The human platelet glycoprotein thrombospondin (TSP) binds specifically and with high affinity to sulfatides (galactosylceramide-1-sulfate). Binding of $^{125}$I-TSP to lipids from sheep and human erythrocytes and human platelets resolved on thin layer chromatograms indicates that sulfatides are the only lipids in the membrane which bind TSP. Binding to less than 2 ng of sulfatide could be detected. TSP failed to bind to other purified lipids including cholesterol 3-sulfate, phospholipids, neutral glycolipids, and gangliosides. Binding of $^{125}$I-TSP was inhibited by unlabeled TSP, by low pH, and by reduction of intersubunit disulfide bonds with dithiothreitol. A monoclonal antibody against TSP (A2.5), which inhibits hemagglutination and agglutination of fixed activated platelets by TSP, strongly inhibited TSP binding to sulfatides. A second monoclonal antibody (C6.7), which inhibits hemagglutination and aggregation of thrombin-activated live platelets, weakly inhibited sulfatide binding. Binding was inhibited by high ionic strength and by some monosaccharide sulfates including methyl-α-D-GlcNAc-3-sulfate. Neutral sugars did not inhibit. Fucoidan, a sulfated fucan, strongly inhibited binding with 50% inhibition at 0.3 μg/ml fucoidan. Other sulfated polysaccharides including heparin and dextran sulfates were good inhibitors, whereas hyaluronic acid and keratan sulfate were very weak.

The platelet glycoprotein thrombospondin (TSP) is secreted from platelet α-granules following platelet activation (1, 2). TSP is also secreted and incorporated into the extracellular matrix by several cultured cell lines including endothelial (3–5) and smooth muscle (5) cells, fibroblasts (5, 6), and type II pneumocytes (7). TSP binds to the surface of activated platelets and is responsible for their hemagglutinating activity (8). Blocking of platelet aggregation by low molecular weight inhibitors of TSP-mediated hemagglutination (9), by a monoclonal antibody to TSP (10), and by polyclonal antibodies to TSP (11) or a heparin-binding fragment of TSP (12) suggests the involvement of TSP in platelet aggregation. Deposition in the extracellular matrix by cultured cells suggests that TSP may also be involved in cell-cell or cell-matrix adhesion.

TSP is proposed to be a trimer of 180-kDa subunits which are linked by disulfide bonds (1, 13). The TSP molecule is an extended structure containing several globular domains, as revealed by rotary shadowing electron microscopy (13, 14). TSP binds fibrinogen (15–17), fibronectin (17, 18), type V collagen (19), heparin (13), and Ca$^{2+}$ (14, 20). Binding of Ca$^{2+}$ is highly cooperative (20) and produces a conformation change in TSP as visualized by rotary shadowing (14). Both Ca$^{2+}$ and Mg$^{2+}$ are required for optimal agglutination of erythrocytes and platelets (21, 22). Protease-resistant fragments of TSP have been isolated which retain the binding sites for collagen V, fibrinogen, and heparin (16, 19, 23, 24). Thus, TSP contains distinct functional domains for each of its binding activities, analogous to those identified in other adhesive glycoproteins such as fibronectin and laminin (25).

Because TSP binds to the surface of activated platelets and mediates erythrocyte agglutination, it has been called the "endogenous lectin" of platelets (8). Purified TSP also agglutinates fixed human erythrocytes (21) and fixed, activated platelets but not fixed, unactivated platelets (22). The binding of TSP to erythrocytes may be a useful model for studying TSP interaction with cells and determining whether carbohydrates are involved.

Recently we demonstrated that sulfated glycolipids on sheep and human erythrocytes bind the adhesive glycoprotein laminin and probably are receptors for laminin-mediated hemagglutination (26). Similarities between the agglutinating properties of laminin and TSP suggested that sulfatides might also be receptors for TSP. We report here that TSP binds with high affinity to sulfatides. This binding is sensitive to inhibition by several agents which block TSP-mediated agglutination.

