Galectin-4 Binds to Sulfated Glycosphingolipids and Carcinoembryonic Antigen in Patches on the Cell Surface of Human Colon Adenocarcinoma Cells

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Galectin-4, a member of the galectin family, is expressed in the epithelium of the alimentary tract. It has two tandemly repeated carbohydrate recognition domains and specifically binds to an SO₃^⁻-3Galβ1-3GalNAc pyranoside with high affinity (Ideo, H., Seko, A., Ohkura, T., Matta, K. L., and Yamashita, K. (2002) Glycobiology 12, 190–208). In this study, we found that galectin-4 binds to glycosphingolipids carrying 3-O-sulfated Gal residues, such as SB1a, SM3, SM4s, SB2, SM2a, and GM1, but not to glycosphingolipids with 3-O-sialylated Gal, such as sLeC4Er, snLeC4Er, GM3, GM2, and GM4, using both an enzyme-linked immunosorbent assay and a surface plasmon resonance assay. A confocal immunocytochemical assay showed that galectin-4 was colocalized with SB1a, GM1, and carcinoembryonic antigen (CEA) in the patches on the cell surface of human colon adenocarcinoma CCK-81 and LS174T cells. This localization was distinct from caveolin/VIP21 staining. Furthermore, immobilized galectin-4 promoted adhesion of CCK-81 cells through the sulfated glycosphingolipid, SB1a. CEA also bound to galectin-4 with K_D value of 2 × 10⁻¹⁰ M by surface plasmon resonance and communoprecipitated with galectin-4 in LS174T cell lysates. These findings suggest that SB1a and CEA in the patches on the cell surface of human colon adenocarcinoma cells could be biologically important ligands for galectin-4.

Galectins are a family of animal lectins defined by their affinity for β-galactoside-containing saccharides and by common amino acid sequence elements. They are involved in regulating diverse biological phenomena, including proliferation, apoptosis, and cell-cell, or cell-matrix interactions (1, 2). Although galectins do not have any secretion signal peptide, they are often found outside of cells (1, 3). Galectin-4 has two carbohydrate recognition domains (CRDs) and is expressed in the epithelium of oral mucosa, esophagus, and intestinal and colonic mucosa (4, 5). It has been reported that galectin-4 is present in certain fractions of rafts in the brush border membrane of pig intestine (6, 7). However, it is not clear how the soluble galectin-4 could be a component of rafts and with which molecules galectin-4 would interact.

We previously reported that galectin-4 binds to SO₃^-3Galβ1-3GalNAc pyranoside with high affinity (8), and this structure occurs not only in O-linked oligosaccharides but also in certain glycosphingolipids (9). In further investigations of endogenous ligands of galectin-4, we were interested in determining whether glycosphingolipids carrying the 3-O-sulfated galactose residues bind to galectin-4, because galectin-8, which also has two conserved CRDs and is 34% identical to galectin-4 at amino acid levels, binds to glycosphingolipids carrying SO₃^-3/sialylα2-3Gal residues (10). Kopitz et al. (11, 12) reported that antibodies against GM1 inhibited galectin-1 binding to neuroblastoma cells, suggesting that galectin-1 binds to GM1 or to glycoprotein receptors in close proximity to GM1 on the cell surface. These reports suggested that glycosphingolipids could also be biologically important ligands for some members of the galectin family.

We also examined whether galectin-4 binds to carcinoembryonic antigen (CEA), and both molecules are colocalized in the cell surface of human colon adenocarcinoma, because CEA is a glycosylphosphatidylinositol (GPI)-anchored glycoprotein (13), which carries 25–27 mol of N-glycans per molecules (14), and is produced by gastrointestinal tumor cells.

We show in this study that galectin-4 binds to glycosphingolipids carrying SO₃^-3Gal residues and CEA, using an enzyme-linked immunosorbent assay (ELISA) and a surface plasmon resonance (SPR) assay. Furthermore, galectin-4 on the cell surface of human colon adenocarcinoma CCK-81 and LS174T cells is colocalized in the patches with SB1a, GM1, and CEA, and this localization differed from caveolin/VIP21 staining on the cell surface, as determined by a confocal immunocytochemical assay.

