Isolation of the von Willebrand Factor Domain Interacting with Platelet Glycoprotein Ib, Heparin, and Collagen and Characterization of Its Three Distinct Functional Sites

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We have used purified proteolytic fragments of von Willebrand factor (vWF) to characterize three related functional sites of the molecule that support interaction with platelet glycoprotein Ib, collagen, and heparin. A fragment of 116 kDa was found to be dimeric and consisted of disulfide-linked subunits which, after reduction and alkylation, corresponded to the previously described 52/48-kDa fragment extending from residue 449 to 728. Fragment III-T2, also a dimer, was composed of two pairs of disulfide-linked subunits, two 35-kDa heavy chains (residues 273-511) and two 10-kDa light chains (residues 674-728). The 116-kDa fragment, but not the constituent 52/48-kDa subunit, supported ristocetin-induced platelet aggregation and retained 20% (on a molar basis) of the ristocetin cofactor activity of native vWF; fragment III-T2 retained less than 5% activity. All three fragments, however, inhibited vWF interaction with glycoprotein Ib. Both 116-kDa and 52/48-kDa fragments inhibited vWF binding to heparin with similar potency, while fragment III-T2 had no effect in this regard. Only the 116-kDa fragment inhibited vWF binding to collagen. These results indicate that dimeric fragments containing two glycoprotein Ib-binding sites possess the minimal valency sufficient to support ristocetin-induced aggregation. The sequence comprising residues 512-673, missing in fragment III-T2, is necessary for binding to heparin and collagen and may be crucial for anchoring vWF to the subendothelium. Immonochemical and functional data suggest that the same sequence, although not essential for interaction with glycoprotein Ib, may influence the activity of the glycoprotein Ib-binding site. Only binding to collagen has absolute requirement for intact disulfide bonds. Thus, the three functional sites contained in the 116-kDa domain of vWF are structurally distinct.

The multimeric glycoprotein von Willebrand factor (vWF) performs an essential role in mediating platelet adhesion and aggregation at sites of vascular injury, particularly under flow conditions characterized by high shear stress (1-3). The molecule is required for normal hemostasis and may be of central importance in the pathogenesis of acute thrombotic occlusion in diseased vessels (4, 5). vWF interacts with two known platelet receptors, the membrane glycoprotein (GP) complexes Ib-IX and IIb-IIIa (6), as well as with components of the vessel wall, among which collagen (7, 8) and heparin (9) have been identified to date. A considerable body of experimental work has led to the definition of distinct functional domains responsible for the activities described above, as well as for mediating the formation of a binonuclear nonequivalent complex with the factor VIII procoagulant protein (10). In particular, the region of vWF subunit comprised between residues Val449 and Lys728 has been shown to be involved in at least three different interactions, namely with GP Ib (11), heparin (9), and collagen (7, 8), all of critical importance for anchoring platelets to the subendothelium. The nature of the GP Ib-binding domain has been elucidated recently in more detail (12), but the specific structures that mediate interaction with heparin and collagen, as well as the possible relationships between these different functional sites, remain to be understood. Progress in this regard is of theoretical as well as practical importance and may lead to the identification of potential targets for anti-thrombotic intervention resulting in inhibition of platelet adhesion to the vessel wall. On the basis of these considerations, we have undertaken the series of experiments described here to obtain additional structural information on the three functional sites of vWF mediating interaction with GP Ib, heparin, and collagen. Our results demonstrate that each of these domains is distinct from the others and establish the role of the vWF sequence comprising residues 512-673 in promoting binding to subendothelial components.

EXPERIMENTAL PROCEDURES AND RESULTS

Effect of Proteolytic Fragments on the vWF-GP Ib Interaction—All three fragments tested inhibited ristocetin-induced aggregation of platelet glycoprotein Ib, collagen, and heparin. The abbreviations used are: vWF, von Willebrand factor; GP, platelet membrane glycoprotein; TPKC, L-1-tosylamido-2-phenyl-ethyl chloromethyl ketone; HPLC, high performance liquid chromatography; Tria buffer, a buffer composed of 0.02 M Tria-HCl, 0.15 M NaCl, pH 7.4; SV8, Staphylococcus aureus V8 protease; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; phosphate buffer, a buffer composed of 0.02 M mono- and disodium phosphate, 0.13 M NaCl, pH 7.4; ELISA, enzyme-linked immunosassay.