**EXPERIMENTAL PROCEDURES**

**Materials**—Calcium-replete TSP was purified from the supernatant of thrombin-activated platelets as described (21). TSP was labeled with Na$^{25}$I (ICN) by the iodogen method (27) to a specific activity of 10 μCi/μg. The iodination was performed in a tube coated with 20 μg of iodogen at 4 °C for 5 min using 30 μg of TSP and 1 mCi of Na$^{25}$I in 0.08 ml of buffer (0.1 M sodium phosphate, pH 6.8, 15% sucrose (w/v), 1 mM CaCl$_2$). After removal of free $^{125}$I by gel filtration on Sephadex G-25, the $^{125}$I-TSP was stored at 4 °C in Tris/BSA (50 mM Tris-HCl, 110 mM NaCl, 5 mM CaCl$_2$, 0.1 mM phenylmethanesulfonyl fluoride, 1% BSA, 0.02% NaN$_3$, pH 8.0). Preparation of monoclonal antibodies to TSP will be described elsewhere (10).2

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1. The abbreviations used are TSP, thrombospondin; BSA, bovine serum albumin; sulfatide, galactosylceramide-1-sulfate.

Thrombospondin Binding to Sulfatides

Dextran sulfates (M, 6000 and 500,000), fucoidan, glucose 6-sulfate, L-ascorbic acid 2-sulfate, cholesterol 3-sulfate, and most phospholipids (synthetic) were obtained from Sigma. Heparin (160 units/mg) was from The Upjohn Co. Other reference lipids and bovine brain sulfatides (galactosylceramide-F-sulfate) were from Supelco. Monosaccharides, galactose and glucose were generously provided by Dr. Alexander Roy (The Australian National University, Canberra City, Australia). Methyl-α-D-glucosamine 2N,30-bisulfate, methyl-α-D-glucosamine 3-sulfate, and methyl-α-D-glucosamine 6-sulfate were provided by Dr. Irwin Leder (National Institutes of Health). The latter two compounds were N-acetylated and purified by gel filtration on Bio-Gel P-2.

Platelet and Erythrocyte Glycolipids—Sheep and human erythrocyte glycolipids were prepared as described previously (25). Glycolipids were isolated from 1 unit of human platelets obtained by platelepheresis of a normal donor. Platelets were recovered from the platelet concentrate containing 0.1 volume of anticoagulant (58 mM citric acid, 75 mM sodium citrate, 136 mM glucose) by centrifugation at 1200 × g for 10 min and extracted twice with chloroform/methanol/water (4:8:3) (28). Sodium acetate (0.4 M) was added to the water for the second extraction (29). The lipid extracts were dried, treated with mild alkali to degrade phosphoglycerolipids (26), neutralized, dialyzed extensively against distilled water, and lyophilized. Neutral and acidic lipids were separated on DEAE-Sepharose in the HCO₃ form as described previously (26). Acidic lipids were eluted sequentially with four column volumes of 0.01, 0.02, 0.05, and 0.5 M NH₄HCO₃ in methanol.

Assay of 125I-TSP Binding to Lipids—A solid-phase radioassay for detection of TSP binding to lipids was developed based on the previously described methods for detection of monoclonal antibody binding to glycolipid antigens (30) and laminin binding to sulfated glycolipids (26). Lipids in methanol (25 μl) were dried onto the wells of 96-well flexible microtiter plates (Falcon 3912). Convol wells were dried with methanol alone. The wells were filled with Tris/BSA and incubated 30 min to block nonspecific binding sites. The buffer was aspirated and 25 μl of 125I-TSP (0.5 μg/ml) in Tris/BSA was added. The plate was covered and incubated 3 h at 4°C. The chromatogram was dried, and exposed to x-ray film (XAR-5, Eastman Kodak) for 8-24 h. The chromatogram was scanned for 125I-TSP binding. Using lipids adsorbed on microtiter plates was used previously (data not shown), and its low concentration precluded detection of TSP binding to uncoated wells was less than 0.5% of total 125I-TSP binding.

To block nonspecifically, the TSP by contaminating proteases in the buffer was added to the water for the second extraction (29). The supernatant fluid was aspirated and the plate was washed three times with cold saline. The wells were cut from the plate and the bound 125I-TSP was counted. For inhibition studies, wells were coated with 100 or 200 ng of 125I-TSP (0.5 pg/ml), in Tris/BSA buffer. Binding at 4°C was rapid at 25°C more slowly. No binding to wells coated with 200 ng of sulfatides, 50 μg each of cholesterol (Sigma grade I) and dipalmitoyl phosphatidylcholine (Sigma, synthetic) to improve reproducibility.

