Recognition Mechanism of Galectin-4 for Cholesterol 3-Sulfate*

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Galectin-4 binds to glycosphingolipids carrying 3-O-sulfated Gal residues, and it co-localizes on the cell surface of human colonic adenocarcinoma cells with glycosphingolipids carrying SO\textsubscript{3}\textsuperscript{-}Gal\textsubscript{β1→3}GalNAc residues (Ideo, H., Seko, A., and Yamashita, K. (2005) J. Biol. Chem. 280, 4730–4737). In the present study, it was found that galectin-4 also binds to cholesterol 3-sulfate, which has no β-galactoside moiety. This characteristic of galectin-4 is unique within the galectin family. The site-directed mutated galectin-4-R45A had diminished binding ability toward cholesterol 3-sulfate, suggesting that Arg\textsubscript{45} of galectin-4 is indispensable for cholesterol 3-sulfate recognition. Gel filtration and chemical cross-linking experiments revealed that some galectin-4 exists as dimers, and this multivalency may be essential for its biologic activity toward cholesterol 3-sulfate, similar to that of galectin-4. We found in this study that cholesterol 3-sulfate-binding character is unique to galectin-4. Because the tissue content and distribution of these sulfated glycosphingolipids on the epithelium of the alimentary tract and in human colon cancer cells bind to and co-localize with galectin-4, it was found that galectin-4 binds to cholesterol 3-sulfate, which could not be separated from SM4 and SM3 by the TLC solvent system in our previous paper (2), although cholesterol 3-sulfate does not have a sugar (galactose) moiety.

Galectins are a family of animal lectins defined by their affinity for β-galactoside and by common amino acid sequence elements (3). It was of interest to determine whether other galectins, in addition to galectin-4, also bind to cholesterol 3-sulfate. As reported previously, galectin-8 binds to glycosphingolipids, including sulfatide (SM4s), SM3, sialyl Lac\textsubscript{4}Cer, SB1a, GD1a, GM3, and sialyl Lac\textsubscript{4}Cer (4). Because SO\textsubscript{3}\textsuperscript{-}3core 1 also binds to a galectin-3-Sepharose column (1) and synthetic sulfated oligosaccharides bind to human splenic galectin-1 (5), we examined whether galectins-1, -3, and -8 have affinity for cholesterol 3-sulfate, similar to that of galectin-4. We found in this study that this cholesterol 3-sulfate-binding character is unique to galectin-4.

Previous site-directed mutagenesis studies have been targeted at carbohydrates containing β-galactoside (6–8). Here, to determine the binding mechanism of cholesterol 3-sulfate, we prepared full-length galectin-4 and site-directed mutants of the N- and C-domains, based on comparisons of the amino acids in the S3 β-sheets of various galectins, and we examined their binding to various ligands.

Although galectins do not have secretion signal peptides, they are often found outside of cells (3, 9). It has been reported that galectin-4 is one of the major components of the detergent-resistant membranes of the brush border membrane of porcine intestine (10–12) and of the HT-29 colon adenocarcinoma cell line (13). Because the involvement of galectin-4 in apical protein delivery has also been reported in HT-29 cells (13), it was of interest to determine which molecules interact with galectin-4 in the cytosol and the regulatory mechanism that is important for protein trafficking in epithelial cells.

EXPERIMENTAL PROCEDURES

Materials—Cholesterol 3-sulfate, asialofetuin, β-lactoglobulin, and fucoidan from Fucus vesiculosus were purchased from Sigma. Sodium dextran sulfate, GM1, GM3, SM4s, phosphati-
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dyethanolamine, phosphatidylinositol, and phosphatidylserine were from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Keratan sulfate sodium salt and heparan sulfate sodium salt were obtained from Seikagaku Co. (Tokyo, Japan), and heparin sodium salt, chondroitin sulfate A sodium salt, and chondroitin sulfate C sodium salt were from Nacalai Tesque Inc. (Kyoto, Japan). Disuccinimidyl suberate (DSS) was obtained from Pierce. The pGEX-6P-1 plasmid, Escherichia coli BL21 strain, glutathione-Sepharose, and PreScission protease were from Amersham Biosciences.

