the TM domains as a driving force for GPIb-IX assembly [8, 9, 12].

In contrast to the wealth of information obtained on GPIb-IX organization and structure, how GPV associates with GPIb-IX in the plasma membrane has not been explored. Only two lines of evidence had been reported earlier to support the interaction of GPV with GPIb-IX. First, GPV was coimmunoprecipitated with anti-GPIbα antibodies from the platelet lysate, and it was accomplished only in the presence of digitonin as the lysis detergent but not in the presence of NP-40 [7, 19]. Since digitonin is considered milder than nonionic detergents such as NP-40 and it can better preserve interactions between ionizable groups in the TM domains [20–22], it suggests that conserved polar residues in the GPV TM domain may be critical to its interaction with GPIb-IX. Second, GPV does not express well in the plasma membrane of transfected cells when expressed alone, but it does when coexpressed with GPIb-IX [19, 23, 24]. This is akin to the aforementioned dependence of GPIX surface expression on GPIbβ, suggesting that efficient surface expression of GPV depends on its interaction with GPIb-IX. In the present study, by systematically analyzing mutational effects on surface expression level of GPV in transfected CHO cells, we show that both TM domains of GPV and GPIbα, especially polar residues in the GPV TM domain, are indispensable for efficient surface expression of GPV and presumably the association of GPV with GPIb-IX.

Materials and methods

Materials

The CHO K1 and human embryonic kidney (HEK) 293T cells were obtained from ATCC (Manassas, VA). CHO cells stably expressing GPIb-IX have been described earlier [25]. WM23, an anti-GPIbα monoclonal antibody (mAb), was kindly provided by Dr. Michael Berndt. The anti-GPV mAb SW16 and the anti-GPIX mAb FMC25 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Millipore (Temacula, CA), respectively. All culture media were purchased from Invitrogen (Carlsbad, CA).
Mutagenesis of TM domains of the GPIb-IX-V complex

The mammalian expression vector pcDNA3.1/Hygro containing GPV cDNA, as well as vector pDX containing GPIba, GPIbβ and GPIX cDNAs, had been described [16, 24]. In the pcDNA3.1/Hygro-GPV vector, two unique restriction sites, SfiI (around residue R460) and BstBI (around residue R535), flanked the gene sequence encoding the GPV TM domain and were therefore used to construct GPV mutants. To replace the predicted TM domain in GPV with a poly-Leu-Ala sequence, a gene fragment encoding the poly-Leu-Ala sequence was synthesized, digested with SfiI and BstBI restriction enzymes, and ligated back into the vector that had been predigested and devoid of the gene sequence encoding the endogenous TM domain. Similar procedure was carried out for site-specific mutagenesis. For GPIba, a pDX-GPIba expression vector with unique BspEI and XbaI sites flanking the GPIba TM domain was used in an earlier study [8]. To construct GPIba mutations in the TM domain, the TM-encoding DNA fragment containing the desired mutations was generated by PCR amplification and subcloned back into the pDX-GPIba vector as a BspEI/XbaI fragment. All the mutations were confirmed by DNA sequencing.

Expression of the GPIb-IX-V complex in transiently transfected CHO K1 cells

pDX vectors containing GPIba, GPIbβ and GPIX cDNAs and pcDNA3.1/Hygro vector containing GPV cDNA were transiently transfected into CHO K1 cells using Lipofectamine 2000 (Invitrogen) as described before [8]. When only the GPIb-IX complex or GPV was transfected, empty vector was included to keep the total amount of DNA a constant. The transfected cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) with 10% fetal bovine serum for an additional 48 hours before being analyzed for protein expression. Surface expression of GPIba, GPIX and GPV was measured by flow cytometry after staining with the antibodies WM23, FMC25 and SW16, respectively. The quantified mean fluorescence intensity (MFI) of each cell population (10,000 cells) was normalized with the value of the wild type CHO-αβIIXV cells being 100% and that of CHO-vector cells 0%.

