Conformational Changes in the A1 Domain of von Willebrand Factor Modulating the Interaction with Platelet Glycoprotein Ibb

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The interaction between von Willebrand factor (vWF) A1 domain and platelet glycoprotein Ibb occurs in the presence of high shear stress or when vWF becomes immobilized onto a surface but not appreciably in the normal circulation. To investigate the structural properties regulating A1 domain function, we have used recombinant fragments prepared either in cyclic form with oxidized Cys509-Cys695 disulfide bond or reduced and alkylated. Interaction with glycoprotein Ibb was assessed by testing inhibition of monoclonal antibody LJ-Ib1 binding to platelets and inhibition of shear-induced platelet aggregation mediated by native vWF. Fragments exposed to pH between 2.5 and 3.5 adopted the molten globule conformation with loosened tertiary structure intermediate between native and completely unstructured state. Maximal receptor binding activity was observed when fragments kept at acidic pH, particularly after reduction of the Cys509-Cys695 disulfide bond, were subjected to quick refolding by rapid pH change over several hours resulted in at least 20-fold lower activity. A specific single point mutation (I546V) resulted in enhanced receptor binding, whereas another mutation (S561G) caused markedly reduced binding. These results provide experimental evidence that conformational transitions can modulate function of the vWF A1 domain in solution.

Platelet adhesion and thrombus formation at sites of vascular injury depend on the function of von Willebrand factor (vWF), particularly in areas of the circulation where blood flow is high shear stress in the normal state. To investigate the structural properties regulating A1 domain function, we have used recombinant fragments prepared either in cyclic form with oxidized Cys509-Cys695 disulfide bond or reduced and alkylated. Interaction with glycoprotein Ibb was assessed by testing inhibition of monoclonal antibody LJ-Ib1 binding to platelets and inhibition of shear-induced platelet aggregation mediated by native vWF. Fragments exposed to pH between 2.5 and 3.5 adopted the molten globule conformation with loosened tertiary structure intermediate between native and completely unstructured state. Maximal receptor binding activity was observed when fragments kept at acidic pH, particularly after reduction of the Cys509-Cys695 disulfide bond, were subjected to quick refolding by rapid pH change over several hours resulted in at least 20-fold lower activity. A specific single point mutation (I546V) resulted in enhanced receptor binding, whereas another mutation (S561G) caused markedly reduced binding. These results provide experimental evidence that conformational transitions can modulate function of the vWF A1 domain in solution.

vWF was initially identified in a monomeric, reduced and alkylated tryptic fragment comprising residues 449–728 of the mature subunit (5) and corresponding essentially to the A1 domain of the molecule (9). It contains both intrachain and interchain disulfide bonds and can be isolated either as a monomer (10) or a dimer (11) after limited proteolytic digestion of vWF; it can also be expressed recombinantly in either form (12–14).

Native vWF in blood does not show detectable binding to platelets. The interaction can be induced by exogenous non-physiologic modifiers, such as ristocetin (15, 16) and botrocetin (17, 18), and by fluid shear stress (19) or can be the consequence of selected mutations in the A1 domain (20); moreover, platelets adhere to vWF immobilized onto a surface (7). It has generally been assumed that the modulation of GP Ibb binding activity involves conformational changes in vWF with exposure of functional sites normally cryptic when the molecule is in solution. In vivo, this may occur when vWF interacts with subendothelial components, like collagen (21), where integrity of the vessel wall is disrupted.

The present studies have been undertaken to test the hypothesis that conformational changes may influence the GP Ibb binding function of vWF. Our results demonstrate that quick refolding following loosening of tertiary structure may lead to increased binding affinity and that single point mutations can either enhance or decrease A1 domain interaction with GP Ibb in the absence of exogenous modifiers. These findings provide initial information on the mechanisms involved in the regulation of receptor recognition sites in the A1 domain of vWF.