Binding of 125I-TSP to lipids separated on thin layer chromatograms was performed as described previously for binding of monoclonal antibodies to glycolipids on thin layer chromatograms (31). Lipids were chromatographed on aluminum-backed silica gel high performance TLC plates (E. Merck) in chloroform/methanol/0.2% aqueous CaCl₂ (60:30:5). The chromatograms were dried, soaked in 1 N NaOH for 15 min to remove polysaccharide from hexane (Polysciences), dried, sprayed with PBS (0.1 M sodium phosphate, 0.15 M NaCl, pH 7.4), and immersed in Tris/BSA for 30 min. The chromatogram was overlayed with 0.5 μg/ml 125I-TSP in Tris/BSA (60 μl/cm²) and incubated in a covered Petri dish for 3 h at 4°C. The chromatogram was washed by dipping in five changes of cold PBS at 1-min intervals, dried, and exposed to x-ray film (XAR-5, Kodak) for 8-24 h. Glycolipids on duplicate plates were visualized by spraying with orcinol-H₂SO₄.

Divalent Cation Dependence of 125I-TSP Binding—Tissue metal were washed from Tris/BSA buffer and washed with distilled water before drying on a dialysis bag filled with Chelex 100 (Bio-Rad). 125I-TSP was demixed by dialysis against the above buffer containing fresh Chelex 100. The 125I-TSP was then dialyzed against the same buffer containing 5 mM ELTA, 2 mM MgCl₂, 2 mM MnCl₂ or 2 mM CaCl₂ and 2 mM MgCl₂. The binding of each preparation to sulfatide was tested using the microtiter plate assay.

Disulfide Requirement for Binding—125I-TSP was reduced by 4°C with 5 mM dithiothreitol under N₂ in the presence of 5 mM Ca²⁺. At various times, aliquots were removed and quenched by the addition of 15 mM N-ethylmaleimide. Binding to sulfatide was measured in microtiter plates coated with 200 ng of sulfatide/well. The effect of reduction on sulfhydryl association was examined by gel filtration of the reduced TSP on a 1.6 × 95-cm column of Sephacryl S-400 (Pharmacia) equilibrated in PBS.

Agglutination Assays—The assays for TSP-mediated agglutination of human erythrocytes and fixed, thrombin-activated human platelets were conducted as described (21, 22).

RESULTS

Binding of Thrombospondin to Erythrocyte and Platelet Membrane Lipids—Direct binding of antibodies and other proteins to lipids resolved on thin layer chromatograms is a convenient method for determining glycolipid binding specificity. We have used this method to examine the lipid binding specificity of laminin and fibronectin and found that erythrocyte sulfatides bound laminin with high specificity, whereas fibronectin bound nonspecifically and with low affinity to all anionic lipids (26). To determine whether lipids in human and sheep erythrocyte and human platelet membranes could serve as receptors for TSP, chromatograms of these lipids were overlayed with 125I-TSP (Fig. 1). 125I-TSP bound avidly to fast-migrating acidic components of both erythrocytes and platelets. The active lipids in sheep erythrocytes (Fig. 1A, lane 2) are identical to those that bind laminin and were identified as monogalactosyl sulfatides (26). TSP binding, however, required approximately 20-fold less lipids to give strong binding than did laminin. Binding to purified sulfatides was confirmed using bovine brain sulfatides (Fig. 1C, lane 6). Binding of 125I-TSP could be detected with less than 2 ng of pure sulfatide as a TLC band.

Based on mobility in two solvent systems and their acid lability, the active glycolipids in human platelets (Fig. 1B, lane 3) were identified as monohexosyl sulfatides. By comparison of TSP binding to a series of dilutions of the platelet lipids and reference sulfatides, we estimate that human platelets contain approximately 2.5 mg of sulfatide/kg of wet weight. Whereas galactose is the only hexose found in monohexosyl sulfatides of other tissues, insufficient material was available to confirm that the platelet sulfatides are galactosyl sulfatides.

