von Willebrand Factor Binds Specifically to Sulfated Glycolipids*

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The human plasma glycoprotein Factor VIII/von Willebrand factor (vWF) binds specifically and with high affinity to sulfatides (galactosylceramide-3-sulfate). vWF does not bind to gangliosides, neutral glycolipids, phospholipids, or cholesterol 3-sulfate. Although the largest oligomers of vWF bind preferentially to sulfatides, vWF monomers and dimers also bind but with reduced affinity. vWF binding is inhibited at high ionic strength or low pH, by some sulfated polysaccharides and by antibodies to vWF. Binding of vWF to sulfatides is probably responsible for its agglutination of aldehyde-fixed erythrocytes and may play a role in vWF-induced platelet adhesion or platelet aggregation.

Human von Willebrand factor (vWF) is a large plasma glycoprotein (1, 2) which binds to human platelets and promotes platelet adhesion to components of the subendothelium including collagen fibrils (3–5) and induces platelet aggregation. These interactions are believed to account for the major role vWF plays in primary hemostasis.

vWF binds specifically to platelets activated with thrombin (6) or ristocetin (7–9) through interactions with the surface glycoproteins Ib/IIIa or Ib, respectively, and binds to purified types I, II, and III collagen (10, 11). Inhibition studies employing monoclonal antibodies to vWF suggest that distinct functional domains on the protein mediate each of these interactions (12, 13). Thus vWF resembles other cell adhesion molecules such as laminin (14) and thrombospondin (15) which contain distinct binding domains for their interactions with cell and matrix components. These three proteins are also similar in that each agglutinates aldehyde-fixed erythrocytes (16–18). We recently demonstrated that laminin and thrombospondin bind specifically to sulfated glycolipids and that agents that inhibit binding also inhibit hemagglutination (19, 20). Thus, binding to sulfatides, which are present on erythrocytes, may account for hemagglutination by laminin and thrombospondin. Hemagglutination by vWF is inhibited by antibodies to vWF (25) (26) except that the incubation with 125I-vWF (2 μg/ml) was for 24 h at room temperature. The supernatant was aspirated and the plate was washed 3 times with cold saline. The wells were cut from the plate and the bound vWF was counted. For inhibition studies, wells were coated with 250 ng of galactosylsulfatide and 125I-vWF at a final concentration of 1 μg/ml was added with the inhibitor to be tested in a total volume of 30 μl.

Binding of 125I-vWF to lipids was developed based on the previously described methods for detection of laminin and thrombospondin binding to lipids (19, 20). Briefly, lipids dissolved in methanol were dried onto 96-well microtiter plates (Costar 3912). Nonspecific binding sites were blocked by incubating the plate in Tris-bovine serum albumin (50 mM Tris-HCl, 110 mM NaCl, 5 mM CaCl2, 1% bovine serum albumin, 0.1 mM phenylmethylsulfonyl fluoride, 0.02% NaN3, pH 7.8). '*'I- or 3H-labeled vWF (1 μl) was added to each well and incubated for 24 h at room temperature. The supernatant was aspirated and the plate was washed 3 times with cold saline. The wells were cut from the plate and the bound vWF was counted. For inhibition studies, wells were coated with 250 ng of galactosylsulfatide and 125I-vWF at a final concentration of 1 μg/ml was added with the inhibitor to be tested in a total volume of 30 μl.

Materials and Methods

Purification of vWF and Fibrinogen—vWF was purified from human cryoprecipitate by chromatography on Sepharose 4B. The ascending limb of the protein peak of the void volume was collected, concentrated by ammonium sulfate precipitation, dialyzed, and used as previously described (21). The protein was labeled either by limited oxidation of terminal galactose residues by galactose oxidase and reduction of the residues with tritiated [3H]potassium borohydride as previously described (21) or by iodination with 125I using immobilized lactoperoxidase (21–23).

The multimeric structure of labeled vWF was analyzed by glyoxyl agarose electrophoresis and autoradiography (24). The intact, non-modified protein was compared to the vWF protein after reduction with 2-mercaptoethanol and alkylation as previously described (21).

Fibrinogen was purified from human plasma by ammonium sulfate precipitation and DEAE-cellulose chromatography as previously described (9). The fibrinogen had an intact subunit structure on polyacrylamide gel electrophoresis after reduction in the presence of sodium dodecyl sulfate and was over 95% clottable. Fibrinogen was iodinated by the same method used for vWF.

