Structural Elements Influencing von Willebrand Factor (vWF) Binding Affinity for Platelet Glycoprotein Ib Within a Dispase-Digested vWF Fragment

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We investigated the structural elements in human von Willebrand factor (vWF) that influence binding affinity for platelet glycoprotein (GP) Ib by using a dispase-digested vWF fragment as a prototype (residues Leu480.Val481-Gly718 of the vWF subunit; Andrews et al., Biochemistry 28:8326, 1989). The major structural features of this fragment are a large A1-loop formed by an intrachain disulfide bond between Cys509 and Cys695 and six O-linked sugar chains. The fragment was chemically modified by (1) reduction and S-carboxamidomethylation (R/A), (2) desialylation (DS), or (3) a combination of both (R/A-DS). The GPIb binding affinity of these fragments was basically evaluated by competitive binding assay with anti-GPIb monoclonal antibody (LJ-Ibl), a receptor blocker for vWF (Sugimoto et al., Biochemistry 30:5202, 1991). Both the prototype and the R/A fragments were also assessed for their function in shear-induced platelet aggregation. Results unambiguously demonstrated that the presence of a disulfide bridge (Cys509-Cys695) within this domain downregulates the affinity of vWF to GPIb. In addition, it was also demonstrated that the terminal sialic acids attached to six O-linked sugar chains within this domain contribute to optimal functional modulation by the antibiotic ristocetin, but not by snake venom botrocetin.

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THE INTERACTION OF von Willebrand factor (vWF) with platelet glycoprotein (GP) Ib is now accepted to be a key event in the initiation of platelet deposition at sites of vessel injury.1 Earlier studies concerning identification of the binding sites responsible for this interaction using proteolytic fragments indicated that the GPIb binding domain of the vWF subunit resides on its amino acid residues 449-728, and that the vWF binding domain of GPIb resides on its N-terminal 45-kD tryptic fragment.2,3 Since then, various approaches have been taken to identify more specifically these functional domains using synthetic peptides and/or recombinant fragments.4-6 However, the most important question, namely, what mechanism modulates or regulates this interaction in vivo, remains to be answered. Because intact vWF in normal circulation never binds to GPIb, a certain rheologic force such as high shear stress or the interaction of vWF with subendothelial component(s) mimicking nonphysiologic modulators such as ristocetin or botrocetin is presumed to be responsible for the vWF binding to GPIb.7

To better understand the conformational and functional transition of vWF, the present study used a dispase-digested vWF fragment8 as a prototype for the GPIb binding domain. Structurally, the fragment (residues 480-481-718 of the vWF subunit) appears to be a monomer containing a large loop formed by one intrachain disulfide bond (Cys509-Cys695) and six O-linked sugar chains.8,9 Concerning functional features, this fragment retains the principal binding affinity for GPIb, heparin, and botrocetin.4 We chemically modified this fragment by reduction followed by S-carboxamidomethylation (R/A), by desialylation (DS), or by both (R/A-DS) and then assessed its binding affinity toward GPIb using a well-characterized monoclonal antibody (MoAb) against GPIb (LJ-Ibl).6-11

MATERIALS AND METHODS

Reagents. Neuraminidase-linked agarose and dispase were purchased from Sigma Chemical Co (St Louis, MO) and Boehringer (Mannheim, Germany), respectively. Iodogen and NHS-LC biotin were from Pierce (Rockford, IL). Peroxidase-labeled avidin was from Zymed (San Francisco, CA). SynChrompack RP-8 column was from SynChrom (Lafayette, IN).

