The Binding Domain of von Willebrand Factor to Sulfatides Is Distinct From Those Interacting With Glycoprotein Ib, Heparin, and Collagen and Resides Between Amino Acid Residues Leu 512 and Lys 673

By Olivier Christophe, Bernadette Obert, Dominique Meyer, and Jean-Pierre Girma

A series of proteolytic fragments of human von Willebrand Factor (vWF) was purified to characterize the functional site that supports its interaction with sulfatides. Splll, an N-terminal homodimer generated by V-8 protease (amino acids [AA] 1 to 1365), bound to sulfatides in a dose-dependent and saturable way. Splll also totally inhibited the binding of vWF to sulfatides and Splll binding was completely abolished by vWF. In contrast, Spl, the complementary C-terminal homodimer (AA 1366 to 2050), did not exhibit any binding affinity for sulfatides. Four purified fragments overlapping the sequence of Splll were also tested for their ability to interact with sulfatides. An N-terminal monomeric 34-Kd fragment (P34, AA 1 to 272) generated by plasmin, a central monomer (Spl, AA 911 to 1365) produced by digestion with V-8 protease, and a tetrameric fragment III-T2 (comprising a pair of the two sequences AA 273 to 511 and AA 674 to 728) produced by secondary digestion of Splll with trypsin did not interact with sulfatides. In contrast, a monomeric 39/34-Kd fragment produced by dispase (AA 480 to 718) bound specifically and with a high affinity to sulfatides and totally displaced vWF or Splll binding. Conversely, binding of the 39/34-Kd species was totally abolished by vWF or Splll. Thus, a functional site responsible for sulfatide binding was localized between AA 480 and 718 and comparison of the binding properties of the 39/34-Kd and III-T2 fragments indicated that the sequence 512 to 673 is necessary for the binding to sulfatides. Further mapping of this new functional domain of vWF, based on experiments of competitive inhibition of binding by either heparin or monoclonal antibodies directed toward vWF, showed that the site interacting with sulfatides is distinct from those involved in binding to platelet glycoprotein Ib, collagen, or heparin. This finding was confirmed by experiments using synthetic peptides which also indicated that the sequence comprising AA 569 to 584 is part of the sulfatide-binding domain or influences its activity.


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bind specifically and with a high affinity to sulfatide (galactosyl ceramide-1-sulfate), a component present in the membrane of cells including platelets and erythrocytes.

In the present report we investigated the sulfatide-binding domain(s) of vWF. For this purpose a series of well-localized proteolytic fragments of vWF was purified and analyzed for their capacity to bind or to inhibit vWF binding to sulfatides in a solid-phase system. Our data indicate that a domain responsible for binding to sulfatides is localized on the 39/34-Kd fragment (AA 480 to 718) of vWF. Other portions of vWF subunit did not exhibit any affinity for sulfatides. The functional site appears distinct from those involved in the binding to heparin and GPIb. The sequence comprising AA 512 to 673 appears necessary for binding to sulfatides and experiments of inhibition of binding by synthetic peptides suggest that the sequence comprising AA 569 to 584 is part of the sulfatide-binding domain or influences its activity.

MATERIALS AND METHODS

Purification of vWF and of proteolytic fragments. Human vWF was purified from lyophilized factor VIII/vWF concentrates (gift from the Centre National de Transfusion Sanguine, Les Ulis, France) essentially as described elsewhere. The purified vWF contains ~200 U vWF Ag/mg of protein and trace amounts (<0.1% wt/wt) of fibrinogen and fibronectin as estimated by radioimmunoassay. Purified vWF in imidazole-HCl buffer (20 mmol/L imidazole-HCl, 0.15 mol/L NaCl, 0.02% NaN3, pH 6.8) was concentrated by precipitation with 40% saturated ammonium sulfate and stored as a paste at ~80°C before use.

