Note

Identification of Galectin-2–Mucin Interaction and Possible Formation of a High Molecular Weight Lattice

Mayumi Tamura,[a] Dai Sato,[a] Moeko Nakajima,[a] Masanori Saito,[a] Takaharu Sasaki,[a] Toru Tanaka,[a] Tomomi Hatanaka,[a,b] Tomoharu Takeuchi,[a] and Yoichiro Arata*[,a,†]

[a] *Faculty of Pharmacy and Pharmaceutical Sciences, Josai University; 1–1 Keyakidai, Sakado, Saitama 350–0295, Japan: and* [b] *Tokai University School of Medicine; 143 Shimokasuya, Isehara, Kanagawa 259–1193, Japan.*
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Galectins comprise a group of animal lectins characterized by their specificity for *β*-galactosides. Galectin-2 (Gal-2) is predominantly expressed in the gastrointestinal tract and has been identified as one of the main gastric mucosal proteins that are proposed to have a protective role in the stomach. As Gal-2 is known to form homodimers in solution, this may result in crosslinking of macromolecules with the sugar structures recognized by Gal-2. In this study, we report that Gal-2 could interact with mucin, an important component of gastric mucosa, in a *β*-galactoside-dependent manner. Furthermore, Gal-2 and mucin could form an insoluble precipitate, potentially through the crosslinking of mucins *via* Gal-2 and the formation of a lattice, resulting in a large insoluble complex. Therefore, we suggest that Gal-2 plays a role in the gastric mucosa by strengthening the barrier structure through crosslinking the mucins on the mucosal surface.

Key words galectin-2; mucin; gastric mucosa; crosslink; galectin

Galectins comprise a type of the animal lectins that specifically bind to *β*-galactosides and are characterized by an evolutionarily conserved eight amino acids in the carbohydrate-binding site.[1–3] Galectins are known to be involved in a wide variety of biological processes including development, cell differentiation, tumor metastasis, apoptosis, RNA splicing, and immune regulation.[4,5] Galectins can be classified into three types in terms of molecular architecture, *i.e.*, proto-type, chimera-type, and tandem repeat-type.[1] Proto-type galectins such as galectin-1 (Gal-1) and galectin-2 (Gal-2) have molecular weights of approximately 14 kDa and are able to form a homodimer in solution. Each monomer possesses a highly conserved single carbohydrate-binding site with affinity for *β*-galactoside. Thus, some of the functions of these proto-type galectins may be due to their potential ability to crosslink two molecules that possess the carbohydrate structure recognized by these galectins.

Among the proto-type galectins, Gal-2 is known to be highly expressed in gastric cells, predominantly in epithelial cells of the rat stomach.[6] In mouse, surface mucous cells and mucous neck cells, which produce mucus, show Gal-2 immunoreactivity, but no reaction was observed in parietal cells and chief cells.[7] *GAL2* mRNA has also been detected in the human stomach.[8] The expression of Gal-2 in the gastrointestinal tract and the amelioration of acute and chronic colitis in mice by Gal-2 overexpression[9] as well as the reduced expression of human Gal-2 associated with lymph node metastasis in gastric cancer[10] and in *Helicobacter*-induced gastric cancer progression[11] have suggested that Gal-2 plays a protective function in the gastrointestinal tract.

One of the major protective components of the mucous barrier of the gastric tract is the glycoprotein mucin secreted by epithelial cells.[12,13] Cell surface-associated mucins are also known to contribute to forming the mucosal barrier and protecting the mucosal surface.[14] In particular, mucin glycoproteins are rich in *O*-linked glycans, with membrane-associated mucins exhibiting *N*-linked glycans as well.[13] It has also been reported that another member of the galectin family, galectin-3 (Gal-3), associates with cell surface mucins and that this association contributes to the ocular surface epithelial barrier.[15–17] As the X-ray crystallographic structure of human Gal-2 revealed that the two binding sites of the homodimer are located at opposite ends of the dimer,[18] allowing sufficient room to bind to larger molecules, it is possible that Gal-2, which is abundant in gastric mucous cells, may similarly potentially form a lattice[19] with secreted mucins or crosslink cell surface mucins to strengthen the mucus barrier and contribute to the protection of the stomach.

In this report, we explored the possibility that Gal-2 may form a crosslinked high molecular weight complex with mucin by testing the interaction of Gal-2 with mucin and also by examining the formation of insoluble precipitate between Gal-2 and mucin using commercially available porcine stomach mucin. For this analysis, a green fluorescent protein (GFP)-tag was added to the C-terminus of the Gal-2 protein and the tagged form of Gal-2 recombinant protein was used to detect the interaction with high sensitivity.

MATERIALS AND METHODS

Chemicals Mucin from porcine stomach (Type III, partially purified powder) was purchased from Sigma (St. Louis, MO, U.S.A.).