The faster moving band in human erythrocyte lipids stained with 125I-TSP (Fig. 1C, lane 3) is also a monohexosyl sulfatide. This component was resolved as a doublet in some experiments. Its mobility and abundance are in agreement with the previous report of monogalactosyl sulfatides in human erythrocytes (32). The slower component in lane 2 and the faintly stained, slow migrating component in lane 2 may be more complex sulfatides. Based on mobility of standards, the former lipid is probably a trihexosyl sulfatide. This band was not present, however, in some human erythrocyte lipid preparations (data not shown), and its low concentration precluded structural analysis.

Binding of TSP to Immobilized Lipids—A solid phase assay using lipids adsorbed on microtiter plates was used previously to examine the binding specificity of the cell adhesion molecules laminin and fibronectin for glycolipids and phospholipids (26). 125I-TSP was examined in the same assay using pure neutral and acidic glycolipids, phospholipids, and cholesterol 3-sulfate (Fig. 2). Only wells coated with sulfatide bound 125I-TSP strongly. Cholesterol 3-sulfate- and phosphatidylinerine-coated wells bound TSP weakly. No binding to other phospholipids, neutral glycolipids, or gangliosides was seen for wells coated with up to 10 μg of lipid. Nonspecific binding to uncoated wells was less than 0.5% of TSP added.

Binding of 125I-TSP to sulfatide was inhibited greater than 90% by a 200-fold excess of unlabeled TSP (100 μg/ml). 125I-TSP binding depended on both the time and temperature of incubation. Binding was rapid at 25°C and was maximal by 60 min. After 5 h, however, binding decreased, suggesting slow proteolysis of the TSP by contaminating proteases in the Tris/BSA buffer. Binding at 4°C was slower, requiring 3 h to reach equilibrium. However, 11% more TSP was bound at 4°C than at 25°C to wells coated with 200 ng of sulfatide, and binding was stable for at least 24 h.
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 and stained with '*I-TSP as described under "Experimental Procedures." Binding to sulfatide was measured using 50-400 ng of TSP (0.3 μg/ml) in the respective buffers was determined after incubation for 3 h at 4 °C.

**TABLE I**

<table>
<thead>
<tr>
<th>Buffer</th>
<th>% 125I-TSP bound*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (5 mM Ca²⁺)</td>
<td>100</td>
</tr>
<tr>
<td>Demetallized</td>
<td>60</td>
</tr>
<tr>
<td>Demetallized + 5 mM EDTA</td>
<td>70</td>
</tr>
<tr>
<td>Demetallized + 2 mM Mg²⁺</td>
<td>100</td>
</tr>
<tr>
<td>Demetallized + 2 mM Mn²⁺</td>
<td>120</td>
</tr>
<tr>
<td>Demetallized + 2 mM Ca²⁺ + 2 mM Mg²⁺</td>
<td>100</td>
</tr>
</tbody>
</table>

* Divalent cations were removed as described under "Experimental Procedures." Binding to sulfatide was measured using 50–400 ng of sulfatide/well and 0.5 μg/ml 125I-TSP. Results are expressed as percent binding obtained with untreated calcium-replete TSP.

30% in the presence of 5 mM EDTA (Table I). Readdition of Mg²⁺ or Ca²⁺ and Mg²⁺ restored binding to the level of the control, whereas addition of Mn²⁺ gave 20% higher binding than the control.
Concentration Dependence of TSP Binding to Sulfatides—The affinity of TSP binding was estimated by measuring the binding of increasing concentrations of labeled and unlabeled TSP to wells coated with 200 ng of sulfatide using the equilibrium conditions defined above. Assuming that the fractions of bound labeled and unlabeled TSP were equal, and subtracting binding of TSP to uncoated wells (<0.5%), a Scatchard plot was constructed (Fig. 4). The nonlinear plot indicates heterogeneous binding. Using a molecular mass for TSP of 450 kDa, the high affinity component has an avidity of \( \approx 10^6 \) M\(^{-1}\). Since binding depends on both sulfatide density and accessibility for TSP binding, this constant is only applicable to one density of sulfatide. With 200 ng of sulfatide/well, the high avidity binding is saturated with only 1 molecule of TSP bound for every 100 molecules of sulfatide. Higher ratios of TSP bound per sulfatide were obtained with the TLC binding assay, suggesting that binding is limited by the area occupied by each TSP molecule.