Digestion of vWF by Staphylococcus aureus V-8 protease (V-8 protease) (Miles, Paris, France) was performed using the enzyme coupled to Sepharose 2B (Pharmacia, Uppsala, Sweden). Proteolytic fragments SpI (monomer, AA 911 to 1365), SpII (dimer, 1366 to 2050), and SpIII (dimer, AA 1 to 1365) were purified as previously described using ion-exchange chromatography on DEAE-Sephacel (Pharmacia) gel filtration on G150 Sephadex (Pharmacia), and immunoadsorption of contaminants onto Sepharose-coupled selected monoclonal antibodies (MoAbs) to human vWF.

Monomeric P34 fragment covering the N-terminal end of vWF (AA 1 to 272) was obtained by digesting purified SpIII (0.5 to 1.0 mg/mL) in 50 mmol/L Na2HPO4, H3PO4 buffer, 80 mmol/L NaCl, pH 7.4, for 24 hours at 22°C with plasmin (Boehringer, Meylan, France) using an enzyme-substrate ratio of 1/20. Proteolysis was terminated with 1 mmol/L (final concentration) of diisopropylfluorophosphate (DFP). The digest was concentrated by lyophilization and dissolved in TBS (25 mmol/L Tris-HCl, 0.15 mol/L NaCl, pH 7.4) containing 6 mol/L guanidinium chloride. It was then submitted to gel filtration on Sepharose 4B and eluted with the same buffer. The protein peak containing the P34 fragment was extensively dialyzed against distilled water and lyophilized. The P34 fragment was finally dissolved in a minimal volume of TBS containing 6 mol/L guanidinium chloride, extensively dialyzed against TBS, and stored at ~80°C before use.

The monomeric 39/34-Kd fragment of vWF (AA 480 to 718) was obtained by digestion of purified vWF with dispase (Boehringer) as described by Andrews et al. vWF (1 mg/mL) in TBS containing 0.2 mmol/L CaCl2 was digested by dispase for 18 hours at 22°C using an enzyme/substrate ratio of 1/5. The reaction was terminated by addition of EDTA (10 mmol/L final concentration). The 39/34-Kd fragment was purified as described by affinity chromatography onto heparin-Sepharose (Pharmacia) in TBS. After extensive washing, the 39/34-Kd fragment was eluted by increasing the concentration of NaCl to 0.5 mol/L and stored at ~80°C before use.

The tetrameric III-T2 fragment comprising the two sequences AA 273 to 511 and AA 674 to 728 was produced by digestion of purified SpIII (1 mg/mL in TBS) by TPCK-trypsin (Millipore, Freehold, NJ) at an enzyme/substrate ratio of 1/50 for 18 hours at 22°C. The reaction was stopped by addition of 2 mmol/L DFP. The digest was chromatographed onto heparin-Sepharose equilibrated in TBS. The pass-through containing the III-T2 fragment was then submitted to gel filtration on Sephadex S 200 HR (Pharmacia) using TBS containing 6 mol/L guanidinium chloride as eluant. After extensive dialysis against TBS and lysisophilization, purified III-T2 fragment was separated by gel filtration onto Sepharose CL-4B eluted with TBS. Purified fragment was stored at ~80°C until use.

The localization within the vWF subunit of the purified proteolytic fragments is indicated on Fig 1.

Characterization of the monoclonal and polyclonal antibodies to vWF. MoAbs to human vWF were produced and characterized as previously described.28,29 Typing showed that all MoAbs were IgG, γ1, κ. IgG fractions were purified from ascites fluids as described.29 A series of 17 MoAbs was used in this study. One, MoAb 8, was directed against SpII fragment. A panel of 16 MoAbs was selected so that the related epitopes were evenly distributed along the SpIII fragment. Among those, four MoAbs recognized functional epitopes of vWF. MoAb 418 is directed against an epitope located on fragment P34 and inhibits F.VIII binding to vWF.31 MoAb 322 blocks the binding of vWF to ristocetin-treated platelets10,15,24 and recognizes the 39/34-Kd dispase fragment and fragment III-T2. MoAb 203, which inhibits vWF binding to collagen, binds to SpI.15 MoAb 724 recognizes the 39/34-Kd dispase species and blocks binding of vWF to sulfatides. A polyclonal antibody to human vWF was raised in rabbits and rendered monospecific as previously described.23 IgG fraction was isolated and specific antibody was immunoadsorbed onto vWF coupled to Sepharose.25 The polyclonal antibody was used as positive control antibody in experi-
ments of inhibition of binding of vWF or vWF fragments to sulfatides by MoAbs. After labeling, polyclonal antibody was also used for estimation of vWF Ag by immunoradiometric assay and for detection of vWF fragments by immunoblotting.