EXPERIMENTAL PROCEDURES

Materials—Galβ1-3GalNAc1-4-O-benzyl (core 1-O-Bn), TRITC-anti-rabbit IgG, and fluorescein isothiocyanate-anti-mouse IgG were core 1, Galβ1-3GalNAc; lactose, Galβ1-4Glc; A-tetra, GalNAcα1-3-Fucα1-2Galβ1-4Glc; LNF-1, Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc; RU, resonance units; Cer, ceramide; GM1, Galβ1-3GlcNAcβ1-4Neu5Acα2-3Galβ1-4Glc-Cer; GM2, GalNAcβ1-4Neu5Acα2-3Galβ1-4Glc-Cer; GM3, Neu5Acα2-3Galβ1-4Glc-Cer; GM4, NeuAcα2-3Galβ1-4Glc-Cer; SB1a, SO₃^-3Galβ1-3GalNAcβ1-4SO₃^-3Galβ1-4Glc-Cer; SB2, SO₃^-3GalNAcβ1-4SO₃^-3Galβ1-4Glc-Cer; SM2a, GalNAcβ1-4SO₃^-3Galβ1-4Glc-Cer; SM3, SO₃^-3Galβ1-4Glc-Cer; SM4, SO₃^-3Gal-Cer.
Galectin-4 binds to sulfated glycosphingolipids in patches

Preparation of sulfoglycosphingolipids—Crude lipids of homogenized CCK-81 cells (5 × 10^6 cells) were extracted once with 19 volumes of chloroform/methanol (2:1, v/v) and then with 20 volumes of chloroform/methanol/water (1:2:8, v/v). The pooled extracts (the total lipid extracts) were dried and dissolved in chloroform/methanol (1:1, v/v). The solution was spotted on a high-performance thin layer chromatography plate (10 × 10 cm, Kieselgel 60, Merck, Darmstadt, Germany) and developed using chloroform/methanol/0.2% CaCl_2 (60:35:7) as the solvent. Lipids were detected by using primuline (5 mg in 100 ml of acetone/water (80/20, v/v)) and visualized with LAS1000 (Fujiﬁlm, Japan). Sulfated glycosphingolipids were monitored at 15 °C. Phospholipids are detected with Dittmer-Lester reagent (16) and ninhydrin reagent for amino groups.

Estimation of Kinetic Constants Based on SPR—The dissociation constants between each of the CRDs of galectin-4 and various carbohydrates were measured using a BIAcore 2000 instrument (Biacore AB, Uppsala, Sweden) as described previously (8). ELISA for binding of galectin-4 to various glycosphingolipids—ELISAs for binding of galectin-4 to various glycosphingolipids using recombinant galectin-4 and anti-galectin-4 were performed in the same manner as described previously (10).

Binding of Galectin-4 to Glycosphingolipids Immobilized on the Surface of BIAcore Sensor Chip—Glycosphingolipids were hydrophobically adsorbed onto the CM5 sensor chip according to Catimel et al. (17). Glycosphingolipids (1 ng/ml) were dissolved in ETOH/MeOH (9:1, v/v), diluted with HBS buffer (10 mM HEPES (pH 7.4), 3.4 mM EDTA, 150 mM NaCl), and injected (80 μl) at a flow rate of 5 μl/min over the unmodified surface.

Purified rhgalectin-4 in HBS buffer was introduced onto the surface at a flow rate of 20 μl/min. The interaction between rhgalectin-4 and glycosphingolipids was monitored at 25 °C, and the kinetic constants were calculated using BIAEvaluation 3.0 software.

Immunofluorescence Analysis—Human colon adenocarcinoma CCK-81 and LS174T cells grown on coverslips were washed with PBS and fixed in 4% paraformaldehyde for 15 min, followed by washing and incubation with 1% BSA for 1 h at room temperature. For double labeling, cells were incubated at 4 °C overnight with anti-galectin-4 and anti-CEA (mouse IgG), anti-caveolin-1/VIP21 (mouse IgG), or anti-caveolin-1/VIP21 (mouse IgG) antibodies in PBS containing 0.1% BSA. After extensive washing, the coverslips were mounted on slides. Fluorescent images were obtained using a confocal laser scanning microscope (LSM 5 PASCAL, Carl Zeiss, Germany). Images were acquired in the multitrack mode to avoid signal cross-talk. The specificity of labeling was assessed by incubation with control primary antibodies.