Portions of this paper (including "Experimental Procedures," part of "Results," and Figs. 7-9) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

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binding of $^{125}$I-vWF to platelet GP Ib, but they differed in their dose-dependent effects (Fig. 1). Thus, IC$_{50}$ values (range of three experiments) were between 0.034 and 0.072 μM with 116 kDa, between 0.3 and 0.66 μM with 52/48 kDa, and between 1.2 and 2.0 μM with III-T2. Molar concentrations were calculated using the apparent molecular mass of each fragment, with intact disulfide bonds for 116 kDa and III-T2. The three fragments also completely inhibited the direct platelet binding of $^{125}$I-labeled asialo-vWF in the absence of ristocetin (Fig. 1). Again, 116 kDa was the most effective with IC$_{50}$ values between 0.42 and 0.65 μM. The corresponding values for 52/48 kDa and III-T2 were between 0.68–1.65 and 3.8–5.1 μM, respectively.

The 116-kDa fragment supported platelet aggregation in the presence of ristocetin and retained approximately 20% of the cofactor activity of purified intact vWF, calculated on a molar basis. Because of the heterogeneous nature of native vWF, molar concentrations were calculated taking into consideration the molecular weight of the constituent subunit (Fig. 2). In contrast, fragment III-T2 possessed less than 5%

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**Fig. 1.** Dose-dependent inhibitory effect of the proteolytic fragments on $^{125}$I-vWF binding to GP Ib. Top panel, binding was measured using formalin-fixed platelets ($1 \times 10^8$/ml) and $^{125}$I-labeled vWF (2 μg/ml) in the presence of 1 mg/ml ristocetin. Tris buffer (control mixture) or proteolytic fragments in the same volume of buffer were added at the concentrations indicated, and the mixtures were incubated for 30 min at 22–25 °C. After incubation, separation of bound from free ligand was achieved by centrifugation of platelets through 20% sucrose, followed by determination of the platelet-associated radioactivity in a γ-scintillation spectrometer. Nonsaturable binding was estimated in the presence of a 50-fold excess of unlabeled vWF and was subtracted from all data points. The platelet-associated radioactivity measured in the presence of Tris buffer was assumed to reflect maximal binding. Residual binding measured in the presence of proteolytic fragments was expressed as percentage of that measured in the control mixture. The latter was consistent with expected values based on the affinity of vWF binding to platelets in the presence of ristocetin, as extensively characterized in previous publications (6, 17). Data points represent the mean and range of three determinations. Bottom panel, binding of asialo vWF was measured in the absence of ristocetin, using platelet-rich plasma with 2 mM EDTA, with a final platelet count of $1 \times 10^8$ and $^{125}$I-asialo-vWF between 10–15 μg/ml.

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**Fig. 2.** Effect of the proteolytic fragments of vWF in supporting platelet aggregation in the presence of ristocetin. Purified native vWF or purified fragments at various concentrations (as indicated) were incubated with washed platelets ($3 \times 10^8$/ml) for 5 min in an aggregometer cuvette at 37 °C. Ristocetin was then added at the final concentration of 1.0 mg/ml, and aggregation was monitored by recording changes in light transmittance. Molar concentrations of vWF were calculated on the basis of the calculated subunit molecular mass (275 kDa); molar concentrations of the fragments were calculated on the basis of their respective molecular mass, with intact disulfide bonds for 116 kDa and III-T2. Note the significant aggregation seen in the presence of 116-kDa fragment, the modest aggregation with III-T2, and the lack of aggregation with the monomeric 52/48-kDa fragment.

Ristocetin cofactor activity, while the 52/48-kDa fragment had no detectable activity (Fig. 2). The high molecular weight fragments separated by gel filtration in the last step of 116-kDa purification (Fraction J, Fig. 7 in the Miniprint) had less than 1% of the ristocetin cofactor activity of native vWF. These results, together with the finding that no intact vWF subunit could be detected in the fractions separated with the final gel filtration step (Figs. 7 and 8 in the Miniprint), ruled out the possibility that contamination by undigested or partially digested vWF could be responsible for the residual ristocetin cofactor activity of the purified 116-kDa fragment.

Unlike intact asialo-vWF, neither the 116-kDa nor the 52/48-kDa fragment could induce platelet aggregation in the absence of other agonists, but both fragments inhibited the aggregation induced by asialo-vWF. Their inhibitory activity was similar and both fragments caused almost complete inhibition of aggregation at the concentration of 1.0 μM.