Preparation of the lentivirus containing the GPV gene

Wild-type or mutant GPV genes were amplified by PCR and subcloned into the pLVX-IRES-Puro vector (Clontech, Mountain View, CA) as a BamHI/EcoRI fragment. The plasmid was then cotransfected, following the manufacturer’s instruction, with psPAX2 and pMD2.G vectors (Addgene, Cambridge, MA) into HEK 293T cells cultured in DMEM complete media at the 3:2:1 ratio. The total amount of DNA used was 8 µg per transfection. Two days after transfection, the culture media containing the GPV-encoding lentivirus were collected and used directly for lentiviral infection. Similar procedure was carried out to generate the GFP-encoding lentivirus using the pLVX-IRES-ZsGreen vector (Clontech).

Expression of GPV in CHO cells by lentiviral infection

CHO K1 cells or CHO cells stably expressing GPIb-IX complex were cultured in a 6-well plate in DMEM complete medium containing necessary antibiotics to 90% confluency as described earlier [25]. The lentivirus containing the GPV gene (1 ml) was mixed with equal volume of DMEM complete medium before being added to the cultured CHO cells. To improve the infection efficiency, 5 µg/ml polybrane (Sigma-Aldrich) was also added to the media and the cells in the culture plate were centrifuged at 1,200 g for 1 h. After 24 h of incubation, the culture media were replaced with fresh media and the cells were incubated for additional 24 h before 5 µg/ml of puromycin was added. The infected cells were cultured for additional 4–7 days before being harvested for flow analysis on a FACS Canto II flow cytometer (BD Biosciences, San Jose, CA). Surface expression of GPV was measured by flow cytometry after staining with the monoclonal antibody SW16. The quantified MFI of
each cell population (10,000 cells) was normalized with the value of stable CHO-αβIX cells infected by GPV-lentivirus as 100% and that of CHO cells infected by empty lentivirus as 0%.

Results

To reproduce the dependence of GPV surface expression on GPIb-IX in our laboratory, GPV cDNA was transiently transfected, either with or without GPIb-IX constructs, into CHO K1 cells. CHO cells have been used extensively in the study of expression, structure and function of the GPIb-IX-V complex [e.g. 16, 24–28]. To minimize unwanted effects of transfection on comparison of protein expression levels among different cells, key parameters of transient transfection, such as the amount of expression vectors and the density and confluence level of cultured cells, were kept constant for all the samples in a single transfection experiment as described in our earlier studies on the GPIb-IX complex [8, 12], such that the transfection efficiency remained the same for all the cells within an experiment. The surface expression levels of GPV and GPIb-IX in CHO cells were measured by flow cytometry using monoclonal antibodies specifically targeting GPIbα, GPIX or GPV (Fig. 1A). Since neither GPIbα nor GPIX can express efficiently by itself in the plasma membrane and both are in the same complex with GPIbβ [16, 17], the difference in fluorescence intensities representing their respective expression levels as shown in Figure 1A is likely due to the difference in monoclonal antibodies used. In CHO cells transfected with cDNAs encoding the GPIb-IX-V complex (referred henceforth as CHO-αβIXV cells), surface expression levels of GPIbα and GPIX, representing the GPIb-IX expression level, were indistinguishable from those in CHO cells transfected with cDNAs encoding only the GPIb-IX complex (CHO-αβIX cells). When CHO cells transfected with only GPV-encoding cDNA (CHO-V cells), little GPV was detected on the cell surface. In comparison, significantly more GPV was detected by flow cytometry on the surface of CHO-αβIXV cells (Fig. 1A). These results are consistent with previous observations that efficient surface expression of GPIb-IX is not affected by the presence of GPV in the cell but that of GPV is dependent on the presence of GPIb-IX [24], which suggests that the GPIb-IX complex interacts with and stabilize GPV during its synthesis and subsequent transport to the plasma membrane.

The next step was to explore the interaction site between GPV and GPIb-IX utilizing the surface expression level of GPV as the indicator, which closely mimics our earlier studies on inter-subunit interactions within the GPIb-IX complex [8, 9, 12]. Although the transfection efficiency could be kept at the same level in any given transfection experiment, it could vary from experiment to experiment. To enable proper comparison of surface expression levels of various GPIb-IX-V subunits obtained from different transfection experiments, CHO cells transfected with sham expression vectors (CHO-vector cells) and CHO-αβIXV cells were included in each transfection experiment. The surface expression levels of individual subunits in a mutant complex were measured by flow cytometry, quantified by the mean fluorescence intensity (MFI) of the entire cell population (10,000 cells, including untransfected cells), and normalized against the values of CHO-αβIXV cells (100%) and CHO-vector cells (0%). For instance, Figure 1B plots the normalized surface expression levels of GPIbα and GPIX in CHO-αβIX cells and that of GPV in CHO-V cells. It shows that the GPV expression level in CHO-V cells is about 10–15% of that in CHO-αβIXV cells.