MoAbs. The two anti-vWF MoAbs 40-1 (IgG) and NMC-4 (IgG) have been previously characterized in detail.11,12 The epitope of MoAb 40-1 resides on a disulfide-linked heterodimer of two peptides residues Cys1786- and Arg1926-Ser2050 of vWF), and that of MoAb NMC-4 on residues Val449-Lys728. NMC-4 inhibits vWF-GPIb interaction in vitro10 and also blocks complex formation between botrocetin and vWF.11 An anti-GPIb MoAb, LJ-Ibl (IgG), totally inhibits the vWF binding to GPIb mediated by ristocetin or botrocetin at the final concentration of approximately 50 μg/mL.12

Purification of proteins. Methods for the purification of human vWF and two-chain botrocetin (hereafter called botrocetin) have been described.13 Bovine vWF was purified by the methods of Mascelli et al.14 A dispase-digested vWF fragment (residues Leu480/Val481-Gly728) was partially purified by heparin-Sepharose affinity chromatography according to the method of Andrews et al.8 The fragment was then highly purified by reversed-phase high-performance liquid chromatography (RP-HPLC) on a SynChromap RP-8 column (10 × 250 mm) equilibrated with 0.1% trifluoroacetic acid with a gradient of acetonitrile from 0% to 60% and a flow rate of 2 mL/min (data not shown). The purified fragment (prototype, see Results) eluted at 45% acetonitrile was lyophilized and stored at −80°C. It was dissolved in 20 mmol/L HEPES-buffered saline, pH 7.4 (HBS), before use.

Gel electrophoresis. Sodium dodecyl sulfate (SDS)-15% polyacrylamide gel electrophoresis (PAGE) was performed as described previously.15 The gels were stained with Coomassie brilliant blue and/or periodic acid Schiff (PAS) reagent.

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Reduction and S-carboxamidomethylation. Reduction and S-carboxamidomethylation (R/A) of polypeptides were performed with dithiothreitol and iodoacetamide as described previously.1

Desialylation. Desialylation (DS) of the prototype or R/A fragment was performed by incubation with neuraminidase-linked agaro-rose (5.0 U/mg protein) in 0.1 mol/L sodium acetate buffer, pH 5.0, at 22°C for 2 hours. The desialylated fragments were separated from neuraminidase-linked agaro-rose by centrifugation at 12,000g for 5 minutes, dialyzed against HBS, and then stored at –80°C until use.

Radioiodination of proteins. Proteins were radioiodinated by the method of Fraker and Speck5 using iodogen with specific radioactivities ranging from 0.5 to 1.5 mCi/mg.

Competitive inhibition based on binding of biotinylated NMC-4 (IgG) to vWF. Each well of a polystyrene microtiter plate was coated with 100 µL of a solution of MoAb 40-1 in 50 mmol/L bicarbonate buffer, pH 9.6 (final concentration, 10 µg/mL), at 37°C for 2 hours. After removal of the solution, the wells were filled with 200 µL of 4% bovine serum albumin (BSA) in 50 mmol/L bicarbonate buffer, pH 9.6, at 37°C for 1 hour to block noncoated areas. They were washed three times with 250 µL of 40 mmol/L phosphate-buffered saline, pH 7.4, containing 0.05% Tween 20 (PBS/T-20) and then incubated with 50 µL of purified vWF (5 µg/mL) at 37°C for 2 hours. After incubation, the wells were washed three times with PBS/T-20.

In the assay, vWF fragments at various concentrations or HBS as a control, biotinylated MoAb NMC-4 (5 µg/mL), and BSA (5 mg/mL) were incubated in a total of 125 µL incubation volume at room temperature for 1 hour. After incubation, two 50-µL aliquots of each mixture were placed into the microtiter plate wells with purified vWF captured through MoAb 40-1. After incubation at 37°C for 1 hour, the wells were washed three times with PBS/T-20 and then incubated with 50 µL of peroxidase-conjugated avidin solution (0.25 µg/mL) each at room temperature for 20 minutes. The wells were then washed three times with PBS/T-20, and 50 µL of 0.8 mg/mL α-phenylenediamine was added as the substrate to each well. The reaction was stopped after 10 minutes with 2 mol/L H2SO4 and the absorbance was measured at 490 nm. Nonspecific binding was estimated in the presence of a 50-fold excess of unlabeled MoAb NMC-4. Binding of 100% indicates specific binding in the absence of any specific inhibitor.

Ristocetin-induced 125I-labeled vWF binding to platelets. The assay method has been previously described in detail.2 125I-labeled vWF, washed platelets, and ristocetin were used at final concentrations of 5 µg/mL, 1 × 108/mL, and 1 mg/mL, respectively. Binding of 100% indicates specific binding in the absence of any specific inhibitor.

