Binding of sulfatide to concanavalin A and *Lens culinaris* agglutinin

Wahida Yousofzai, Miwa Kashimoto, Hideko Tanaka, Isamu Matsumoto, Kyoko Kojima-Aikawa, and Tetsuyuki Kobayashi

Department of Biological Sciences, Graduate School of Humanities and Sciences, Ochanomizu University, 2-1-1 Ohtsuka, Bunkyo-Ku, Tokyo 112-8610, Japan

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SUMMARY

Lectins, carbohydrate binding proteins of non-immune origin, are characterized by a high degree of specificity in binding to some saccharides in preference to others. Some lectins are reported to interact with sulfated glycosaminoglycans, but little is known about an interaction between lectins and sulfatide. Here, we examined the binding of sulfatide to leguminous plant lectins, such as *Lens culinaris* agglutinin (LCA), concanavalin A (Con A), peanut agglutinin, soybean agglutinin, *Solanum tuberosum* agglutinin, *Ulex europaeus* agglutinin, and *Bauhinia purpurea* agglutinin. A solid phase binding assay using horse radish peroxidase-labeled lectins showed that LCA and Con A could bind to sulfatide coated on microtiter plates, but failed to bind to galactosylceramide. The binding of these lectins to sulfatide was inhibited in the presence of excess amounts of either methyl α-D-mannopyranoside or D-glucose, whereas it was not affected by D-galactose. Furthermore, both Con A and LCA bound to sulfatide which was incorporated into lipid vesicles and the binding was inhibited by addition of methyl α-D-mannopyranoside. These results suggest that Con A and LCA have a binding site of sulfatide whose affinity is affected by the specific binding of saccharides.

Keywords: lectin, sulfatide, concanavalin A (Con A), *Lens culinaris* agglutinin (LCA)

INTRODUCTION

Lectins are carbohydrate binding proteins of non-immune origin found in all types of living organisms. Plant lectins, which are the best studied lectin group, have been used to study the complex carbohydrates of various cells, taking account of their ability to bind certain specific sugar structures.

Data on protein-carbohydrate interactions are especially interesting given that protein-carbohydrate recognition is a major form of biological and cell communication (1).

An interaction between lectins and sulfated glycosaminoglycans, such as heparin, heparan sulfate, keratan sulfate and chondroitin sulfate was also reported and the involvement of the sulfate groups in glycosaminoglycans in their binding was suggested (2-4). These findings raised the possibility that some lectins bind to sulfatide, a sulfated glycosphingolipids, but an interaction between sulfatide and lectins has been remained to be elucidated.

Sulfatide, 3-*O*-sulfo-β-D-galactosylceramide, is widely distributed throughout mammalian tissues including the brain, kidney, and gastrointestinal tract (5-9), and is particularly prevalent in both neuronal and myelin membranes within the nervous system (6). Sulfatide has been considered potential targets for autoimmune neurological disease pathogenesis (10). Besides, sulfatide is thought to have important functions in blood coagulation systems (11, 12). Several functions, such as interaction with membrane component including receptors, proteins involved in bacteria-host binding and ion channels, have been also attributed to sulfatide (6, 13). The sulfatide binding proteins, such as cardiotoxin (14) and annexin V (15), are useful as molecular tools for studying the functions of sulfatide.

In this paper, we examined the binding of a number of leguminous plant lectins to sulfatide, and showed that concanavalin A (Con A) and *Lens culinaris* agglutinin (LCA) interact with sulfatide coated on microtiter plates or incorporated into liposomes. Both Con A and LCA are known to bind methyl α-D- mannopyranoside and D-glucose. Inhibition of their binding to sulfatide was observed in the presence of either methyl α-D-mannopyranoside or D-glucose.

MATARIALS AND METHODS

Materials

Sulfatide from bovine brain and phosphatidylcholine from egg yolk (Type XVI-E) were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Non-labeled Con A and horse radish peroxidase-labeled lectins, such as Con A, LCA, peanut agglutinin (PNA), soybean agglutinin (SBA), *Solanum tuberosum* agglutinin (STA), *Ulex europaeus* agglutinin-I (UEA-I) and *Bauhinia purpurea* agglutinin (BPA) were purchased from Seikagaku Corp. (Tokyo, Japan). Non-labeled LCA was obtained from J-Oil Mills, Inc. (Tokyo, Japan), and *o*-phenylenediamine from Nacalai Tesque Inc. (Kyoto, Japan). Microtiter plates (Immulon 1B) were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Methyl α-D-mannopyranoside, D-glucose and D-galactose were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Lipid-binding Assay on Microtiter Plates

