Identification of the Amino Acid Residues of the Platelet Glycoprotein Ib (GPIb) Essential for the von Willebrand Factor Binding by Clustered Charged-to-Alanine Scanning Mutagenesis*

Atsuya Shimizu‡‡,†, Tadashi Matsushita‡†, Takahisa Kondo‡†, Yasuya Inden‡†, Tetsuhiito Kojima‡‡, Hidehiko Saito‡‡‡,‡‡‡, and Makoto Hirai‡‡

From the ‡First Department of Internal Medicine, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Department of Medical Technology, Nagoya University School of Health Sciences, 1-1-20, Daiko-Minami, Higashi-ku, Nagoya 461-8673, *‡Nagoya National Hospital, Sanomaru-4-1-1, Naka-ku, Nagoya 461-0001, and †‡AiCh Blood Disease Research Foundation, Moriyanma-ku, Nagoya 463-0074, Japan

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GPIb shown similar phenotype. These findings indicated the interaction of the soluble fragment containing the N-terminal chain of the GPIb; VWF, von Willebrand factor; LRR, leucine-rich repeat; and Asp 175 specifically decreased both ristocetin- and Arg 571 of VWF A1 domain, whose mutations had 125I-labeled human VWF. Mutations at Glu 128, Glu 172, and Glu128, but HPL7 binding to Glu 121 has no effect on VWF binding of the glycoprotein (GP) 1 and 6D1 inhibited ristocetin- and botrocetin-induced VWF binding, suggesting that these sites are important for VWF binding of platelet GPIb. Monoclonal antibody 6D1 inhibited ristocetin- and botrocetin-induced VWF binding, and a mutation at Glu128 specifically reduced the binding to 6D1. In contrast, antibody HPL7 had no effect for VWF binding, and mutant E121A reduced the HPL7 binding. Mutations at His12 and Glu14 decreased the ristocetin-induced VWF binding with normal botrocetin-induced binding. Crystallographic modeling of the VWF-GPIb complex indicated that Glu128 and Asp127 form VWF binding sites; the binding of 6D1 to Glu 125 interrupts the VWF binding of Glu128, but HPL7 binding to Glu 121 has no effect on VWF binding. Moreover, His12 and Glu14 contact with Glu121 and Arg287 of VWF A1 domain, whose mutations had shown similar phenotype. These findings indicated the novel binding sites required for VWF binding of human GPIb.

Platelet adhesion is an initial step in thrombus formation and is dependent on the binding of the glycoprotein (GP)1

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‡ Present address: Dept. of Humoral Regulation, Division of Regulation of Organ Function, Research Institute of Environmental Medicine, Nagoya University, Chikusa-ku, Nagoya 464-8601, Japan.

† To whom correspondence should be addressed. Tel.: 81-52-744-2145; Fax: 81-52-744-2161; E-mail: tmatsu@med.nagoya-u.ac.jp.

‡‡ Amino acid residues of human GPIbα are numbered from the starting histidine as + 1.
Expression and Characterization of Recombinant GPIbα Chain—Human 293T cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen). Cells were transfected with pcDNA/hGPIbFLAG containing either wild type or mutant GPIbα by using LipofectAMINE according to the manufacturer’s recommendation. In mock transfection, pcDNA1/neo was used. Twenty-four h after transfection, cells were washed with phosphate-buffered saline (PBS) and then incubated with the serum-free medium (Opti-Mem-1; Invitrogen). Cells were harvested and detached by trypsinization. The harvested cells were incubated with PBSA and 2 mg/ml (final) 4% BSA. The supernatant was concentrated using Centriprep-10 (Millipore). GPIbα expression was confirmed by Western blot analysis using rabbit polyclonal anti-human GPIbα antibodies. The MAb inhibition assay for the ristocetin- or botrocetin-induced binding was performed as described previously (17). After GPIbα expression, the cells were harvested and used for the MAb inhibition assay.