The GPV TM domain is critical to its efficient surface expression in the presence of GPIb-IX

To test whether the GPV TM domain is critical to the GPIb-IX-dependent surface expression of GPV, the gene sequence encoding the GPV TM domain (residues F505–I528)
was replaced with that encoding a poly-Leu-Ala sequence of the same length (Fig. 2A). Since the poly-Leu-Ala sequence is mostly hydrophobic and does not contain any known TM-TM association motifs, poly-Leu-Ala peptide forms a stable α-helix in the lipid bilayer and stays as a monomer [29]. Replacing the GPV TM domain with the poly-Leu-Ala sequence was designed to remove any interaction motif in the GPV TM domain that may mediate its interaction with GPIb-IX. After the mutant GPV containing the poly-Leu-Ala TM domain (GPV\textsubscript{pLA}) was transiently transfected into CHO cells, with or without GPIb-IX-encoding vectors, surface expression level of GPV\textsubscript{pLA} as well as that of GPIb-IX was measured by flow cytometry and quantitated as described above. As expected, surface expression levels of GPIb\textalpha and GPIX were not significantly affected by the presence of GPV\textsubscript{pLA} in the cell (CHO-αβIX vs. CHO-αβIX\textsubscript{pLA} cells in Fig. 2B). However, the relative expression level of GPV\textsubscript{pLA} in CHO-αβIX\textsubscript{pLA} cells was only 9.8±5.7% of the expression level of GPV in CHO-αβIX cells, significantly less than the relative expression level of GPIb-IX (Fig. 2C). These results indicate that the enhancing effect of GPIb-IX on GPV surface expression requires the GPV TM domain, suggesting that GPIb-IX may stabilize GPV through its interaction with the GPV TM domain.

To eliminate the possibility that the decrease in GPV expression as a result of TM replacement is due to an intrinsic property of the poly-Leu-Ala sequence rather than disruption of an interaction motif in the GPV TM domain, we proceeded next to identify site-specific mutations in the GPV TM domain that can recapitulate the effect of TM replacement. Considering the α-helical structure of a TM domain, it is reasonable to assume that the GPV TM domain interacts with another subunit via residues clustered on one side of the helix. To quickly screen for mutations, residues in the GPV TM domain were divided into seven “sides”, each of which corresponds to one side of the TM helix and arbitrarily assigned as side a–g (Fig. 3A). All the residues on each side, one at a time, were changed to either Leu or Ala, whichever matched the poly-Leu-Ala sequence in GPV\textsubscript{pLA} (Fig. 3B). Each of the seven corresponding mutant subunits, denoted as GPV\textsubscript{a} to GPV\textsubscript{g}, was transfected transiently into CHO cells, along with or without GPIb-IX-encoding cDNAs. Expression levels of GPIb\textalpha and GPIX in mutant cells were measured by flow cytometry and expressed as a percentage of that in wild-type CHO-αβIX\textsubscript{c} cells as described above (Fig. 3C). When expressed alone, all 7 side-scanning GPV mutants, like the wild-type, exhibited little surface expression in transiently transfected CHO cells (data not shown). On the other hand, when expressed in the presence of GPIb-IX, the expression levels of GPV mutants varied substantially, with CHO-αβIX\textsubscript{c} cells exhibiting the lowest GPV expression level (Fig. 3C). Out of the 7 side-scanning GPV mutants, relative surface expression levels of 3 GPV mutants (CHO-αβIX\textsubscript{a}, CHO-αβIX\textsubscript{b}, CHO-αβIX\textsubscript{c} cells) were significantly lower than those of GPIb-IX in the same transfected cells, indicating that the stabilizing effects of GPIb-IX on these GPV mutants were compromised by these mutations. In comparison, no significant difference in relative expression levels of GPIb-IX and GPV were observed in CHO-αβIX\textsubscript{c}, CHO-αβIX\textsubscript{d}, CHO-αβIX\textsubscript{f} and CHO-αβIX\textsubscript{g} cells.