EXPERIMENTAL PROCEDURES

Expression and Purification of Escherichia coli-derived Monomeric Recombinant vWF Fragments—The two fragments designated rVWF508–704 and rVWF445–733 were expressed in E. coli BL21-D3 using plasmids containing the T7 RNA polymerase promoter (22, 23), as described previously (24, 25). Fragments, prepared either with reduced and alkylated or oxidized Cys residues, were purified by reverse-phase high performance liquid chromatography (24), concentrated by solvent evaporation, dialyzed against 2 mM acetic acid titrated with HCl to pH 3.5, and stored at −70 °C (or at 4 °C for shorter periods of time) until used.

Expression and Purification of Mammalian Cell-derived Monomeric Recombinant vWF Fragment—The DNA construct with a Cys→Ala substitution at position 462, directing secretion in Chinese hamster ovary K1 cells of a monomeric molecule designated rVWF41–733C462A, has been described previously (13, 14). Secreted proteins were precipitated with 80% ammonium sulfate, dissolved with 20 mM Tris-HCl, pH 8.0, dialyzed against the same buffer at 4 °C, and purified by anion exchange chromatography on a Mono-Q column (Pharmacia Biotech Inc.) with a 50-min linear elution gradient from 0 to 1 M NaCl. Eluted fractions that contained antigen reacting with the monoclonal antibodies LJ-RG46 and LJ-52K2 (see below) were pooled and subjected to reverse-phase chromatography on a Poros R2/H column (PerSeptive Biosystems) using a 20-min 12–70% acetonitrile gradient in 0.1% trifluoroacetic acid and 2% 2-<m>propyl alcohol. The purified fragment was concentrated by solvent evaporation or reduced and alkylated (10) and...
then concentrated after repeating the reverse-phase chromatography. Finally, the fragments were dialyzed against 2 M acetic acid titrated with HCl to pH 2.5 and stored at −70 °C (or 4 °C for shorter periods of time) until used.

Site-directed Mutagenesis—Two amino acid substitutions identified in patients with the typical phenotype of type 2B von Willebrand disease (26, 27) were expressed in the recombinant A1 domain 508–704 construct. One of the mutations, Ile546→Val, has not been reported before; the other, Pro734→Leu, has been found in at least one family (28, 29). Moreover, we also expressed the Gly561→Ser mutation known to cause type B von Willebrand disease (30), a subtype of type 2M (31). Site-directed mutagenesis was performed using uracil-containing templates (23) with a 50% probability of recombination. The normal code sequence for rWF 508–704 and oligonucleotide primers with the desired nucleotide substitution (underlined) within the rWF noncoding strand sequence: 5′-GACCCACTTCTGGAGAGCGCCAGCGCTCCATC-3′, changing the codon of Ile546→Val; 5′-TAGGGCTGGGAGCTGTGACTC-3′, changing the codon of Gly561→Ser; 5′-GCCGCAGCCTCGTACACGTTCCGGC-3′, changing the codon of Pro734→Leu. Occurrence of the desired mutation was verified by sequence analysis, and the mutated fragments were then cloned and expressed in E. coli BL21-D3, as described above.

Refolding Procedures—Fragments exposed to acidic pH were reneutralized to neutral pH either rapidly, by direct mixing with an appropriate buffer (quick refolding), or progressively, by dialysis at 4 °C against 2 M ammonium acetate or 45% acetonitrile containing 0.1% trifluoroacetic acid. Samples were introduced into the analyzer at a rate of 4.0 μl/min.

Dynamic Light Scattering—The molecular weight of molecules in solution and thus their state of aggregation was determined on the basis of the hydrodynamic radius derived from the translational diffusion coefficient measured with a dp-801 molecular size detector (Biotech Inc.). A monochromatic laser light of 780 nm wavelength from a 30 mW power source illuminated samples introduced into the measuring cell at a concentration of 1.5 mg/ml. The intensity fluctuations of the scattered light, due to the Brownian motion of molecules in solution, were analyzed to deduce the radii of the moving molecules using a connected digital signal processor and computer. The mammalian cell-derived fragments were predominantly monomeric in solution at pH <3.5 but exhibited a tendency to form aggregates with increasing pH.