Inhibition of VWF Binding to GPIbα by Anti-GPIb MAb—To analyze MAb inhibition for the ristocetin-induced VWF binding of wild type GPIbα, 4 µg/ml VWF, 0.7 µg/ml wild type rGPIbα, and 10 µg/ml each anti-GPIb antibody were incubated for 1 h in the presence of 2 ng/ml ristocetin and 3% BSA in a total volume of 50 µl at 37 °C. After 30 min, the solution was transferred to anti-FLAG M2 MAbs-coated plate wells and incubated; the solution was then washed, followed by measurement by the γ-scintillation counter as in the ristocetin-induced binding assay. Negative control assays were performed by using concentrated conditioned media from mock-transfected 293T cells. For both assays, nonspecific binding was determined by subtracting the total radioactivity from that of mock control.

Western Blotting Analysis of Wild Type Recombinant GPIbα—Approximately 25–30 ng of wild type rGPIbα was diluted 2-fold with gel-loading buffer and separated by SDS-polyacrylamide gel electrophoresis in 10% acrylamide gels under non-reduced and reduced conditions. After transfer onto polyvinylidene difluoride membrane (Bio-Rad), proteins were incubated with anti-FLAG M2 MAb or various anti-GPIb MAb (6D1, HPL7, AN51, and S2Z) and then incubated with peroxi-
dase-conjugated anti-mouse immunoglobulin G. Blots were developed by using ECL-Western blotting detection systems.
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Design and Expression of rGPIbα Mutants—A functional domain that retains VWF binding activity resides in the N-terminal 45-kDa fragment between residues His1 and Arg293 of GPIbα (21, 22). The segment that was targeted in this study consists of 287 amino acid residues between His1 and Asp287 of human GPIbα (16). This segment contains several regions including the N-terminal flank, LRRs, the C-terminal flank region, the anionic sulfated tyrosine region, and a part of the macroglycopeptide region (Fig. 1) (4, 23).

To simplify the production and analysis of mutant constructs, a clustered charged-to-alanine scanning strategy was chosen. Charged amino acids usually are at or near the protein surface, and alanine was selected as the replacement residue because it is the most common amino acid in proteins, is compatible with all types of secondary structures, and does not impose new structural effects related to hydrogen bonding, unusual hydrophobicity, or steric bulk (24). The recombinant GPIbα fragment 1–302 contained 62 charged residues, including 19 single mutations and 19 clustered mutations. Only one mutant D106A was not secreted, and D106K was constructed and analyzed instead.

RESULTS

Fig. 1. Amino acid residues of human GPIbα targeted for charged-to-alanine scanning mutagenesis. The amino acid sequence shown includes residues 1–287 of the α chain of human GPIb. The functional elements of GPIbα are indicated below the sequence. Seven short LRRs have been predicted in human GPIbα (28), but the crystal structure (5) suggested that amino acid sequences from Phe201 contain LRR-8 and the C-terminal flank region. Charged residues His, Arg, Lys, Glu, and Asp were targeted for the mutagenesis. Segments that contain mutations in each construct are indicated by boxes. For convenience, the mutant proteins were named according to the residue number of the mutated amino acids in the mature GPIbα. If more than one charged amino acid was mutated, the range of residue numbers and the number of alanine substitutions are indicated. For example, in mutant (172–175)2A, the two residues Glu172 and Asp175 were changed to alanine. As a result, 38 mutants were constructed, including 19 single mutations and 19 clustered mutations. Only one mutant D106A was not secreted, and D106K was constructed and analyzed instead.

Fig. 2. Western blotting analysis of wild type recombinant GPIbα. Wild type or mutant rGPIbα was separated by SDS-polyacrylamide gel electrophoresis in 10% acrylamide gels under non-reduced (A) or reduced (B) conditions. After transfer onto polyvinylidene difluoride membrane by electroblotting, the membrane was incubated with anti-FLAG M2 MAb or several anti-GPIb MAbs (6D1, HPL7, AN51, and SZ2).

wild type rGPIbα. Negative control assays were performed by using concentrated conditioned media from mock-transfected 293T cells.

Crystallographic Structure Representations—The PDB file of the structural coordinate was downloaded from the Protein Data Bank (www.pdb.org). The coordinate of isolated human GPIbα (1GWB) (20) was obtained from Dr. Jonas Emsley.3 Protein ribbon representation of the complex of GPIbα was prepared with the program PyMol (www.pymol.org).