Synthetic peptides. Peptides were synthesized by the method of Merrifield et al. and purified by high-performance liquid chromatography using a reverse-phase system. Four peptides were used in this study. Peptides Cys 474 to Pro 488 (CQEPGGLWPPTDAP) and Ser 692 to Pro 708 (SYLCDLAPEAPPPTLPP) were a gift from Dr J. Diaz (Sanofi Recherche, Monpellier, France). Both peptides were previously shown to inhibit vWF binding to ristocetin-treated platelets.35 Peptides Lys 569 to Val 584 (KDRKPSELRRIASQV) and Ser 152 to Val 166 (SHPSVCKKRVTILV) were provided by Dr G. Marguerie (INSERM U 217 Grenoble, France). The four peptides had free N- and C-terminal ends and S-carboxymethylated cysteins.

Radiolabeling of proteins. vWF, proteolytic fragments, and antibodies to vWF were labeled with Na[125I] (Amersham, Les Ulis, France) using Iodo-Gen (Pierce Chemical Co, Rockford, IL) as described from Fraker and Speck.36 Specific radioactivity was from 0.03 to 2 μCi/μg for proteins used as ligands to determine isotherms of binding to sulfatides. It was ~0.5 to 10 μCi/μg for competitive inhibition of binding of [125I]ligands to sulfatides and for the determination of the purity of proteins by gel electrophoresis. For Western blots the specific radioactivity of antibodies was ~2.5 μCi/μg. The labeled proteins were stored at 4°C and used within 5 days for vWF, 2 days for vWF proteolytic fragments, and 2 months for antibodies.

Binding of [125I]vWF or fragments to sulfatides. A solid-phase assay was developed according to the method of Roberts et al.37 using sulfatides coated on 96-well microtiter plates (Dynatech, Marnes-La-Coquette, France), with slight modifications. Aliquots (100 μL) of diluted sulfatides (Sigma, La Verpilliere, France) in 100% methanol were introduced in the wells of microtiter plates and dried under nitrogen. Wells treated with methanol alone in the absence of sulfatides were used as controls. Nonspecific binding was determined to be 12 pg/well (120 pg/mL). This concentration was used in all of the following experiments. Kinetics of binding of [125I]vWF (0.5 μCi/mL) to coated sulfatides (12 μg/well) were studied at 22°C between 30 minutes and 48 hours. At selected times the reaction was stopped by aspiration of the supernatants and wells were counted. Results showed that a maximum binding was reached after 6 hours and that no significant modification of the equilibrium occurred for 48 hours.

Inhibition of binding of [125I]vWF or [125I]vWF fragments to sulfatides of unlabeled proteins, synthetic peptides, heparin or antibodies was performed using a modified binding assay. [125I]-ligand at a final concentration of 0.5 μg/mL was premixed with serial dilutions of unlabeled competitor with final concentrations varying from 0.5 to 250 μg/mL for vWF or vWF fragments, from ~6 to 1,200 μg/mL for peptides and from 15 to 2,000 μg/mL for heparin. IgG at a final concentration between 7.5 and 120 μg/mL was preincubated with [125I]-ligand for 30 minutes at 37°C before starting the binding assay. After centrifugation for 5 minutes at 10,000g the supernatants were incubated onto sulfatide-coated wells and the assay was continued as described previously. Results were expressed by comparison with the specific binding of [125I]-ligand estimated in the absence of competitor.