Synthesis of 3′-Sulfo-Core 1 by Gal 3-O-Sulfotransferase-2 (Gal3ST-2)—Sulfation of core 1 was performed using recombinant Gal3ST-2. The cDNA encoding the catalytic region of human Gal3ST-2 was amplified by PCR using an expression vector as the template, as reported previously (14, 15). The oligonucleotide primers were 5′-ttgtaatGGGGGCCAGGCCTGGGG-3′ (forward primer) and 5′-ttggccggccAGGGGCCTCGTC-3′ (reverse primer). The sequences in lowercase letters indicate the restriction sites. The amplified cDNAs were used as the templates for PCR. The amplified cDNAs were digested with EcoRI and NotI and cloned into pPIC9 (Invitrogen). The amplified cDNAs were used as the templates for PCR. The amplified cDNAs were digested with EcoRI and NotI and cloned into pPIC9 (Invitrogen). The amplified cDNAs were digested with EcoRI and NotI and cloned into pPIC9 (Invitrogen). The amplified cDNAs were digested with EcoRI and NotI and cloned into pPIC9 (Invitrogen). The amplified cDNAs were digested with EcoRI and NotI and cloned into pPIC9 (Invitrogen).

Plate Maxisorp Surface, Nalge Nunc International K.K.). After washing with PBS, 50 μl of 3% BSA in PBS were added as a blocking solution, and the plate was left overnight at 4 °C. After washing with PBS, 50 μl of various concentrations of galectins in the blocking solution were added to each well, and the plate was left for 1 h at room temperature. The plate was washed several times with washing buffer (0.01% Tween 20 in PBS), and anti-galectin-4 or anti-His6 antibody (clone BMG-His-1; Roche Applied Science) diluted in the washing buffer was added. After incubation for 1 h at room temperature, the plate was washed, and anti-rabbit IgG conjugated with horseradish peroxidase (HRP) (GE Healthcare) or alkaline phosphatase (The Binding Site Ltd., Birmingham, UK) was added. p-Nitrophenylphosphoric acid disodium salt (pNPP) in 0.1 M carbonate buffer, pH 9.6, and o-phenylenediamine (Nacalai Tesque Inc., Kyoto, Japan) in 10 mm acetate buffer, pH 5.0, were used as the substrates for alkaline phosphatase and HRP, respectively.

For the galectin-4 binding assay, the results using anti-His6 antibody were almost the same as the results using anti-galectin-4 antibody. However, the anti-His6 antibody resulted in a lower background than the anti-galectin-4. Because the detection limit of anti-galectin-4 toward galectin-4 was lower than that of the anti-His6 antibody, anti-galectin-4 detected a small amount of nonspecific binding of galectin-4 to the plate, which occurred even after BSA blocking. Furthermore, the anti-His6 antibody did not recognize the carbohydrate recognition sites of the galectins. Therefore, the binding could be directly compared without considering the recognition specificity and binding activity of the antibody. Thus, we used the anti-His6 antibody for the binding assays.

Co-sedimentation Assays—Co-precipitation of galectin-4 with lipid vesicles was performed according to Kojima et al. (17). Five μg of lipids were dissolved in TBS and sonicated at 0 °C for 15 min to prepare lipid vesicles. Lipid solutions (20 μl) containing 5 μg of lipids and 1.5 μg of galectin-4 were incubated at 37 °C for 30 min and then centrifuged at 15,000 × g for 5 min. The supernatants were discarded, and the pellets were subjected to SDS-PAGE, and the proteins were stained with Sypro Orange™ (Bio-Rad) or Cooassie Brilliant Blue R-250.

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Inhibition Assay Based on ELISA—The inhibition assay between galectin-4 and cholesterol 3-sulfate was performed using ELISA as described above. Fifty μl of galectin-4 (1 μg/ml) with various concentrations of inhibitors in 1% BSA in PBS were applied to cholesterol 3-sulfate-coated plates (1 μg/well), and the relative binding abilities of galectin-4 were measured using anti-His₄ antibody.

Binding of Galectins to Immobilized Asialofetuin on the Surface of a BIACore Sensor Chip—The binding of galectins to asialofetuin was measured by a surface plasmon resonance (SPR) assay using a BIACore 2000 instrument. Asialofetuin was immobilized on a CM5 sensor chip by the amine-coupling method. Various concentrations of galectins were diluted in HBS buffer (10 mM HEPES, pH 7.4, 3.4 mM EDTA, 150 mM NaCl, 0.005% (v/v) surfactant P-20) and injected onto the sensor chip at 20 μl/min. The sensor surface was regenerated with 0.1 M lactose.

Estimation of Kinetic Constants Based on SPR—The dissociation constants between the galectin-4-N- or -C-domains and carbohydrates were measured using a galectin-4-domain-immobilized sensor in a BIACore 2000 instrument, as described previously (1).