Competitive inhibition based on the binding of 125I-labeled LJ-Ib1 (IgG) to GPIb. Inhibition assay was performed according to the method of Sugimoto et al.6,11 vWF fragments at various concentrations, 125I-labeled MoAb LJ-Ib1 (IgG, 10 µg/mL), and washed platelets (1 × 108/mL) were incubated at room temperature for 30 minutes in the absence or presence of ristocetin (1 mg/mL) or botrocetin (5 µg/mL). After incubation, platelets were centrifuged through 20% sucrose to separate free ligand, and the platelet-bound radioactivity was determined after gamma-scintillation counter. Nonspecific binding was estimated in a mixture containing the same reagents and a 50-fold excess of unlabeled MoAb LJ-Ib1. Binding of 100% indicates specific binding in the absence of any specific inhibitor.

Direct binding of 125I-labeled bovine vWF to platelets. 125I-labeled bovine vWF (5 µg/mL) and human washed platelets (1 × 108/mL) were incubated with or without competitive inhibitors at room temperature for 30 minutes in the absence of ristocetin or botrocetin. Platelet-bound radioactivity and nonspecific binding were determined essentially as described above. Binding of 100% indicates specific binding in the absence of any specific inhibitor.

Shear-induced platelet aggregation (SIPA). The procedures used to measure SIPA have been previously described in detail.7 Trisodium citrate as an anticoagulant was used at a final concentration of 0.011 mol/L. Platelet-rich plasma (PRP) from a normal individual was prepared as described.7 In each experiment, vWF fragment at various concentrations, anti-vWF MoAb NMC-4 (10 µg/mL), an anti-GPIb MoAb LJ-Ib1 (50 µg/mL), or HBS as a control was added to PRP. The platelet count of the mixtures was then adjusted to 300 × 103/µL using homologous instead of analogous platelet-poor plasma (PPP). A shear stress gradient (6 to 108 dynes/cm2) was applied to the PRP mixture. After exposure to 6 dynes/cm2 for an initial 15 seconds, shear stress was increased to 12 dynes/cm2 over the next 90-second period (low shear stress). Stress was then increased linearly from 12 to 108 dynes/cm2 over the following 120 seconds, and finally kept constant at 108 dynes/cm2 for the last 125 seconds (high shear stress).

RESULTS

Characterization of the dispase-digested vWF fragment (prototype) and its chemically modified forms. On SDS-15% PAGE analysis under nonreducing conditions, the prototype fragment purified by RP-HPLC exhibited an electrophoretic mobility faster than its R/A counterpart (Fig 1). However, their mobilities were almost identical and showed a molecular mass of 39/34 kD under reducing conditions. The prototype fragment was fully bioologically active in terms of its inhibitory action on ristocetin- or botrocetin-induced vWF binding to GPIb and the binding of botrocetin to vWF,9 indicating that one disulfide bond formed between Cys509 and Cys695 within the molecule, and its biologic function remained unchanged even after purification by RP-HPLC at pH 3.2 and with 45% acetonitrile (data not shown). After DS, both the prototype and the R/A fragments showed electrophoretic mobilities faster than their counterparts with the sugar chains intact on SDS-PAGE analysis. The total loss of PAS staining activity after neuraminidase treatment of the fragments was confirmed on the relevant SDS-PAGE gels (data not shown). These four fragments are used through out this report with the following abbreviations: (1) prototype, (2) R/A, (3) DS, and (4) R/A-DS fragments.