In transient transfection, not all the cells were transfected and expressed GPV (Fig. 1, 2). To test whether the presence of untransfected cells obscures the enhancing effect of GPIb-IX on the surface expression of GPV, we proceeded next to transfact CHO cells using a lentiviral transfection system. The GFP-encoding lentivirus, generated from HEK 293T cells as described in Materials and Methods, was used to optimize the transfection protocol to achieve >90% transfection efficiency in CHO cells (Supplement Figure 1). Following the same transfection protocol, lentiviruses encoding either the wild-type or selected mutant GPV gene were added to CHO K1 cells and CHO cells stably expressing GPIb-IX (stable CHO-αβIX cells). Empty lentivirus that did not contain GPV gene was used as a negative control. Since lentiviral infection did not significantly alter the surface expression level of GPIb-IX in stable CHO-αβIX cells (Fig. 4A), the surface expression level of GPV and its
mutants in infected cells were measured by flow cytometry and directly compared. Unlike transiently transfected cells, nearly all cells treated with GPV-encoding lentivirus expressed GPV on the surface, whereas those treated with empty lentivirus did not, confirming high transfection efficiency of the lentiviral system (Fig. 4B). In infected cells, wild-type and all other mutant GPV was expressed at a similar level (Fig. 4B,C). In infected stable CHO-αβIX cells, wild-type GPV was expressed at a level significantly higher than that in infected CHO cells, whereas GPV_{pLA} was expressed at the same level as in infected CHO cells (Fig. 4C). Furthermore, surface expression levels of side-scan mutants GPV_{c}, GPV_{d}, GPV_{f} and GPV_{g} in infected stable CHO-αβIX and CHO cells were similar to those of GPV, and surface expression levels of the other mutants GPV_{a}, GPV_{b}, and GPV_{e} were similar to those of GPV_{pLA} (Fig. 4C). These results were remarkably consistent with those obtained in transiently transfected cells. Thus, although the presence of untransfected cells and relatively low expression level of GPV in transiently transfected cells may increase the noise in our measurement, the mutational effects on GPV surface expression can be measured and analyzed in transiently transfected cells. Overall, we have identified specific mutations in the TM domain of GPV are critical to the efficient surface expression of GPV in the presence of GPIb-IX. It is noteworthy that three critical sides (a, b, e) are clustered on one side of the TM helix, consistent with the expected lateral interactions between TM α-helices.

**Polar residues in the GPV TM domain are critical to its interaction with the GPIb-IX complex**

TM domains contain mostly hydrophobic residues due to their obvious need to partition into the hydrophobic cell membrane. Polar residues in the TM domain are rare but often with functional significance [30]. There are 3 residues in the GPV TM domain that contain polar groups in their side chains: Y509, Q516 and T520. Earlier observations that the GPIb-IX-V complex is stable only in digitonin-lysed but not NP-40-lysed cells [7, 19], which was confirmed in our laboratory (data not shown), suggest that polar-polar interactions between TM helices that are better preserved in digitonin than NP-40 or Triton X-100 are present between GPV and GPIb-IX. Consistently, the three polar residues in the GPV TM domain are located on sides b and e (Fig. 3A), 2 of the 3 sides that were identified as the likely interfacial region between GPV and GPIb-IX. To test directly whether these polar residues in the GPV TM domain are critical to efficient surface expression of GPV, they were mutated to Ala, individually or together, as illustrated in Fig. 5A. Each mutant GPV construct was transiently transfected into CHO cells, along with or without GPIb-IX-encoding genes, and surface expression levels of GPIb-IX-V subunits were measured by flow cytometry, quantified and normalized against those in CHO-αβIXV cells (Fig. 5). When expressed alone, all 4 GPV polar mutants produced little surface expression as the wild-type GPV. Compared to wild-type GPV, GPV_{Y509A} and GPV_{T520A} were expressed at a significantly lower level in the presence of GPIb-IX. Although GPV_{Q516A} was also expressed at a lower level, the difference was not statistically significant in our experiments. Furthermore, when all three polar residues were mutated to Ala in CHO-αβIXV_{YQT,A} cells, surface expression level of GPV_{YQT-A} was only 40% of that of wild-type GPV in CHO-αβIXV cells, even lower than that of any of the single mutants (Fig. 5B), suggesting that the effects of mutating polar residues may be additive. Together, these results showed that polar residues in the GPV TM domain are important for efficient surface expression of GPV in the presence of GPIb-IX, probably by mediating the interaction between them.