Circular Dichroism Spectra—These measurements were carried out on a Cary 61 spectropolarimeter modified by replacing the original Perkin-Elmer Cary 60 spectrophotometer with a 50 kHz photoelastic modulator (Hinds International, model FS-SPEM-80) and the linear polarizer (AVIV Inc.). A lock in amplifier (EGG Princeton Applied Research, model 128) was used to integrate the phase detected output of the original end-on photomultiplier tube and preamplifier. System automation, multiple scan signal averaging, and base line subtraction were accomplished with a computer interfaced directly with the spectropolarimeter and the amplifier. The system software and custom hardware interfaces were designed by Allen MicroComputer Services, Inc., and the Department of Chemistry Computer Facility of the University of California at San Diego. Recombinant WVF fragments were tested at a concentration of 0.5-1 μg/ml in 2 M acetic acid titrated with HCl to pH 2.5 (for the fragment derived from mammalian cells) or 3.5 (for the rWF 508–704) or in 2 M ammonium acetate titrated to pH 7.0 with ammonium hydroxide. Measurements were carried out in a 0.5-mm quartz cell kept at 20 °C in a thermostatically controlled holder. Spectra between the wavelength limits of 185-260 nm were obtained as the average of 20 repeated measurements at 0.1-nm intervals with dynode voltage below 450 mV. After correction for spurious signals generated by the solvent according to our acid/base's instructions, spectra were expressed as mean residue ellipticities based on concentrations determined by amino acid analysis of samples (5-10 μg) subjected to acid hydrolysis.

Inhibition of Anti-GP Ib Monoclonal Antibody Binding to Platelets by Recombinant WVF Fragments—The inhibition of Lb1 binding to platelets can be assumed to reflect the affinity of interaction between rWF fragments and GP Ib expressed as a percentage of the fragment binding to platelets minus the corresponding control mixture and used to calculate the concentration of rWF fragment required to inhibit 50% of antibody binding to GP Ib (IC50).
RESULTS

Characterization of Purified Recombinant vWF A1 Domain Fragments

A schematic representation of the fragments used in these studies is shown in Fig. 1. After reduction of the Cys^{509}, Cys^{695} intrachain disulfide bond, all purified fragments exhibited slower migration in SDS-PAGE and markedly reduced reactivity with the monoclonal antibody NMC-4 (Fig. 2).

Conformational Changes in the Recombinant GP Ib Binding Domain of vWF

The occurrence of conformational changes in the isolated GP Ib binding domain of vWF was evaluated by measuring intrinsic fluorescence and CD spectra either at acidic pH or after slow refolding at pH 7. The results reported here were obtained with the mammalian-derived rWF^{441-730}C^{462}A at pH 2.5, but essentially identical findings were observed with the E. coli-derived fragments at pH 3.5. The different susceptibility to the denaturing effect of increasing [H^+] can be explained by the protective effect of carbohydrate chains present in the mammalian but not in the bacterially derived fragments. Emission spectra of rWF^{441-730}C^{462}A reflect the presence of eight Tyr residues and a single Trp residue located at position 550 of the native vWF sequence within the Cys^{509}Cys^{695} loop. Excitation at 280 nm demonstrated a difference in the wavelength of the light emitted with maximal intensity by the fragment kept at pH 2.5, \( \lambda_{max} \approx 340 \text{ nm} \), as compared with the one refolded at pH 7.0. \( \lambda_{max} \approx 335 \text{ nm} \) (Fig. 3). Moreover, the fragment at low pH exhibited greater fluorescence intensity at ~303 nm (Fig. 3), indicative of more efficient Tyr emission due to decreased energy transfer to the Trp residue as a consequence of increased distance. Excitation at 295 nm resulted in emission spectra dominated by the contribution of the Trp residue, with a clear difference in the wavelength of the light emitted with maximal intensity by the fragment kept at low pH, \( \lambda_{max} \approx 345 \text{ nm} \), as compared with the one refolded at pH 7.0. \( \lambda_{max} \approx 335 \text{ nm} \) (Fig. 3). The shift to red in the emission spectrum indicates that the indole ring in the side chain of Trp is located in a more hydrophilic environment near the surface of the molecule as a consequence of conformational changes induced by acidic pH.

