Localization of a Factor VIII Binding Domain on a 34 Kilodalton Fragment of the N-Terminal Portion of von Willebrand Factor

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Factor VIII (F.VIII) was tested for its ability to bind in solid phase system to von Willebrand Factor (vWF) or fragments obtained with Staphylococcus aureus V-8 protease, ie, SpII (N-terminal), Spl (central), and SplI (C-terminal). Bound F.VIII was estimated in situ by clotting and chromogenic assays. F.VIII bound in a dose-dependent manner to immobilized vWF and SplII but not to Spl or SplI. Binding was inhibited by 0.25 mol/L CaCl₂ as well as by an excess of vWF or SplII. Accordingly, immobilized F.VIII specifically bound ¹²⁵I-vWF and SplII but not SplI or Spl. Twelve monoclonal antibodies (MoAbs) directed towards SplII, specifically blocking binding of F.VIII to vWF or SplII, were used for the mapping of plasmic or tryptic fragments of vWF or SplII. We thus established that a F.VIII binding domain of vWF is located on a 34 kilodalton (kd) fragment of the N-terminal portion of vWF, between residues 1 and 910, and that it is distinct from the GPIb and collagen binding domains.

HUMAN von Willebrand Factor (vWF) mediates adhesion of platelets to the subendothelium of the vessel wall and serves as Factor VIII (F.VIII) carrier, stabilizing its activity. Results of proteolytic degration of vWF have shown that the basic 270 kilodalton (kd)-subunit of vWF contains distinct functional domains. Staphylococcus aureus V-8 protease (V-8 protease) hydrolyses vWF-subunit at Glu 1,365 producing two fragments, SplI, a dimer of the C-terminal portion (residues 1,366 to 2,050) containing a binding domain for GPIIb/IIIa and SplII, a dimer of the N-terminal portion of vWF (residues 1 to 1,365). SplII contains binding domains for GPIb and for heparin, both being localized between residues 449 and 728 and two distinct domains interacting with collagen (residues 542 to 622 and 948 to 998). The latter is in the central part of the vWF-subunit fragment SplI, (residues 911 to 1,365) obtained by secondary digestion of Spl with V-8 protease. The localization of the domain of vWF that binds to F.VIII is unknown. Using fragments of vWF obtained with V-8 protease we demonstrate here that a F.VIII binding domain is located on a 34 kd species of the N-terminal part of SplII, between amino acid residues 1 and 910 of vWF-subunit.

MATERIALS AND METHODS

Purification of human vWF and fragmentation. Human vWF was purified according to Thorell and Blomback. It was digested with V-8 protease (Miles Laboratories, Inc, Naperville, IL) and proteolytic fragments Spl, SplI and SplII were purified as reported. Purified vWF or SplII were digested for 24 hours in 25 mmol/L Tris-HCl, pH 8, 0.15 mol/L NaCl with Trypsin-TPCK (Worthington, Freehold, NJ) at 22°C, or with plasmin at 37°C. Plasmin was produced by incubating human Glu-plasminogen (gift of Dr E. Anglés-Cano, INSERM, U. 143, Paris) for one hour at 37°C. Following washing with 25 mmol/L sodium barbital-acetate buffer, pH 7.3 (Michaels buffer) containing 0.3% BSA and 0.05% Tween 20 (washing buffer), potential traces of F.VIII were eliminated by incubating for 30 minutes at 37°C with 0.25 mol/L CaCl₂ in Michaels buffer and washing. F.VIII (0 to 2 U/mL in 3% BSA-Michaels buffer) was then incubated for one hour at 37°C. Unbound F.VIII was eliminated by washing and bound F.VIII was estimated in situ. Nonspecific binding of F.VIII was eliminated using fibrinogen, fibronecin, or albumin-coated tubes. In control experiments, selected MoAbs (IgG) were previously coated to the tubes at a concentration of 20 µg/mL and vWF or fragments were immobilized as already described. In both systems, the amount of immobilized vWF or fragments was determined at various steps of the assay by either mixing ¹²⁵I-vWF or fragments with the unlabeled protein or by reacting for three hours at 37°C the content of the tubes with a specific ¹²⁵I-MoAb IgG to Spl II, or SplI in % BSA-Michael's buffer, followed by washing and counting. Inhibition of F.VIII binding was tested by incubating 2 U/mL of F.VIII premixed with increasing amounts (0 to 10 µg/mL) of vWF, SplI, or SplII as competitor. MoAbs (IgG) to vWF were used at a concentration of 20 µg/mL.

