Identification of Functional Domains on von Willebrand Factor by Binding of Tryptic Fragments to Collagen and to Platelets in the Presence of Ristocetin

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With the use of monoclonal antibodies that inhibit the ristocetin-induced binding of von Willebrand factor (VWF) to platelets and the binding to collagen, we have previously identified two distinct tryptic fragments. To prove that these fragments contain the platelet binding or the collagen binding domain, we investigated the direct binding of tryptic fragments of 125I-VWF to platelets in the presence of ristocetin and to collagen fibrils. During the course of the tryptic digestion, there was a rapid and parallel decrease in binding to platelets and collagen. In the first ten minutes, binding decreased >50%; a further decrease to 19% and 29%, respectively, was noted at 90 minutes, but no further decrease was observed thereafter.

FVIII-VWF is a complex of two molecules: factor VIII (FVIII), the procoagulant molecule absent in hemophilia A, and von Willebrand factor (VWF) absent in von Willebrand's disease.1 VWF is present as a series of multimers in which the repeating protomer is a dimer of the 250-kd primary subunit.2,4 VWF is required for the adhesion of blood platelets to collagen and to subendothelium at high shear rates.2,4 This adhesion-enhancing function of VWF is affected by binding of VWF to subendothelium or to collagen followed by interaction of blood platelets with this bound VWF.5,6 The interaction of blood platelets with bound VWF occurs through the platelet membrane glycoprotein Ib, absent in the Bernard-Soulier syndrome.8,9 The interaction of VWF bound to collagen or to subendothelium with blood platelets is in this respect similar to the binding of VWF to platelets in the presence of ristocetin. This ristocetin-induced binding is thus used as a model for the VWF activity, although both activities are not always parallel.10

The study of the structure–function relationship of a large multimeric molecule such as VWF is hampered by the fact that only low residual activity remains after disruption of the molecule.11 In a previous report, we used two monoclonal antibodies against VWF to identify the platelet and collagen binding domains on VWF.12 The platelet binding domain was associated with a 116-kd tryptic fragment, and the collagen binding domain was associated with a 48-kd tryptic fragment. Here we describe the direct binding of tryptic fragments of VWF to collagen and to platelets in the presence of ristocetin. These data demonstrate that the peptides previously identified by the monoclonal antibodies bind to collagen and to platelets. It becomes evident that VWF is a multidomain molecule with distinct separated binding domains for collagen and blood platelets.

MATERIALS AND METHODS

All chemicals were purchased from commercial sources and were of the highest purity grade available. Benzamidine, N-ethylmaleimide (NEM), and soybean trypsin inhibitor (type I-S) were from Sigma Chemical Co (St Louis). L-ε-aminocaproic acid was from Nogepha (Alkmaar, the Netherlands). L-((tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK)-treated trypsin was obtained from Worthington Biochemical Corp (Freehold, NJ). Trichloroacetic acid (TCA) and sodium deoxycholate were from Merck AG (Darmstadt, F.R.G.). Carrier-free Na 125I (13–17 Ci/mg) was purchased from Amersham International Ltd (Amersham, England). Iodo-Gen was from Pierce Chemical Co (Rockford, Ill). Ristocetin sulfate was obtained from H. Lundbeck and Co (Copenhagen). Acrylamide, bisacrylamide, sodium dodecyl sulfate (SDS), N,N,N',N'-tetramethyl-ethylenediamine (TEMED), N-mercaptoethanol, and ammoniumpersulfate were electrophoresis-grade reagents obtained from Bio-Rad Laboratories (Richmond, Calif).

Purification and radiolabeling of FVIII-VWF. FVIII-VWF was purified from human cryoprecipitates by gel filtration on Sepharose CL-4B.11 The FVIII-VWF in the void volume fractions was precipitated by dialysis against 1.6 mol/L of ammonium sulfate, pH 7.0, at 4 °C. The precipitated protein was collected by centrifugation 10 min, 10,000 g, 4 °C). The pellet was dissolved in 0.05 mol/L of Tris-HCl, and 0.1 mol/L of NaCl, pH 7.4, and further dialyzed against the same buffer. Radiolabeling with 125I was performed with the Iodo-Gen method.14 Free 125I was removed by dialysis at 4 °C against 0.05 mol/L Tris and 0.1 mol/L of NaCl, pH 7.4. The labeled preparations had a specific activity of 26 μCi/mg, and free 125I was < 5%.