Design and Expression of rGPIbα Mutants—A functional domain that retains VWF binding activity resides in the N-terminal 45-kDa fragment between residues His1 and Arg293 of GPIbα (21, 22). The segment that was targeted in this study consists of 287 amino acid residues between His1 and Asp287 of human GPIbα (16). This segment contains several regions including the N-terminal flank, LRRs, the C-terminal flank region, the anionic sulfated tyrosine region, and a part of the macroglycopeptide region (Fig. 1) (4, 23).

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3 J. Emsley, personal communication.
ing arginine, lysine, aspartate, glutamate, and histidine, and was changed singly or in small clusters to alanine. The 62 charged residues in the targeted region were covered in a total of 38 constructs of rGPIb/H9251, including 19 clustered mutants and 19 single mutants (Fig. 1). For convenience the mutants were named according to the residue number of the mutated amino acids in the mature GPIb/H9251. If more than one charged amino acid was mutated, the range of residue numbers and the number of alanine substitutions are indicated. For example, in construct E128A, one glutamate at position 128 was changed to alanine; in construct (172–175)2A, the two residues Glu172 and Asp175 were changed to alanine (Fig. 1).

Mutants were expressed in human kidney 293T cells and secreted as soluble proteins with FLAG tag at the C-terminal end. Cells were transfected with each construct, and serum-free media were analyzed for the expression of mutant rGPIb/H9251. Because a construct containing the single mutation D106A was not secreted, D106K, where negatively charged Asp was changed to positively charged Lys, was constructed and analyzed. All other mutant proteins were expressed and secreted efficiently (data not shown).

**Western Blotting Analysis of Wild Type Recombinant GPIbα**—The present study employed four MAbs, 6D1 (25), HPL7, AN51 (26), and SZ2 (27), directed against human GPIbα. We performed Western blotting analysis of wild-type rGPIbα by using the four MAbs (Fig. 2). The binding of anti-FLAG M2 antibody was studied simultaneously, and it bound both reduced and non-reduced forms of wild-type rGPIbα with ~45-kDa molecular masses (Fig. 2). Under non-reduced condition, all anti-GPIb antibodies reacted with wild-type rGPIbα, and ~45-kDa bands were visible (Fig. 2A). When proteins were reduced, 6D1, HPL7, and AN51 lost the binding, whereas SZ2 bound normally (Fig. 2B). Therefore, the binding of the antibodies 6D1, AN51, and HPL7 was sensitive to reduction, suggesting that these antibodies are protein conformation-dependent. The binding of SZ2 was not sensitive to protein reduction.

**Effect on VWF Binding by Monoclonal Antibodies for GPIbα**—The four MAbs, 6D1, HPL7, AN51, and SZ2, were tested for their ability to inhibit the ristocetin- or botrocetin-induced binding of VWF to rGPIbα (Fig. 3A). We also tested the inhibition for plasma VWF binding to fixed human platelets (Fig. 3B). MAb 6D1 completely inhibited both rGPIbα-VWF and platelet-VWF interaction in the presence of ristocetin or botrocetin (Fig. 3, A and B). SZ2 inhibited botrocetin-induced rGPIbα-VWF interaction by 30% and botrocetin-induced plate-
let-VWF interaction by 60% (Fig. 3, A and B). AN51 and HPL7 had no effects on VWF binding induced by either ristocetin or botrocetin (Fig. 3).

**Binding of Monoclonal Antibodies to Mutant rGPIbα—**Mutant binding to each MAb was measurable by a specific ELISA. The MAb epitope might be related to the important amino acid residues for binding to VWF, and we analyzed the binding of the mutants to a panel of the four MAbs (Fig. 4).