Binding of [125I]vWF to ristocetin-treated platelets or to collagen and inhibition by MoAbs. Binding of [125I]vWF to ristocetin-treated platelets and inhibition of the binding by MoAbs to vWF were performed using fresh washed platelets (10⁶ cells/mL) and 1 mg/mL of ristocetin (Lunbeck and Co, Copenhagen, Denmark) as previously described.11 Binding of [125I]-vWF to collagen and its inhibition by MoAbs to vWF were performed using human fibrillar type III collagen (Sigma) according to the method already described.15

SDS-gel electrophoresis and Western blots. SDS-polyacrylamide slab gels with a linear gradient (3.5% to 20%) of polyacrylamide were performed in a discontinuous buffer system according to the method of Laemmli using a 3.5% polyacrylamide stacking gel. SDS-agarose slab gels (0.1% SDS, 6 mol/L urea, 1% acrylamide, 1% agarose, 6 mol/L EDTA in 0.1 mol/L Tris-phosphate buffer, pH 7) were performed as previously described using agarose Sea-kem HGT (Eurobio, Les Ulis, France). Markers included low mol wt standards (Pharmacia), purified IgG (mol wt 160 Kd), fibronectin (mol wt 440 Kd), fibrinogen (mol wt 340 Kd), and IgM (mol wt 900 Kd). Gels were stained with Coomassie blue and dried. Radioactive material was shown by autoradiography of the dried gels performed at ~80°C using Kodak X-Omat 5 films and X-Omatic cassettes (Eastman Kodak Co, Rochester, NY). Alternatively, polyacrylamide gels were electroblotted onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany).38 Transferred proteins were incubated with [125I]-MoAb or polyclonal anti-vWF IgG. After extensive washing with 0.15 mol/L NaCl, the reactive proteins were displayed by autoradiography.

RESULTS

Localization of a sulfatide binding site on proteolytic fragments of vWF by direct binding. Table 1 compares the capacity of binding to coated sulfatides (12 μg/mL) of [125I]vWF and of [125I]-proteolytic fragments used at a final concentration of 0.5 μg/mL. Results expressed as percent of bound to total radioactivity show that SpIII fragment significantly bound to sulfatides (B/T ~8%) when compared with nonspecific binding estimated on uncoated wells (B/T ~0.2%). The binding affinity of SpIII appeared lower than that of intact vWF at the same concentration (B/T ~32%). In contrast, [125I]-SpII was unable to bind to sulfatides (B/T <0.2%). Among the four other fragments tested (P34, 39/34-Kd fragment, III-T2, and SpI), only the 39/34-Kd fragment was capable of interacting with sulfatides (B/T ~4%) as compared with nonspecific binding (B/T ~0.15%). In contrast, binding of P34, III-T2, and SpI was not significantly distinct from the nonspecific binding on repeated testing.

Sulfatide-bound 39/34-Kd fragment was analyzed after...
Table 1. Comparison of the Capacity of vWF and its Proteolytic Fragments To Bind to Sulfatides

<table>
<thead>
<tr>
<th>121-Ligand (0.5 μg/mL)</th>
<th>No. of Experiments</th>
<th>121-Ligand Bound % of Total (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>vWF</td>
<td>15</td>
<td>32 ± 1.4</td>
</tr>
<tr>
<td>Spili</td>
<td>12</td>
<td>8.2 ± 1.3</td>
</tr>
<tr>
<td>Spil</td>
<td>6</td>
<td>0.14 ± 0.1</td>
</tr>
<tr>
<td>Spl</td>
<td>6</td>
<td>0.10 ± 0.1</td>
</tr>
<tr>
<td>39/34 Kd</td>
<td>10</td>
<td>4.1 ± 1</td>
</tr>
<tr>
<td>P34</td>
<td>5</td>
<td>0.16 ± 0.1</td>
</tr>
<tr>
<td>Ill-T2</td>
<td>3</td>
<td>0.10 ± 0.1.</td>
</tr>
</tbody>
</table>