Effect of Disulfide Bond Reduction on Sulfatide Binding—The TSP-mediated enhancement of fixed erythrocyte and agglutination of fixed, activated platelets are inhibited following reduction of intersubunit disulfide bonds with dithiothreitol (21, 22). Reduction with dithiothreitol also inhibited binding of \(^{125}\)I-TSP to sulfatides (Fig. 5). To correlate loss of activity with the extent of disulfide bond reduction, TSP was reduced for various times with 5 mM dithiothreitol and the reaction was quenched by addition of excess N-ethylmaleimide. In control experiments, direct alkylation with N-ethylmaleimide did not alter sulfatide binding. Loss of binding to sulfatides was first order with time of reduction, with a 50% reduction in binding obtained after 1.5 h (Fig. 5).

It was shown previously by sodium dodecyl sulfate gel electrophoresis that dithiothreitol reduced the intersubunit disulfide bonds of TSP (21). It is not clear, however, whether the subunits dissociate or remain associated noncovalently following reduction with dithiothreitol (21, 22). We therefore examined the effect of dithiothreitol on the molecular size of \(^{125}\)I-TSP using gel filtration under nondenaturing conditions (Fig. 5). Native TSP eluted primarily as a peak with a partition coefficient \( K_v \) of 0.16 on a Sephacryl S-400 column with a small amount of material eluting in the void volume, presumably aggregated TSP. After reduction for 5.5 h, these peaks were lost and a single peak \( K_v = 0.35 \), coeluting with a horse spleen ferritin standard \( M, 440,000 \), appeared. With limited reduction, a mixture of the two species was obtained. Whereas the high axial ratio reported for intact TSP (14) could account for the high mobility of \(^{125}\)I-TSP on gel filtration (Fig. 5A), whether the peak in Fig. 5C represents monomeric TSP subunits remains to be determined. However, these results demonstrate a decrease in molecular size following intersubunit disulfide bond reduction and suggest that the loss of sulfatide binding activity as well as agglutinating activities may be due to a decrease in valence rather than a disruption of the binding site(s).

Inhibition of Sulfatide Binding by Monoclonal Antibodies—The inhibition of \(^{125}\)I-TSP binding to sulfatides by several monoclonal antibodies to TSP was examined using the solid-phase radioassay (Fig. 6). Monoclonal antibody A2.5 inhibited TSP binding to sulfatides by 50% at a concentration of 2 ng/ml. This antibody, which binds the \( \text{NH}_2 \)-terminal heparin-binding domain of TSP, also inhibited the hemagglutination of fixed erythrocytes and agglutination of fixed thrombin-activated platelets by TSP. A second antibody, C6.7, also inhibited TSP binding to sulfatides, although much higher concentrations of this antibody (6 \( \mu \)g/ml) were required to give 50% inhibition. This antibody also inhibited hemagglutination by TSP and inhibited the aggregation of thrombin-activated live platelets but not the agglutination of thrombin-activated fixed platelets (10). A third monoclonal antibody to TSP, C4.4, which did not inhibit agglutination, did not inhibit binding of \(^{125}\)I-TSP to sulfatides.

**Fig. 4.** Concentration dependence of thrombospondin binding to sulfatides. Binding to sulfatide (200 ng/well) was determined at 4 °C. Bound TSP was calculated by subtraction of nonspecific binding to uncoated wells (<0.5%) and is presented as a Scatchard plot (44).

**Fig. 5.** Effect of disulfide bond reduction on sulfatide binding and molecular size of thrombospondin. \(^{125}\)I-Thrombospondin was partially reduced using dithiothreitol, quenched with N-ethylmaleimide, and assayed for binding to sulfatide (200 ng/well) as described under "Experimental Procedures." Left panel, sulfatide binding is presented as the percent of that obtained without reduction as a function of time of reduction. Right panels, elution profiles for gel filtration of unreduced TSP (A) or TSP reduced with dithiothreitol for 1 h (B) and 5.5 h (C) on Sephacryl S-400. The arrows indicate the void volume \( V_v \) determined using a ferritin aggregate and the elution volume of a horse spleen ferritin standard \( V_r \).