Cell Adhesion Assay—ELISA plates were pre-coated with rhgalectin-4 in PBS for 16 h at 4 °C, followed by blocking with 1% BSA for 2 h at 37 °C. CCK-81 cells and Chinese hamster ovary-K1 cells on tissue culture plates were detached with 0.25% trypsin and 0.02% EDTA in PBS. After washing with culture medium, cells were resuspended in serum-free medium containing 10 mM EDTA and re-seeded onto the galectin-4-coated plates. After incubating at 37 °C for 30 min, the plates were washed three times with PBS, and the adherent cells were stained with 0.2% crystal violet in PBS containing 20% methanol for 15 min at 22 °C. Excess dye was washed with water, and the bound cells were solubilized in 1% SDS for 1 h at 22 °C and quantified by measuring the absorbance at 595 nm with a spectrophotometer. All assays were performed in triplicate.

Binding of CEA to Galectin-4 Immobilized on the Surface of BIAcore Sensor Chip—The affinity of CEA and galectin-4 was measured by SPR assay using BIAcore 2000 instrument. Galectin-4 was immobilized on a CMS sensor chip by the amine-coupling method. The coupling density (RU) of various concentrations of CEA was determined by BIAcore HBS buffer (10 mM HEPES, pH 7.4, 3.4 mM EDTA, 150 mM NaCl) 150 μl 0.05% (v/v) surfactant P-20 and injected onto the sensor chip at 20 μl/min. The sensor surface was regenerated by 0.1 M lactate.

Immunoprecipitation—1 × 10^7 of LS174T cells were solubilized 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)) for 1 min at 4 °C and harvested. The postnucleoprotein supernatants were collected following centrifugation at 15,000 rpm for 10 min. Aliquots of the supernatants were incubated with 60 μl of an anti-galectin-4 conjugated-Sepharose 4B (5.5 mg of IgG/ml gel). Following immunoprecipitation, the beads were washed five times with lysis buffer and boiled with Laemmli sample buffer for 5 min. Samples were subjected to SDS-PAGE (12.5% acrylamide) followed by blotting onto a nitrocellulose membrane. The blots were incubated with anti-CEA and anti-galectin-4 antibodies followed by

Preparation of Antibody—Antiserum against human galectin-4 was raised in rabbit according to standard procedures. Purified rhgalectin-4 (8) (1–1.5 mg) was injected into a rabbit (initial and two boosts) at intervals of 3 weeks.

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**RESULTS**

Carbohydrate Binding Specificities of Galectin-4 N-domain and C-domain to Oligosaccharides—Galectin-4 has two conserved CRDs, which share 34% amino acid identity. Because we found that N- and C-domains of galectin-8 have distinct carbohydrate-binding specificities (10), we prepared GST-fused recombinant proteins corresponding to the N-domain and the C-domain of galectin-4 (GST-N-domain and GST-C-domain, respectively) and compared their binding affinities toward oligosaccharides that had showed some binding ability to the full-length galectin-4 in our previous study (8). Binding of various oligosaccharides to the immobilized recombinant proteins was measured by SPR. As summarized in Table II, the GST-N-domain and the GST-C-domain showed similar affinity toward three oligosaccharides, SO$_3$$^-$$\beta$Gal-β1→3GalNAc, A-tetra, and LNF-I. On the other hand, the GST-C-domain showed a 12-fold higher affinity than the GST-N-domain for the SO$_3$$^-$$\beta$Gal-β1→3core 1-O-Bn oligosaccharide. These results indicated that the two domains of galectin-4 have different carbohydrate-binding specificities with respect to SO$_3$$^-$$\beta$Gal-β1→3core 1-O-Bn, which is a better ligand for the GST-C-domain than for the GST-N-domain.

**Galectin-4 Binds to Sulfated Glycosphingolipids**—We found that galectin-4 bound SO$_3$$^-$$\beta$Gal-β1→3GalNAc with high affinity (8), and this structure occurs in certain glycosphingolipids. To investigate the interaction of galectin-4 with various glycosphingolipids (shown in Table I), we first measured the binding of galectin-4 to glycosphingolipid-coated plates, using an antibody against galectin-4. As shown in Fig. 1, galectin-4 bound strongly to SB1a, SM3, SM4s, and SB2, and bound weakly to GM1, SM2a, and GD1a. GaCer, LacCer, GM1, GM2, GM4, sLeCer, and smLeCer were not recognized by galectin-4, at least at the concentrations tested. These results indicated that galectin-4 recognizes the glycosphingolipids carrying 3-O-sulfated Gal residue, because this moiety is common among SB1a, SM3, SM4s, SB2 and SM2a. However, glycosphingolipids with a 3-O-sialylated Gal, such as sLeCer, snLeCer, GM3, GM4, and sLe4Cer, were not good ligands for galectin-4. This is in agreement with the fact that galectin-4 has a high affinity for SO$_3$$^-$$\beta$Gal-β1→3GalNAc but does not recognize 3'sialyl lactose (8). Galectin-4 also bound to GM1; this could be explained by the fact that galectin-4 has a weak affinity for the Galβ1→3GalNAc structure (8).