The binding of lectins to lipids on microtiter plates was measured according to the method as

described (15). Serial dilutions of either sulfatide or galactosylceramide dissolved in methanol were added (50 μl/well) to each well in 96-well microtiter plates (Immulon 1B) and dried at 37°C for 2 h. The plates were then blocked with 5% BSA in TBS (100mM Tris-HCl, 150mM NaCl, pH 7.4) (300 μl/well), and incubated for 2 h at room temperature. One hundred μl of the solution of lectins (3 μg/ml), Con A, LCA, SBA, STA, UAE, BPA or PNA labeled with HRP, was added to each well, followed by incubation for 2 h in the presence of 1% BSA. The wells were washed with TBS and developed by the addition of 100 μl of 0.04% *o*-phenylenediamine and 30% H_2O_2 in 100mM citrate-phosphate buffer (pH 5.5). Color development was stopped by the addition of 50 μl of 4N H_2SO_4. The absorbance of each well was then read at 490nm.

For the binding studies of Con A and LCA to sulfatide in the presence of monosaccharide, the wells coated with 3 μg/well of sulfatide were incubated with 0.3 μg/well of HRP-labeled Con A or LCA in the presence of 100 mM of either methyl α-D-mannopyranoside, D-glucose or D-galactose.

Preparation of Lipid Vesicles

The multilamellar vesicles consisted of 5 mol% sulfatide and 95 mol% phosphatidylcholine from egg yolk were prepared for the binding studies. Ten μl of 1 mM sulfatide and 190 μl of 1 mM phosphatidylcholine in chloroform/methanol (2:1, v/v) were placed in Pyrex glass tubes, and the organic solvent was removed using a rotary evaporator. For control experiments, 200 μl of 1 mM phosphatidylcholine in chloroform/methanol (2:1, v/v) alone was used. The lipid film was thoroughly dried to remove residual organic solvent by placing the samples on a vacuum pump overnight. The dried lipid film was dispersed in 100 μl of TBS at room temperature and agitated vigorously using a vortex mixer. The test tubes containing lipid suspension were placed in a bath sonicator and gently sonicated for 1 min. The suspension was transferred to a microcentrifuge tube and centrifuged for 10 min at 15,000 rpm. The supernatant was removed and the pellet resuspended in TBS was used for the binding studies.

Binding Assay of Lectins to Sulfatide in Lipid Vesicles

To assess the binding activity of Con A and LCA to sulfatide in membranes, lipid vesicles composed of egg yolk phosphatidylcholine with or without sulfatide being 5 mol% of total lipid were prepared in TBS as described above. Stock solutions of Con A and LCA were pre-centrifuged at 15,000 rpm at 4°C for 10 min before use to remove insoluble forms. The Con A or LCA solution was added to the liposome solutions in the presence or absence of 100 mM methyl α-D-mannopyranoside and allowed to incubate for 2 h at 37°C. After incubation, the solutions were centrifuged at 15,000 rpm for 10 min at 4°C and resultant pellets were washed three times with TBS. Pellets containing the lipid vesicles and bound lectins were solubilized with sodium sodium dodecyl sulfate (SDS) loading buffer (10% glycerol (v/v), 2%SDS (w/v), 100 mM 2-mercaptoethanol and 0.1% bromphenol blue (w/v)), and then analyzed using 12% gels of SDS-PAGE, followed by staining with Coomassie Brilliant Blue.

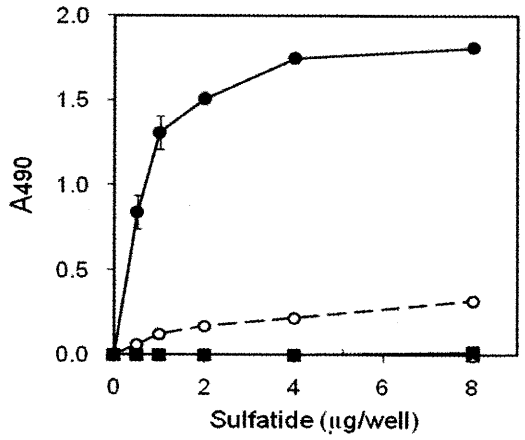

Fig. 1. **Binding of leguminous plant lectins to sulfatide on microtiter plates.** Microtiter wells coated with a various amount of sulfatide were incubated with 0.3 μg of lectins labeled with HRP, followed by assay with *o*- phenylenediamine. Each value represents the mean of three determinations ± S.D. ●, LCA; ○, Con A; ■, PNA; □, *SBA*; ▲, STA; Δ, UEA; x, BPA.