Site-directed Mutagenesis of Full-length Galectin-4 and Galectin-4-N- and C-domains—Site-directed mutagenesis was performed using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA), according to the manufacturer’s instructions. Nucleotide sequences were analyzed using an Applied Biosystems PRISM 310 Genetic Analyzer.

Size-exclusion Chromatography—Gel-filtration chromatography was performed using Superose 12 (GE Healthcare), equilibrated with PBS containing 2 mM EDTA. Galectin-4 (200 μl, 100 μg/ml) was applied to the column at a flow rate of 0.3 ml/min at room temperature, and 0.15-ml fractions were collected. The absorbance of the eluate at 280 nm was monitored with an ATTO Bio-mini UV monitor AC-5200 (Atto Co., Tokyo, Japan). Fractions containing proteins were boiled in SDS sample buffer with 2-mercaptoethanol, subjected to SDS-PAGE, immunoblotted with anti-galectin-4 antibody followed by HRP-conjugated anti-rabbit antibody (GE Healthcare), and visualized with the ECL system (GE Healthcare). The column was calibrated using blue dextran, BSA (67 kDa; GE Healthcare), β-lactoglobulin (35 kDa), and ribonuclease A (13.7 kDa; GE Healthcare). The binding assay to cholesterol 3-sulfate was performed using galectin-4 antibody and anti-His₄ antibody.

Chemical Cross-linking—For each reaction, 1 μl of fresh 20 mM DSS solution prepared in dimethyl sulfoxide (Me₂SO) was added to a 40-μl aliquot of galectin solution (100 μg/ml) for a final concentration of 0.5 mM, followed by incubation at room temperature for 30 min. Reactions were terminated by the addition of 2 μl of 1x Tris-HCl, pH 7.5, for a final concentration of 50 mM, followed by incubation at room temperature for 15 min. Aliquots of the reaction mixtures were boiled in SDS sample buffer with 2-mercaptoethanol, subjected to electrophoresis, and stained with Coomassie Brilliant Blue R-250.

Extraction of Protein and Lipids from Porcine Esophagus and Intestine—Porcine esophagus and intestine were obtained from a slaughterhouse. Several grams of porcine esophagus and intestine were cut into small pieces and homogenized in lysis buffer (0.5% Triton X-100, 25 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2 mM EDTA, 1:100 (v/v) Halt protease inhibitor mixture (Pierce)). The homogenates were cleared by centrifugation at 500 × g for 10 min to remove tissue debris and centrifuged at 30,000 × g for 15 min at 4 °C. The resulting pellets were resuspended in the same buffer, re-extracted at 37 °C for 30 min, and centrifuged with at 30,000 × g for 30 min. Crude lipids from the resulting pellets were extracted and analyzed as follows.

Crude lipids of homogenized porcine esophagus and intestine were extracted once with 5 volumes of chloroform/methanol (2:1, v/v). The pooled extracts (total lipid extracts) were dried and dissolved in chloroform/methanol (1:1, v/v). The solution was spotted for TLC (10 × 10 cm, silica gel 60; Merck) and developed using chloroform/methanol/acetone/acidic acid/water (8:2:4:2:1, by volume) as the solvent to separate cholesterol 3-sulfate and SM4. Sulfated lipids were detected by azure A (18). Extracted proteins were subjected to SDS-PAGE and immunoblotting with anti-galectin-4 antibody as described above.

RESULTS

Galectin-4 Binding to Cholesterol 3-Sulfate—Galectin-4 binds to glycosphingolipids carrying 3-0-sulfated Gal residues derived from colon epithelial cells (2). Because the mobility of cholesterol 3-sulfate corresponded to that of authentic SM4, which was stained by the azure A method on TLC using chloroform/methanol, 0.2% CaCl₂ (60:35:7) as the solvent, we re-examined whether cholesterol 3-sulfate and various lipids bound to galectin-4. First, we measured the binding abilities of galectin-4 to various lipid-coated plates using an antibody against galectin-4 (Fig. 1A). Galectin-4 bound, not only to SM4 and GM1, but also to cholesterol 3-sulfate, although this molecule has no carbohydrate moieties. In contrast, galectin-4 did not bind to cholesterol. The binding abilities of galectin-4 to these lipids were also examined by co-sedimentation assays. After incubation of galectin-4 with the respective lipid vesicles, the lipid-associated galectin-4 was precipitated by centrifugation. As shown in Fig. 1, B and C, galectin-4 bound to cholesterol 3-sulfate, SM4, GM1, phosphatidylinositol, and phosphatidylserine, but not to phosphatidylethanolamine, phosphatidylcholine, or cholesterol, consistent with the ELISA results described above.