We also measured the binding of GST-N- and C-domains to glycosphingolipids. As shown in Fig. 2, both domains bound to glycosphingolipids tested; however, the affinity of GST-C-domain toward 3-O-sulfated glycosphingolipids SB1a and SM3 was higher than that of GST-N-domain. This characteristic of galectin-4 is different from galectin-8, of which only the N-domain has high affinity for glycosphingolipids (10).

The binding of galectin-4 to immobilized glycosphingolipids was further examined by SPR. As shown in Fig. 3, galectin-4 bound to SB1a and GM1 dose dependently and dissociated slowly (Fig. 3, A and B), but not to GM3 (Fig. 3C). The $K_D$ value to SB1a was 18.2 times lower than that of GM1. These results were in good agreement with the results of the ELISA.

**Table II**

| Oligosaccharide | Rhgalectin-4$^a$ | GST-N-domain | GST-C-domain |
|----------------|----------------|---------------|---------------|
| Galβ1→4Glc (lactose) | $8.6 \times 10^{-4}$ (1.0)$^b$ | $1.0 \times 10^{-4}$ (8.6) | $7.8 \times 10^{-5}$ (11) |
| SO$_3$$^-$$\beta$Gal-β1→4Glc-O-pNP | $7.5 \times 10^{-5}$ (11) | ND | ND |
| Galectin-4 | $5.2 \times 10^{-5}$ (17) | $3.9 \times 10^{-5}$ (22) | $2.4 \times 10^{-5}$ (36) |
| Fucα1→2Galβ1→3GlcNacβ1→4Glc (LNF-I) | $9.6 \times 10^{-5}$ (9.0) | $8.9 \times 10^{-5}$ (9.7) | $1.2 \times 10^{-4}$ (7.2) |
| Galβ1→3GalNAc (core 1) | $2.2 \times 10^{-4}$ (3.9) | ND | ND |
| SO$_3$$^-$$\beta$Gal-β1→3GalNAc1-O-Bn(SO$_3$$^-$$\beta$Gal-β1→3core 1-O-Bn) | $3.4 \times 10^{-6}$ (250) | $2.3 \times 10^{-5}$ (37) | $2.0 \times 10^{-6}$ (430) |

$^a$ Ido et al. (8).

$^b$ The relative binding abilities in the parentheses were calculated by dividing the $K_D$ value for lactose binding to rhgalectin-4 by the $K_D$ values for oligosaccharides.

$^c$ ND, not determined.
Galectin-4 is specifically expressed in intestine, colon, and stomach (5), we next investigated whether these sulfoglycosphingolipids exist in these tissues. Our preliminary experiment showed that sulfoglycosphingolipids, including SM4, SM3, SM2, and SB1a, exist in chloroform-methanol extracts of mouse intestine, and galectin-4 and SB1a were stained in normal human colon epithelium in preliminary immunohistochemical study (data not shown). For further study, we searched human colon adenocarcinoma cell lines that express galectin-4. Among eight cell lines tested, we found that CCK-81 cells produce higher levels of galectin-4.