121-fragments or vWF was incubated at the final concentration of 0.5 μg/mL (50 ng/well) into wells coated with 12 pg of sulfatides, and the binding assay was performed as described in Materials and Methods. Nonspecific binding estimated using uncoated wells was less than 0.2% for all of the 121-fragments and ~1.5% for 121-vWF. Specific radioactivity was 2 × 10⁶ cpm/μg for vWF, 9 × 10⁵ cpm/μg for Spili, 9.4 × 10⁵ cpm/μg for Spil, 4.3 × 10⁵ cpm/μg for Spl, 10.4 × 10⁴ cpm/μg for P34, 6 × 10⁴ cpm/μg for fragment 39/34 Kd, and 3.9 × 10⁴ cpm/μg for fragment Ill-T2.

extraction, by SDS-PAGE and compared with the starting 121-ligand (Fig 2). The material bound to sulfatides had the same pattern and mobility as the purified ligand that appeared as a doublet with mol wt of 39 and 34 Kd. No other band could be detected even after prolonged autoradiography.

The presence of a sulfatide-interacting domain on SpIII and the 39/34-Kd fragment was confirmed by studying the concentration dependence of their binding to sulfatides in comparison with 121-vWF. With the three ligands (Fig 3), results interpretable as specific and saturable binding to sulfatides were observed. The total amount of bound vWF (Fig 3A) increased from 3.7 to 425 ng/well as concentrations of added 121-vWF increased from 0.1 to 100 μg/mL (0.01 to 10 μg/well). Nonspecific binding linearly increased to 140 ng/well at the highest concentration of vWF tested.

When 121-SPIII was used as ligand (Fig 3B), the total amount of bound 121-SPIII varied from 9.2 to 252 ng/well when the added concentration increased from 2.2 to 360 μg/mL (0.22 to 36 μg/well). Nonspecific binding linearly increased from 0.45 to 67 ng/well in that range of concentrations.

Similarly, binding of the 39/34-Kd fragment also appeared concentration dependent and saturable (Fig 3C). The total amount bound varied from 5.8 to 200 ng/well when the concentration increased from 2.5 to 500 μg/mL, whereas the nonspecific binding reached 20 ng/well at the highest concentration used.

The binding parameters derived by Scatchard analysis from the specific binding curves for vWF (Fig 3D), SpIII (Fig 3E), and the 39/34-Kd fragment (Fig 3F) indicated that the three ligands interacted with a single class of sites (r = .95, .90, and .96, respectively). At saturation, the binding was 280 ng/well for 121-vWF, 200 ng/well for SpIII, and 240 ng/well for 39/34-Kd species (ie, 23, 16.7, and 20 ng/μg of sulfatides). The apparent constants of dissociation (kD) were ~1.9 × 10⁻⁸, 3.3 × 10⁻⁷, and 3.2 × 10⁻⁶ mol/L, respectively, assuming a mol wt of 275, 170, and 39 Kd for the subunit of each ligand.

Localization of a sulfatide binding site on proteolytic fragments of vWF by competitive inhibition. Purified fragments SpIII, SpII, SpI, and fragment 39/34 Kd were compared with vWF for their ability to inhibit binding of the labeled ligands to sulfatides. When 121-vWF was used as ligand (Fig 4), unlabeled vWF completely inhibited the binding at the concentration of ~100 μg/mL. Half-maximum inhibition was reached with 4 μg/mL (15 nmol/L). Assuming a mol wt of 275 Kd for the subunit, the apparent kD that derived was 2.9 × 10⁻⁸ mol/L. In addition, we observed (Fig 4) that SpIII completely inhibited the vWF binding and the 39/34-Kd fragment almost completely abolished it at the highest concentration used (250 μg/mL). Half-maximum
Fig 3. Binding of purified $^{125}$I-vWF, $^{125}$I-SpIII, and $^{125}$I-39/34-Kd fragment to sulfatides. Aliquots (100 µL) of increasing amounts of $^{125}$I-vWF (A) (final concentration 0.1 to 100 µg/mL, specific radioactivity $\sim 6 \times 10^7$ cpm/µg), $^{125}$I-SpIII (B) (final concentration 2.2 to 380 µg/mL, specific radioactivity $\sim 40,000$ cpm/µg), or $^{125}$I-39/34-Kd fragment (C) (final concentration 1.2 to 500 µg/mL, specific radioactivity $\sim 100,000$ cpm/µg) were incubated at 22°C on microtiter plates coated with sulfatides (12 µg/well). After 18 hours, free $^{125}$I-ligand was separated from sulfatide-associated radioactivity by aspiration and washing. Specific binding ($\Delta$) was estimated by subtracting nonspecific binding (0) from total binding (0). Nonspecific binding was estimated by the radioactivity bound onto uncoated wells. Results show the mean ± SD for triplicate determinations. Scatchard plots derived from the specific binding curves are shown on (D) for vWF, (E) for SpIII, and (F) for the 39/34-Kd fragment.