**Effect of Proteolytic Fragments on vWF Binding to Immobilized Heparin—**The 116-kDa fragment and its 52/48-kDa constituent subunit inhibited vWF binding to heparin by more than 80% when present at a concentration of 10 μM. In contrast, fragment III-T2 had no effect at concentrations as high as 50 μM (Fig. 3). At variance with the inhibitory effect
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**Fig. 3.** Dose-dependent inhibitory effect of the proteolytic fragments on $^{125}$I-vWF binding to immobilized heparin. $^{125}$I-vWF (2 μg/ml) was incubated with 5% (v/v) heparin-Sepharose gel in the presence of proteolytic fragments at various concentrations, as indicated, or Tris buffer in the control mixture. Bound radioactivity is expressed as percentage of that measured in the control mixture. Data points represent the mean and range of three determinations.

**Fig. 4.** Dose-dependent inhibitory effect of the proteolytic fragments on $^{125}$I-vWF binding to fibrillar collagen type I. The binding of $^{125}$I-vWF (2 μg/ml) to bovine fibrillar collagen type I was measured in the presence of fragments at various concentrations, as indicated, or the same volume of Tris buffer in the control mixture. Residual binding in the presence of fragments is expressed as percentage of that measured in the control mixture. Data points represent the mean and range of two determinations.

on vWF binding to GP Ib, 116 kDa with intact disulfide bonds as well as the reduced and alkylated 52/48-kDa fragment inhibited vWF binding to heparin with similar efficacy. The IC$_{50}$ values (range of three experiments) were between 5.6–7.5 μM in both cases.

**Fig. 5.** Binding of monoclonal antibodies to insolubilized proteolytic fragments of vWF. Polystyrene microtiter wells were coated with a solution of purified fragment (○, 116 kDa; ▲, 52/48 kDa; ●, III-T2) at the concentration of 0.6 μM. Purified IgG of the three monoclonal antibodies tested (NMC-4, RG-46, and 52-K2) was then added in increasing concentrations, as indicated on the abscissa, and binding to the insolubilized fragments was detected in an ELISA assay using a peroxidase-conjugated goat anti-mouse IgG antibody. The intensity of the color developed after the addition of the peroxidase substrate o-phenylenediamine was evaluated by measuring light absorbance at 482 nm and was taken to represent the amount of monoclonal antibody bound in each well. All experimental points were tested in duplicate, and the values reported represent the calculated mean of the two observations.

**Effect of Proteolytic Fragments on vWF Binding to Fibrillar Collagen**—The 116-kDa fragment inhibited vWF binding to bovine collagen type I by 80% when present at a concentration of 8 μM, while neither the 52/48-kDa fragment nor III-T2 showed any inhibitory effect at similar or greater concentrations (Fig. 4). The IC$_{50}$ values obtained with the 116-kDa fragment (range of three experiments) were 1.8–2.5 μM.

**Effect of Proteolytic Fragments on vWF Interaction with Selected Monoclonal Antibodies**—These studies were performed to characterize the immunochromical structure of the three proteolytic fragments of vWF by testing their reactivity with three monoclonal antibodies of known epitope specificity (12). All fragments reacted in a similar manner with antibodies RG-46 and 52-K2, which recognize two distinct epitopes located between residues 474–488 and 694–708 of the vWF subunit, respectively. The results were the same whether the interaction was tested directly by measuring antibody binding to insolubilized fragment (Fig. 5), or indirectly by measuring the inhibitory effect of preincubation with the fragments on antibody binding to insolubilized vWF (Fig. 6). In contrast, only the 116-kDa fragment reacted well with antibody NMC-4 (Figs. 5 and 6), which has previously been shown to recognize an epitope formed by residues in the two segments of subunit between residues 474–488 and 694–708 existing only when disulfide bonds in vWF are intact (12). Fragment III-T2 reacted much less than the 116-kDa fragment with this antibody, and the interaction was detectable only as inhibition of antibody binding to vWF; fragment 52/48 kDa failed to react at all (Figs. 5 and 6).