The experiments were repeated five times under the same conditions and the intensity of the monomer Con A band (27 kDa) in each lane was quantified by an image analyzer, LAS-3000 (Fujifilm Corp., Tokyo, Japan).

RESULTS

Binding of Con A and LCA to Sulfatide on Microtiter Plates

In this study, we examined the interaction of sulfatide with seven kinds of leguminous plant lectins such as Con A, LCA, SBA, STA, UAE, BPA, and PNA, using a solid phase binding assay system; various concentrations of sulfatide were absorbed on a plastic microtiter plate surface, and then solid-phase binding to various lectins labeled with HRP was examined. LCA showed a strong binding activity to sulfatide in a dose dependent manner and a significant binding of Con A to sulfatide was also detected (Fig. 1). No binding was observed to any lectins tested other than LCA and Con A. On the other hand, Con A and LCA failed to bind to galactosylceramide, which is the analog of sulfatide lacking a sulfate group (Fig. 2A, 2B). These results suggested that Con A and LCA recognize the sulfate group of sulfatide upon binding.

Since a specific interaction of Con A and LCA with methyl α-D-mannopyranoside and D-glucose is well known, effects of these saccharides on sulfatide binding were examined. The binding of Con A to sulfatide on microtiter plates was inhibited in the presence of excess amounts of either methyl α-D-mannopyranoside or D-glucose, whereas it was not affected by galactose (Fig. 3). Similar results were observed with LCA. This could be attributed to a conformational change of the lectins upon saccharide binding, thereby leading to reduction in binding affinity to sulfatide.

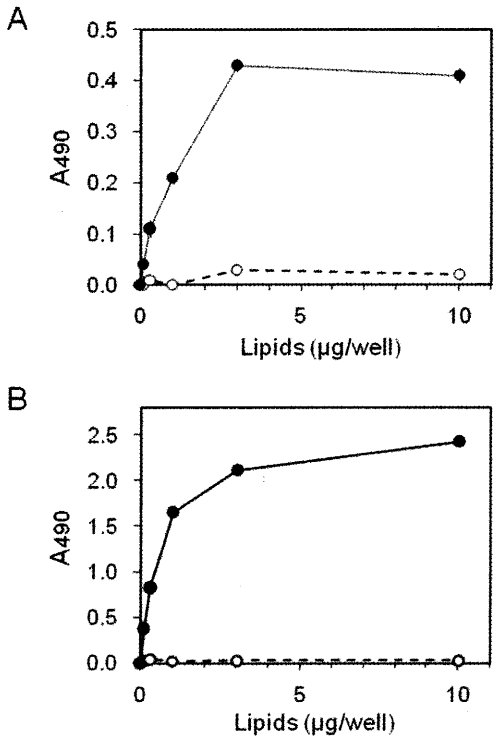

Fig. 2. **Con A (panel A) and LCA (panel B) bind to sulfatide but not galactosylceramide.** Wells of microtiter plate were coated with serial dilutions of either sulfatide (•) or galactosylceramide (○), and dried. After blocking with 5% BSA, either HRP-labeled Con A or LCA (0.3 μg/well) was added. After washing with TBS (pH 7.4), the plates were assayed with *o*- phenylenediamine.

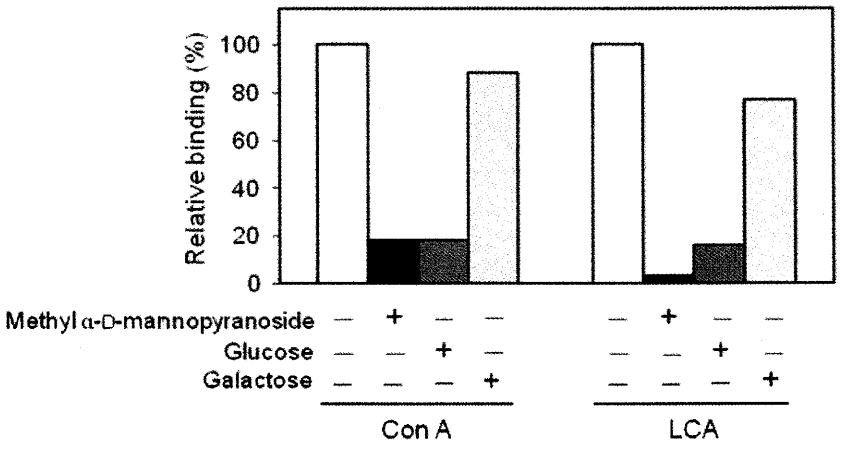

Fig. 3. **Inhibition of the binding of sulfatide to immobilized Con A and LCA by methyl α-D-mannopyranoside and glucose.** The 96-well plate was coated with sulfatide (3 μg/well) and incubated with HRP-labeled Con A or LCA (0.3 μg/well) in the presence of 100 mM of either methyl α-D-mannopyranoside, D-glucose or D-galactose. After washing with TBS (pH 7.4), the plates were assayed with *o*- phenylenediamine. Data are expressed as relative percentages to the absorbance in the absence of monosaccharide.