Both cyclic and reduced and alkylated rWF^{441-730}C^{462}A maintained either at physiologic or acidic pH showed similar CD spectra, indicative of the presence of \( \alpha \) helix as well as \( \beta \) strand secondary structures (Fig. 4). A slight difference in mean residue ellipticity at <205 nm in the spectrum of the fragments kept at pH 2.5, particularly after reduction and alkylation, indicated some increase in the content of unordered structure. Overall, the CD and intrinsic fluorescence spectra were typical of the molten globule intermediate between the native and the completely unfolded states (42).

Interaction of Recombinant vWF A1 Domain Fragments with Platelet GP Ib

Effect of Conformational Transitions and of the Intramolecular Disulfide Bond between Cys^{509} and Cys^{695}—The interaction of rWF fragments with platelet GP Ib was evaluated by

![Schematic representation of the recombinant vWF fragments](image-url)
the ability to inhibit binding of the anti-GP Ibα monoclonal antibody, LJ-Ib1, in the absence of any exogenous modulator. The results shown here in detail (Fig. 5) were obtained with E. coli-derived rvWF508–704, but similar findings were observed with the other two fragments used for these studies. Slowly refolded molecules, whether cyclic or reduced and alkylated, had the lowest GP Ibα binding activity (high IC50). The apparent affinity of rvWF508–704 increased by at least 3-fold (lower IC50) when the cyclic fragment was exposed to acidic conditions and then quickly refolded by neutralizing the pH at the time of mixing with the platelets; the corresponding change was approximately 20-fold when the procedure was performed on the reduced and alkylated fragment (Fig. 5). Of note, the GP Ibα binding activity of slowly refolded fragments could be increased by exposure to acidic conditions and quick refolding but decreased again after a second slow refolding. The IC50 values obtained in this assay with all the fragments tested are shown in Table I.

The results obtained by measuring inhibition of LJ-Ib1 binding to platelets were confirmed by testing directly the ability of the rvWF fragments to inhibit shear-induced aggregation, a phenomenon that is strictly dependent on native vWF binding to GP Ibα. Also in this case, quickly refolded fragments kept at acidic pH until just before the assay were more effective inhibitors than the corresponding slowly refolded ones, and the greatest inhibitory effect was obtained with reduced and alkylated fragments. Results similar to the ones shown here for E. coli-derived rvWF508–704 (Fig. 6) were obtained with all the fragments prepared for this study.

Effect of Single Point Mutations on the Receptor Binding

Function of the Recombinant A1 Domain Fragment rvWF508–704—Two distinct single point mutations identified in patients with typical type 2B von Willebrand disease phenotype had different effects on the GP Ibα binding activity of rvWF508–704. The I564V substitution resulted in enhanced inhibition of LJ-Ib1 binding (Fig. 7), suggesting that the mutation conferred greater affinity for the receptor. The difference between this mutant and the normal control was apparent in cyclic as well as reduced/alkylated fragments but was particularly evident after slow refolding of cyclic molecules that, if carrying the mutation, had a 2.8-fold lower IC50 for inhibition than the normal control fragment (Fig. 7). In contrast, the P574L substitution had essentially no effect on the interaction of cyclic fragments with GP Ibα, whether refolded slowly or quickly; unexpectedly, however, reduced/alkylated fragments carrying this mutation had even lower affinity for the receptor than the normal control fragment (Fig. 7). In the latter case, the IC50 for inhibition of LJ-Ib1 binding to platelets was between 1.9- and 3-fold greater for the mutant than the normal fragment. Finally, the S561G substitution, thought to be responsible for defective vWF binding to platelets in type 2M subtype B von Willebrand disease, resulted in a markedly reduced interaction between rvWF508–704 and GP Ibα (Fig. 7).