Binding of vWF to Lipids—A solid phase assay for the binding of vWF to lipids was developed based on the previously described methods for detection of laminin and thrombospondin binding to lipids (19, 20). Briefly, lipids dissolved in methanol were dried onto 96-well microtiter plates (Falcon 3912). Nonspecific binding sites were blocked by incubating the plate in Tris-bovine serum albumin (50 mM Tris-HCl, 110 mM NaCl, 5 mM CaCl2, 1% bovine serum albumin, 0.1 mM phenylmethylsulfonyl fluoride, 0.02% NaN3, pH 7.8). '*'I- or 3H-labeled vWF (25 μl) was added to each well and incubated for 24 h at room temperature. The supernatant was aspirated and the plate was washed 3 times with cold saline. The wells were cut from the plate and the bound vWF was counted. For inhibition studies, wells were coated with 250 ng of galactosylsulfatide and 125I-vWF at a final concentration of 1 μg/ml was added with the inhibitor to be tested in a total volume of 30 μl.

Binding of 125I-vWF to lipids separated on thin layer chromatograms was performed as described previously for 125I-thrombospondin binding (20). The binding of 125I-vWF (2 μg/ml) was for 24 h at room temperature. Lipids from sheep and human erythrocytes and human platelets were prepared as previously described (19, 20).

Dissolution Cation Dependence of 125I-vWF—Trace metals were removed from Trit/bovine serum albumin buffer prepared without calcium by stirring overnight with a dialysis bag filled with Chelex 100 (Bio-Rad). 125I-vWF was demetalized by dialysis against the above buffer containing fresh Chelex 100. The 125I-vWF was then dialyzed against the same buffer containing 5 mM EDTA, 2 mM MgCl2, 2 mM MnCl2, or 2 mM CaCl2 and 2 mM MgCl2. The binding of each preparation of vWF to sulfatide was tested using the microtiter plate assay.

Results

Using direct binding of proteins to lipids separated on thin layer chromatograms, we previously demonstrated that laminin and thrombospondin bind specifically to sulfated glycolipids whereas plasma fibronectin binds nonspecifically and with low affinity to all anionic glycolipids and phospholipids tested (19, 20). The same method was used to examine the
binding of vWF to lipids isolated from erythrocytes and platelets (Fig. 1). \(^{125}\)I-vWF bound avidly to fast-migrating acidic lipids from human and sheep erythrocytes and human platelets. These lipids are identical to those labeled with \(^{125}\)I-thrombospondin and were previously identified as sulfated glycolipids (19, 20). Galactosyl sulfatides were detected in all three cell extracts. Binding to galactosyl sulfatide was confirmed by specific binding to 20 ng of purified bovine brain sulfatides (Fig. 1C, lane 5). The faster migrating bands in human erythrocytes have identical mobilities to the three forms of galactosyl sulfatide purified by Hansson and co-workers (25). In addition, two slower migrating lipids were labeled in human erythrocytes (Fig. 1A, lane 2). The same lipids were labeled with \(^{125}\)I-thrombospondin staining (20). Solvolysis of the human erythrocyte lipids under conditions which selectively remove sulfate esters, 8 mM \(\text{H}_2\text{SO}_4\) in 9:1 dimethyl sulfoxide/methanol for 90 min at 90 °C (26), abolished binding of \(^{125}\)I-vWF to both galactosyl sulfatide and the slower migrating lipids. Thus, the slower migrating lipids are probably complex sulfated glycolipids. In this assay, about 3 ng of sulfatide is required to detect binding of vWF.

Binding of vWF to sulfatides could also be determined using lipids adsorbed on microtiter plates (Fig. 2). Only sulfatide-coated wells bound vWF. \(^{125}\)I-vWF did not bind to wells coated with up to 10 \(\mu\text{g}\) of neutral glycolipids, gangliosides, phospholipids, or cholesterol 3-sulfate. Approximately 0.5–0.8% of the added \(^{125}\)I-vWF bound nonspecifically to uncoated wells, whereas up to 60% of the added radioactivity was bound to sulfatide-coated wells.

Specific binding to sulfatides was also found using vWF labeled in the carbohydrate moieties with tritium (21). Tritiated vWF bound to sulfatides but not to other neutral or acidic glycolipids adsorbed on microtiter plates. Using 14 \(\mu\text{g/ml}\) of tritiated vWF, 10% of the added radioactivity was bound at saturating sulfatide (1 \(\mu\text{g/well}\)). Binding of both tritiated and iodinated vWF to sulfatides was abolished by 100 \(\mu\text{g/ml}\) of unlabeled vWF. Thus, high affinity binding to sulfatides is not an artifact of iodination.