**The GPIb-IX complex enhances GPV expression through the GPIba TM domain**

Since a single-span TM domain of a membrane receptor is physically located in the membrane bilayer, it should interact only with other TM domains in a complex. We reasoned that the GPV TM domain is most likely to interact with other TM helices in GPIb-
Previous studies suggested that GPV interacts with GPIbα [24]. We investigated here whether the GPIbα TM domain is critical to the interaction with GPV and thus surface expression of GPV.

In an earlier study we showed that replacement of the GPIbα TM domain with either a poly-Leu or a poly-Leu-Ala sequence led to approximately 90% reduction in the expression of GPIb-IX on the surface of transfected CHO cells [8]. This is due to the disruption of interactions among GPIbα, GPIbβ and GPIX TM domains that leads to aberrant assembly of the receptor complex [11, 12]. Since our analysis of the decrease in GPV surface expression requires an adequate expression level of the co-expressing GPIb-IX, side-scanning mutagenesis, instead of whole domain replacement, was carried out in the GPIbα TM domain. Like GPV, the TM helix of GPIbα was divided into 7 sides, assigned as side a to g, and all the residues in each side were mutated to Leu, one side at a time (Fig. 6A,B). Each GPIbα side mutation gene, denoted as GPIbαa to GPIbg respectively, was co-transfected with wild-type GPIbβ and GPIX genes into CHO cells. In the absence of GPV, these GPIbα mutants exhibited wild-type-like expression on the cell surface, albeit at slightly lower levels (Fig. 6C,D). The expression level of GPIX in cells expressing GPIbα side mutants was also similar to that in CHO-αβIX cells. When the side-scanning GPIb-IX mutant complex was co-transfected with wild-type GPV into CHO cells, the surface expression level of GPV varied substantially (Fig. 6E). While in many cells recorded similar relative expression levels of GPIbα and GPV, in 2 mutant cells (CHO-αβIXV and CHO-αeβIXV) the relative expression level of GPV was significantly lower than that of GPIbα, indicating that sides b and e of the GPIbα TM domain are critical to the enhancing effect of GPIb-IX on GPV expression. Interestingly, in one mutant cell (CHO-αfβIXV) the relative expression level of GPV became higher than that of GPIbα. (Fig. 6E). This could be explained as the mutation enables GPIb-IX to stabilize GPV further, likely by strengthening the interaction between them. Since the actual number of GPIbα is significantly more than that of GPV on the cell surface (Fig. 1), it is likely that there is enough GPIbαf mutant protein on the cell surface to support enhanced GPV expression. Overall, these results showed that residues on sides b, e and f of the GPIbα TM domain are important for ability of GPIb-IX to support surface expression of GPV. The fact that these sides form a continuous surface on the side of GPIbα TM helix suggests that this surface may mediate its interaction with GPV.

**Discussion**

GPV is one of the most expressed glycoproteins on the platelet surface. It was first identified as missing in BSS patients and subsequently demonstrated to interact non-covalently with GPIb-IX [7, 19, 24, 31, 32]. However, the molecular basis underlying the association of GPV and GPIb-IX has remained unclear, partly due to the uncertainty on the organization and structure of GPIb-IX and partly due to the difficulty in directly monitoring the relatively weak association between GPV and GPIb-IX. Taking advantage of the observation that all the subunits in GPIb-IX are required for its efficient surface expression [16], we have established tightly controlled transient transfection experiments and were able to utilize the surface expression level of individual GPIb-IX subunits in transfected mammalian cells as an effective indicator for receptor complex assembly [8]. Coupling it with systematic mutational analysis, we had found earlier that TM domains of GPIb-IX are critical to efficient surface expression and assembly of the complex [8, 12]. Further biophysical characterization of isolated GPIb-IX TM peptides revealed that they interact specifically with one another and form a parallel 4-helical bundle complex [5, 11]. Mutational effects on GPIbβ-enhanced surface expression of GPIX, in combination with crystal structures of GPIb-IX-derived extracellular domains, recently helped to shed critical insights on the assembly mechanism of GPIb-IX and on the pathogenesis of Bernard-Soulier syndrome [9, 13]. In the present study, the same approach was applied to explore the association of GPV
and GPIb-IX since efficient surface expression of GPV depends on its interaction with GPIb-IX. We show here that specific mutations in the GPV or GPIbα TM domains can largely abolish the dependence of GPV surface expression on GPIb-IX in transfected mammalian cells. Our results demonstrate for the first time the importance of GPV and GPIbα TM domains in mediating efficient surface expression of GPV and thus likely the interaction between GPV and GPIb-IX.