DISCUSSION

The interaction between vWF A1 domain and GP Ibα has been characterized previously with the use of ristocetin (15, 16)

FIG. 2. SDS-PAGE and dot blot analysis of the recombinant vWF fragments. A, dot blot analysis. An aliquot of each fragment was applied onto a nitrocellulose membrane in a circular area. After drying, the membrane was treated with a solution of the anti-vWF monoclonal antibody NMC-4 (5 μg/ml) followed by fluorographic detection of the bound antibody using an alkaline phosphatase-conjugated rabbit antimouse IgG. Note that the antibody reacts well with the cyclic fragments containing the intact Cys509–Cys695 disulfide bond (C) but only minimally after reduction and alkylation of Cys residues (R/A). B, SDS-PAGE analysis. Fragments were analyzed in a 10% gel under nonreducing conditions. Proteins in the gel were stained with Coomassie Brilliant Blue. The numbers to the right indicate the molecular masses of standard proteins (in kDa). Note the slower migration and apparent size heterogeneity of the mammalian cell-derived fragments. Results similar to the ones shown here for E. coli-derived rvWF441–730C462A as compared with the corresponding E. coli-derived rvWF445–733, composed of approximately the same number of residues. These findings are compatible with the presence of variable carbohydrate side chains in the mammalian cell-derived molecule, as in native vWF (5, 13).

FIG. 3. Intrinsic fluorescence spectra of rvWF441–730C462A, cyclic or reduced and alkylated, at acidic pH or after slow refolding at neutral pH. The fragment was tested at concentrations between 0.5 and 1 μM in either 2 mM acetic acid titrated with HCl to pH 2.5 or 2 mM ammonium acetate, pH 7.0. Excitation wavelength of either 280 or 295 nm was used; the latter minimizes the contribution of Tyr emission.
and botrocetin (17, 18), two molecules without known physiologic counterpart that modulate activity but have no defined effects on vWF structure. Here, we show that slowly refolded recombinant fragments with intact Cys509–Cys695 disulfide loop behave like the A1 domain of native plasma vWF, which typically cannot interact with GP Ibα in the absence of modulators or mechanical shear forces. In particular, both \( \text{rvWF}^{441-730} \text{C462A} \) and \( \text{rvWF}^{445-733} \) inhibited antibody LJ-Ib1 binding to platelets with IC50 well above the 20 nm approximate plasma concentration of vWF subunit, tantamount to the lack of measurable interaction shown by the latter. In contrast, reduced and alkylated fragments refolded quickly after exposure to acidic pH attained an apparent affinity for GPIbα that, if exhibited by circulating vWF, would result in binding to platelets. Our findings, therefore, although based on experimental conditions that cannot be directly relevant in vivo, establish a correlation between specific conformational characteristics of vWF A1 domain and function.

It has been suggested previously that isolated recombinant fragments expressed in bacteria or mammalian cells reflect the biological activities of native vWF (12, 13, 20, 24, 43). This concept is further supported by the demonstration provided here that mutations causing enhanced or reduced interaction of

![Circular dichroism spectra of \( \text{rvWF}^{441-730} \text{C462A} \), cyclic or reduced and alkylated, at acidic pH or after slow refolding at neutral pH.](image)

**FIG. 4.** Circular dichroism spectra of \( \text{rvWF}^{441-730} \text{C462A} \), cyclic or reduced and alkylated, at acidic pH or after slow refolding at neutral pH. The fragment was tested at concentrations between 0.5 and 1 μM in either 2 mM acetic acid titrated with HCl to pH 2.5 or 2 mM ammonium acetate, pH 7.0. Far ultraviolet circular dichroism spectra were recorded at wavelength intervals of 0.1 nm ranging from 260 to 185 nm. Each measurement was repeated 20 times. The results were averaged and then corrected for spurious signals generated by the solvent. The spectra were converted to mean residue ellipticities.