The effects of incubation time, temperature, pH, and divalent cations on \(^{125}\)I-vWF binding were examined to determine optimal assay conditions. Binding at both 4 and 23 °C was relatively slow with 50% of equilibrium binding attained at approximately 3 h. Binding at 4 °C after 24 h, however, was only 50% of that obtained at 23 °C. Binding was optimal between pH 7.5 and 8.5. At lower pH, binding progressively decreased. At pH 6.0, wells coated with 4 \(\mu\text{g}\) of sulfatide gave the same binding as obtained at pH 8.0 with wells coated with 100 ng of sulfatide. As shown in Fig. 2, binding to sulfatide at higher surface densities was depressed in the absence of divalent cations, and the addition of 5 mM EDTA, 5 mM \(\text{MgCl}_2\), or 5 mM \(\text{MnCl}_2\) to the demetallized vWF had no effect. In contrast, addition of 5 mM \(\text{CaCl}_2\) to the vWF restored the original binding curve. Based on these results, all subsequent assays were conducted at room temperature, pH 7.8, with 5 mM \(\text{Ca}^{2+}\).

The concentration dependence for binding of vWF to sulfatides indicates that binding is saturable and of relatively high affinity (Fig. 3). Half-saturation was obtained with 1.8 \(\mu\text{g/ml}\) free \(^{125}\)I-vWF (8 \(\times\) 10\(^5\) M monomers). However, binding appears to be heterogeneous as 50 \(\mu\text{g/ml}\) vWF was required to achieve saturation.

Since FVIII/vWF consists of a family of oligomers varying in molecular weight from approximately 0.8 to 18 \(\times\) 10\(^6\) Da, heterogeneity in sulfatide binding could reflect differences in valence and hence avidity of binding among the oligomers. Examination of the \(^{125}\)I-vWF bound to sulfatide-coated wells by glyoxal-agarose electrophoresis after solubilization in sodium dodecyl sulfate (Fig. 4) revealed that the largest oligomers which don’t migrate into the agarose were selectively bound to sulfatides. In order to compare the binding of high and low molecular weight oligomers, vWF was fractionated by gel filtration (21), the high and low molecular weight fractions were iodinated, and their binding was examined.

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**FIG. 1. Binding of von Willebrand factor to thin layer chromatograms of erythrocyte and platelet lipids.** Alkali-stable lipids from erythrocytes and platelets were separated into neutral and acidic fractions by DEAE-Sepharose chromatography. The fractions were chromatographed on silica gel high performance TLC plates (Merck) and stained with \(^{125}\)I-vWF as described under "Materials and Methods." (Panels A and B, lanes 1–4, and panel C, lanes 1–5) or with orcinol-\(\text{H}_2\text{SO}_4\) to visualize glycolipids (panels A and B, lanes 5–8, and panel C, lanes 6–10). Panel A, human erythrocyte acidic lipid fractions eluted with 0.01 M \(\text{NH}_4\text{HCO}_3\) (lanes 1 and 5), 0.02 M \(\text{NH}_4\text{HCO}_3\) (lanes 2 and 6), 0.05 M \(\text{NH}_4\text{HCO}_3\) (lanes 3 and 7), and 0.5 M \(\text{NH}_4\text{HCO}_3\) (lanes 4 and 8). Lipids from 40 mg of tissue wet weight were applied on lanes 1–4 and 500 mg on lanes 5–8. Panel B, human platelet acidic lipid fractions as in panel A. Lipids from 10 mg of platelets were applied on lanes 1–4 and 100 mg on lanes 5–8. Panel C, sheep erythrocyte acidic lipid fractions (lanes 1–4 and 6–9) as in panel A and reference bovine brain sulfatide (20 mg, lane 5, and 2 \(\mu\text{g}\), lane 10). Lipids from 25 mg of erythrocytes were applied on lanes 1–4 and 500 mg on lanes 6–9. The migration of reference glycolipids is indicated in the right margin for lactosyl ceramide (CDH), Galα1-4Galβ1-4GlcCer (CTH), and NeuAcα2-3Galβ1-4GlcCer (GM3).
wells were incubated with Tris/bovine serum albumin buffer and then duplicate on wells of microtiter plates by drying from methanol. The binding to uncoated wells and is presented as a function of free vWF concentration at equilibrium. Their titration curves with varying sulfatide concentrations were similar. The high molecular weight fraction bound to sulfatides was bound than of the high molecular weight fraction. vWF was also reduced and alkylated to give a mixture of monomers and dimers. These results indicate that larger oligomers bind sulfatide with higher affinity but more of the lower molecular weight forms can bind to surface adsorbed sulfatides.