**DISCUSSION**

Previous experiments have established that the region of vWF subunit comprising residues 449–728 contains the elements necessary and sufficient for interaction with GP Ib (11), heparin (9), and collagen (7, 8). Now, we demonstrate that this region of the molecule represents a distinct structural domain that can be isolated as a dimeric proteolytic fragment with intact interchain disulfide bonds. As such, it maintains functional integrity with regard to mediating platelet aggre-
between residues 512-673. This segment of sequence, there-

between the fragment T 96 kDa employed previously (8) and

constituent subunit, inhibited vWF binding to collagen. Binding
domains are distinct, since only fragment 116 kDa

thelium. It is clear, however, that the heparin and collagen

interaction indicates interaction of that fragment with a given monoclonal

antibody in solution. Therefore, the latter function, but not heparin binding, must

functional conformation is maintained by native disul-

fide bonds located externally to the binding site. Alternatively, the interacting site may be located outside of residues 512–

673, although obviously within residues 449–728, but elements in the former sequence may contribute in a crucial manner to

stability of the functional conformation. It is important to remember that vWF contains a second collagen-binding do-

main, located between residues 911–1114 (8), which does not interact with heparin and whose possible structural homolo-
gies and functional integration with the domain discussed here remain to be understood.

Interaction of vWF with heparin, like binding to collagen, appears to involve one or more sites located between residues 512–673. Native conformation of the molecule, however, is not relevant for heparin binding, since both the 116-kDa fragment, with intact disulfide bonds, and the reduced and alkylated 52/48-kDa fragment were equally effective in their inhibitory activity. The direct interaction between heparin and the 52/48-kDa fragment has been demonstrated previously (9). Thus, it is likely that limited linear sequences within residues 512–673 represent the necessary and sufficient structural elements that mediate vWF binding to heparin. It remains to be established whether any correlation exists be-

between the heparin-binding site in the 116-kDa domain and that described by others (16) in the amino-terminal region of vWF, within the first 272 residues.

In a previous publication (12) we have provided the demonstra-
tion, based on the use of synthetic peptides and mono-
clonal antibodies, that the GP Ib-binding domain of vWF is formed by elements present in at least two discontinuous sequences located between residues 474–488 and 694–708, respectively. The present results are in agreement with those findings, since all three proteolytic fragments studied, which contain the two segments of sequence identified as being functionally important, inhibited vWF binding to GP Ib. Moreover, the two fragments with intact disulfide bonds, 116
kDa and III-T2, which are dimeric in nature, also supported ristocetin-induced aggregation. Thus, a divalent fragment capable of binding to two GP Ib receptors on different platelets can act as a bridging protein and mediate platelet-to-platelet contact, a VWF function for which the present results establish a minimum size and valency. The requirement for bivalency appears to be crucial in this regard, since the monovalent fragment 52/48 kDa, which inhibited VWF binding to GP Ib more effectively than the divalent fragment III-T2, could not support platelet aggregation or agglutination. As previously reported for the 52/48-kDa fragment (11), fragments 116 kDa and III-T2 displayed the ability to interact with GP Ib without need for the presence of ristocetin, as shown by the fact that they could inhibit platelet binding of asialo-VWF occurring in the absence of any other agonist.

Fragment 116 kDa interacted with GP Ib with higher affinity than fragment III-T2, particularly in the presence of ristocetin. Moreover, the monoclonal antibody NMC-4 reacted well with fragment 116 kDa, but very poorly with III-T2. The reasons for these findings are not immediately apparent, since both fragments possess the two sequences constituting the GP Ib-binding domain and NMC-4 epitope (Fig. 3 in the Miniprint), as well as intact disulfide bonds that should maintain these discontinuous sequences in appropriate conformation (12). Thus, it appears that, in addition to correct disulfide bonding, elements in the VWF sequence 512–673 influence immunological conformation and functional activity of the GP Ib-binding site. This effect may involve residues that, in addition to those identified previously and located at positions 474–488 and 694–708, participate in the interaction with GP Ib and antibody NMC-4, perhaps only after initial contact has been established through the two sequences identified above. Alternatively, the VWF sequence 512–673 may modulate the functional and immunological conformation of the GP Ib-binding site without directly participating in the corresponding interactions.

In conclusion, our present studies demonstrate that a distinct structural domain of VWF, the disulfide-bonded dimeric 116-kDa tryptic fragment comprising the sequence from Val19 to Lys78, exhibits three activities of the native molecule, those responsible for interaction with GP Ib, heparin, and collagen. Within this domain, we have identified some of the structural features that support each activity. In particular, the sequence comprising residues 512–673 appears to have an important role in the function of this domain, essential for binding to heparin and collagen, and subsidiary for binding to GP Ib. Our results may provide clues for future efforts aimed at defining more precisely the chemical bases of VWF involvement in platelet thrombus formation.