For each antibody, the relative absorbance value was expressed as the percentage for wild type rGPIbα (Fig. 4). Mutants D83A, H86A, D106K, and (149–152)3A constitutively decreased binding to MAbs 6D1, HPL7, and AN51, whereas these mutants normally bound to SZ2. From the Western blotting analysis, the binding of 6D1, AN51, and HPL7 for wild type rGPIbα was lost by reduction, whereas SZ2 binding was not affected (Fig. 2), suggesting that the binding of the former three MAbs is dependent on protein conformation. Therefore, it is suggested that Asp83, His86, Asp106, Lys149, Glu151, and Lys152 are included in amino acids that are important for protein conformation of GPIbα.

In contrast, MAb 6D1 specifically reduced the binding to mutant E125A by 63.5% of wild type rGPIbα, and MAb HPL7 showed 9.7% binding to a mutant R121A (Fig. 4). These mutants showed normal binding to other antibodies. It is thus suggested that the epitope of 6D1 and HPL7 is included in Glu125 and Arg121, respectively.

SZ2 showed decreased binding to (274–277)2A (13.2%) and to (281–283)3A (14.8%) (Fig. 4), suggesting that the binding of SZ2 is dependent on the C-terminal side of the rGPIb fragment between Asp274 and Asp283 and that the epitope is included in residues Asp274, Asp277, Glu281, Glu282, and Asp283. This result is consistent with the study on human-canine chimera proteins by Shen et al. (28) that mapped the epitope of SZ2 into the anionic sulfated tyrosine region. Fig. 4 does not clearly demonstrate epitopes of MAb AN51.

**Binding of rGPIbα to VWF—**Thirty-eight mutants were tested for the VWF binding induced by ristocetin or botrocetin. To determine the assay condition, we first evaluated the specific 125I-labeled VWF binding of varying doses of immobilized rGPIbα. For ristocetin-induced binding, wild type rGPIbα was tested in the presence of 2 mg/ml ristocetin. Wild type rGPIbα (1.4 µg/ml) immobilized on anti-FLAG M2 MAb-coated plates appeared to give half-maximum binding of VWF (Fig. 5A). Thus, varying VWF concentrations were tested for immobilized wild type rGPIbα (1.4 µg/ml), and 50 µg/ml 125I-labeled VWF gave half-maximum binding (Fig. 5B). Based on these control experiments, the binding of 50 µg/ml 125I-labeled VWF to 1.4 µg/ml each mutant GPIbα was tested and compared.

The botrocetin-induced VWF binding assay was tested in the presence of 10 µg/ml botrocetin. Wild type rGPIbα (0.7 µg/ml) immobilized on anti-FLAG M2 MAb-coated plates appeared to give optimum binding of VWF (Fig. 5C). Varying VWF concentrations were tested for 0.7 µg/ml wild type rGPIbα, and 1.25 µg/ml 125I-labeled VWF gave the optimum binding (Fig. 5D). This condition was used for comparison of the mutants.

To summarize binding data in graphic form, values obtained for each rGPIbα mutant were normalized to the corresponding values obtained for wild type rGPIbα in the presence of ristocetin or botrocetin (Fig. 6). Twenty-six mutants, representing 40 charged amino acids residues, had essentially normal VWF binding. Mutants E128A and (172–175)2A decreased the VWF binding in the presence of ristocetin or botrocetin, whereas they showed normal bindings to the MAb panel (Fig. 4). For ristocetin-induced binding, E128A and (172–175)2A reduced the binding by 9.2 and 12.7%, respectively. For botrocetin-induced binding, E128A and (172–175)2A decreased by
vations may suggest that there are distinct mechanisms of VWF-GPIb interaction between botrocetin-induced and ristocetin-induced binding.

For botrocetin-induced binding, two mutants selectively decreased the binding: (217–218)2A and (285–287)2A reduced by 27.7 and 21.7% of wild type rGPIb/H9251, respectively. Mutant (217–218)2A slightly decreased ristocetin-induced binding by 42.8% of wild type, but (285–287)2A showed normal binding. These mutants bound normally to the MAb panel (Fig. 4), and the mutated amino acid residues are located in the C-terminal flank region and the sulfated tyrosine region. On the other hand, Fig. 4 indicates that mutations at Asp274, Asp277, Glu281, Glu282, and Asp283 included epitopes of MAb SZ2 that partially inhibited botrocetin-induced VWF binding of GPIb (Fig. 3). Taken together, it is suggested that botrocetin-induced VWF-GPIb interaction is dependent on the C-terminal side of the GPIb fragment.