We first examined what kinds of glycosphingolipids exist in human colon adenocarcinoma CCK-81 cells. Chloroform-methanol extracts were separated by TLC and visualized with both primuline (Fig. 4A, lane 1) and Azure A (Fig. 4A, lane 2). Mobilities of primuline staining-positive bands a, b, c, d, e, f, and g (Fig. 4, lane 1) corresponded to those of authentic phosphatidylethanolamine (and SM4), SM3, phosphatidylaminositol, phosphatidylserine (and SM2), GM3, SB1a, and GM1, respectively, and bands a, b, c, d, and f were also stained with Azure A reagent (Fig. 4, lane 2). Faint or no bands were detected by primuline staining at the positions for authentic GM1 and GD1a. When the bands a to g were extracted and individually coated on ELISA plates, galectin-4 bound to each of these glycosphingolipids a, b, and f, which correspond to authentic SM4, SM3, and SB1a, respectively, and did not bind to bands c, d, e, and g (Fig. 4B). When we detected the phospholipids with Dittmer-Lester reagent, bands a, c, and d were also stained (data not shown) and bands a and d were also stained with ninhydrin reagent. Sufficient amounts of authentic phosphatidylaminositol and phosphatidylserine were Azure A-positive, suggesting that primuline-positive bands a, c, and d contain phosphatidylethanolamine, phosphatidylaminositol, and phosphatidylserine, respectively. Galectin-4-binding component in band a should be SM4, because galectin-4 did not bind to phosphatidylethanolamine (data not shown). These results indicated that galectin-4 reactive-sulfated glycosphingolipids are prominent in human colon adenocarcinoma CCK-81 cells, in comparison with sialylated glycosphingolipids on the basis of comparative primuline staining. The similar results for lipid compositions were also obtained in LS174T cells (data not shown).
Galectin-4 binds to sulfated glycosphingolipids in patches on the cell surface of CCK-81 and/or LS174T cells—Subsequently, we studied whether sulfated glycosphingolipids are colocalized with galectin-4 on the cell surface of human colon adenocarcinoma cell lines. Interestingly, when we stained the cell surface of CCK-81 and LS174T cells with anti-galectin-4, galectin-4 was localized in patches on the cell surface (Fig. 5). The anti-SB1a stained the same area as anti-galectin-4, suggesting the colocalization with galectin-4 (Fig. 5, A and D).

Anti-SB1a antibody, which specifically binds to sulfated glycosphingolipid containing a SO$_3^{-}$-3Galβ1→3GalNAc residue, has weak cross-reactivity with SM3 (18). When the proteins of CCK-81 cell lysates were separated by SDS-PAGE followed by transfer to nitrocellulose membranes, and were stained with anti-SB1a antibody using standard techniques, no positive bands were detected. Furthermore, methanol fixation of CCK-81 cells on the coverslips greatly reduced the SB1a staining, and dipping the coverslips into chloroform/methanol (2:1) for a short time completely diminished this staining (data not shown), thus supporting that anti-SB1a antibody specifically reacts to sulfated glycosphingolipids, but not to glycoproteins.

Anti-GM1 antibody also stained the similar area as anti-galectin-4 (Fig. 5B), although the relative content of GM1 was less than that of SB1a (Fig. 4). However, the localization of caveolin-1/VIP21 was different from that of galectin-4 (Fig. 5D). Galectin-4 was localized in the similar area as anti-galectin-4 (Fig. 5C). These results suggest that galectin-4 is localized in patches, which involve SB1a, GM1, and CEA on the cell surface of CCK-81 and/or LS174T cells.

**Immobilized Galectin-4 Bound to CCK-81 Cells through 3-O-Sulfated Glycosphingolipids**—Sequentially, we investigated whether the galectin-4 on the cell surface of CCK-81 cells is related to cell adhesion. Galectin-4 immobilized on plates promoted the adhesion of CCK-81 cells in a dose-dependent manner in Ca$^{2+}$-free condition (Fig. 6A), whereas plates without galectin-4 failed to support cell adhesion (Fig. 6B). Galectin-4 did not bind to Chinese hamster ovary cells (Fig. 6A), which contain LacCer and GM3 as glycosphingolipids (19) and glycoproteins with Neu5Acα2→3Gal residues at the non-reducing termini (20), consistent with the fact that galectin-4 has no affinity toward Neu5Acα2→3Gal residues (8). Anti-SB1a effectively inhibited the binding of CCK-81 cells to galectin-4-coated plates (Fig. 6B), suggesting that galectin-4 binds to CCK-81 cells through 3-O-sulfated glycosphingolipids without Ca$^{2+}$ and stimulates adhesion of CCK-81 cells.