Construction of a Wild-Type Recombinant Gal-2 Expression Plasmid A DNA fragment encoding rat Gal-2 was amplified by PCR using rat stomach first strand cDNA as a template and KOD plus DNA polymerase (TOYOBO, Osaka, Japan). After the preheating step of 95°C for 3 min, a 30-cycle PCR was conducted under the following conditions: denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 68°C for 1 min. First strand cDNA was prepared from

† Present address: *Faculty of Pharma-Science, Teikyo University; 2–11–1 Kaga, Itabashi-ku, Tokyo 173–8605, Japan.*

* To whom correspondence should be addressed. e-mail: arata@pharm.teikyo-u.ac.jp

total RNA isolated from primary-cultured rat gastric epithelial cells.[20] All procedures were approved by the Institutional Animal Care and Use Committee at the University of Josai Life Science Center and followed the Guide for the Care and Use of Laboratory Animals published by the NIH. Forward and reverse primer sequences containing *Nde*I and *Bam*HI sites (underlined), respectively, were 5′-CAT ATG TCG GAG AAA TTC GAG GTC AC-3′ and 5′-GGA TCC TCA CTC GAG CTT GAA GG-3′.

The PCR product was ligated into the pGEM-TX vector,[21] the plasmid was digested with *Nde*I and *Bam*HI, and the excised DNA fragment was inserted into the corresponding restriction sites of the pET21a vector (Novagen, Merck KGaA, Darmstadt, Germany) for protein expression.

Construction of a GFP-Tagged Form of Gal-2 DNA fragments encompassing the coding region of Gal-2 were amplified by PCR using cloned pGEM-TX-Gal-2 as a template and a forward primer containing a *Hin*dIII site covering the initiation codon used for amplification (5′-AAG CTT CAC CAT GTC GGA GAA ATT C-3′) and a reverse primer containing a *Bam*HI site downstream of the desired stop codon (5′-GGA TCC TCG AGC TTG AAG-3′). KOD plus DNA polymerase (TOYOBO) was used for the PCR reaction as follows: preheating at 95°C for 3 min, and 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 68°C for 40 s. The PCR product was ligated into the pGEM-TX vector and the *Gal-2* DNA with GFP tag on its C-terminus was constructed using the pAcGFP-N1 (Clontech, Mountain View, CA, U.S.A.) vector.

Purification of Recombinant Galectins Expression and purification of recombinant protein of the wild-type or GFP-tagged form of Gal-2 (Gal-2-GFP) were conducted using a pET21a vector as described previously.[22–24] In brief, *Escherichia coli* cells were grown at 37°C in 250 mL of 2×YT medium containing 125 μg/mL ampicillin and subjected to protein expression at 20°C overnight by adding isopropyl-β-thiogalactopyranoside to give a final concentration of 0.4 mM. The cells were harvested, suspended in 10 mL ethylenediaminetetraacetic acid-phosphate buffered saline (EDTA-ME-PBS) (10 mM PO_4^{3-}, 0.14 M NaCl, 2.7 mM KCl, 1 mM EDTA, pH 7.4 (EDTA-PBS) with 4 mM 2-mercaptoethanol), and disrupted by sonication. The debris was removed by centrifugation and the extracts were applied to a β-galactoside-immobilized column[25] and eluted with EDTA-ME-PBS containing 0.1 M lactose.

For Gal-2-GFP, the cells expressing the recombinant protein were disrupted by sonication and the debris was removed by centrifugation. The extracts were applied to a β-galactoside-immobilized column[25] and eluted with EDTA-ME-PBS containing 0.1 M lactose. Recombinant Gal-2-GFP was specifically eluted with 0.1 M lactose and the eluted protein was detected as a single band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at the molecular weight of 42 kDa. The fluorescence derived from the GFP-tag was also observed in the lactose-eluted fractions, demonstrating that the sugar-binding ability of Gal-2-GFP remained the same as that of wild-type Gal-2.

Preparation of a Gal-2 Immobilized Column Wild-type Gal-2 was immobilized onto HiTrap NHS-activated HP 1 mL columns (GE Healthcare, Chicago, IL, U.S.A.) following the manufacturer's instructions. Specifically, 4.2 mg protein was immobilized onto the resin in each column.

Binding Mucin to a Gal-2 Immobilized Column in Different pH Conditions The binding of porcine mucin to Gal-2 immobilized column was determined in different pH conditions. Mucin was added to the column, followed by washing and elution with 0.1 M lactose using EDTA-PBS (pH 7.4) or acetic acid–sodium acetate buffer at pH 5.5, 5.0, or 4.7.