**Fig. 6.** Inhibition of thrombospondin binding to sulfatide by monoclonal antibodies. Binding of \(^{125}\)I-TSP (0.3 \( \mu \)g/ml) to sulfatides was determined in the presence of the indicated dilutions of ascites fluid containing monoclonal antibodies A2.5 (●), C6.7 (○), or C4.4 (△). The antibody concentrations in the three ascites fluids were 0.2–0.3 ng/ml.
Inhibition of TSP Binding to Sulfatides by Carbohydrates—
Various polysaccharide and monosaccharide sulfates were tested for inhibition of \(^{125}\)I-TSP binding to immobilized sulfatides (Table II). None of the monosaccharides were potent inhibitors. The best inhibitor, methyl-\(\alpha\)-GlcNAc-3-sulfate, gave 50% inhibition of \(^{125}\)I-TSP binding at 50 mM and was 3.7-fold more potent than chloride anion. Other sugar sulfates including galactose 3-sulfate, the sugar present in sulfatides, were less active. The neutral monosaccharides galactose, glucose, and N-acetylglucosamine did not inhibit.

Polysaccharides varied greatly in their inhibitory activity. The most potent inhibitor was fucoidan, which gave 50% inhibition at 0.3 \(\mu\)g/ml. Fucoidan is a polymer of \(\alpha\)-fucose linked primarily \(\alpha\)-1→2 with sulfates on the three or four hydroxyls (33). Heparin and dextran sulfates were also good inhibitors, whereas hyaluronic acid was weakly inhibitory and keratan sulfate was inactive.

Effect of Fucoidan on Agglutination by TSP.—The effects of glycosaminoglycans on the TSP-mediated agglutination of fixed erythrocytes (21) and fixed activated platelets (22) have been examined previously. In both assay systems, heparin was a far more effective inhibitor of the agglutinating activities of TSP than other glycosaminoglycans. Since fucoidan was a more potent inhibitor of TSP-sulfate binding than heparin, the polysaccharide was examined for its ability to inhibit the agglutinating activities of TSP. In the erythrocyte assay (21), 2 \(\mu\)g/ml fucoidan inhibited the hemagglutinating activity of 10 \(\mu\)g/ml TSP. 1 \(\mu\)g/ml heparin inhibited the hemagglutinating activity of the same amount of TSP. Using fixed activated platelets, however, 250 \(\mu\)g/ml fucoidan increased the minimum concentration of TSP required for agglutination 2-fold but did not inhibit agglutination by 10 \(\mu\)g/ml TSP. In contrast, 7.5 \(\mu\)g/ml heparin inhibited platelet agglutination by 10 \(\mu\)g/ml TSP. Thus, fucoidan selectively inhibits TSP-mediated hemagglutination but not platelet aggregation, whereas heparin inhibits both red cell and platelet agglutination.

The inhibition by fucoidan of binding of monoclonal antibodies to TSP was similar to that of heparin.\(^2\) Although 50% inhibition of antibody A2.5 binding to TSP was obtained with 10 \(\mu\)g/ml fucoidan, 10 \(\mu\)g/ml was required to inhibit 90%. Fucoidan did not significantly inhibit binding of antibody A6.1 to TSP. Antibody A6.1 binds to a 68-kDa fragment of TSP which does not bind the antibodies C6.7 or A2.5.\(^2\)

**Table II**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>(K_{0.50\text{ inhibition}})</th>
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<tbody>
<tr>
<td></td>
<td>(\mu)g/ml</td>
</tr>
<tr>
<td>Cl(^-)</td>
<td>185</td>
</tr>
<tr>
<td>SO(^4)</td>
<td>116</td>
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<tr>
<td>Citrate(^a)</td>
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<tr>
<td>Methyl-(\alpha)-GlcNAc-3-SO(_4)</td>
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<tr>
<td>Methyl-(\alpha)-GlcNAc-6-SO(_4)</td>
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<tr>
<td>Methyl-(\alpha)-Glc-2,3-bis-SO(_4)</td>
<td>51</td>
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<td>L-Ascorbate 2-sulfate</td>
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<tr>
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<td>&gt;80</td>
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<tr>
<td>Fucoidan</td>
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<tr>
<td>Dextran sulfate (M, 500,000)</td>
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<td>Heparin</td>
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<tr>
<td>Dextran sulfate (M, 5000)</td>
<td>28</td>
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<tr>
<td>Hyaluronic acid</td>
<td>140</td>
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<tr>
<td>Keratan sulfate</td>
<td>&gt;1000</td>
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</table>