Previous evidences have indicated that the interaction of GPV with GPIb-IX may be relatively weak. In particular, it could only be detected in digitonin-lysed cells, which explains the absence of GPV in the GPIb-IX complex purified from Triton X-100-lysed platelets [7, 33, 34]. We were able to confirm this observation in our lab (data not shown). Such relatively weak interaction is difficult to duplicate in vitro with recombinant fragments of the GPIb-IX-V complex in membrane-mimicking conditions. Since GPV surface expression is dependent on the presence of GPIb-IX in the cell, we utilized the relative surface expression level of GPV as an indicator for the interaction of GPV with GPIb-IX. Indeed, residues identified in this study as critical to efficient surface expression of GPV are consistent with previously reported constraints on the interaction between GPV and GPIb-IX. First, the sides of the GPV TM domain that are critical to efficient surface expression of GPV (sides a, b, e as shown in Fig. 3A) are clustered together, forming a continuous surface on the GPV TM helix amenable to lateral association with other helices in the membrane. Coincidentally, sequence alignment of GPV orthologues available from the Ensembl database showed that most of the GPV TM residues conserved across the species are located in the sides a, b, e and f (Supplemental Figure 2). In particular, all 3 polar residues in the GPV TM domain are located in sides b and e. Further mutational analysis demonstrated the importance of polar residues to efficient surface expression of GPV, which fits very well with the well-documented differential effects of digitonin and Triton X-100 on GPV association with GPIb-IX. Second, like that of the GPV TM domain, side-scanning mutagenesis of the GPIbα TM domain identified a continuous surface spanning 2–3 sides of the helix (sides b, e and f as shown in Fig. 6A) as important to likely interaction with the GPV TM domain. Remarkably, this surface does not overlap with sides c and g, which contain residues to mediate interactions with GPIbβ and GPIX TM domains [5, 8]. Instead, sides b, e and f of the GPIbα TM domain point away from GPIbβ and GPIX TM domains [12], and thus are available for interaction with GPV.