Fig 4. Inhibition of binding of $^{125}$I-vWF to sulfatides by unlabeled vWF or Spl, Spll, Splll, and 39/34-Kd fragment. $^{125}$I-vWF (final concentration 0.5 µg/mL, specific radioactivity $\sim 10^6$ cpm/µg) was premixed with increasing concentrations of unlabeled protein (0 to 250 µg/mL, final concentration). After centrifugation, the mixture (100 µL) was incubated at 22°C into sulfatide-coated wells (12 µg/well) and the binding assay was performed as described in Materials and Methods. In the absence of competitor, the total binding was 30% of the applied radioactivity. Nonspecific binding of $^{125}$I-vWF was less than 2% and did not significantly vary with the concentration of unlabeled protein added. Results (mean ± SD, n = 3) are expressed as the percentage of inhibition of specific binding estimated in the absence of competitor. $^{125}$I-vWF was incubated with vWF (●), SplII (○), SplIII (□), Spl (▲), or the 39/34-Kd fragment (■).

Inhibition was observed at 10.6 µg/mL and 47 µg/mL for SplIII and the 39/34-Kd fragment, respectively. In contrast, even used at 250 µg/mL, SplII and SplI were unable to compete for the binding of $^{125}$I-vWF to sulfatides. Similarly, experiments using $^{125}$I-SpIII and $^{125}$I-39/34-Kd fragments as ligands confirmed the presence of binding sites of vWF to sulfatides on SplIII as well as on the 39/34-Kd fragment (not shown). At 250 µg/mL of competitor, binding of $^{125}$I-SpIII was abolished to more than 85% by vWF or SplIII. The respective half-maximum inhibition was reached at 10.5 µg/mL (39 nmol/L of vWF) and 14.5 µg/mL (85.3 nmol/L of SplIII), and the apparent kd of $^{125}$I-SpIII binding by unlabelled SplIII was 4.3 $\times$ 10$^{-7}$ mol/L. More than 85% of binding of $^{125}$I-39/34-Kd fragment was inhibited in the presence of 250 µg/mL of either vWF or 39/34-Kd fragment. The half-maximum inhibition was reached with 4.2 µg/mL of vWF (15.6 nmol/L) and 11.5 µg/mL of 39/34 Kd (290 nmol/L). The apparent kd of $^{125}$I-ligand binding by the unlabeled 39/34-Kd fragment was 2 $\times$ 10$^{-8}$ mol/L.

When heparin was tested as competitor at a final concentration ranging from 15 to 2,000 µg/mL, no significant effect was detected on the binding of $^{125}$I-vWF, SplIII, and the 39/34-Kd fragment to sulfatides (not shown). Among the series of MoAbs to vWF to vWF tested at a final concentration between 0.1 and 120 µg/mL, only MoAb 724 totally abolished the binding of $^{125}$I-vWF and the 39/34-Kd fragment to sulfatides. Half-maximum inhibition was reached with 0.8 and 4 µg/mL, respectively. No other MoAbs exhibited the capacity to inhibit the binding of the $^{125}$I-ligands to sulfatides, in particular MoAb 322, known to...
block the binding of vWF or of its fragments to ristocetin-treated platelets. Conversely, MoAb 724, which by immunoblot recognized the 39/34-Kd fragment but not III-T2 species, was unable to inhibit binding of vWF to ristocetin-treated platelets and to collagen (data not shown). In contrast, a polyclonal antibody to vWF used as control totally abolished binding of $^{125}$I-vWF to sulfatides at the lowest concentration used (7.5 µg/mL), but had no significant effect on the binding of the vWF fragments, even at 120 µg/mL.