In addition to D83A and H86A, mutants H203A, (222–225)2A, and (269–272)3A also increased the botrocetin-induced VWF binding by near 200% of wild type rGPIb (Fig. 6B). Such a gain-of-function phenotype has been also observed in platelet-type von Willebrand disease (29, 30), and the location of the mutations found in this disease was recently explained by the crystallographic analysis of VWF A1-GPIb complex (5). The crystallographic interpretation is required to assess whether the similar mechanism underlies the phenotype of our mutants.

**DISCUSSION**

*Interaction between GPIbα and VWF—Crystallographic structure of human GPIbα-VWF complex was recently determined by Huizinga et al. (5). This recombinant GPIbα was expressed in BHK cells and harbored the mutations N21Q and N159Q to remove N-glycosylation sites. In addition to the previous studies by way of total deglycosylation experiment (6) or by expression in insect cells (7), the successful co-crystallization implies that the two N-glycosylation sites may not necessarily be required for GPIbα binding to VWF. The Protein Data Bank data file (1M10) was converted into graphical form and is shown in Fig. 7, providing a framework for interpreting the effects of mutations on VWF binding of recombinant human GPIbα.*

From the VWF binding experiments, two mutants, E128A and (172–175)2A, reduced both the ristocetin- and botrocetin-induced VWF binding (Fig. 6). In Fig. 7, Glu128 is located at the central position of the concave LRR domain, and it appears to
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Figure 7. Amino acid residues with decreased binding to VWF and MAbs. The schematic ribbon representation of the GPIbα-VWF A1 complex was constructed from the coordinate 1M10 (5) and shows the location of amino acid side chains (sticks). Lys599 of the VWF A1 domain is a previously identified GPIbα binding site and is shown in magenta. A, in the stereo image, residues are shown with the loss of VWF binding either both by ristocetin and botrocetin (blue, Glu125 and Asp175) or in selective loss of ristocetin-induced binding (orange, His12 and Glu14). The proposed counterpart of these GPIbα residues in VWF A1 (blue, Lys608 and Phe603; orange, Arg571 and Glu613, respectively) is shown in the same color. On GPIbα, Glu125, mutated along with Asp175, is not located at the important position and is shown by light blue. The epitope of MAbs 6D1 (Glu613) and HPL7 (Arg121) is shown with gray. Four clustering amino acid residues of GPIbα in yellow, magenta, green, and salmon pink are not indicated in number but are indicated in C. B, 150° clockwise rotation from bottom of the view in A is shown, and the location of residues Lys149, Glu151, and Lys152 is indicated, whose mutations showed decreased binding to the conformation-dependent panel of MAbs (Fig. 4) and to VWF in the presence of ristocetin (Fig. 6A). The coloring of other amino acid side chains is the same as in A. A close contact between Glu125 and Lys149 is expressed by a dotted line. C, close-up view of 45° clockwise rotation of the view in A. A transparent surface image of VWF A1 is depicted, and several residues involved in VWF-GPIbα interaction are shown as well as A. Mutations at residues Asp83, His85, and Asp106 decreased the binding to conformation-dependent MAbs (Fig. 4) and to VWF in the presence of ristocetin (Fig. 6A). They are shown along with Ser85, which appears to be located in the center of these residues. Location of these residues is also indicated without numbering in A but not in B. See “Discussion” for details.

a Amino acid residues of human von Willebrand factor are numbered from the starting methionine as +1. The previous numbering is indicated in parentheses. For old numbering of mutants, the N-terminal Ser of the mature processed VWF subunit was used as +1.
H86A, D106K, and (149–152)3A constitutively decreased the binding to conformation-dependent MAbs (Fig. 6), whereas these bound normally to SZ2 (Fig. 6), which also binds the reduced form of GPIba.