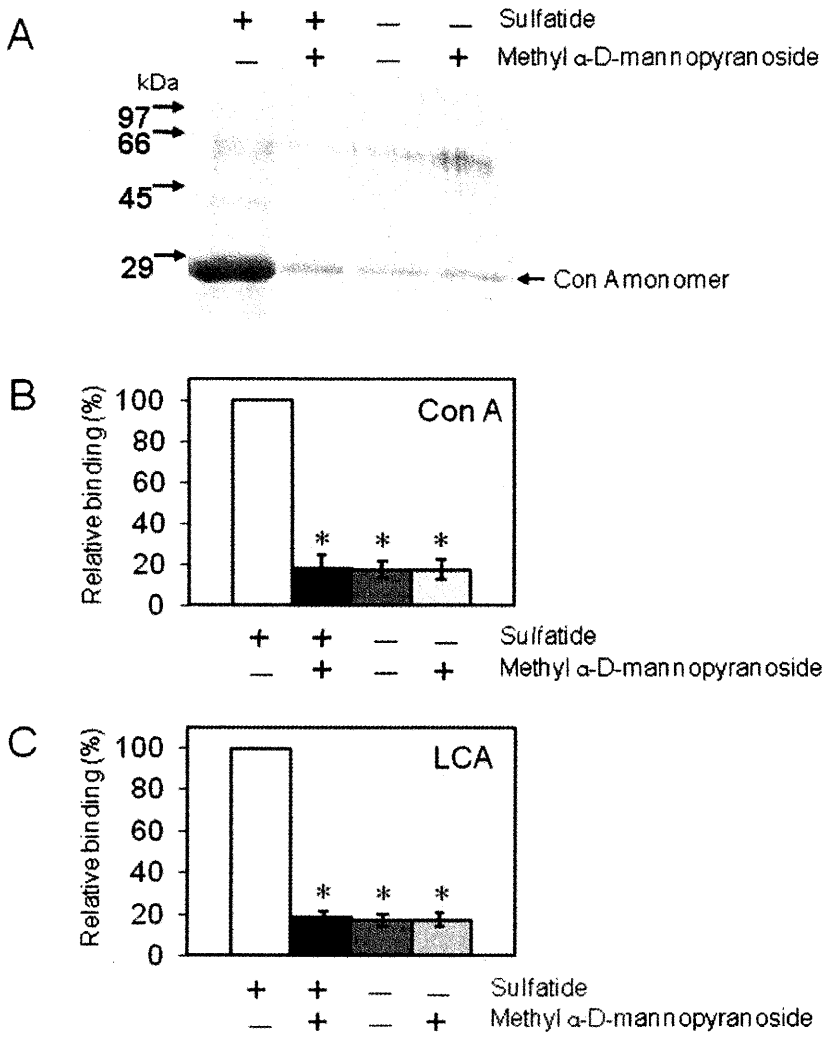

Fig. 4. **Centrifugation/SDS-PAGE-detected binding of Con A to sulfatide-containing vesicles.** (A) Detection of Con A associated with sulfatide-containing vesicles by SDS-PAGE. Con A was incubated with lipid vesicles composed of egg yolk phosphatidylcholine with or without 5 mol% sulfatide in the presence or absence of 100 mM methyl α-D-mannopyranoside. The pellet obtained after centrifugation was analyzed on SDS-PAGE. (B) The intensity of the monomer Con A band (27 kDa) in each lane of gels was quantified by an image analyzer. The binding activity was expressed as a percentage relative to the intensity in the lane of sulfatide-containing vesicles in the absence of methyl α-D-mannopyranoside. Each value represents the mean of five determinations ± S.D. The differences between the means were compared by Student's *t* test (*, $p<0.01$). (C) The binding activity was expressed as a percentage relative to the intensity of the monomer LCA band in the lane of sulfatide-containing vesicles in the absence of methyl α-D-mannopyranoside. Each value represents the mean of five determinations ± S.D. The differences between the means were compared by Student's *t* test (*, $p<0.01$).