Comparison of Galectins-1, -3, -4, and -8 Binding to Cholesterol 3-Sulfate—Because the strong binding of galectin-4 to cholesterol 3-sulfate was observed, we next studied whether other members of the galectin family could also bind to cholesterol 3-sulfate. The binding abilities of His-tagged galectin-1, -3, and -4 were compared using an anti-His tag antibody and an HRP-conjugated secondary antibody. The binding ability of galectin-8 was measured using anti-galectin-8 antibody and an HRP-conjugated secondary antibody. When each galectin (1 μg/ml) in 50 μl of 1% BSA in PBS was applied to various amounts of cholesterol 3-sulfate-coated plates, only galectin-4 bound to cholesterol 3-sulfate (Fig. 1D, □). Galectins-1, -3, -8 did not bind to cholesterol 3-sulfate (Fig. 1D, □, ▲, △, respectively), even at 10 μg/well of cholesterol 3-sulfate. When various concentrations of each galectin were applied to the cholesterol 3-sulfate-coated plates, galectin-4 bound to cholesterol 3-sul-
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FIGURE 1. Binding of galectin-4 to various lipids. A, the relative binding of galectin-4 (90 ng) to lipoid-coated plates. The amount of bound galectin-4 was measured using an anti-galectin-4 antibody and alkaline phosphatase-conjugated anti-rabbit IgG and pNPP as described under “Experimental Procedures.” Backgrounds without galectin-4 were subtracted. B and C, binding of galectin-4 to suspended lipids. The mixtures of galectin-4 and each lipid were incubated, followed by centrifugation. The pellets were solubilized with Laemmli sample buffer and subjected to SDS-PAGE. The galectin-4 was visualized with Sypro Orange or Coomassie Brilliant Blue R-250 (C). Cho, cholesterol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; Sch, cholesterol 3-sulfate. D and E, the relative binding of galectin-4 ( ), galectin-1 ( ), galectin-3 ( ), and galectin-8 ( ) to cholesterol 3-sulfate. Fifty μl of galectin (1 μg/ml) were applied to plates coated with different amounts of cholesterol 3-sulfate (Δ). Different concentrations of galectins-1, 3, 4, and 8 were applied to the cholesterol 3-sulfate-coated plate (1 μg/well) (E). The amounts of bound His-tagged galectins-1, 3, and 4 were measured using an anti-His tag antibody and HRP-conjugated anti-mouse antibody. The amount of bound galectin-8 was measured using anti-galectin-8 antibody and HRP-conjugated anti-rabbit antibody as described under “Experimental Procedures.” Backgrounds without galectins were subtracted.

In a dose-dependent manner (Fig. 1E, ). However, galectins-1, -3, and -8 did not bind (Fig. 1E, , ). Although the GST-galectin-8-N-domain bound to SM3, GM3, and SM4 (4), it did not bind to cholesterol 3-sulfate. These results indicate that galectin-4 has a unique ability to bind cholesterol 3-sulfate, unlike galectins-1, -3, and -8.

Inhibition of Galectin-4 Binding to Cholesterol 3-Sulfate by Anionic Polysaccharides—The binding ability of galectin-4 to cholesterol 3-sulfate was divalent cation-independent, because the binding was not influenced by the addition of 10 mM EDTA (data not shown).

To clarify the basis for the specificity of galectin-4 binding to cholesterol 3-sulfate, carbohydrates and anionic polysaccharides were tested as inhibitors using ELISA. When 1.4 pmol (50 ng/50 μl) of galectin-4 and various concentrations of inhibitors were applied to the cholesterol 3-sulfate-coated plates, the IC50 values for dextran sulfate, fucoidan, heparin, keratan sulfate, A-tetra, lactose, and choledrin sulfite were calculated to be 0.04, 0.07, 15, 500, 1 × 104, and >1 × 104 μg/ml, respectively (Fig. 2). The IC50 value of dextran sulfate is 0.04 μg/ml, which means ~2 ng in 50 μl contain at least 3.4 pmol of 3-O-sulfated glucose (calculated as one 3-O-sulfated glucose per 2 glucose units). On the basis of such a stoichiometry, the best inhibitors of galectin-4 binding to cholesterol 3-sulfate were dextran sulfate and fucoidan, which have sulfate residues at the C-3 and/or C-2 positions of glucose and fucose, respectively. Heparin sulfate has sulfate residues at the C-4 or C-6 positions of N-acetylgalactosamine, and keratan sulfate has sulfate residues at the C-6 position of galactose and N-acetylglucosamine. Heparin has a sulfate residue at C-2, -3, or -6 of glucosamine. These results suggest that galectin-4 strongly recognizes 3-O- or 2-O-sulfated glycan, but not 4-O- or 6-O-sulfated glycans, irrespective of their monosaccharide compositions. These structural differences may reflect their inhibitory strengths toward the galectin-4/cholesterol 3-sulfate interaction. Similarly, it has been reported that the binding of 200 ng/ml of thrombospondin to sulfatide was inhibited by 2.3 ng/ml of dextran sulfate (19).