Fig. 7B indicates that Lys152 is projected toward VWF and may participate in VWF binding. This finding may be consistent with the decreased ristocetin-induced binding of (149–152)3A (Fig. 6A). Indeed, Huizinga et al. (5) have suggested that Lys152 has close contact with Asn504 of VWF A1, and thus the importance of Lys152 of GPIba for VWF binding is not excluded (Fig. 7B). In contrast, Lys149 is located close to His203, which increased botrocetin-induced VWF binding, and is shown in orange. Mutations at Glu285 and Asp287 decreased botrocetin-induced VWF binding but are not covered in the figure. Residues Asp274 and Asp280 are colored in gray, and residues 274–280 are colored in gray to indicate the proposed MAb SZ2 epitope. Sulfated tyrosine residues 276, 278, and 279 are shown with sticks in light gray. The positions of VWF were used to adjust the VWF-GPIba complex. Fig. 8 indicates that the C-terminal flank region in A1–botrocetin–GPIba complex could be interpreted. To address the functional role of GPIba for the interaction with botrocetin or VWF A1, the coordinate by Ulf et al. (5) was first superimposed onto the structure of the VWF-GPIba complex (Protein Data Bank code 1M10). The position of VWF was used to adjust the VWF–botrocetin complex (Protein Data Bank code 1JK) (32), thus virtually representing A1-botrocetin-GPIba trimeric structure (Fig. 8).

Botrocetin is a non-physiological modulator to initiate VWF to bind GPIb. VWF A1 forms a complex with botrocetin via Arg1399 (636), Lys1430 (667), Arg1329 (629), and Arg1395 (632) (31), and botrocetin-A1 complex binds GPIb tightly. However, botrocetin alone does not bind GPIb (33), and it has been thought to introduce the conformational changes of VWF. Mutant (217–218)2A decreased the botrocetin-induced binding, and (285–287)2A selectively decreased the botrocetin-induced binding, suggesting the possible role of the C-terminal regions of the GPIba fragment for association with the botrocetin-VWF complex. Fig. 8 indicates that the C-terminal flank region including Arg217 and Arg218 appears to interact with the β subunit of botrocetin. Remarkably, Arg218 is closely projected on the surface of subunit β, partly explaining the (217–218)2A phenotype. On the other hand, the amino acid stretch from Asp274 is projected toward the B chain of botrocetin (Fig. 8). Mutations at Glu285 and Asp287, both of which are not mapped on Fig. 8, may also participate in the association with botrocetin, interpreting the (285–287)2A phenotype. In the C-terminal anionic region, tyrosine residues 276, 278, and 279 are sulfated...
and have been proved important for botrocetin-mediated VWF-GPIb interaction (34, 35). These facts may propose another role of the C-terminal regions of the GPIbα fragment for association with the botrocetin-VWF complex.

In terms of sulfation of three tyrosines, studies on other glycoproteins have shown that 293 cells, parents of 293T cells, had considerable ability of sulfation (36, 37). The important feature of tyrosine sulfation consensus sequences has been the amino acid residue directly before a tyrosine to be sulfated, and it can be acidic or neutral (38). Bundgaard et al. (38) recently indicated that the charge of position –1 of the tyrosine is critical and can be Asp, Asn, or Ala. However, the degree of sulfation was partly influenced by the residues in positions –2 and –3, although the C-terminal side has not been studied (39). In mutant (269–273)3A, positions –7, –6, and –4 of Tyr276 are mutated to Ala from Asp, Glu, and Asp, respectively. In mutant (274–277)2A, positions +1 and +2 of Tyr276 are mutated from Asp to Ala. These facts suggest that our mutations do not completely disrupt the consensus sequences, but deficit in sulfation is still possible.