![Inhibition of anti-GP Ibα monoclonal antibody LJ-Ib1 binding to platelets by \( \text{rvWF}^{508-704} \text{cyclic or reduced and alkylated.}\)](image)

**FIG. 5.** Inhibition of anti-GP Ibα monoclonal antibody LJ-Ib1 binding to platelets by \( \text{rvWF}^{508-704} \) cyclic or reduced and alkylated. Platelet-rich plasma was mixed with 10 mM HEPES buffer, pH 7.4, a constant volume (always <12.5 μl) of various concentrations of vWF fragment and 10 μg/ml of \( ^{125} \text{i} \)-labeled monoclonal LJ-Ib1 IgG in 10 mM HEPES buffer, pH 7.4. The fragment was either added into the experimental mixture from a solution in 2 mM acetic acid at pH 3.5 (this was called "quick" refolding because the fragment was brought to pH 7.0–7.4 by direct mixing with the other test reagents) or refolded before testing by slow pH change from acidic to 7.0 ("slow" refolding). The control mixture contained either 2 mM acetic acid titrated to pH 3.5 with HCl or 2 mM ammonium acetate, pH 7.0, instead of vWF fragment at the corresponding pH. The total volume of each mixture was 125 μl, and the final platelet count was 1 × 10^9/ml; after adding all reagents, the pH of each mixture was between 7.0 and 7.4. After incubation at room temperature for 30 min, platelets were separated by centrifugation through a layer of 20% sucrose, and the radioactivity of LJ-Ib1 bound to platelets was measured in a γ-scintillation counter. Nonspecific binding of \( ^{125} \text{i} \)-labeled LJ-Ib1 was estimated with the addition of a 100-fold excess of unlabeled LJ-Ib1 and was always ≤10% of total binding; the corresponding value was subtracted from all data points. The results are expressed as the mean ± S.E. of percent residual binding relative to the corresponding control mixture (n = 7 for cyclic fragments and n = 6 for reduced/alkylated fragments).

|TABLE 1| Inhibitory effect of recombinant vWF fragments on LJ-Ib1 binding to platelet glycoprotein Ibα. |
|---|---|
|The concentration of each fragment (in μmol/liter) necessary to inhibit 50% of antibody binding (IC50) is reported as the mean value ± standard deviation calculated from the indicated number of experiments (n). Note that the pH for slow refolding of the E. coli-derived \( \text{rvWF}^{445-733} \) was limited to 5.0 to avoid precipitation. The mammalian cell-expressed fragment could not be tested at concentrations >2.0 μM because of limited availability of the purified product. |
|---|---|---|
| | | | | |
|IC50 | Slow refolding | Quick refolding | n |
|---|---|---|---|
| \( \text{rvWF}^{441-730} \text{C462A} \) (mammalian expression) | | | |
| Cyclic | >2.0 | >2.0 | 4 |
| Reduced/alkylated | >2.0 | 0.200 ± 0.059 | 4 |
| \( \text{rvWF}^{508-704} \) (E. coli expression) | | | |
| Cyclic | 1.165 ± 0.528 | 0.355 ± 0.146 | 7 |
| Reduced/alkylated | 0.583 ± 0.162 | 0.029 ± 0.006 | 6 |
| \( \text{rvWF}^{445-733} \) (E. coli expression) | | | |
| Cyclic | 5.284 ± 2.216 | 2.270 ± 0.980 | 4 |
| Reduced/alkylated | 0.583 ± 0.447 | 0.126 ± 0.081 | 3 |
native vWF with GP Ibα produce similar functional alterations when expressed in monomeric rVWF508–704. It is of interest that only one of the two type 2B mutations tested resulted in increased receptor binding activity in the absence of modulators, suggesting that different amino acid substitutions known to be associated with type 2B von Willebrand disease (29) have distinct structural and functional consequences. Indeed, it is not known to date whether all type 2B mutations result in
enhanced multimeric vWF binding to platelets in the absence of modulators and/or shear stress (44), and, in at least one specific case, it has been shown that expression of a dimeric A1 domain fragment in mammalian cells may be necessary to demonstrate heightened activity (20). Equally noteworthy is the fact that expression of the type 2M subtype B mutation in monomeric rVWF 508–704 demonstrates markedly decreased function in the absence of modulators, whereas native or recombinant multimeric vWF with the same mutation shows reduced activity with ristocetin but normal with botrocetin (30). Our results provide experimental evidence that the defect, in this case, is intrinsically related to structural changes affecting A1 domain receptor binding function and not necessarily modulator-dependent. Interestingly, GP Ibα binding activity, although well below normal, could be elicited in the subtype B mutant rVWF 508–704 by quick refolding after denaturation, suggesting that the procedure leads to exposure of “hidden” functional sites similar to what may be caused by complex formation with botrocetin.