Roles of the Lys44 and Arg55 Residues in Galectin-4-N-domain Binding—We next investigated which amino acids of galectin-4 were responsible for its binding to cholesterol 3-sulfate.

X-ray crystal structures of human galectins-2, -3, -7, and -10 showed that these galectins have very similar tertiary structures (20–22). Site-directed mutants of galectins have been studied only with β-galactoside. The amino acids that are critical for interaction. Similarly, it has been reported that the binding of 200 ng/ml of thrombospondin to sulfatide was inhibited by 2.3 ng/ml of dextran sulfate (19).

FIGURE 2. Inhibition curves of galectin-4 binding to cholesterol 3-sulfate by carbohydrates and anionic polysaccharides. The relative binding abilities of 50 ng of galectin-4 in the presence of lactose ( ), A-tetra ( ), heparin-β ( ), keratan sulfate ( ), chondroitin sulfite A ( ), chondroitin sulfite C ( ), fucoidan ( ), and dextran sulfate ( ) were measured as described under “Experimental Procedures.” Background without galectin-4 was subtracted. The relative binding abilities were calculated by dividing the values of galectin-4 binding with inhibitors by the values without inhibitor.
Galectin-4, like galectin-8, consists of two tandemly repeated carbohydrate recognition domains. Amino acids in the S3 \( \beta \)-sheet are likely to be involved in the binding of sialic acid/sulfate residues, because galactose-3-O-linked nonreducing terminal moieties were deduced to interact with amino acids in the extended clefts formed by the S3 \( \beta \)-sheet of galectins. We investigated which amino acids in the galectin-4-N-domain interact with sulfate residues. Comparison of the amino acids in the S3 sheets of galectins showed that only the galectin-4-N-domain had the successive basic amino acids Lys\(^{44} \) and Arg\(^{45} \) (Fig. 3). Therefore, we constructed two mutated galectin-4s, K44A and R45A, and we analyzed their carbohydrate-binding abilities.

The binding of oligosaccharides to the wild type and mutant (K44A and R45A) galectin-4-N-domains, which were individually immobilized on CM5 sensor chips, was measured using the SPR assay (Fig. 4). The wild type and mutant N-domains showed similar affinities toward A-tetra (Fig. 4, A–C) and other neutral oligosaccharides (data not shown), indicating that the binding ability toward these oligosaccharides did not differ much between the wild type and the mutants. Interestingly, SO\(_3\)-core 1 did not bind to the R45A mutant (Fig. 4F), suggesting that Arg\(^{45} \) is important for sulfate residue recognition.

**The Binding Ability of the K226V Mutant of the Galectin-4-C-Domain to Sulfated Oligosaccharides**—The galectin-4-C-domain has two basic amino acids, Lys\(^{219} \) and Lys\(^{226} \), in the S3 \( \beta \)-sheet (Fig. 3). A comparison of the amino acids in the S3 \( \beta \)-sheets of galectins showed that Lys\(^{219} \) is conserved in galectin-1, the galectin-4-N-domain, and the galectin-8-C-domain, whereas Lys\(^{226} \) is not conserved among galectins. Because our earlier study revealed that galectin-1 and the galectin-8-C-domain did not bind to SM4 (4), Lys\(^{219} \) seems not to be responsible for the binding to sulfated glycosphingolipids. Therefore, we constructed a mutated galectin-4-C-domain with Val\(^{226} \) replacing Lys\(^{226} \) (K226V). We constructed and purified the wild type and mutated GST-galectin-4-C-domains and purified the GST-free form of the galectin-4-C-domains, because the galectin-4-C-domains could not be expressed via the expression plasmid for the N-domain. However, the wild type and mutant C-domains showed similar affinities toward neutral and sulfated oligosaccharides (data not shown), indicating that the binding ability for these oligosaccharides does not differ much between the wild type and the mutant. These results suggest that Lys\(^{226} \) is not directly involved in sulfate-residue recognition.