Tryptic digestion of FVIII-VWF. FVIII-VWF was digested with trypsin as previously described.12 TPCK-trypsin was added to a solution of 125I FVIII-VWF in 0.05 mol/L of Tris-HCl, 0.1 mol/L of NaCl, pH 7.4. The incubation was performed at 37 °C at an enzyme-substrate ratio of 1:2.2 (mol/mol). Before the addition of trypsin, the samples were centrifuged, and further dialyzed against the same buffer. Radiolabeling with 125I was performed with the Iodo-Gen method.14 Free 125I was removed by dialysis at 4 °C against 0.05 mol/L of NaCl, pH 7.4. The labeled preparations had a specific activity of 26 μCi/mg, and free 125I was < 5%.
COLLAGEN AND PLATELET BINDING PEPTIDES OF VWF

150 g, 20 °C). Platelets were washed three times by centrifugation as previously described. After being washed, the platelets were resuspended in Tyrode's buffer (140 mmol/L of NaCl, 2.7 mmol/L of KCl, 0.42 mmol/L of NaH2PO4, 12 mmol/L of NaHCO3, pH 7.4, containing 1 mmol/L of CaCl2, 5 mmol/L of glucose, and 3.5 mg/mL of human albumin. 125I-VWF or tryptic fragments of 125I-VWF were diluted to a concentration of 20 μg/mL in Tyrode’s solution containing CaCl2 and albumin. A volume of 0.25 mL of this solution was first mixed with 4 μL of a ristocetin solution (200 mg/mL of human albumin). VWF or tryptic fragments of VWF were solubilized in sample buffer to avoid overloading the gel with protein. SDS-PAGE was performed on 3% to 30% polyacrylamide gradient gels essentially according to Laemmli. The gels were run at 200 V (constant voltage) for 20 hours, stained with Coomassie brilliant blue R-250, and dried. Autoradiography was performed with Kodak X-Omat AR5 film (Eastman Kodak Co, Rochester, NY) and DuPont Cronex Lightning-plus intensifying screens (DuPont Instruments, Wilmington, Del). A mixture of low mol wt protein markers (Pharmacia, Uppsala, Sweden) and high mol wt protein markers (Bio-Rad) was run in parallel.

RESULTS

Binding of 125I-VWF to platelets and collagen during tryptic degradation. Figure 1 shows the binding of 125I-VWF to washed platelets in the presence of ristocetin or to collagen during the course of the digestion of 125I-VWF with trypsin. Before digestion, and 10 minutes, 90 minutes, 6 hours, and 24 hours after the onset of digestion, samples were taken from the incubation mixture, inhibitors were added, and the binding of 125I-VWF to platelets and collagen was determined. During the first 90 minutes, binding to both platelets and collagen rapidly declined with no further decrease thereafter. After ten minutes, the binding to platelets in the presence of ristocetin was decreased to 47% of the initial value, and the binding to collagen was decreased to 48%. From ten minutes to 90 minutes, the ristocetin-induced binding further decreased to 19% of the starting value, and the binding to collagen declined to 29%. After 24 hours, the binding to platelets was 15% of the binding before digestion and only two times above the control (platelets without ristocetin). The binding to collagen was 26% of the starting value and four times above the control.

The rapid and parallel decline of the binding to platelets and collagen was accompanied by a degradation of the VWF molecule. The tryptic degradation pattern of VWF is shown in Fig 2 and has been described previously. After ten minutes, a 36-to 34-kd doublet appeared, and high mol wt material entered the gel. At 90 minutes, fragments of 180, 116, and 48 kd appeared. At 24 hours, two fragments of 116 and 48 kd were predominantly present.

Tryptic fragments of 125I-VWF binding to platelets in the presence of ristocetin. At different times during the tryptic digestion of 125I-VWF, samples were taken from the incubation mixture and incubated with washed platelets in the presence of ristocetin. As a control, the incubation of 125I-VWF and platelets was performed without ristocetin. After incubation for 30 minutes at room temperature, the mixture was layered on top of 1 mL of 20% (wt/vol) sucrose in Tyrode’s buffer and then centrifuged (one minute, 10,000 g). The supernatant was aspirated, and the platelet pellet was counted in a γ counter. The bound 125I-VWF was eluted by resuspending the platelet pellet in Tyrode’s containing 10 mmol/L of EDTA, immediately after centrifugation. The suspension was centrifuged and the supernatant contained >60% of the bound radioactivity. The eluted material was concentrated by TCA precipitation: 0.1 mL of 0.15% (wt/vol) sodium deoxycholate was added to 1 mL of the supernatant; after ten minutes, 0.1 mL 72% (wt/vol) TCA was added, and the precipitate was collected by centrifugation. More than 90% of the radioactivity was precipitated by TCA. The TCA pellet was boiled for five minutes in sample buffer (0.0625 mol/L of Tris-HCl, pH 6.8, 3% (wt/vol) sodium dodecyl sulfate (SDS) 10% (vol/vol) glycerol, 0.05% (wt/vol) bromophenol blue) and applied to a SDS polyacrylamide gradient gel. The platelet pellet was not directly solubilized in sample buffer to avoid overloading the gel with protein. Analysis of bound fragments by immunoprecipitation was performed as described previously.