The segment of sequence containing Trp550 has been implicated in interacting with the modulator botrocetin (18), thus, indirectly, in the control of binding to GP Ibα, a role also suggested by the observation that a Trp550 → Cys mutation causes enhanced interaction with the receptor in type 2B vWF (20). Our results now demonstrate that perturbations in the position of the side chain of Trp550 correlate with an increase of between 1 and 2 orders of magnitude in GP Ibα binding affinity, presumably consequent to surface exposure of a previously masked active site. Transition from low to high affinity appears to require loosening of native tertiary structure followed by quick refolding. During this process, exposed hydrophobic regions in the molten globule conformation created by partial unfolding at acidic pH (42) may form initially “incorrect” intramolecular associations, leading to a transient non-native structure with unmasked GP Ibα interaction sites. This state may be followed by rearrangement of the transient hydrophobic interfaces during slow refolding, resulting in the final native structure with masked interaction sites. In this regard, the Cys509–Cys695 disulfide bond can be viewed as a fixed tertiary structural element that tends to maintain native intramolecular interactions even after exposure to low pH; hence the impossibility for the cyclic fragments to achieve maximal GP Ibα binding activity. Because quick refolding, in the context of these studies, was initiated by a change from acidic to neutral pH coincident with functional measurements, formation of the intermediate and active conformation may occur rapidly and be relatively short lived. Whether refolding under these conditions was also affected by the presence of the receptor on platelets acting as a template for inducing optimal binding conformations cannot be determined at present. If this happened, the fragments used for CD and intrinsic fluorescence measurements, refolded quickly in solution in the absence of platelets, may have been structurally different from those used in functional tests.

The present study, in agreement with previous results from our laboratory (5, 24) but in apparent contrast with conclusions reached by others (45), clearly indicates that the Cys509–Cys695 disulfide loop is not strictly required to preserve A1 domain interaction with GP Ibα. Disruption of the bond was proven unequivocally by molecular mass determinations, thus eliminating any concern about its possible resistance to the reducing procedures utilized (45). Rather, methodological differences with respect to the use of exogenous modulators are the most likely cause of the conflicting findings. It has been reported that full-length recombinant vWF multimers containing Cys → Ser mutations at positions 509 and 695 fail to bind to platelets in the presence of ristocetin (45), suggesting that the Cys509–Cys695 disulfide bond may be needed for the expression of activity induced by this modulator. However, loss of function in this case may also result from major conformational alterations within the A1 domain, possibly caused by abnormal folding of the large vWF subunit in the absence of an important stabilizing structural element, and/or as a consequence of disulfide bond rearrangement. These factors appear to be irrelevant in the case of isolated A1 domain fragments, providing a more direct assessment of the involvement of the Cys509–Cys695 loop in the expression of function.