Specific Binding to Sulfatide in Liposomal Membranes

The interaction of Con A and LCA with sulfatide in membranes was examined by measuring the association of these lectins with lipid vesicles *via* centrifugation of lectins mixed with multilamellar vesicles containing sulfatide. The amount of bound lectins in the lipid vesicle-containing pellet was determined by polyacrylamide gel electrophoresis in SDS. The protein band with molecular weight of 27,000 which corresponds to a monomer of Con A was detected by sedimentation with sulfatide-containig vesicles (Fig. 4A).

The binding is strongly inhibited by addition of methyl α-D-mannopyranoside, and no binding was observed to egg yolk phosphatidylcholine vesicles without sulfatide (Figs. 4A and 4B), which is consistent with the observations reported previously (16). LCA showed the similar binding characteristics (Fig. 4C). These observations indicate that both Con A and LCA can bind specifically to sulfatide incorporated into membranes under the conditions used.

DISCUSSION

The interaction of sulfatide with seven kinds of legume lectins such as Con A, LCA, SBA, STA, UAE, BPA, and PNA was examined. It was demonstrated from our present study that among these lectins tested, LCA and Con A can bind to sulfatide on microtiter plates. Legume lectins are a large family of homologous proteins displaying a broad range of different carbohydrate specificities. Con A and LCA are grouped together, because they show similar preference for mannose/glucose. The binding of Con A and LCA to sulfatide on microtiter plates or in lipid vesicles was inhibited by methyl α-D-mannopyranoside or glucose. These observations indicate that the affinity to sulfatide at its binding site on Con A and LCA is strongly influenced by the specific binding of monosaccharides. In addition, Con A and LCA did not bind to galactosylceramide, suggesting that a sulfate group in sulfatide is involved in the recognition and binding.

An interaction between sulfated glycosaminoglycans and lectins was reported at low pH and low ionic strength (3, 4), whereas the present study demonstrated that the association of LCA and Con A with sulfatide occurred in TBS at pH 7.4. It is known that Con A exists at pH 7.4 as a tetramer of four identical continuous polypeptides of 237 amino acid residues each and the tetramer dissociates to form dimmers at lower pH. It was suggested that sulfatide can bind to the tetramer form of Con A.

In addition to the sugar binding site, a number of legume lectins possess a hydrophobic binding site, which has a low affinity for hydrophobic ligands like indoacetic acid and 8-anilinonaphthalene-1-sulfonate (ANS) (17) and is positioned near the sugar binding site. Isothermal titration calorimetric studies revealed that ANS binds to Con A at pH 5.2 or 2.5, where it exists as a dimer, but no binding was observed with the tetrameric form of Con A at pH 7.2 (18). Therefore, the present study has demonstrated existence of a binding site of sulfatide which is distinct from the

known binding site of hydrophobic ligands in Con A.

Several proteins have been reported to bind sulfatide *in vitro*. The adhesive proteins in extracellular matrix such as thrombospondin (19), and antistatin (20), which is a leech salivary protein and an inhibitor of coagulation, have been shown to bind sulfatide. Properdin, a plasma glycoprotein which stabilizes the C3 convertase during the activation of the alternate pathway of complement, also binds to sulfatide and certain sulfated glycoconjugates (21). Based on the structure of these proteins, a consensus sequence (Cys-Ser-Val-Thr-Cys-Gly-X-Gly-X-X-X-Arg-X-Arg) has been proposed to represent a sulfatide binding domain (20). Neither Con A nor LCA has this consensus sequence, suggesting that a sulfatide-binding domain in Con A and LCA is distinct from that in properdin, thrombospondin, and antistatin.

Some lipid binding proteins induce membrane disruption such as hemolysis. Our unpublished results on the permeabilization activity of Con A showed that Con A did not induce calcein leakage from sulfatide-containing liposomes. These results suggest that Con A binding to sulfatide in membranes does not induce membrane disruption.

The extensive study of binding characteristics of lectins to sulfatide will be useful for exploring biological functions of sulfatide, which is not only a major species of glycosphingolipids in the central and peripheral nervous system (6), but also involved in a number of important cellular events such as the pathology of Alzheimer's disease (22), cytokine production in mononuclear cells (23), insulin secretion in pancreatic β cells (24) and infection of human immunodeficiency virus type 1 (25).

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Wahida Yousofzai, Miwa Kashimoto, Hideko Tanaka, Isamu Matsumoto, Kyoko Kojima-Aikawa, and Tetsuyuki Kobayashi
Department of Biological Sciences, Graduate School of Humanities and Sciences, Ochanomizu University, 2-1-1 Ohtsuka, Bunkyo-Ku, Tokyo 112-8610, Japan
E-mail: kobayashi.tetsuyuki@ocha.ac.jp