MAb SZ2 partially inhibited botrocetin-induced VWF binding to GPIb but had no effect on ristocetin-induced VWF binding (Fig. 3). Fig. 4 indicates that SZ2 showed reduced binding to mutations at several residues in the C-terminal anionic region of the GPIbα fragment: Asp274, Asp277, Glu281, and Glu282, whereas mutations at Asp269, Glu270, and Asp272 showed normal SZ2 binding. Ward et al. (27) reported previously that SZ2 immunoprecipitated a peptide Tyr276-Glu282 (27). A later study using recombinant human-canine chimeric GPIb suggested that the SZ2 epitope is between Gly268 and Glu262 (28). Dong et al. (40) reported that SZ2 failed to bind mutants of the sulfated three tyrosines, suggesting that the sulfated tyrosines are also the key residues for SZ2. A possibility remains that (274–277)2A is not completely sulfated, and decreased SZ2 binding of (274–277)2A may be related to the sulfation. Above all observations suggest that in addition to sulfated tyrosines, the reactivity of SZ2 may be dependent on the wide range of C-terminal amino acids between 274 and 283, rather than between 268 and 273, thereby possibly expressing inhibitory activity for interaction between GPIbα and VWF-botrocetin. Although direct association between botrocetin and GPIb has not been proven, botrocetin complexed with VWF A1 may interact weakly with the C-terminal regions of the VWF binding domain of GPIbα, and this interaction may aid the tight trimer complex formation. Further studies are required for the involvement of this region in GPIbα VWF binding.

Mutant (269–273)3A showed normal binding to SZ2, but Fig. 6 indicates that it displayed increased botrocetin-induced VWF binding. In Fig. 8, the C-terminal regions contain two disulfide bonds, Cys209-Cys248 and Cys211-Cys264, and residues beyond Asp269 extend outward from Cys260 resembling a hinge that turns through 180°. Asp269 and Glu275 are located at the first hinge, while Asp272 is located before the second hinge comprising residues Asp271–Asp277 (Fig. 8). In solution, this structure may be in equilibrium with a more extended conformation through flexibility around the hinges. In this context, it may be possible that mutation at three acidic amino acids may affect the conformation of the flexible hinges, thus shifting the C-terminal amino acids, probably enhancing the interaction with botrocetin B chain.

On the basis of structure of VWF-GPIbα, the flexible disordered loop from Val227 to Ser241 (β switch) undergoes a conformational change on complex formation resulting in the gain-of-function phenotype of platelet-type von Willebrand disease (5). Asp222 and Glu225 are located at the bottom of the β switch (Fig. 8B) and thus may be shifted by the conformational change of the β switch. Asp222 and Glu225 are closer to botrocetin than VWF A1, and the interaction between botrocetin and mutant GPIbα (222–225)2A may be influenced (Fig. 6B). On the other hand, His201 has close contact with Val203 located in the β switch (Fig. 8A). Substitution by alanine may disrupt this contact, probably influencing the β switch movement. However, it is not known how the conformational shifting introduced by H203A affects the interaction with botrocetin; this has to be clarified by the crystallographic analysis of the VWF-GPIbα-botrocetin complex.

Although mutants D83A, H86A, D106K, and (149–152)3A decreased the binding to conformation-dependent MABs and the ristocetin-induced VWF binding (Figs. 4 and 6), botrocetin-induced binding was normal (D106A and (149–152)A) or increased (D83A and H86A) (Fig. 6B). Usually, recombinant proteins with defective conformation may lead to the binding defect for their ligand. In the case of VWF A1, such recombinant proteins had shown reduced binding both to GPIb and botrocetin, probably because the formation of the GPIbα binding site and the formation of the botrocetin binding site are conformationally related (31). However, rGPIbα with apparently defective conformation also abolished ristocetin-induced binding to VWF but did bind the botrocetin-VWF complex. Such botrocetin-mediated “rescue” might be supported by the sulfated anionic region to interact with botrocetin, and the rescue might occur regardless of the protein conformation of GPIbα. Particularly in the case of D83A, H86A, D106K, and (149–152)3A, the region of LRRs is mutated, and the function of the C-terminal region may not be affected. Additionally, interpretation of the tertiary structure of the VWF-GPIbα-botrocetin complex should be awaited to explain the enhanced botrocetin-induced VWF-GPIbα interaction.

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Identification of the Amino Acid Residues of the Platelet Glycoprotein Ib (GPIb) Essential for the von Willebrand Factor Binding by Clustered Charged-to-Alanine Scanning Mutagenesis

Atsuya Shimizu, Tadashi Matsushita, Takahisa Kondo, Yasuya Inden, Tetsuhito Kojima, Hidehiko Saito and Makoto Hirai

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