Whether GPIbβ and GPIX TM domains participate directly in the interaction with GPV was not explored in this study. Scanning mutagenesis of GPIbβ and GPIX TM domains had been carried out in earlier studies to elucidate the molecular basis for the GPIb-IX TM domain complex [8, 12]. Many of GPIbβ and GPIX TM mutations disrupted proper assembly of GPIb-IX and resulted in a significant decrease of its surface expression level. Thus, it would have been difficult to unambiguously attribute the effects of GPIbβ or GPIX TM mutations to their disruption of a direct interaction with GPV rather than their disruption of GPIb-IX assembly and expression. Furthermore, the GPIbβ or GPIX extracellular domains in the GPIb-IX complex form a clover-like structure, stemming from their neighboring TM domains [13]. Such structural arrangement at the membrane surface precludes the GPIbβ or GPIX TM domain from having direct contact with GPV TM helix, or a severe steric hindrance would develop between GPIbβ and GPIX extracellular domains with the GPV counterpart (Fig. 7). Considering that the membrane-proximal region of GPIbα (about 40–50 residues) is significantly smaller than well-folded GPIbβ and GPIX extracellular domains (each about 120 residues), the GPIbα TM domain in the complex should be more accessible to GPV association (Fig. 7). This is consistent with the side-scanning mutagenesis results reported here that suggest GPV and GPIbα TM domains to interact directly.
Based on the expression levels of GPV and GPIb-IX in platelets measured by antibody binding, it was suggested that they associate in platelets at a stoichiometry of 1:2 [7]. It was further postulated that GPV is sandwiched between two GPIb-IX complexes [24]. Strictly speaking, however, there has been no direct evidence addressing the stoichiometry of the GPIb-IX-V complex, partly due to the technical difficulty in keeping the weakly associated GPIb-IX-V complex intact and free of impurities. That GPV and GPIbα are constantly being shed from the platelet surface [35] would also add additional uncertainty to any measurement. While this study was not designed to address the stoichiometry question, our results are not consistent with a model in which the GPV TM helix interacts with 2 GPIbα TM helices. This is because the aforementioned size constraints imposed by GPIb-IX extracellular domains would require the two GPIbα TM helices to interact with the GPV TM helix from the opposite sides, but the 3 sides of the GPV TM helix identified as the likely binding interface with GPIbα TM domain are too small to support simultaneous association with 2 GPIbα TM helices. Nonetheless, our results do not rule out the possibility that GPV interacts with one GPIbα subunit through their TM domains and with another GPIbα subunit through other domains, since the TM domain may not be the only domain that participates in GPV interaction with GPIb-IX. Multiple mutations in the GPV TM domain produced 60% reduction but not a complete loss in surface expression levels of GPV (Fig. 3,5). Elucidation of exact details of GPV association with GPIb-IX would require further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Efficient surface expression of GPV depends on the presence of GPIb-IX. CHO K1 cells were transiently transfected with GPV cDNA in the absence or presence of GPIb-IX genes. Two days after transfection, surface expression levels of GPIbα, GPIX and GPV were detected by flow cytometry with antibodies WM23, FMC25 and SW16, respectively. (A) Overlaid flow cytometry histograms from the same transfection experiment comparing the surface expression levels of GPIbα, GPIX and GPV in various transfected CHO cells. Identities of transfected cells are denoted by the transfected subunits. The overlaid histograms are representative of 3–5 independent experiments. (B) Bar plots of surface expression levels of GPIbα (open bar), GPIX (slashed bar) and GPV (black bar), quantified as relative mean fluorescence intensity (MFI). The fluorescence intensity measured from 10,000 cells was normalized to the value in CHO-αβIXV cells as 100% and that in CHO-vector cells 0% (not shown). The data are presented as the mean ± S.D. calculated from 3–5 independent experiments. Groups were compared using the non-paired t test; **, p < 0.001.
Figure 2.
Replacing the GPV TM domain with an unrelated sequence reduces its surface expression in the presence of GPIb-IX. (A) Sequences of wild-type or poly-Leu-Ala-containing GPV TM domains. The GPV TM domain sequence, marked by the starting and ending residue numbers, was replaced by a poly-Leu-Ala sequence in GPV$_{pLA}$. The extracellular and cytoplasmic domains remained unchanged in GPV$_{pLA}$. (B, C) Overlaid histograms and quantitative bar plots showing the surface expression level of the TM-replaced GPIb-IX-V complex. GPV and GPV$_{pLA}$ was each transfected into CHO cells in the absence or presence of GPIb-IX, and surface expression levels of GPIb$_\alpha$, GPIX and GPV were detected by flow cytometry with antibodies WM23, FMC25 and SW16, respectively. The legends follow that
used in Figure 1. The data are presented as the mean ± S.D. calculated from 3–5 independent experiments. Groups were compared using the non-paired t test; **, $p < 0.001$. 