**The Arg\(^{45} \) Residue of Galectin-4 Is Important for Cholesterol 3-Sulfate Recognition**—The results so far described suggest that the Arg\(^{45} \) residue of galectin-4 is involved in cholesterol 3-sulfate recognition. To further confirm this, we prepared full-length galectin-4 mutants, including K44A, R45A, and K226V, and we compared their binding abilities to cholesterol 3-sulfate. The wild type and all of the mutants bound to the asialofetuin column and were eluted with lactose. When the full-length galectin-4 and K44A, R45A, and K226V were introduced to the wild type (A and D) and mutant K44A (B and E) and R45A (C and F) galectin-4-N-domains immobilized on surfaces, at a flow rate of 20 \( \mu \)l/min for 180 s. The relative response (RU) was determined by subtracting the blank values obtained on the nonimmobilized surface from the values obtained on the galectin-4-N-domain-immobilized surfaces.
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K226V mutants (1 μg/ml) in 50 μl of 1% BSA in PBS were applied to plates coated with various amounts of cholesterol 3-sulfate, the wild type and the K44A and K226V mutants all bound to the same extent (Fig. 5A, ●, ■, and □, respectively). In contrast, the R45A mutant had diminished binding to cholesterol 3-sulfate (Fig. 5A, ○). When various concentrations of the wild type galectin-4 and the K44A, R45A, and K226V mutants were applied to the cholesterol 3-sulfate-coated plate (1 μg/well), galectin-4 and the K44A and K226V mutants bound to cholesterol 3-sulfate in a dose-dependent manner (Fig. 5B, ●, ■, □, respectively). However, the full-length galectin-4-R45A mutant had diminished binding ability to cholesterol 3-sulfate (Fig. 5B, ○). These results strongly support that Arg45 in full-length galectin-4 is indispensable for cholesterol 3-sulfate recognition.

**The Galectin-4 Dimer Exists in Solution** — The apparent molecular weight of galectin-4 in solution was determined using a Superose-12 gel-filtration column (Fig. 6A). The galectin-4 eluted at the positions of peaks I and II, which were calculated to be 48 and 24 kDa, respectively. SDS-PAGE analysis showed that both peaks I and II migrated as 34-kDa proteins, close to the calculated size of 37 kDa of His-tagged galectin-4 (Fig. 6C). The difference in the behaviors on Superose-12 column chromatography and SDS-PAGE may be because of some hydrophobic interaction between galectin-4 and Superose 12, because some hydrophobic interaction has been noted between Superose 12 and compounds such as membrane proteins and lipoproteins which elute later than predicted (manufacturer’s handbook). Peaks I and II would correspond to the dimer and monomer of galectin-4, respectively. Galectin-8 eluted as a single peak at the position calculated to be 35 kDa, close to its theoretical monomeric size of 34 kDa.

The binding abilities of the respective fractions to cholesterol 3-sulfate (Fig. 6A, ●) and asialofetuin (Fig. 6B) were also measured. The dimeric form of galectin-4 showed relatively higher binding to both cholesterol 3-sulfate (Fig. 6A, ●) and asialofetuin (Fig. 6B) than the monomeric form. This implies that a substantial amount of full-length galectin-4 dimerizes in solution, enhancing its carbohydrate-binding ability. The His-tag-free galectin-4 also eluted as two peaks at positions slightly smaller than peaks I and II (data not shown).

We also probed the dimerization state of galectin-4 by chemically cross-linking with DSS (Fig. 6D). After cross-linking with DSS, SDS-PAGE showed that full-length galectin-4 formed a dimer in solution (Fig. 6D), indicating that dimeric galectin-4 has four CRDs in solution. In contrast, most of the galectin-8, before and after cross-linking with DSS, migrated to the same position as the monomeric form on SDS-PAGE (Fig. 6D). The formation of a multivalent form of galectin-4 might enhance its binding ability to cholesterol 3-sulfate and may have a role in various biological functions.