The identification of GP Ibα interaction sites in the A1 domain of vWF is a topic of ongoing research. A recent study based on the mutation of charged residues to Ala has highlighted the role of the segment of sequence 596–645 (46) in receptor binding: experiments with synthetic peptides have lead to similar conclusions for residues 474–488 and 694–708 in one study (33) or residues 514–542 in another (47). The reasons for these conflicting results are not clear at present, but all these findings are likely to be only partially informative because each method may identify functionally important segments of sequence but cannot exclude the potential involvement of others. The issue is further complicated by the still undefined effects of the modulators used, because the interaction sites induced in the presence of ristocetin or botrocetin have never been proven to be identical and are likely to be at least partially distinct (48). Relevant to the present studies, E. coli-expressed fragments terminating at residue 696 but still comprising the Cys509–Cys695 disulfide loop have previously been found to exhibit GP Ibα binding function similar to rVWF 508–704 when oxidized but to have no measurable activity after disulfide bond reduction (24). These results imply that residues on the carboxyl-terminal side of Cys695 are necessary for GP Ibα binding but only after disruption of the intramolecular disulfide loop (24). Thus, the GP Ibα interaction sites expressed by cyclic or reduced and alkylated A1 domain fragments in the absence of exogenous modulators may be partially different, and residues in the sequence 697–704 may be responsible for the enhanced binding activity of reduced and alkylated fragments. Incidentally, it has also been shown that recombinant vWF fragments truncated at residue 696 fail to interact with the monoclonal antibody NMC-4 (24), even though they contain all the residues indicated by others as necessary for expression of the corresponding epitope within the Cys509–Cys695 loop (46). This is further indication that residues 697–704 may participate in molecular recognition events involving the A1 domain of vWF. From the sum of all results available to date, it appears that A1 domain interaction with GP Ibα is

### Table II

| Fragment Description | IC<sub>50</sub> Value | n | Method |
|----------------------|----------------------|---|--------|
| Normal               |                      |   | Slow refolding |
| Cyclic              | 1.60 ± 0.34          | 3 | 4.00    |
| Reduced/alkylated    | 0.38 ± 0.03          | 2 | 4.00    |
| 1546V                | 0.56 ± 0.05          | 3 | 4.00    |
| Reduced/alkylated    | 0.21 ± 0.02          | 2 | 4.00    |
| PS74L                | 1.61 ± 0.18          | 3 | 4.00    |
| Reduced/alkylated    | 0.72 ± 0.02          | 2 | 4.00    |

**Inhibitory effect of normal or mutant rVWF 508–704 on Lj-1b1 binding to platelet glycoprotein Ibα**

The concentration of each fragment (in μmol/liter) necessary to inhibit 50% of antibody binding (IC<sub>50</sub>) is reported as the mean value ± standard deviation calculated from the indicated number of experiments (n).
complex and likely to involve discontinuous sites that individually may play functional roles of varying relevance depending on conformational conditions and/or the presence of exogenous modulators.

It has recently been proposed that VWF binding to GP Ib αx is influenced by three segments of sequence (residues 497–511, 540–578, and 687–698) that “inhibit” the interaction by repressing an endogenous “botrocetin-like” modulator site corresponding to residues 520–534 and 626 (46). Our results agree with the concept that the sequence 540–578, comprising Trp550, may control A1 domain function and that regions flanking the Cys509–Cys695 loop, particularly on the amino-terminal side, may act as negative control elements and cause decreased affinity in receptor binding. Furthermore, carbohydrate chains in the mammalian cell-derived fragment, also located on both sides of the A1 loop (49), appear to provide additional negative regulation of function. Of note, reduction of the intrachain disulfide bond in association with partial denaturation/quick refolding had the most pronounced effect in enhancing the disulfide bond in association with partial denaturation/quick refolding. Of note, reduction of the intrachain disulfide bond, although their GP Ib αx activity of “long” fragments with flanking regions surrounding the disulfide loop, maintained approximately 1 order of magnitude lower than that of the “short” fragment composed essentially of the loop itself. This indicates that selected sequences within and/or in proximity of the A1 loop may conformationally constrain and/or sterically hinder functional receptor recognition sites. Forthcoming information on the three-dimensional structure of the VWF A1 domain will allow verification of these hypothetical interpretations of our findings.

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