Galectin-4 Binding to CEA by SPR and Coimmunoprecipitation of CEA with Anti-galectin-4 Antibody in LS174T Cells—We next examined whether CEA binds to galectin-4. CEA bound to galectin-4-immobilized sensor chip dose-dependently with a $K_D$ value of 2 x $10^{-9}$ M and was eluted with 0.1 M lactose (Fig. 7A). CEA did not bind to galectin-4 when it was injected with 10 mM lactose (data not shown). Because CEA carries 25-27 mol of tri- and tetra-antennary complex type of N-glycans per molecule (14), the clustered non-reducing terminal Galβ1→4/3GlcNAc and Fucα1→2Galβ1→3/4-GlcNAc residues seem to bind to galectin-4, although the $K_D$ value

**Fig. 4.** Separation of sulfated glycosphingolipids from CCK-81 cells on TLC plates and relative binding ability to galectin-4. A, lane 1, primuline staining of lipids from CCK-81 cells; lane 2, azure A staining of lipids from CCK-81 cells. Positions of authentic phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), GM3, GM1, GD1a, SM4, SM3, SM2, and SB1a are indicated by arrows. The solvent system for TLC was chloroform/methanol/0.2% CaCl$_2$ (60:35:5). B, relative binding ability of bands a–g to galectin-4. Lipids of bands a–g were extracted from silica gels with chloroform/methanol (1:1) and were evaporated and dissolved in an equal volume of methanol (100 μl). Ten microliters were coated on ELISA plate. Relative binding ability was measured using 50 μl of rhgalectin-4 (50 nM) and anti-galectin-4 antibody according to the “Experimental Procedures.”

**Fig. 5.** Double immunofluorescence labeling of the cell surface. CCK-81 and/or LS174T cells washed with PBS were fixed with 4% paraformaldehyde for 15 min, stained with anti-galectin-4 (A–E), anti-SB1a (A and D), anti-GM1 (B), anti-caveolin-1 (C), and anti-CEA (E), followed by appropriate secondary antibodies, and examined by confocal laser microscopy. The merged confocal images are shown in the third column. Corresponding differential interference contrast images are shown in the fourth column.
trypsin and 0.02% EDTA, washed with PBS, and seeded (7 E
hamster ovary cells (○) and Chinese

ELISA plates were precoated with 0.1 ml of the indicated concentra-

binds to LS174T cells through 3-
sulfated glycosphingolipids

O

C

C

cell adhesion to plates coated with galectin-4. A, 96-well
ELISA plates were precoated with 0.1 ml of the indicated concentra-
tions of galectin-4 at 4 °C overnight. CCK-81 cells (○) and Chinese
hamster ovary cells (○) were detached from culture plates with 0.25% trypsin and 0.02% EDTA, washed with PBS, and seeded (7 × 10^5 each) in serum-free medium with 10 mM EDTA onto the coated wells. Following incubation at 37 °C for 30 min, bound cells were measured according to the “Experimental Procedures.” B, 96-well ELISA plates were pre-coated with 0.1 ml of 10 μg/ml of galectin-4 at 4 °C overnight. CCK-81 cells detached from culture plates (3 × 10^5 each) were preincubated with serum-free medium with 10 mM EDTA and anti-SB1a antibody (20 μg/ml) or anti-galectin-4 antibody (30 μg/ml) at 22 °C for 30 min. Then cells were seeded on galectin-4-coated wells. Following incubation at 37 °C for 30 min, wells were washed, and the amount of adherent cells was measured according to the “Experimental Procedures.”

A.

Absorbance at 595 nm

Gaal-4 (μg/ml)

CCK-81 cells

CHO cells

0

2.0

1.0

0.5

0

0.5

1.0

1.5

2.0

CCK-81 cells

CHO cells

B.

Added antibody

Anti-SB1a

Anti-Galectin-4

Anti-SB1a

Anti-Galectin-4

0

Gaal-4 coated

CeA

D.

Absorbance at 595 nm

of each Gaβ1→4(3)GlcNAc residue binding to galectin-4 is rather higher than Gaβ1→3GalNAc (8).

We could not detect the distinct CEA band in CCK-81 cell lysates by anti-CEA antibody, because CEA in CCK-81 cells may be immediately released into the medium (21). We could detect CEA in LS174T cell lysates and in the immunoprecipitated fraction by anti-galectin-4 antibody (Fig. 7B).