Inhibition of binding of vWF, SpIII, and the 39/34-Kd fragment to sulfatides by synthetic peptides.

In these experiments, selected synthetic peptides were tested to identify the domain of vWF, SpIII, and the 39/34-Kd fragment involved in binding to sulfatides. Three sequences overlapped that of the 39/34-Kd fragment: AA 474 to 488, 569 to 584, and 692 to 708. A fourth peptide (AA 152 to 166) was used as negative control. Figure 5 shows that among the four competitors tested only peptide 569 to 584 had a significant capacity to inhibit binding of the three $^{125}$I-ligands in a dose-dependent manner. More than 80% of the three bindings was abolished at the highest concentration of peptide used (1,200 µmol/L). Half-maximum inhibition was reached with 182, 200, and 255 µmol/L of peptide for $^{125}$I-vWF, $^{125}$I-SpIII, and $^{125}$I-39/34-Kd fragment, respectively. In contrast, peptides 474 to 488 and 692 to 708 had no significant inhibitory effect when compared with the control peptide 152 to 166.

DISCUSSION

vWF mediates platelet adhesion to the subendothelium. This capacity has been related to the presence of multiple functional sites along the vWF subunit interacting either with components of the subendothelium like collagen or heparinlike molecules, or with GPs of the platelet membrane, ie, GPIX and GPIlb/IIIa. vWF has also been shown to be capable of inducing agglutination of aldehyde-fixed erythrocytes, and this property has been related to the presence of a specific site of vWF involved in binding to sulfatides (galactosylceramide-I-sulfate) located on the membrane. However, the portion of the vWF molecule implicated in its interaction with sulfatides has not yet been elucidated. Thus, we attempted to precisely localize the sulfatide-binding domain within the sequence of vWF subunit; for this, we prepared a series of well-defined fragments of vWF and compared them with vWF in a solid-phase assay for their capacity to interact with immobilized sulfatides.

Direct-binding experiments and Scatchard plots derived from the specific binding curves confirmed results already reported in the literature, that vWF binds to sulfatides in a specific and saturable fashion. The specificity and saturability of the binding are further supported by the capacity of unlabeled vWF to inhibit competitively the binding of $^{125}$I-vWF with the same apparent affinity. In addition, specificity of the binding is indicated by the failure of related proteins such as SpII, the homodimeric fragment (AA 1366 to 2050), to interact with sulfatides by either direct binding or competitive inhibition studies.

The localization of a sulfatide-binding domain on SpIII, a homodimer of the N-terminal portion of vWF subunit (AA 1 to 1365) generated by digestion with V-8 protease, was established by showing that the labeled fragment binds specifically and with a high affinity to sulfatides in a dose-dependent and saturable way. This finding was con-
firmed by experiments of competitive inhibition showing that unlabeled SpIII inhibits 125I-SpIII binding with the same apparent affinity. 125I-vWF binding can also be totally abolished by SpIII and 125I-SpIII binding by vWF. Thus, our data strongly suggested that the two species have the interactive site for sulfatides in common.

A more precise localization of the specific domain for sulfatides relied on the use of purified monomeric fragments overlapping the sequence of SpIII, P34 (AA 1-272),13 SpI (AA 911 to 1365),10 and 39/34-Kd (AA 480 to 718)13 generated by secondary digestion of SpIII by plasmin or V-8 protease or of vWF by dispase, respectively. Among these only the 39/34-Kd fragment showed the ability to specifically bind to sulfatides. In addition, characterization of the sulfatide-bound material by gel electrophoresis confirmed that the 39/34-Kd fragment did bind specifically and that no other protein accounted for the binding. The presence of a binding site of vWF on the 39/34-Kd fragment was confirmed by experiments of competitive inhibition, 125I-39/34-Kd fragment binding was totally inhibited by unlabeled 39/34-Kd fragment with a similar apparent affinity. This binding was also totally abolished by vWF and binding of 125I-vWF by the 39/34-Kd fragment. Therefore, our data, which clearly showed that fragments P34 and SpI are devoid of affinity for sulfatides, also strongly suggested the absence of interactive sites outside the sequence 480 to 718 of vWF subunit.