Immunoreactivity of the four fragments with anti-vWF MoAb NMC-4. NMC-4 is one of the best inhibitors of vWF-GPIb binding in vitro. Although the epitope of this antibody resides on residues 449-728 of the vWF subunit, its immunoreactivity with vWF is dependent on a conformation formed by a disulfide-bridged (Cys509 and Cys695) loop.10

On Western blotting analysis, none of the four fragments reacted with NMC-4 (data not shown), although the tryptic 97- to 116-kD fragment is highly immunoreactive.13

However, in a liquid-phase competitive binding assay, the prototype fragment clearly inhibited the interaction of NMC-4 with vWF in a dose-dependent manner (Fig 2). However, the R/A-DS fragment exhibited much lower apparent immunoreactivity toward NMC-4. Moreover, the DS and R/A fragments showed lower immunoreactivity toward NMC-4 than their respective counterparts, suggesting that both native terminal sialic acids in the sugar chains and the disulfide-bridged (Cys509-Cys695) loop are important for maintaining the three-dimensional structure required for the optimal immunoreactivity of NMC-4.14

Interaction of the four fragments with GPIb in the presence or absence of vWF modulators. Using the competitive

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inhibition assay based on the binding of $^{125}$I-labeled LI-Ib1 (IgG) to GPIb, relative binding affinity of the four fragments to GPIb was measured as described in Materials and Methods.

In the absence of exogenous vWF modulator, both the R/A and the R/A-DS fragments (with or without desialylation) exhibited dose-dependent inhibition at closely similar IC50 ranges (1.1 to 1.2 μmol/L). In contrast, the nonreduced fragments (prototype and DS) had only minimal effects (Fig 3). However, in the presence of ristocetin (1 mg/ml), both the prototype and R/A fragments showed lower IC50s (approximately 0.2 μmol/L), whereas the desialylated fragments (DS and R/A-DS) showed much higher values (1 to 10 μmol/L). Moreover, in the presence of botrocetin (5 μg/ml), another vWF modulator, all four fragments showed closely similar IC50s (around 0.05 μmol/L).

Effect of the four fragments on ristocetin-induced $^{125}$I-vWF binding to GPIb. The results obtained above strongly suggested that ristocetin cannot properly modulate the function of the desialylated vWF fragments (Fig 4).

In the presence of regular concentration of ristocetin (1 mg/ml), the IC50s of both the R/A and prototype fragments ranged from 0.2 to 0.3 μmol/L. However, the two desialylated fragments (DS and R/A-DS), did not show 50% inhibition of $^{125}$I-vWF binding to GPIb even at 10 μmol/L.

Effect of the four fragments on direct $^{125}$I-bovine vWF binding to GPIb. Both the R/A and the R/A-DS fragments exhibited a dose-dependent inhibition on $^{125}$I-bovine vWF binding to GPIb with almost the same IC50s (0.4 and 0.5 μmol/L). However, the nonreduced fragments did not show any significant inhibition on that binding (Fig 5).

Effects of the prototype and R/A fragments on SIPA. The functional importance of the disulfide-bridged (Cys509-Cys695) loop structure of vWF was also evaluated by its inhibition of SIPA. Neither the prototype (Fig 6A) nor the R/A fragment (Fig 6B) influenced platelet aggregation under
Inhibitory effect of proteolytic fragments on anti-GPIb MoAb (LJ-Ibl) binding to platelets. (Upper panel) Washed platelets (final count, 1 x 10⁸/mL) were incubated with various concentrations of fragments and ¹²⁵I-labeled LJ-Ibl (10 µg/mL) for 30 minutes at room temperature. After incubation, platelet-bound radioactivity was determined as described in Materials and Methods. Data points indicate the mean ± 2SD obtained in three separate experiments. (Middle and lower panels) The same experiments as above performed in the presence of 1 mg/mL ristocetin (middle) and 5 µg/mL botrocetin (lower).

DISCUSSION

In 1981, De Marco and Shapiro demonstrated that desialylated vWF spontaneously binds to platelets and initiates their aggregation. Since then, many studies on the vWF-GPIb interaction have focused on the carbohydrate moieties and multimeric structures of vWF. Our study and others have shown that a restricted region of the vWF subunit is responsible for this binding. Notably, the reduced and alkylated 52/48-kD fragment (residues 449-728 of the vWF subunit) spontaneously binds to GPIb, thereby blocking the interaction between vWF and GPIb in the absence (or presence) of exogenous vWF modulators. This fragment lacks direct aggregability toward platelets, presumably because of its monovalency. In contrast, the unreduced dimeric tryptic 97- to 116-kD vWF fragment (a homodimer of residues 449-728), like native vWF, does not bind to GPIb unless vWF modulators such as ristocetin or botrocetin are present, but does induce platelet aggregation in the presence of vWF modulators. These results suggest that the presence of one intrachain disulfide bond (Cys509-Cys695) and possibly three sets of interchain disulfide bonds (at Cys459, Cys462, and Cys464) within this domain are crucial for the regulation of the vWF-GPIb interaction.