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Figure 3.
Side-scanning mutagenesis of the GPV TM domain. (A) The helical wheel view of the GPV TM domain. Residues in the TM domain were divided into seven sides, each of which was assigned from a to g. The sides identified as important are boxed in green. (B) TM sequences of the wild-type (GPV) and seven side-scanning mutants (GPV<sub>a</sub> - GPV<sub>g</sub>). Residue numbers are on the top. The mutations in each sequence are underlined. (C) Bar plot of the surface expression levels of GPIbα (open bar), GPIX (slashed bar) and GPV (black bar), quantified as relative MFI, in side-scanning mutant cells. The data are presented as the mean ± S.D. from 3–6 independent experiments. Groups were compared using the non-paired t test; *, p < 0.05; **, p < 0.001.
Figure 4.
The TM domain of GPV is important for its surface expression in lentiviral-transfected CHO cells. The lentivirus containing a copy of GPV gene or empty lentivirus was used to infect CHO K1 cells or CHO cells stably expressing GPIb-IX (stable CHO-αβIX cells). After infected cells were cultured for 4–7 days, surface expression levels of GPIbα, GPIX and GPV were detected by flow cytometry with antibodies WM23, FMC25 and SW16, respectively. (A) Overlaid flow cytometry histograms comparing the surface expression levels of GPIbα (left panel) and GPIX (right panel) in stable CHO-αβIX cells before (black trace) and after (blue) GPV-containing lentivirus infection. The grey-filled histograms were obtained using CHO K1 cells before infection. The histograms are representative of 2
independent experiments. (B) Overlaid flow cytometry histograms comparing surface expression levels of GPV (black trace) and GPV_{PLA} (red) in lentiviral-transfected CHO K1 cells (left panel) or stable CHO-αβIX cells (right panel). The grey-filled histograms were obtained using the empty lentivirus. The histograms are representative of 3–4 independent experiments. (C) Bar plots of surface expression levels of GPV and its TM mutants as illustrated in Figure 2A and 3B, quantified as relative MFI, in lentiviral-transfected CHO-αβIX cells (white bar) and CHO K1 cells (grey bar). The data are presented as the mean ± S.D. from 2–4 independent experiments. Groups were compared using the non-paired t test; *, p < 0.01.
Figure 5.
The polar residues in the GPV TM domain are critical to its interaction with GPIb-IX. (A) TM sequences of the wild-type GPV and polar-residue mutants, with the subscript describing mutations within. In each sequence, the actual mutations are underlined. (B) Bar plot of surface expression levels of GPV polar mutants when they are expressed alone in transfected cells. The level is measured by flow cytometry, quantified and expressed as a percentage of that in CHO-αβIXV cells. (C) Bar plot of the surface expression levels of GPIbα (open bar), GPIX (slashed bar) and GPV (black bar), quantified as relative MFI, in transfected cells expressing GPV polar mutants. The data are presented as the mean ± S.D.
from 3–4 independent experiments. Groups were compared using the non-paired *t* test; *, *p* < 0.05.
Figure 6.
Side-scanning mutagenesis of the GPIbα TM domain. (A) The helical wheel view of the GPIbα TM domain. Residues in the TM domain were divided into seven sides, each of which was assigned from a to g. The sides identified as important are boxed in magenta. (B) TM sequences of the wild-type (GPIbα) and seven side-scanning mutants (GPIbα<sub>a</sub> - GPIbα<sub>g</sub>). Residue numbers are on the top. The mutations in each sequence are underlined. (C) Overlaid flow cytometry histograms showing surface expression levels of GPIbα in transiently transfected CHO cells expressing various GPIbα TM mutants. The wild-type trace is in black, and the seven mutants are rainbow colored as indicated in (B). The overlaid histograms are representative of 3-4 independent experiments. (D) Bar plot of the surface
expression levels of GPIbα (open bar) and GPIX (slashed bar), quantified as relative MFI, in cells transfected with GPIb-IX genes. (E) Bar plot of the surface expression levels of GPIbα (open bar), GPIX (slashed bar) and GPV (black bar), quantified as relative MFI, in transfected with GPIb-IX-V genes. The data are presented as the mean ± S.D. from 3–5 independent experiments. Groups were compared using the non-paired t test; *, p < 0.01; **, p < 0.001.
Figure 7.
The GPIbα TM domain is accessible to GPV association. (A) A ribbon diagram showing a structural model of GPIb-IX complex, adapted from a recent report [13]. The model contains the membrane-proximal stalk region and TM domain of GPIbα (magenta), extracellular and TM domains of GPIbβ (light and dark blue) and GPIX (orange). The membrane-distal portion of GPIbα extracellular domain and all the cytoplasmic domains are not shown. (B) The same model was rotated by 90 degrees horizontally, showing the accessibility of GPIbα TM domain to direct association of GPV TM domain (green) as well as the inaccessibility of GPIX TM domain.