It has been recently established that the 39/34-Kd fragment comprises several functional domains of vWF, including unrelated binding domains for heparin, collagen, and platelet GPIb.13 Thus, it was of interest to analyze whether the site presently determined to be involved in binding to sulfatides may overlap one of the others.

Our data using inhibition of binding to sulfatides by MoAbs to vWF, synthetic peptides, and heparin bring a line of evidence to support that the sulfatide-interacting site is independent from the others. The lack of inhibition of binding to sulfatides of vWF, SpIII, or the 39/34-Kd fragment by MoAb 322, known to specifically block vWF binding to ristocetin-treated platelets, strongly suggested that vWF binding sites for GPIb and sulfatides were unrelated. Additional evidence was derived from our data of inhibition of binding to sulfatides using MoAb 724 specific for the 39/34-Kd fragment. This MoAb identified as a strong inhibitor of vWF binding to sulfatides was totally unable to affect vWF binding to ristocetin-treated platelets. In addition, our observation that MoAb 724 had no effect on vWF binding to human fibrillar collagen also supports that the collagen binding and sulfatide binding domains of vWF are distinct. The existence of a specific site for sulfatides was further illustrated by experiments of competitive inhibition performed using synthetic peptides derived from the sequence of vWF subunit. Our data showed that two peptides extending between residues 474 to 488 and 692 to 708, and previously characterized as potent inhibitors of vWF binding to GPIb in the presence of ristocetin,12,13 were unable to interact with the binding of vWF, SpIII, or the 39/34-Kd fragment to sulfatides. Similarly, our data showed that fragment III-T2, a tetramer comprising the two sequences AA 273 to 511 and AA 674 to 72813 generated by secondary digestion of SpIII by trypsin and known to interact with GPIb,13 was unable to bind to sulfatides and to MoAb 724. Thus, it appears from our experiments using either MoAbs or synthetic peptides that the sulfatide binding site of vWF is distinct from those interacting with either GPIb or collagen.

There is also evidence that the sulfatide binding domain is distinct from that involved in heparin binding. Thus, our results showed that heparin, even used at high concentration, was unable to inhibit binding of vWF, SpIII, or 39/34-Kd species, confirming previous data from Roberts et al14 using vWF. Further localization of the sulfatide-binding domain was derived from the comparison of the binding capacity to sulfatides of the 39/34-Kd fragment (AA 480 to 718) and III-T2 fragment (AA 273 to 511, 674 to 728), indicating that the sequence 512 to 673, missing in the III-T2 species, was required for vWF binding to sulfatides. We then established that the peptide lying between AA 569 and 584 strongly inhibited the interaction between sulfatides and vWF or its two fragments. This finding confirms the similarity of binding properties to sulfatides of vWF, SpIII, and the 39/34-Kd fragment and the identity of the binding sequences of the three species as already indicated by our experiments of reciprocal competitive inhibition of binding. Sulfatides have been identified on the platelet membrane.26 In addition, recent data from Data et al27 established that unstimulated platelets adhere to sulfatides and that this adhesion is mediated by vWF. Therefore, the new functional domain that we identified may play a role in the interaction of vWF with its platelet receptors, and our results indicate that the sequence 569 to 584 of vWF is part of the interactive site for sulfatides or may influence its activity.

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REFERENCES


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The binding domain of von Willebrand factor to sulfatides is distinct from those interacting with glycoprotein Ib, heparin, and collagen and resides between amino acid residues Leu 512 and Lys 673

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