Laminin binds specifically to sulfated glycolipids
(sulfatides/fibronectin/agglutination/gangliosides)

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Communicated by Phillips W. Robbins, October 15, 1984

ABSTRACT Previous studies of the agglutination of erythrocytes by the basement membrane glycoprotein laminin have suggested that laminin binds to gangliosides [Kennedy, D. W., Rohrbach, D. H., Martin, G. R., Momoi, T. & Yamada, K. M. (1983) J. Cell. Physiol. 114, 257-262]. Based on the following evidence, however, we find that laminin binds specifically to sulfatides, not gangliosides. Monogalactosyl sulfatides, purified from sheep erythrocytes with a yield of 4.3 mg/kg of packed cells, bind laminin with high affinity as did authentic bovine brain sulfatide (galactosylceramide-1-sulfate). The binding activity of these lipids and of total erythrocyte lipids was stable to alkaline and neuraminidase treatment but labile to dilute acid under conditions that destroy sulfatides but not gangliosides. Of various glycolipid and phospholipid standards tested, only sulfatides bound laminin with high affinity. Sulfatide binding and agglutinating activities of proteolytic fragments of laminin indicated that the globular end regions of the 200-kDa subunits are required for both activities. Thus, monogalactosylsulfatides, and possibly other more complex sulfated glycolipids, are probably involved in the agglutination of erythrocytes. These results also suggest a physiological function of sulfatides in cell adhesion. The agglutination of erythrocytes by fibronectin is also inhibited by gangliosides [Yamada, K. M., Kennedy, D. W., Grotendorst, G. R. & Momoi, T. (1981) J. Cell. Physiol. 109, 343-351]. Fibronectin, however, did not bind to sulfatides with high affinity but rather bound with low affinity to all anionic lipids tested, including phospholipids, gangliosides, and sulfatides.

Laminin is a major basement membrane glycoprotein that may participate in the attachment of cells to the basement membrane network through binding to heparan sulfate, type IV collagen, entactin, and the cell surface (1-5). Specific domains of the laminin molecule are involved in its interaction with cells and matrix components (1, 6, 7). In addition to cell attachment (2, 8, 9), laminin is also involved in related phenomena including cell growth and differentiation (10), morphogenesis (11), cell migration (12), neurite outgrowth (13), and cancer metastases (4). A protein receptor, which binds laminin with a Kd of 2 × 10−9 M, has been purified from the plasma membrane of some laminin-dependent cell lines (14-16). Monoclonal antibodies to the receptor blocked laminin binding to these cells (17).

Laminin agglutinates aldehyde-fixed sheep, human, and rabbit erythrocytes (18, 19). Inhibition studies of hemagglutination suggested that gangliosides and some anionic phospholipids might also be receptors for laminin (19). We have tested this hypothesis by measuring binding of laminin to erythrocyte membrane lipids, gangliosides, and other glycolipids. We report here that sulfatidic glycolipids, but not gangliosides or anionic phospholipids, bind laminin specifically and with high affinity. In contrast, fibronectin, another cell attachment protein that agglutinates erythrocytes (20), binds with low affinity to all anionic lipids tested.

MATERIALS AND METHODS

Materials. Laminin was purified from 0.5 M NaCl extracts of mouse Engelbreth Holm Swarm tumor by DEAE-cellulose chromatography (2) and 4.0 M NaCl precipitation. Protein-derase-derived fragments of laminin were purified and examined by rotary shadowing electron microscopy as described (6, 21). Antibodies to laminin were raised in New Zealand White adult rabbits and purified by affinity chromatography. These antibodies did not crossreact with type IV collagen or heparan sulfate proteoglycan of the tumor matrix. Human plasma fibronectin was prepared by gelatin affinity chromatography (22). Goat anti-human fibronectin was from Cappel Laboratories (Cochranville, PA). Protein A, DEAE-Sepharose CL-6B, and Sephadex G-25 were from Pharmacia. BioSil HA (minus 325 mesh) was from Bio-Rad. Lipid standards were obtained from Supelco (Bellefonte, PA) and Sigma. Sheep blood was from the National Institutes of Health Animal Farm (Poolsville, MD). Protein A and fibronectin were labeled with Na125I (ICN) by the Iodo-Gen method (23) to specific activities of 40-60 and 18 μCi/μg, respectively (1 Ci = 37 GBq). Laminin was iodinated using immobilized lactoperoxidase (Enzymobeads, Bio-Rad).