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REFERENCES


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UVF Interaction with GP Ib, Heparin, and Collagen

EXPERIMENTAL DESIGN

UVF interaction with GP Ib, Heparin, and Collagen: The interaction of UVF with GP Ib was studied using an ELISA assay. UVF was incubated with anti-GPIb antibody and the complex was then incubated with a secondary antibody conjugated to horseradish peroxidase. The absorbance was measured at 490 nm.

UVF interaction with Heparin: UVF was incubated with different concentrations of heparin and the absorbance was measured at 490 nm.

UVF interaction with Collagen: UVF was incubated with different concentrations of collagen and the absorbance was measured at 490 nm.

RESULTS

UVF interaction with GP Ib: The absorbance increased with increasing concentrations of GP Ib.

UVF interaction with Heparin: The absorbance increased with increasing concentrations of heparin.

UVF interaction with Collagen: The absorbance increased with increasing concentrations of collagen.

DISCUSSION

It is concluded that UVF interacts with GP Ib, Heparin, and Collagen. Further studies are needed to understand the mechanism of interaction.
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Figure 8. Electrophoretic analysis of the purified proteolytic fragments of vWF. Protein (10 μg per lane) were separated by electrophoresis in polyacrylamide gels containing 7 M urea, and stained with Coomassie Brilliant Blue. Samples were either untreated (NR) or reduced with 1 M dithiothreitol (DTT). The four lanes on the left are from a 4-7% polyacrylamide gel, the two lanes in the middle are from a 10% gel, and the two lanes on the right are from a 7-5% gradient gel. Purified vWF is shown in the first two lanes to demonstrate that no intact molecule was present in the fractions obtained after the final gel filtration step (see Figure 1). This is true also for fractions 116 and 52/48, which represent the purified 116 kDa fragment (NR) and its constituent 52/48 kDa (fraction 11), respectively. Following reduction, the 116 kDa fragment is evident, while the 10 kDa small chain is only poorly resolved and can be seen just above the dye front. Molecular masses were calculated from the relative rate of migration of standard proteins.

The amino terminal and carboxyl terminal residues of the 52/48 kDa fragment, representing the constituent subunit of the 116 kDa fragment, as well as those of the two chains constituting fragment 111-12, have been reported in previous publications (11, 12). These fragments have overlapping amino acid sequence between residues Val499-Arg511 and Ala499-Gly512 (residue numbers correspond to those in the mature vWF sequence) reported in reference [14]. In contrast, residues Ser552-671 are present only in fragments 116 kDa and its constituent subunit 52/48 kDa, while residues 271-279 are typically present only in fragment 111-12 (Figure 9). In occasional preparations of 116 kDa fragment, a constituent chain of apparently 50 kDa was seen, in addition to the previously described 52/48 kDa subunit, whose sequence extends from residues 383-499, differing from that of the heavy chain of fragment 111-12 (13). Therefore, these preparations of fragment 116 kDa, functionally indistinguishable from all the others, contained variable amounts of a subunit comprising residues 271-279. All the fragments described here are the result of tropic clearance at distinct bonds in the same region of the vWF molecule. Cleavage occurs differently depending on the conditions of proteolytic digestion. When intact vWF is used as a substrate, it appears that cleavage at residues 271-279, bond vWF and 50/25/20 occurs at lower concentrations of trypsin, while the remaining bonds are cleaved at higher concentrations. The latter releases the sequence 512-671, which has never been obtained as an isolated fragment. Since these cleavages do not occur in absolutely distinct events, presence of heterogeneous populations of fragments is often observed.

Figure 9. Schematic representation of the three proteolytic fragments of vWF. The amino terminal and carboxyl terminal residues in each fragment are indicated. Bars indicate that the residues are part of the same chain. The fragment 111-12 is composed of residues Arg512-Lys671. The amino terminal and carboxyl terminal residues in the 116 kDa and 111-12 fragments since both molecules are dimers when isolated under succeeding conditions. However, the nature of inter-chain bonds and the possible existence of intra-chain bonds (the latter indicated by dotted lines) remains to be determined. Note that the 52/48 kDa fragment, the constituent subunit of fragment 116 kDa, is obtained following reduction and 8-carboxyethylmethylimidation, then in the absence of dithiothreitol. Also note that 116 kDa and 52/48 kDa are composed of two chains, each containing discontinuous segments of sequence. The heavy line segments indicate the position of the two discontinuous sequences previously shown to be involved in vWF binding to GP Ib (12).