**Cholesterol 3-Sulfate Is the Major Galectin-4-binding Sulfated Lipid in the Esophagus** — It has been reported that the molar percentage of cholesterol 3-sulfate in the total sulfolipids in various rabbit digestive tract tissues decreases in the order of esophageal epithelium (100%), gastric mucosa (92%), and jejunal mucosa (35%) (23). A 37-kDa protein, which was later identified as galectin-4, was also reported in the porcine esophagus (24, 25). To elucidate whether endogenous galectin-4 binds, not only sulfatide but also to cholesterol 3-sulfate, we examined whether galectin-4 and cholesterol 3-sulfate co-existed in the Triton X-100-insoluble fraction of the porcine esophagus at 37 °C in comparison with the intestine. First, total lipid extracts were separated by TLC and visualized with azure A (Fig. 7A). The mobility of the major azure A-staining bands of the porcine esophagus was also evaluated (* in Fig. 7A). In the case of the intestine, the major azure A-staining bands corresponded to those of authentic cholesterol 3-sulfate. An unidentified azure A-positive band was also detected (* in Fig. 7A). When azure A-staining bands were extracted and individually coated on ELISA plates, galectin-4 bound to each of these sulfated lipids (data not shown).

The porcine galectin-4 in the esophagus and intestine were recovered in the Triton X-100-insoluble fraction at 37 °C (Fig. 7B). The main sulfated lipids co-existing with galectin-4 in the insoluble fractions were identified as cholesterol 3-sulfate for the esophagus and SM4 for the intestine (Fig. 7C). These results suggested that cholesterol 3-sulfate and SM4 bind to galectin-4 in the microdomains of esophageal epithelium, respectively.

**DISCUSSION**

In this study, we clearly demonstrate that galectin-4, a member of the galectin family, binds to cholesterol 3-sulfate. This characteristic of galectin-4 is unique in the galectin family. Galectin-4 binding to cholesterol 3-sulfate is not a simple ionic interaction, because proteoglycans, including keratan sulfate containing sulfated-6-galactose, and chondroitin sulfates A and C containing sulfate-6 or -4-N-acetylgalactosamine residues, are not good inhibitors of galectin-4...
though they have no galactose moieties. The binding site of galectin-4 to cholesterol 3-sulfate seems to be located in or near the carbohydrate recognition domain, because lactose and A-tetra also slightly inhibit this binding. It appears that galectin-4 recognizes the sulfate residue attached to certain structures in the target molecule.

Amino acids facing the carbohydrate-binding sites in the S4, S5, and S6 β-sheets are conserved among various galectins. However, those in the S2 and S3 β-sheets are not conserved among galectins. Accordingly, we focused on specific amino acids in the S3 β-sheet of both the N- and C-domains, to determine the amino acid(s) responsible for sulfate-residue recognition. When the Arg45 residue in the S3 β-sheet of galectin-4 is converted to Ala45, the cholesterol 3-sulfate-binding ability is reduced, indicating that this specific amino acid residue in the S3 β-sheet is indispensable for the specific recognition of cholesterol 3-sulfate. Similar results have been observed in the galectin-8 N-domain. When the Gln47 residue in the S3 β-sheet of the galectin-8 N-domain is converted to Ala47, the specific affinity for sulfated or sialylated glycans is selectively lost (4). Further examination of the galectin-4 N-domain and the galectin-8 N-domain by x-ray-crystallographic analysis may help to resolve their precise recognition mechanisms.

The sulfate substitution at the C-3 position of β-galactosyl residues in oligosaccharides increases the binding to not only galectin-4 but also to galectin-3 (1) and galectin-1 (5). This enhancement of binding ability by 3-O-sulfation of β-galactose residues in oligosaccharides may be due to the specific Arg or Lys residue in the S3 β-sheet. However, galectins-1 and -3 do not bind to sulfated glycosphingolipids as galectin-4 does. The galectin-4 N- and C-domains themselves dissociate from sulfated glycosphingolipids very quickly (data not shown). These results suggest that recognition of 3-O-sulfated β-galactose is not enough
for binding to sulfated lipids. Experiments using chemical cross-links have revealed that oligomerization of the GST-galectin-4-C-domain enhances the binding to glycosphingolipids. Lys on the S3 β-sheet influences the oligomerization and its mutation reduces the binding to glycosphingolipids and asialofetuin. The GST-galectin-8-N-domain and GST-galectin-8 also bind to sulfated and sialylated glycosphingolipids. However, GST-free galectin-8 dissociates from its ligands very quickly (data not shown), because only the N-domain has affinity for sulfated and sialylated ligands, even though full-length galectin-8 has two CRDs.