Sheep Erythrocyte Glycolipids. Glycolipids were isolated from 2 liters of sheep blood. Ghosts were prepared by hypotonic lysis of washed erythrocytes (24) and were extracted twice with chloroform/methanol/water, 4:8:3 (vol/vol) (25). The combined extracts were evaporated to dryness and treated with 100 ml of 0.2 M NaOH in methanol for 1 hr at 37°C. The reaction mixture was neutralized with an equal volume of 0.2 M HCl in methanol, evaporated to 1/4th vol, dialyzed extensively against distilled water, and lyophilized. Neutral and acidic lipids were separated on a 3.5 × 10 cm column of DEAE-Sepharose, bicinecatal form, equilibrated with chloroform/methanol/water, 30:60:8 (vol/vol) (26). After elution of neutral lipids with the same solvent (600 ml), acidic lipids were eluted with 600 ml each of 0.01 M, 0.02 M, 0.05 M, 0.1 M, and 0.5 M NH4HCO3 in methanol. Active lipids in the 0.01 M NH4HCO3 fraction were further purified by silicic acid chromatography on a 0.8 × 92 cm column of Bio-Sil HA. Glycolipids were applied to the column in chloroform/methanol, 96:4 (vol/vol) and eluted with a linear gradient (2 liters) from chloroform/methanol, 9:1 (vol/vol) to chloroform/methanol/water, 47:47:6 (vol/vol). Purity of the isolated glycolipids was determined by TLC on silica gel high performance plates (Merck) developed with chloroform/methanol/0.25% KCl, 5:4:1 (vol/vol), chloroform/methanol, 4:1 (vol/vol), or 1-propanol/15% ammonia/water, 60:9.5:11.5 (vol/vol). Glycolipids were visualized with orcinol/H2SO4. Other lipid components were visualized using 0.25% ninhydrin in ethanol or by charring.
Analytical Procedures. Purified erythrocyte sulfatides were quantified by the dye binding assay of Kean (27) using bovine brain sulfatide (galactosylsphingosine-1-sulfate, Supelco) as a standard. Sugar composition was determined by GLC analysis of alditol acetates (28) on a column packed with SP 2340 (Supelco). Isolated glycolipids were desulfated using 0.05 M HCl in dry methanol (29) at room temperature for 16 hr. Glycolipids were treated with Clostridium perfringens neuraminidase (0.1 unit/ml; Sigma, type VI) in 50 mM sodium acetate/0.15 M NaCl/5 mM CaCl$_2$, pH 5.5.

Laminin Binding to Lipids. A solid-phase RIA for detection of laminin binding to membrane lipid was developed based on previously described methods using monoclonal antibodies (30, 31) for detection of glycolipid antigens. Laminin binding to lipids separated on thin layer chromatograms was detected by immunostaining based on a method using monoclonal antibodies (31) for the detection of glycolipid antigens.

RESULTS

As reported by Kennedy et al. (19) laminin agglutinates glutaraldehyde-fixed sheep erythrocytes and trypsinized fixed human erythrocytes (Table 1). Treatment of the cells with neuraminidase had no effect on sheep erythrocyte agglutination by laminin but increased the agglutinability of human erythrocytes, suggesting that neuraminidase-sensitive sialyl residues are not involved in binding laminin. EDTA partially inhibited agglutination at 2 mM, consistent with the results of Ozawa et al. (18) who also reported that Ca$^{2+}$ is required for agglutination. Kennedy et al. (19) reported no inhibition by EDTA.

![Figure 1](https://example.com/figure1.png)

A total lipid extract of sheep erythrocytes was tested for laminin binding by solid phase RIA. Initially, $^{125}$I-labeled laminin was used but it was less active by a factor of at least 10 than an agglutinin than unlabeled laminin and did not bind to fixed erythrocytes or isolated lipids (data not shown). Therefore, laminin binding was measured indirectly using anti-laminin antiserum and $^{125}$I-labeled protein A. When this assay was used, the binding of laminin to 1 $\mu$g of erythrocyte total lipid extract was easily detected (Fig. 1). In control experiments in which incubation with laminin was omitted, protein A bound at the same level as seen in uncoated wells. Alkali treatment, which destroys most phosphoglycerolipids, increased binding. However, mild acid treatment of the alkali-stable lipids under conditions in which sulfatides are hydrolysed but gangliosides are stable (0.05 M HCl in methanol, 25°C) decreased binding by a factor of approximately 6. Treatment of alkali-stable lipids coated on microtiter wells with neuraminidase (0.1 unit/ml, 1 hr, 37°C) had no effect (data not shown).

To identify the lipids that bind laminin, alkali-stable lipids were separated into neutral and acidic fractions and analyzed on silica gel high performance TLC plates, and the binding of laminin was detected by immunostaining. Laminin bound strongly to several components in the fraction eluted with 0.01 M NH$_4$HCO$_3$ (Fig. 2A, lane 2) and weakly to two components in the fraction eluted with 0.05 M NH$_4$HCO$_3$ (Fig. 2A, lane 4). Diffuse staining of slower migrating components in the 0.01 M NH$_4$HCO$_3$ fraction and in the neutral fraction (Fig. 2A, lane 1) was also observed in a control experiment in which incubation with laminin was omitted and that therefore represents nonspecific binding of antibody or protein A to lipids. Specific binding of laminin to the 0.01 M NH$_4$HCO$_3$ fraction was lost after mild acid treatment of the lipids. This loss coincided with loss of the triplet of orcinol-positive bands seen in Fig. 2B, lane 2. These lipids were purified by silicic acid chromatography (Fig. 2B, lane 7) and retained their ability to bind laminin (Fig. 2A, lane 7). The active lipids were obtained in a yield of 4.3 mg/kg of packed sheep erythrocytes.