\(^a\)Concentration giving 50% inhibition of \(^{125}\)I-TSP binding (0.25 \(\mu\)g/ml to 200 ng of sulfatide in the solid-phase radioassay as described under "Experimental Procedures."

**DISCUSSION**

The following evidence suggests that TSP agglutinates erythrocytes by binding to sulfated glycoconjugates on their surfaces. TSP binds with high affinity to sulfatides, but not to other membrane lipids. This binding is specifically blocked by monoclonal antibodies A2.5 and C6.7 which also inhibit TSP-mediated hemagglutination. Sulfatide binding and hemagglutinating activity are both lost following reduction of TSP with dithiothreitol. Finally, the relative potencies of polysaccharides to inhibit both activities are similar. Heparin and fucoidan are potent inhibitors, whereas hyaluronic acid and keratan sulfate are weak.

One difference between sulfatide binding and hemagglutination is in the requirement for divalent cations. Whereas substantial binding to sulfatides occurs in the presence of EDTA, hemagglutination is inhibited (21). The basis for this difference may be the effect of the Ca\(^{2+}\)-induced conformation change on binding. Ca\(^{2+}\)-free TSP may bind sulfatide immobilized on a surface but be unable to bind simultaneously to sulfatides on two adjacent erythrocytes. A divalent cation-dependent self association or conformation change could, therefore, enhance the hemagglutinating activity of TSP. Alternatively, EDTA may alter the exposure of sulfated glycoconjugates on the erythrocyte membrane, and Ca\(^{2+}\) and Mg\(^{2+}\) binding may increase the accessibility of sulfatides in the membrane.

Whereas sulfatides are present on human and sheep erythrocyte membranes and can bind TSP, sulfated carbohydrates on additional structures including erythrocyte membrane glycoproteins or proteoglycans may also be receptors for TSP. Carbohydrate receptors for other hemagglutinins, such as those for ABO blood group-specific antibodies and lectins, are present both on glycolipids and membrane glycoproteins (34). The role of sulfatides in agglutination of fixed, activated platelets by TSP is less clear. Whereas inactivation of the TSP platelet agglutinating activity following dithiothreitol reduction and inhibition by monoclonal antibody A2.5 and heparin are consistent with sulfatide binding, the failure of fucoidan to inhibit fixed, activated platelet agglutination is not. Sulfatides are present on platelets and bind TSP when resolved on TLC. The concentration of sulfatides in human platelets (2.5 mg/kg of cells) is higher than their reported concentration in human erythrocytes (0.5 mg/kg) (32). Thus, the sulfatide density should be sufficient for agglutination. Sulfatides in the platelet membrane, however, may not be accessible for TSP binding. The accessibility of other glycolipids in the platelet membrane is altered following activation with thrombin (35). The accessibility of trihexosylceramide and globoside to labeling by reduction with NaB\(_4\)H\(_4\) following treatment with galactose oxidase or periodate decreased and the accessibility of the ganglioside GM\(_1\) (NeuNAc\(_2\)-2-Sgal\(_1\)-4GlcCer) increased. The effect of thrombin on exposure of sulfatides was not examined.

The observation that sulfatide binding is more sensitive to inhibition by fucoidan than heparin suggests that two distinct sites on TSP may be involved in sulfatide binding and platelet aggregation.
agglutination. This is consistent with previously reported differences between hemagglutination and fixed platelet agglutination by TSP (21, 22). Heparin is a more potent inhibitor of hemagglutination than of platelet agglutination. In contrast, fibrinogen partially inhibited fixed platelet agglutination but did not affect hemagglutination. There are also differences in the sensitivity of hemagglutination and platelet agglutination to inhibition by the monoclonal antibody C6.7 (10). The overlap in inhibitory activities suggests that two distinct sites are located in the same region of the TSP molecule close to the monoclonal antibody A2.5-binding site. However, the inhibition by A2.5 could be conformational, as was proposed to account for the inhibition of antibody A2.5 binding to TSP in the presence of C6.7.