Assay of F.VIII. Bound F.VIII was estimated in situ using a one-stage activated partial thromboplastin time by adding to the tubes 100 µL of Michaelis buffer, 100 µL of F.VIII deficient plasma (Diagnostica Stago, Asnières, France), 100 µL of 5% kaolin and 100 µL of cephalin. Following three minutes at 37°C, coagulation was initiated by 100 µL of 25 mmol/L CaCl₂. A chromogenic assay
(Coatest factor VIII, KabiVitrum, Stockholm) was performed as described by the manufacturer except that 300 μL of Michaelis buffer were added before 100 μL of the other reagents. Results were compared with those using 0 to 10 μL of normal pool plasma (1 U/mL of F.VIII) in 100 μL of buffer for the clotting assay and in 300 μL for the chromogenic assay.

**Binding of vWF or vWF degradation fragments to F.VIII.** Following precoating of MoAb D4H1 (IgG) at a final concentration of 60 μg/mL, washing and incubation of F.VIII (8 U/mL) for two hours at 37°C, serial dilutions of 125I-vWF, SpI, SpII, or SpIII in 3% BSA-Michaelis buffer were incubated in the tubes for one hour at 37°C and 18 hours at 4°C. Following washing, the bound material was estimated by counting. Nonspecific binding was determined in the absence of F.VIII.

**RESULTS**

**Binding of F.VIII to immobilized vWF or vWF fragments.** The amount of coated vWF or SpI, SpII, or SpIII was consistent, dose-dependent, and saturable, reaching a maximum of ~500 ng of protein bound per tube out of 2 μg applied in 500 μL. Under these conditions, purified F.VIII specifically bound to coated vWF and to SpIII in a dose-dependent and saturable manner (Fig 1) with a maximum reached at ~0.5 U/tube of F.VIII. Comparison of these results with the control curve obtained using serial dilutions of normal pool plasma in uncoated tubes (Fig 1, insets) showed that a maximum of ~0.01 U of purified F.VIII was bound per coated tube. F.VIII was unable to bind to immobilized SpII or SpI as well as to BSA, fibrinogen or fibronectin (Fig 1). Control experiments showed that bound F.VIII could be totally removed by incubating with 0.25 mol/L CaCl2. Bound F. VIII was markedly inhibited by MoAb to F.VIII D4H1 at concentrations ≥30 μg/mL and completely neutralized by the oligoclonal Ab to F.VIII (results not shown). Measurement of vWF or fragments using 125I-MoAbs 9 or B203 indicated that the amount of coated protein was constant throughout the various steps of the assay. Thus the increase of clotting times was not related to a loss of coated protein. In addition, specific binding of F.VIII to vWF or to SpIII and absence of binding to SpII or to SpI was observed when the proteins were immobilized through a MoAb to vWF coated to the tube. Specificity of F.VIII binding to vWF or to SpIII was confirmed in experiments of competitive inhibition (Fig 2). Results were similar whether the substrate was coated vWF (Fig 2A) or SpIII (Fig 2B). By contrast SpII or SpI had no significant effect on F. VIII binding even at a concentration of 10μg/mL.

**Binding of 125I-vWF or vWF fragments to antibody-bound**

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**Fig 1.** Binding of purified F.VIII to immobilized vWF or vWF degradation fragments. Bound F.VIII was estimated in situ using either a one-stage clotting method (A) or a chromogenic assay (B) as described in Materials and Methods. Binding of F.VIII was estimated using tubes coated with BSA (○), SpI (□), SpII (●), or SpIII (▲). Insets show the control dose-response curves obtained using various amounts of normal pool plasma (NPP) in uncoated tubes.