The major lipids that bound laminin were identified as monogalactosyl sulfatides on the basis of their chemical and physical properties. The mobility of the erythrocyte lipids on TLC was slightly less than that of brain sulfatides (Fig. 2B, lane 8). Decreased mobility was also noted for human erythrocyte sulfatides and was attributed to differences in spinogosine and fatty acid composition (32). The purified erythrocyte...
cyte lipids and reference bovine brain sulfatides were desulfated by mild acid treatment (Fig. 2C, lanes 1–4). Both were converted to neutral compounds that migrated faster on TLC. Both the erythrocyte lipids and the bovine brain sulfatides bound laminin whereas their desulfation products did not (Fig. 2C, lanes 5 and 6). The erythrocyte lipids reacted as sulfatides in the colorimetric assay of Kean (27). Their infrared spectrum (solid film) showed characteristic bands for sulfatides including an amide absorption at 1650 cm$^{-1}$ and sulfate absorption at 1220 and 830 cm$^{-1}$. GLC analysis of alditol acetates established that galactose was the only sugar present in the erythrocyte sulfatides.

The binding of laminin to reference lipids was examined to further define its specificity. As shown in Fig. 3, sulfatides bound laminin at lower concentrations than any of the phospholipids or glycosphingolipids tested. Of the two other sulfated lipids tested, cholesterol 3-sulfate bound laminin weakly and sodium dodecyl sulfate not at all. Both phosphatidylserine and phosphatidylethanolamine bound laminin weakly, whereas no binding was detected to other phospholipids at levels up to 10 μg per well. Binding to phosphatidylethanolamine and phosphatidylserine was confirmed by overlaying laminin on these lipids separated on high performance TLC. Laminin staining coincided with the lipids as detected by ninhydrin staining of a second plate developed under the same conditions but at least 10 μg of phospholipid was required to give laminin staining comparable with that obtained with 500 ng of sulfatide.

Laminin binding specificity for lipids was also examined by immunostaining. No binding was detected to neutral glycolipids including mono-, di-, and trihexosyl ceramides and globoside, brain gangliosides GM3, GM2, GM1, GD3, GD1b, and GQ1b, and any of the gangliosides in an upper phase lipid extract of human meconium. It did, however, bind to meconium sulfatides. Human erythrocyte acidic lipids exhibited a pattern similar to that seen with sheep erythrocyte acidic lipids.

The concentration dependence for laminin binding to sulfatides or to cholesterol 3-sulfate is shown in Fig. 4. Binding of laminin to sulfatide (100 ng per well) is saturable with half-maximal binding occurring at a laminin concentration of 4 μg/ml (~5 nM). Control experiments wherein either laminin antisera or protein A concentrations were increased 2-fold did not affect the curve, indicating that saturable binding is not an artifact resulting from limiting antibody or protein A. Binding to cholesterol 3-sulfate, however, is low affinity and not saturated at 100 μg of laminin/ml.

Binding of proteolytic fragments of laminin to sulfatide was also examined (Fig. 5). An α-thrombin fragment of laminin that lacks the 400-kDa subunit of laminin (6) gave saturable binding to sulfatide, although with slightly lower affinity than intact laminin. The chymotryptic fragment, however, which lacks the globular end regions of the short arms as well as the 400-kDa subunit (6, 21), did not bind to sulfatide. The proteolytic fragments were tested for hemagglutinin activity with fixed sheep erythrocytes. The α-thrombin fragment was slightly less active than intact laminin, requiring a 2.5-fold higher concentration for agglutination. The chymotryptic fragment was inactive at the highest concentration tested (100 μg/ml).

Since fibronectin also agglutinates glutaraldehyde-fixed erythrocytes (20) and this agglutination is inhibited by gangliosides (33), fibronectin was compared with laminin in the assays described above. Fibronectin binding was detected by using either 125I-labeled fibronectin or indirectly by using anti-fibronectin antibodies and 125I-labeled protein A. When the latter method was used, 10 ng of fibronectin could be detected if coated directly on microtiter wells. In contrast to the specific high-affinity binding of laminin to sulfatides, fibronectin did not bind with high affinity to any lipids in these assays. No binding of fibronectin to wells coated with up to 10 μg of phospholipid, sulfatide, globoside, sphingomyelin, or gangliosides GM3, GM2, or GD1a was
detected by solid-phase RIA.