Binding to sulfatides was inhibited by simple anions and by some sulfated polysaccharides (Table I). Chloride anion inhibits binding 50% at 210 mM above isotonicity. A simple sulfamate ester and inorganic sulfate were only slightly more inhibitory. The most effective polysaccharide inhibitor was the high molecular weight dextran sulfate, which inhibited binding of 1 pg/ml '*'I-vWF to sulfatide was abolished. A goat antiserum to vWF was also examined as an inhibitor of binding to sulfatides. At a final antiserum dilution of 1:100, binding of 1 μg/ml 125I-vWF to sulfatide was abolished. A final antiserum dilution of 1:3000 inhibited binding 50%.

Because vWF and fibrinogen share a common receptor on thrombin-activated platelets, fibrinogen was tested for binding to various lipids. The vWF which bound to uncoated microtiter well and then eluted was eluted from the sulfatides in solid phase assay. Lane 3 is the vWF which bound to and was eluted from the sulfatides (18,000 cpm). Lane 4 is the vWF bound to an uncoated microtiter well and then eluted (200 cpm). Lane 5 is the supernatant vWF (30,000 cpm) corresponding to lane 4. The vWF in the supernatant after incubation with the sulfatides lacks the largest multimers (lane 2) while there is a preponderance of the larger multimers in the eluted vWF (lane 3). No vWF binds to uncoated plates and the full complement of multimers is seen in the supernatant.

Their titration curves with varying sulfatide concentrations were similar. The high molecular weight fraction bound to 250 ng of sulfatide with slightly higher affinity. At saturation, however, 50% more of the low molecular weight oligomers were bound than of the high molecular weight fraction.

50% at 10 μg/ml. The sulfated fucan, fucoidan, also inhibited binding, whereas other sulfated polysaccharides and hyaluronate inhibited weakly or were inactive.

A goat antiserum to vWF was also examined as an inhibitor of binding to sulfatides. At a final antiserum dilution of 1:100, binding of 1 μg/ml 125I-vWF to sulfatide was abolished. A final antiserum dilution of 1:3000 inhibited binding 50%.

Because vWF and fibrinogen share a common receptor on thrombin-activated platelets, fibrinogen was tested for binding to various lipids. 125I-Fibrinogen bound very weakly to cholesterol 3-sulfate and sulfatide, with less than 3% of the added fibrinogen bound to wells coated with 2.5 μg of lipid. In contrast, more than 50% of 125I-vWF bound to sulfatide.

**TABLE I**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>I_{50} a</th>
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<tr>
<td>Anion (mM) b</td>
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</tr>
<tr>
<td>Cl−</td>
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</tr>
<tr>
<td>Cyclohexylsulfamate</td>
<td>200</td>
</tr>
<tr>
<td>SO4−</td>
<td>150</td>
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<tr>
<td>Polysaccharides (µg/ml)</td>
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<td>Dextran sulfate (M₀ = 500,000)</td>
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</tr>
<tr>
<td>Fucoidan</td>
<td>100</td>
</tr>
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<td>Keratan sulfate C4</td>
<td>700</td>
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<tr>
<td>Heparin</td>
<td>1000</td>
</tr>
<tr>
<td>Dextran sulfate (M₀ = 5,000)</td>
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</tr>
<tr>
<td>Chondroitan sulfate</td>
<td>&gt;1000</td>
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<tr>
<td>Hyaluronic acid</td>
<td>&gt;1000</td>
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a Concentration of inhibitor producing 50% inhibition of binding using 1 μg/ml 125I-vWF.
b Sodium salts.

**FIG. 2.** Binding of von Willebrand factor to purified glycolipids and phospholipids. Serial dilutions of lipids were coated in duplicate on wells of microtiter plates by drying from methanol. The wells were incubated with Tris/bovine serum albumin buffer and then with 1 μg/ml of 125I-vWF for 24 h at room temperature. The per cent of vWF bound is presented as a function of lipid/well for sulfatides (●), sulfatides assayed in calcium-free Tris/bovine serum albumin (○), phosphatidylserine (■), phosphatidylethanolamine (◇). phosphatidylinositol (▲), phosphatidylcholine (△), globoside (▼), ganglioside GM₁ (Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4GlcCer, ▲), ganglioside GD₁ (NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4GlcCer, ◇), cholesterol 3-sulfate (○), and sphingomyelin (●).