Binding of LS174T Cells to Galectin-4 was Inhibited by CEA—Sequentially, it was examined whether the expression of CEA on LS174T cells influences the cell adhesion toward galectin-4. LS174T cells also bound to the galectin-4-coated plate dose-dependently, but the bound cells were much lower than those of CCK-81 cells (Fig. 7C, filled circles). Anti-SB1a antibody effectively inhibited the binding of LS174T cells to galectin-4-coated plates (Fig. 7C), suggesting that galectin-4 also binds to LS174T cells through 3-O-sulfated glycosphingolipids without Ca²⁺. After the galectin-4-immobilized plates were preincubated with CEA at 37 °C for 2 h, LS174T cells diminished the binding ability via sulfated glycosphingolipids to the galectin-4-coated plates (Fig. 7D), suggesting that the exogenous CEA suppresses the binding of galectin-4 molecule to sulfated glycosphingolipids. These results suggest that galectin-4 binds to both SB1a and CEA in the specific patches on the cell surface of CCK-81 and/or LS174T cells and modulates their adhesion.

DISCUSSION

We have clearly demonstrated in this study that galectin-4 binds strongly to glycosphingolipids carrying SO₃~3Gal, including SM4, SM3, and SB1a, but not to glycosphingolipids carrying Siaα2→3Gal, including Slc4Cer, snLc4Cer, GM3, GM2, and GM4, and that these sulfated glycosphingolipids exist in human colon adenocarcinoma cells (Fig. 4), and mouse intestine and human colon epithelium (data not shown). We also found that (sulfated)-glycosphingolipids and galectin-4 are colocalized on the cell surface of human colon adenocarcinoma cells by an immunocytochemical assay (Fig. 5, A and D). The galectin-4/SB1a immunoreactive area is distinct from the anti-caveolin/VIP21 immunoreactive area (Fig. 5C), but similar to anti-GM1 immunoreactive area (Fig. 5B), suggesting that galectin-4 is localized in a kind of glycosphingolipids-rich patches on the cell surface of CCK-81 cells and LS174T cells. CEA was also stained in a galectin-4-rich area (Fig. 5E). It is known that GPI-anchored proteins are specifically targeted to apical membranes of polarized epithelial cells (22), and many GPI-anchored proteins are sorted to glycolipid-enriched membrane subdomains (23). Accordingly, this galectin-4/SB1a-rich domain may be a kind of platform for GPI-anchored glycoproteins in colon adenocarcinoma cells.

Moreover, galectin-4 immobilized on plates promoted adhesion of CCK-81 cells through sulfated glycosphingolipids (SB1a) (Fig. 6), suggesting that secreted galectin-4 modulates cell-cell adhesion via sulfated glycosphingolipids on the cell surface of CCK-81 cells. Sulfated glycosphingolipids are known to bind specifically to diverse adhesion proteins such as laminin, selectins, and anti-coagulant factors in vitro (9). For example, Suzuki et al. (24) reported that L-selectin binds to SM4, SM3, SB2, and SB1a. These facts imply that secreted galectin-4 modulates the interaction of these proteins with sulfated glycosphingolipids, because galectin-4 interacts with the membrane through adhesion to sulfated glycosphingolipids.

Galectin-4 also binds to LS174T cells through sulfated glycosphingolipids (Fig. 7C), and CEA suppressed galectin-4 binding to sulfated glycosphingolipids on LS174T cells (Fig. 7D). Because galectin-4 has much higher affinity for the SO₃~3-Galβ1→3GalNAc residue of SB1a than for Galβ1→3(4)GlcNAc and Fuca1→2Galβ1→3(4)GlcNAc residues in glycoproteins (Table II), the binding between galectin-4 and sulfated glycosphingolipids may function for the cell adhesion of epithelial cells in the gastrointestinal tract. It has been reported that CEA mediates homotypic as well as heterotypic intercellular adhesion (25). However, the expression patterns of CEA in malignant cells suggested that CEA functions as a cell-contact inhibitory molecule rather than as an adhesion molecule (26). Accordingly, the overexpression of CEA on the surface of tumor cells suppresses the galectin-4 binding to the sulfated glycosphingolipids and may facilitate migration and motility of tumor cells, i.e. metastases formation.

Binding of galectins-1 and -3 to CEA in cell lysates of adenocarcinoma cells has been reported (27, 28). However, galactin-1 is abundant in muscles, neurons, thymus, kidney, and placenta (1), and galectin-3 is abundant in lung, artery, thymus, and spleen (29); expression of these proteins in intestine and colon is quite low in comparison with galectin-4. In fact, we...
could not detect galectin-3 on the cell surface of LS174T cells by immunofluorescence analysis. Because Danielsen et al. (30) showed that galectin-4 is present in the detergent-insoluble complex of pig intestine, galectin-4 may be one of the major ligands for CEA in the epithelium of alimentary tracts.