TSP and laminin (26) bind with high specificity to sulfatides in sheep erythrocyte lipids. Comparing binding to reference lipids, both proteins strongly prefer sulfatide to the other common sulfated membrane lipid, cholesterol 3-sulfate. The specificity of TSP for sulfatide may be even greater than that of laminin in that phosphatidylserine and phosphatidyl-ethanolamine bound laminin better than TSP. The concentration dependences for TSP and laminin binding indicate similar affinities for sulfatide. Whereas detection of sulfatides after thin layer chromatography using TSP is 20-50-fold more sensitive than using laminin, different detection methods rather than different affinities may be responsible. Labeled TSP retains sulfatide binding whereas iodinated laminin does not.

Based on inhibition by small molecules, binding of TSP is not highly sugar-specific and ionic interactions are important. Choline iodide inhibits binding 50% at 185 mM over isoelectric conditions. Divalent and trivalent anions are slightly better inhibitors than choline. The best inhibitor, methyl-α-D-GlcNAc3-sulfate, is only severalfold more potent than choline anion. Moreover, uncharged anionic sugars including glucose 1-phosphate, l-ascorbate 2-sulfate, and D-galacturonate are almost as inhibitory. None of the glucose sulfates, however, were as active as the corresponding galactose sulfates. Based on these data, the high specificity for sulfatides over other anionic lipids such as phospholipids, gangliosides, and cholesterol 3-sulfate cannot be ascribed to specific recognition of galactose, and the classification of TSP as a "platelet lectin" on the basis of erythrocyte agglutination is inconsistent with the definition of lectin activity (36).

In addition to its specificity for binding sulfated glycolipids, TSP is remarkably selective for inhibition by sulfated polysaccharides (Table II). Fucoidan and keratan sulfate differ at least 2000-fold in their potency as inhibitors. The large range of activities may be due in part to differences in size and hence valence of the inhibitors. This is supported by the greater activity of high molecular weight dextran sulfates relative to low molecular weight forms. Fucoidan, however, has a lower molecular weight ($M_r \approx 1 \times 10^3$) than the larger form of dextran sulfate but is a better inhibitor. Valence should also depend on the degree of sulfation, yet fucoidan has a lower degree of sulfation than some less active inhibitors such as heparin. These data and the specificity of lipid binding can be rationalized if the binding site on TSP recognizes more than one sulfated structure, and a particular spatial orientation of these structures is required for high affinity binding. A possible function of galactose in sulfatides is to present sulfate esters in this orientation.

Based on inhibition by monoclonal antibodies and sulfated polysaccharides, the sulfatide-binding site of TSP is essential for erythrocyte agglutination. This site or a closely linked site is probably also involved in the agglutination of fixed, acti-vated platelets by TSP. The TSP-sulfate interaction alone does not support aggregation of live platelets since the monoclonal antibody that most strongly inhibits this interaction, A2.5, does not inhibit platelet aggregation. Moreover, heparin and fucoidan, both of which inhibit TSP binding to sulfatides, do not effectively inhibit platelet aggregation at comparable concentrations. However, TSP-sulfate interactions may modulate TSP binding to platelets and may account for the low levels of TSP binding to unactivated platelets (22, 37).

As the function of TSP in other cells which produce and secrete TSP is unknown, the significance of its binding to sulfatides which may be in the membranes of those cells is unclear. The potent inhibition by fucoidan of TSP binding, however, suggests several functions for TSP or related sulfatide-binding proteins in specific cell interactions which are inhibited by fucoidan. These include lymphocyte adhesion to high endothelial venules (58) and recirculation (39), sperm-egg adhesion (40, 41), tumor cell growth in vivo (42), and the maintenance of monolayer morphology by cultured endothelial cells (43). Monoclonal antibodies that specifically block sulfatide binding by TSP may mimic the effects of fucoidan and will be useful for elucidating the role of TSP in these systems.

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