Binding of tryptic fragments to collagen. In this study, we used fibrils of collagen type III. The isolation of collagen type III from human umbilical arteries and the preparation of fibrils have been described previously. 125I-VWF or tryptic fragments of 125I-VWF were diluted to a concentration of 20 μg/mL in 0.05 mol/L of Tris-HCl, 0.1 mol/L of NaCl, pH 7.4, containing 1% (wt/vol) bovine serum albumin (BSA). A volume of 0.25 mL of this solution was mixed with 0.2 mL of a suspension of collagen fibrils (1 mg/mL in 0.02 mol/L of Na2HPO4). As a control, 0.2 mL of sodium phosphate buffer was added. The mixture was incubated for 30 minutes at 37 °C and centrifuged (15 minutes, 10,000 g). The collagen pellet was washed once with 0.05 mol/L of Tris-HCl, 0.1 mol/L of NaCl, pH 7.4, and then resuspended in 0.1 mL of the same buffer containing 1% (wt/vol) SDS which eluted >85% of bound radioactivity. After centrifugation, the supernatant was electrophoresed on a SDS polyacrylamide gradient gel. Analysis of bound fragments by immunoprecipitation was performed as described previously.

Polyacrylamide gel electrophoresis (PAGE). SDS-PAGE was performed on 3% to 30% polyacrylamide gradient gels essentially according to Laemmli. The gels were run at 200 V (constant voltage) for 20 hours, stained with Coomassie brilliant blue R-250, and dried. Autoradiography was performed with Kodak X-Omat AR5 film (Eastman Kodak Co, Rochester, NY) and DuPont Cronex Lightning-plus intensifying screens (DuPont Instruments, Wilmington, Del). A mixture of low mol wt protein markers (Pharmacia, Uppsala, Sweden) and high mol wt protein markers (Bio-Rad) was run in parallel.
fragments were again eluted from the platelets and analyzed on a polyacrylamide gradient gel. The autoradiogram in Fig 3 showed that the fragments that became bound to the platelets had a lower mol wt than did the corresponding starting material. At ten minutes, the starting material predominantly showed fragments with mol wts >200 kd, whereas the bound material showed fragments of <200 kd. At 24 hours, a single fragment of 50 kd bound to the platelets (Fig 3A).

We have previously demonstrated that CLB-RAg 35, a monoclonal antibody that inhibits the ristocetin-induced binding of VWF to platelets, immunoprecipitated a fragment of 116 kd after 24 hours of digestion. After reduction, the epitope for CLB-RAg 35 was confined to a 52- to 56-kd fragment, close to the 50-kd fragment identified here, suggesting that the tryptic fragments bound to the platelets became reduced, probably by endogenous reducing substances from the platelets.

To avoid reduction, we performed the incubation with platelets in the presence of 10 mmol NEM. The bound fragments were again eluted from the platelets and analyzed on a polyacrylamide gradient gel. The autoradiogram in Fig 3A shows the bound fragments with platelets and ristocetin in the presence of NEM.
3B shows that in this case reduction was prevented; the fragments were of a size similar to that of the starting material. The fragments that became directly bound to platelets in the presence of ristocetin showed predominance of a band at 180 and 116 kd after six-hour digestion and of 180-kd, 116-kd, and some 48-kd material after 24-hour digestion. The predominant 116-kd material bound to platelets immunoprecipitated with CLB-RAg 35 (Fig 4).

Tryptic fragments of $^{125}$I-VWF binding to collagen. At different times of digestion, samples were also incubated with fibrils of collagen type III. After centrifugation, the bound radioactivity was eluted from the collagen fibrils with SDS and analyzed on a 3% to 30% polyacrylamide gradient gel. At ten minutes, only high mol wt fragments were bound to collagen. At 90 minutes, high mol wt fragments (>180 kd) and a 48-kd fragment were identified (not shown). At six hours of digestion, a single fragment of 48 kd bound to collagen (Fig 4). This fragment was recognized by immunoprecipitation with CLB-RAg 201, a monoclonal antibody that inhibits the binding of VWF to collagen. A similar fragment bound to fibrils of collagen type I (not shown).