**FIG. 3.** Concentration dependence of the binding of von Willebrand factor to sulfatides. Binding to sulfatides (250 ng/well) was determined following equilibration for 24 h at 23°C. Specific vWF bound (mean ± 1 S.D., n = 6) was calculated by subtraction of binding to uncoated wells and is presented as a function of free vWF concentration at equilibrium.

**FIG. 4.** Glyoxyl agarose gel electrophoresis. Lane 1 is radiolabeled 125I-vWF (30,000 cpm). Lane 2 is the supernatant of the radiolabeled vWF (20,000 cpm) which has been incubated with sulfatides in solid phase assay. Lane 3 is the vWF which bound to and was eluted from the sulfatides (18,000 cpm). Lane 4 is the vWF bound to an uncoated microtiter well and then eluted (200 cpm). Lane 5 is the supernatant vWF (30,000 cpm) corresponding to lane 4. The vWF in the supernatant after incubation with the sulfatides lacks the largest multimers (lane 2) while there is a preponderance of the larger multimers in the eluted vWF (lane 3). No vWF binds to uncoated plates and the full complement of multimers is seen in the supernatant.
using the same protein concentration. No binding of fibrinogen was detected using up to 10 μg of phospholipids, neutral glycolipids or gangliosides. Nonspecific binding to wells coated with serum albumin was 0.7%.

**DISCUSSION**

vWF binds specifically and with high affinity to sulfatides adsorbed on plastic or thin layer chromatograms. Binding of 125I-vWF is saturable and completely inhibited by both unlabeled vWF and antiserum to vWF. Binding to sulfatides is probably responsible for the ability of vWF to agglutinate aldehyde-fixed erythrocytes as suggested for two other sulfatide-binding proteins, laminin (19) and thrombospondin (20), which are also agglutinins for aldehyde-fixed erythrocytes. The three proteins bind to the same sulfated glycolipids in erythrocytes and platelets, and binding to sulfatides in each case is inhibited by high ionic strength and low pH. Although vWF binds with high affinity to erythrocyte sulfatides, other sulfated glycoconjugates including erythrocyte membrane glycoproteins and proteoglycans may also be receptors for vWF-mediated agglutination.

Binding of both vWF and thrombospondin (20) to sulfatides shows a partial dependence on divalent cations. EDTA, however, inhibits thrombospondin binding only at low concentrations of sulfatides, whereas vWF binding is inhibited significantly only at high sulfatide densities. Furthermore, Ca²⁺, Mg²⁺, and Mn²⁺ are equally stimulatory for thrombospondin, but only Ca²⁺ stimulates vWF binding. Further work is needed to determine whether the effect of Ca²⁺ on vWF binding is due to Ca²⁺ binding to the protein or to the sulfatide monolayer.

The binding of vWF to sulfatides differs from the binding of thrombospondin in certain respects: 1 mg/ml of heparin has little effect on the binding of vWF (Table I) whereas 10 μg/ml of heparin inhibits the binding of thrombospondin 50% (20); of the sulfated polysaccharides tested, high molecular weight dextran sulfate is the best inhibitor of vWF (Table I) whereas ficoidan is the best inhibitor of thrombospondin (20); finally, vWF reduced with dithiothreitol still binds to sulfatides whereas reduced thrombospondin does not.

The characteristics of vWF binding to sulfatides resemble those of vWF binding to platelets; binding to both targets is heterogeneous and larger vWF multimers bind with high affinity while smaller multimers bind with high capacity and low affinity. Both vWF-facilitated platelet adherence to subendothelium (27) and vWF binding to sulfatides are stimulated by calcium. The specific binding of vWF to sulfatides isolated from platelets suggests that these lipids may contribute to vWF-mediated platelet-platelet interaction or the vWF-mediated adhesion of platelets to surfaces. There is extensive evidence, however, that the glycoproteins Ib and IIb/IIIa on the platelet surface are required for interactions with vWF (6–9). The failure of antibodies that block thrombospondin binding to sulfatides to inhibit thrombin-induced platelet aggregation also suggests that sulfatides are not accessible on the platelet membrane (20, 28). Thus, interaction of vWF with sulfatides alone probably cannot account for its binding to platelets, but binding to sulfatides exposed following vascular injury could play a role in vWF-mediated adhesion of platelets to subendothelium.

**REFERENCES**