Several groups of investigators have shown that type IIB vWF molecules, characterized by their enhanced binding to GPIb, have a single amino acid substitution or insertion
The present study, we used a monomeric dispase vWF fragment. Because this monomeric fragment has been shown to be the minimal functional unit of the GPIb binding domain. In this region on the vWF-GPIb interaction. The different structural features of the four fragments were first characterized in three ways, ie, by (1) SDS-PAGE analysis, (2) PAS staining, and (3) immunoreactivity with anti-vWF MoAb NMC-4. Their relative binding affinity to GPIb was then evaluated by the LJ-Ib1 (a receptor blocker for vWF) binding assay to GPIb. The results clearly demonstrated that, in the absence of any vWF modulator, the R/A fragments, regardless of their desialylated status, showed much higher affinity for GPIb than their counterparts with the disulfide bond intact. Specifically, the presence of a disulfide-bridged loop structure in this domain downregulates the vWF binding affinity to GPIb. Direct binding studies using 125I-bovine vWF also supported this concept. The dose-dependent inhibition of platelet aggregation under a high shear stress by the R/A fragment visualized this scenario, and conducted a final conclusion on this issue.

In the presence of 1 mg/mL ristocetin, the prototype and R/A fragments showed closely similar binding affinity toward GPIb, in contrast to the respective desialylated fragments, which showed much lower affinity for GPIb. These results were derived from two different competitive assays based on LJ-Ib1 and conventional vWF binding to GPIb. Interestingly, in the presence of botrocetin, these four fragments almost equally inhibited LJ-Ib1 binding to GPIb with much lower IC50s (by two orders magnitude) than in the absence of botrocetin. Because botrocetin is known to bind to vWF, producing an activated complex with binding ability to GPIb, the results indicate that neither the disulfide-bridged structure nor the terminal sialic acids are critical for botrocetin's modulation of vWF function.

In summary, this study shows that the intrachain disulfide bond formed by Cys509-Cys695 plays a key role in preventing the random binding of native vWF to platelet GPIb in normal circulation. Experiments using the nonphysiologic vWF modulators ristocetin and botrocetin suggest that the activated complexes formed between the four different fragments and botrocetin may have a similar conformation for GPIb binding, whereas the interaction of the four fragments with ristocetin is more complex because of the strong influence of the terminal sialic acids present in the fragments.

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Fig 6. Effect of the prototype or R/A fragment on SIPA. A shear stress gradient as indicated by dotted lines (dynes/cm²) was applied on PRP as described in Materials and Methods. (A) The prototype fragment was present at the final concentration of 10 μmol/L, and MoAb NMC-4 or LJ-Ib1 was added at the final concentration of 10 μg/mL or 50 μg/mL, respectively. (B) SIPA was measured under the same shear stress gradient as described above in the presence of the R/A fragment at the various final concentrations in micromoles.

within the GPIb binding domain. This suggests that these mutant molecules may have some conformational difference from normal vWF. Furthermore, independent testing by two different groups of a panel of overlapping synthetic peptides encompassing residues 449-728 of vWF showed the GPIb binding domain to be located on two discrete regions. One group located the domain on two discontinuous regions (residues 474-488 and 694-708) and the second on residues 514-542. Gralnick et al have demonstrated that a recombinant vWF fragment (residues 504-728) with a single intrachain disulfide bond (Cys509-Cys695), expressed in E coli, inhibits vWF-GPIb interactions and platelet adhesion at high shear stress. Subsequently, Sugimoto et al have reported that a similar recombinant vWF fragment (residues 508-696) with an intrachain disulfide bond (Cys509-Cys695) is fully active in regard to binding ability to GPIb, but any segmental lack within this loop region diminished the original activity.


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