On the other hand, there are several reports that sulfated glycosphingolipids serve as receptors for bacterial or viral toxins. A human respiratory pathogen, *Bordetella pertussis*, binds to SM4 and glycosphingolipids containing a GalNAc–H1–Gal sequence (31). Influenza A viruses bind to sulfates (32), and neutrophil-activating protein of *Helicobacter pylori* binds to SM4 and some sulfated glycosphingolipids are expressed in mammal gastrointestinal tract (9). We also found that SB1a exists in mouse intestine and human colon epithelium (data not shown). Because galectin-4 is specifically expressed in the gastrointestinal tract, galectin-4 may play a role in host defense by masking the sulfated glycosphingolipids from bacterial or viral toxins.

The anti-SB1a antibody was originally obtained using the acidic glycolipid mixture prepared from human hepatocellular carcinoma cells (PLC/PRF/5) as immunogens. Although the SB1a antigen is a relatively minor glycolipid in hepatic cells, the anti-SB1a antibody strongly stained the surface of PLC/PRF/5 cells (18). Analysis of the glycolipids extracted from hepatocellular carcinoma tissues and cirrhotic livers of patients and from a normal liver revealed that SB1a is expressed in some hepatocellular carcinoma tissues (5 of 17 cases) but not expressed in the cirrhotic liver and normal liver (34). We found that SB1a was expressed on the surface of human colon adenocarcinoma cells (CCK-81 and LS174T cells) (Fig. 5) but not expressed on the surface of another type of human colon adenocarcinoma cell, M7609 (data not shown). These data suggest that the surface expression or disappearance of SB1a might be related to the respective characters of carcinoma.

Plant-derived lectins have been used for as lymphocyte-stimulating reagents, and some endogenous mammalian lectins also play an important role in immune responses by cross-linking certain cell surface glycoreceptors (35, 36). Furthermore, it is thought that glycosphingolipids, which are abundant in detergent-resistant membranes, influence signal transduction by modulating the binding of exogenous effectors to cell surface receptors (37–40). Accordingly, secreted galectin-4 may be able to cross-link cell surface ligands and thereby may mediate or modulate cell responses. It has been recently reported that galectin-4 stimulates interleukin-6 production by intestinal CD4+ T-cells under inflammatory conditions (41). Galectin-4 bound to sulfated glycosphingolipids and promoted cell adhesion, whereas CEA modulated this interaction by binding to galectin-4 on the cell surface of colon adenocarcinoma cells. Whether these glycoconjugates are involved in galectin-4-dependent cell responses and/or cell anoikis should be resolved in the next step.

![FIG. 7. Binding of CEA to galectin-4 and cell adhesion assay in LS174T cells. A, various concentrations of CEA were introduced onto the galectin-4-immobilized (7200 RU) surface for 180 s at a flow rate of 20 μl/min. The relative response was determined by subtracting the blank values on the non-immobilized surface from the values on the galectin-4-immobilized surface. B, immunoprecipitation of CEA with galectin-4. LS174T cell lysates were immunoprecipitated with the anti-galectin-4 antibody. The immunoprecipitate was subjected to SDS-PAGE, blotted, and stained with anti-CEA and anti-galectin-4 antibody. C, 96-well ELISA plates were precoated with 0.1 ml of the indicated concentrations of galectin-4 at 4 °C overnight. The detached LS174T cells were incubated with anti-SB1a antibody (20 μg/ml) at 22 °C for 30 min or without antibody and seeded onto the galectin-4-coated wells (3 × 10⁴ each). Following incubation at 37 °C for 30 min, the bound cells were measured according to the “Experimental Procedures.” D, 96-well ELISA plates were precoated with 0.1 ml of 10 μg/ml of galectin-4 at 4 °C overnight and sequentially incubated with 0.1 ml of the indicated concentrations of CEA at 37 °C for 2 h, followed by blocking with 1% BSA for 2 h at 37 °C. The detached LS174T cells were seeded on CEA-galectin-4-coated wells. Following incubation at 37 °C for 30 min, wells were washed and the amount of adherent cells was measured according to the “Experimental Procedures.”](http://www.jbc.org/content/199/23/4736.f5)
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