**Fig 2.** Competitive inhibition of F.VIII binding to vWF- [A] or SpIII-coated tubes (B) by increasing concentrations of competitor vWF (△), SpI (□), SpII (●), or SpIII (▲). Bound F.VIII was estimated by its clotting activity. [★] indicates the control clotting time when using BSA-coated tubes.
F.VIII. Using F.VIII immobilized onto antibody coated tubes, a dose-dependent binding of $^{125}$I-SpIII or $^{125}$I-vWF was observed, eight and 35 times higher than the control, respectively. $^{125}$I-SpI or $^{125}$I-SpI did not bind to F.VIII under the same conditions (not shown).

Displacement of MoAbs to vWF by F.VIII was observed (Fig 3) derived from the larger immunoreactive digestion. We therefore established that the 34 kd fragment consistently blocked binding of F.VIII to coated vWF or SpII (95% to 98% inhibition). They did not bind to SpI but recognized a small number of intermediates among those produced during the limited digestion of vWF and of SpIII with trypsin or plasmin (Fig 3). Kinetic studies of the digestion established that the 34 kd fragment consistently observed (Fig 3) derived from the larger immunoreactive fragments and represented the smallest species immunodetectable (not shown). The other MoAbs to SpIII as well as MoAbs to SpII had no inhibitory effect on F.VIII binding to vWF or SpIII (not shown).

DISCUSSION

The location on vWF of the domain specific for F.VIII is unknown. In this study we estimated in a solid phase system the interaction between purified F.VIII and vWF or its fragments produced by V-8 protease (SpI, SpII, and SpIII). Our data demonstrate that a vWF-domain interacting with F.VIII is located on the N-terminal part of the subunit. Our findings also strongly suggest that there is no other F.VIII binding domain on the rest of the molecule. We have thus established that F.VIII binds in a specific and dose-dependent manner to immobilized vWF and SpIII, a dimer of the N-terminal portion of vWF-subunit (residues 1 to 1,365). Conversely, F.VIII does not bind to SpII, a dimer of the C-terminal end of vWF-subunit (residues 1,366 to 2,050) nor to SpI, the C-terminal part of SpIII (residues 911 to 1,365). Thus, the binding domain for F.VIII on the vWF-subunit is localized in the N-terminal part of SpIII, between residues 1 and 910. Control experiments in which accessibility to the bound protein was modified by using fragments immobilized on previously coated MoAbs indicated that the absence of a binding site for F.VIII on SpI and SpII was not related to steric hindrance. In addition binding experiments of F.VIII binding to coated vWF or SpIII by vWF, SpI, SpII, or SpIII clearly confirmed that solely SpIII was able to substitute for vWF and interact with F.VIII. Similarly, when F.VIII was immobilized through a specific MoAb, significant binding of labeled vWF and SpIII was observed, contrasting with a total lack of binding of SpII.

Additional evidence for the presence of a F.VIII binding domain on a portion extending between amino acid residues 1 and 910. We observed that these MoAbs recognize a 34 kd fragment produced in the early phase of digestion of vWF and SpIII by trypsin or plasmin. Recent data from Hamilton et al. demonstrated that a 34 kd fragment containing the N-terminal sequence of vWF-subunit was produced by early cleavage with these enzymes. We thus assume that the F.VIII binding domain of vWF is located on the previously identified 34 kd N-terminal fragment.

NOTE ADDED IN PROOF

Subsequent to the submission of this manuscript to BLOOD, the paper "A Major Factor VIII Binding Domain Resides within the Amino-Terminal 272 Amino Acid Residues of von Willebrand Factor" by P.A. Foster, C.A. Fulcher, T. Marti, K. Titani, and T.S. Zimmerman appeared in the Journal of Biological Chemistry, 262:8443, 1987.

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REFERENCES


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