Gel filtration analysis and the chemical cross-linking experiment reveal that a portion of the galectin-4 exists in dimeric form, indicating that a portion of galectin-4 exists as a complex with four CRDs. The dimeric form of galectin-4 has a stronger affinity for ligands, suggesting that this high affinity binding is because of its multivalency. Furthermore, some hydrophobic interaction with the column was observed for galectin-4 but not for galectin-8. These results may partly explain the specific binding character of galectin-4 toward cholesterol 3-sulfate, which is unique within the galectin family.

Although cholesterol 3-sulfate is widely distributed in human tissues, its physiological roles are not well understood (for a review see Ref. 26). Cholesterol 3-sulfate has emerged as an important component of cell membranes, protecting erythrocytes from osmotic lysis and regulating sperm capacitation. It also can control the activities of serine proteases, which are involved in blood clotting, fibrinolysis, and epidermal cell adhesion. In addition, it regulates the activities of selective protein kinase C isoforms and modulates the specificity of phosphatidylinositol 3-kinase, which is involved in signal transduction. The possible exchange of cholesterol for cholesterol 3-sulfate inside cells could represent a mode of regulation of the intraacellular cholesterol level, because it has been shown that cholesterol 3-sulfate inhibits cholesterol esterification (27) and that cholesterol 3-sulfate can potently modulate hydroxymethylglutaryl-CoA, which is the rate-limiting enzyme for cholesterol synthesis (28).

Galectin-4 is abundant not only in the epithelium of the alimentary tract but also in tissues which have little sulfated glycosphingolipid (29). Therefore, cholesterol 3-sulfate, instead of sulfated glycosphingolipids, could be a ligand for galectin-4 in those tissues. In support of this, the cholesterol 3-sulfate content decreases progressively in the esophagus, stomach, duodenum, and jejunum, whereas the sulfatide content increases progressively in these same organs (23). Mouse, rat, and cod intestines do not contain SM4 and, instead, are enriched for cholesterol 3-sulfate, suggesting that cholesterol 3-sulfate can replace SM4 (29). Because the intracellular distribution and biological function of galectin-4 in these respective tissues may depend on which molecules bind to galectin-4, the interaction between galectin-4 and cholesterol 3-sulfate is particularly interesting. The development of a cholesterol 3-sulfate-specific probe might answer these questions.

Because the rate of intermembrane exchange for cholesterol 3-sulfate is about 10-fold faster than that for cholesterol in liposomal membranes (30), galectin-4 binding to cholesterol 3-sulfate may affect the secretion mechanism of galectin-4 from the plasma membrane. Galectin-4 was first discovered as a 17-kDa protein in rat intestine and was identified as the rat galectin-4 C-domain. Full-length rat galectin-4 was found by Western blotting only from freshly isolated intestine that was homogenized in 4 M guanidine- HCl to inactivate all proteases (31). This suggests that the linking region of galectin-4 is susceptible to protease digestion. Our present study shows that full-length galectin-4 has much greater affinity for its ligands. Accordingly, the cleavage of galectin-4 into each domain by endogenous proteases may be one of the regulatory mechanisms controlling galectin-4 lectin activity.

It has been reported that the sulfogalactose moiety of sulfoglycosphingolipids serves to mimic tyrosine phosphate, suggesting that vicinal sulfate or phosphate in either an aryl or sugar ring can present for ligand binding (32). Because carbohydrate specificity is achieved through a combination of hydrogen bonding to sugar hydroxyl groups and hydrophobic interaction between an apolar patch of the sugar face and aromatic amino acid side chains (33), the cholesterol moiety in cholesterol 3-sulfate, as an apolar molecule, may mimic the apolar character of galactose. Lectins often bind to natural polysaccharides with high affinities. In contrast, their interactions with oligosaccharides are far weaker (33). Increased affinity for oligosaccharides results from the clustering of simple binding sites in oligomers of the lectin polypeptides (33). This geometry of the oligomeric lectin gives it the ability to distinguish among or cross-link glycoconjugates. We have clearly demonstrated that galectin-4 binds strongly to cholesterol 3-sulfate, which lacks the β-galactoside moiety. Because the data concerning
interactions between oligosaccharides and lectins reveal only one aspect of the lectin activity, we will reinvestigate the binding ability of galectin family members to various compounds, including sulfate, sialic acid, and phosphate residues and lipids. This will help address the question of why so many galectins with different localizations and functions exist in organisms from *Caenorhabditis elegans* to humans (3).

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