DISCUSSION

In a previous study, we identified a 116-kd tryptic fragment of VWF that contained the platelet binding domain and a 48-kd fragment that contained the collagen binding domain. These tryptic fragments were immunoprecipitated by the monoclonal antibody CLB-RAg 35 that inhibits the ristocetin-induced binding of VWF to platelets, and the monoclonal antibody CLB-RAg 201, that inhibits the binding of VWF to collagen. Thus, the 116-kd and 48-kd fragments contain the epitopes for either CLB-RAg 35 or CLB-RAg 201. However, because of steric hindrance, the actual binding domains may be different from these epitopes. The binding domains may even reside on different fragments. To prove that the previously identified fragments contain the proper binding domains, we investigated the direct binding of tryptic fragments of $^{125}$I-VWF to platelets in the presence of ristocetin and to collagen.

There is a preferential binding of the larger multimers of VWF to platelets in the presence of ristocetin and to collagen. Disruption of the VWF structure by limited proteolytic digestion will therefore diminish the binding affinity. As shown in Fig 1, there was a rapid and parallel decrease in the binding of VWF to platelets and to collagen after the onset of tryptic digestion. After 90 minutes of digestion, the decrease stabilized, but the residual binding still remained above the control value. The rapid decrease in binding to platelets in the presence of ristocetin and the low residual binding is in agreement with the study of Martin et al.

In that study, the ristocetin cofactor activity decreased to 25% of the starting value after ten minutes of tryptic digestion, and the residual activity was only 4% after 90 minutes.

To analyze which fragments were bound to platelets, the bound radioactivity was eluted and electrophoresed on polyacrylamide gradient gels by resuspension of the platelet pellet in buffer. Analysis of the bound fragments revealed that these were substantially smaller as compared with the starting material, probably because of reduction. The 50-kd fragment which bound after 24 hours of digestion was similar to the one obtained after reduction of the 116-kd fragment immunoprecipitated by CLB-RAg 35. This reduction of the bound fragments may be caused by endogenous reducing substances from the platelets, such as reduced glutathione. Reduction was prevented by adding 10 mmol/L of NEM to the incubation mixture, which had no effect on the binding itself. Another possibility, which may explain why smaller fragments were found bound to platelets than were present in the corresponding starting material, is the action of calcium-
activated protease. The cleavage of VWF by this protease has recently been reported.\textsuperscript{20} Calcium-activated protease of platelets is sulfhydryl-dependent and is inhibited by NEM.

In the presence of NEM, the bound fragments were of a size similar to that of the starting material. After ten minutes, high mol wt material and a 36- to 34-kd fragment were present. After 90 minutes and 6 hours, 180-kd and 116-kd fragments and fragments >180-kd were bound. After 24 hours of digestion, some 180-kd and 48-kd fragments were bound, but the prominent band was the 116-kd band. This 116-kd fragment, bound to platelets in the presence of ristocetin, was specifically immunoprecipitated by CLB-RAg 201 and is similar to the 116-kd tryptic fragment described by Martin et al,\textsuperscript{11} which contained some residual ristocetin cofactor activity. From these combined observations, we may conclude that the domain on VWF involved in the ristocetin-induced binding to platelets resides on a 116-kd tryptic fragment.

The fragments bound to fibrils of collagen type III were eluted with SDS and analyzed on polyacrylamide gradient gels. The bound tryptic fragments were similar to those previously identified by CLB-RAg 201.\textsuperscript{12} After ten minutes of digestion, only high mol wt material was bound. After 90 minutes, a 48-kd fragment was bound in addition to high mol wt material. After six hours, a single fragment of 48 kDa was bound to collagen. From these observations, we conclude that this 48-kd tryptic fragment contains not only the epitope for CLB-RAg 201, but also the collagen binding domain.

From electron microscopic and spectroscopic studies, it is clear that the VWF molecule is a long flexible filament\textsuperscript{21} with discrete ordered conformational domains linked by regions of random polypeptide chain.\textsuperscript{22} Limited proteolysis offers the opportunity to identify different domains, as demonstrated here and in previous studies.\textsuperscript{11,12} Using functional binding assays, we identified the domain involved in ristocetin-induced binding to platelets and the collagen binding domain, which were located on distinct tryptic fragments of 116-kd and 48 kDa, respectively. This is in agreement with the data previously obtained with the use of monoclonal antibodies,\textsuperscript{12} thereby demonstrating the usefulness of these antibodies in mapping the domain structure. The exact localization of the domains remains to be determined. Studies using limited proteolysis with different enzymes and identification of overlapping fragments, as well as protein sequencing and cloning studies, are currently in progress in different groups and will solve this issue in the future.

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REFERENCES


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