Weak binding of 125I-fibronectin to lipids separated on thin layer chromatograms could be detected by the overlay technique (Fig. 2D, lanes 1–5). All negatively charged lipids stained weakly with fibronectin, including standard brain gangliosides, sulfatides, and phosphatidylserine. With the exception of phosphatidylserine, which gave detectable staining at 1 μg, at least 5–10 μg of anionic lipid was required to bind fibronectin. Thus binding of fibronectin to gangliosides is not specific for carbohydrate structure or sialic acid content.

**DISCUSSION**

Laminin binds specifically and with high affinity to sulfatides immobilized on plastic surfaces and thin layer chromatograms. Sheep erythrocyte sulfatides therefore may be receptors for laminin-mediated agglutination. Support for this hypothesis is the fact that the ability to agglutinate sheep erythrocytes and to bind to sulfatides is lost following proteolytic removal of the globular end regions of the short arms of laminin. Both activities are also lost following iodination of the intact molecule.

Based on recovery of purified sulfatides from sheep erythrocytes and assuming a cell volume of 35 μm³ (34), the sulfatide density is ~1.1 × 10⁵ molecules per cell. Blood group antigens responsible for agglutination of erythrocytes by some antibodies and lectins occur at comparable densities (35). Human erythrocytes contain less sulfatide than do sheep erythrocytes, ~4 × 10⁴ molecules per cell (32). This lower number is consistent with the weaker agglutination of human erythrocytes by laminin (Table 1). Sulfatides may not be the only receptor for laminin on erythrocytes, as sulfate esters occur in other glycoconjugates. If sulfated glycopro-
teins are involved, however, the sulfated sugars must be on trypsin-resistant molecules.
Laminin binds weakly to two phospholipids, phosphatidylyserine and phosphatidylethanolamine. Whereas these are major components of the erythrocyte membrane, both are largely if not exclusively on the cytoplasmic face of the membrane and are not exposed in intact erythrocytes (36).
In addition to sulfatides, laminin binds to other sulfated glycoconjugates, including heparin (5). Sulfatides and heparin differ in both sugar composition and position of sulfation. By studying the binding of proteolytic fragments of laminin, the relationship between these two activities may be examined. A proteolytic fragment thought to be derived from the long arm of laminin was isolated by Ott et al. (7) by chromatography of limited protease digests on heparin-agarose. Large fragments similar to the α-thrombin-derived fragment, which lack the long arm, were largely unretarded on heparin-agarose (7). These results suggest that a heparin binding domain is located on the long arm. Binding of laminin to sulfatides, however, is only slightly reduced for the α-thrombin fragment relative to intact laminin but abolished when the globular domains of the short arms are removed. These results suggest that laminin may have two sites that interact with sulfated sugars. The end of the long arm, which probably contains a high-affinity heparin binding site, may contribute weakly to sulfatide binding. High-affinity binding sites for sulfatides are probably located in the globular end regions of the short arms. These sites may also bind heparin weakly, as a large fragment (SI-4) containing the short arms, produced by digestion with staphylococcal protease, was partially retarded on a heparin column (7).

The failure of human plasma fibronectin to bind with high affinity to sulfatides also suggests that heparin and sulfatide binding need not be related. Fibronectin binds heparin and specific domains responsible for this activity have been mapped (37, 38). Our results indicate that fibronectin binds weakly to all anionic lipids with no specificity for sulfated lipids. Therefore, the presence of a heparin binding site is not sufficient for high-affinity binding to sulfatides. Binding of fibronectin to phospholipids and aggregation of lipid vesicles have been reported to be nonspecific for phospholipid type (39). Because of this lack of specificity, the hypothesis that lipids are the cell receptors for fibronectin cannot readily account for the cell specificity of fibronectin as an attachment factor.

The relevance of sulfatide binding to the biological properties of laminin remains to be determined. The observation that untreated sheep and human erythrocytes are not agglutinated suggests that the sulfatides are not accessible on these cells. Sulfatides have been isolated from many tissues, are especially rich in brain and kidney, and occur with a variety of carbohydrate structures and sulfation positions (40). Laminin stains several slower migrating lipids in sheep erythrocyte extracts (Fig. 2A). Binding to some of these bands is lost following acid desulfation (unpublished data), suggesting that other more complex sulfated lipids may also bind laminin. Whether these lipids differ in their affinity for laminin binding remains to be determined. The cell surface protein that has been identified as a laminin receptor mediates some actions of laminin (14-16). This receptor and sulfatides may act cooperatively in cell attachment. Alternatively, the protein receptor may mediate attachment to the basement membrane, whereas sulfatides could be important in cell–cell interactions. The fact that laminin is multivalant for sulfatide binding is consistent with the latter function.