Quantitation of Mucin The content of mucin in solution was determined using a modified method from Matsuno *et al.*[26] Separate 5 μL aliquots of mucin solution at the concentrations of 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL were dotted onto polyvinylidene fluoride (PVDF) membranes that had been immersed in methanol for 10 s and then in EDTA-PBS for 10 min prior to dotting and left at room temperature until the solutions had dried completely. Samples were also dotted onto pretreated PVDF membranes. The membranes were stained with 0.1% alcian blue solution for 1 h and areas without mucin were destained with methanol 3 times for 5 min each. A standard curve was prepared using the Volume Tools of Image Lab™ software (BioRad, Berkeley, CA, U.S.A.) by analyzing the stained PVDF membranes.

Analysis of the Interaction between Mucin and Gal-2-GFP Using a 96-Well Plate A 200-μL aliquot of 0.25 mg/mL porcine mucin (50 μg of porcine mucin) was immobilized onto the bottom of wells of a 96-well plate for 1 h at 37°C and washed with EDTA-PBS. Then 200 μL of 0.25 mg/mL bovine serum albumin was added for blocking for another 1 h at 37°C. After another step of washing with EDTA-PBS, 50 μL of Gal-2-GFP solution was added and incubated at 4°C for 1 h. After washing, the fluorescence was measured using a Spectra Max M5e microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.) (Ex: 475 nm, Em: 505 nm). By using different concentrations of Gal-2-GFP, a standard curve was prepared and using this curve, the amount of bound Gal-2-GFP was determined.

Detection of the Formation of a High Molecular Weight Lattice in Solution by Measuring Turbidity at 600 nm When solutions of Gal-2 and porcine mucin were mixed at certain concentrations, insoluble particles precipitated, potentially owing to the two compounds producing a lattice structure as Gal-2 forms a dimer and potentially has the ability to crosslink two mucin molecules, resulting in the formation of a high molecular weight lattice that could become insoluble and precipitate. A total of 50 μL of 1.0 mg/mL mucin in EDTA-PBS was added to the wells of a 96-well plate followed by the addition of 50 μL Gal-2-GFP at the concentrations of 0, 0.25, 0.5, 0.75, and 1.0 mg/mL. After incubation of the 96-well plate for 2 h in the dark at room temperature, the turbidity at 600 nm was measured for each well. To check the inhibition by lactose or sucrose, 25 μL of 2.0 mg/mL mucin in EDTA-PBS was added to the wells of a 96-well plate followed by the addition of 50 μL of either 0.2 M lactose or sucrose in EDTA-PBS, along with 25 μL Gal-2-GFP at the concentration of 2 mg/mL to bring the total volume of the solution in each well to 100 μL.

To check the formation of insoluble particles at different pH, EDTA-PBS (pH 7.4) or acetic acid–sodium acetate buffer (pH 4.7) were used. A total of 50 μL of 1.0 mg/mL mucin in EDTA-PBS (pH 7.4) or acetic acid–sodium acetate buffer (pH 4.7) was added to the wells of a 96-well plate followed by the addition of 50 μL Gal-2 at 1.0 mg/mL in EDTA-PBS (pH 7.4)

or acetic acid–sodium acetate buffer (pH 4.7). After incubation for 2 h in the dark at room temperature, the turbidity at 600 nm was determined in each well.

RESULTS

Binding of Mucin to a Gal-2-Immobilized Column and Its Elution with Lactose Interaction between Gal-2 and mucin were analyzed by adding mucin solution to a Gal-2 immobilized column. As can be seen in Fig. 1(a), after the elution of excess mucin from the column (white triangle in Fig. 1(a)), adsorbed mucin, which was held in the column even after excessive washing, was specifically eluted after the addition of EDTA-PBS containing 0.1 M lactose (shown as a black triangle in Fig. 1(a)). This result suggested that mucin is recognized by Gal-2 *via* β-galactoside-containing carbohydrate chain(s). Adsorption of mucin to a Gal-2-immobilized column and elution with lactose was determined in different pH conditions (7.4, 5.5, 5.0, and 4.7). The results show that the adsorption of mucin to the immobilized Gal-2 was substantially reduced at pH 4.7 (Fig. 1(b)).

Gal-2 forms a dimer and shows hemagglutination activity; this activity is inhibited when lactose is added to the mixture.[23,24] As the addition of mucin inhibited this hemagglutination activity (data not shown), we suggest that the interactions between Gal-2 and mucin may occur *via* the carbohydrate structure(s) on mucin molecules.

Detection of an Interaction between Mucin and Gal-2 Using a GFP-Tagged Form of Gal-2 To detect the interaction between Gal-2 and mucin at higher sensitivity albeit without using a high amount of recombinant Gal-2 protein, we prepared a GFP-tagged form of Gal-2 (Gal-2-GFP) that has a GFP tag on its C-terminus. A DNA fragment coding the Gal-2 protein was inserted into a GFP vector and the recombinant protein was expressed. The recombinant proteins were adsorbed onto a β-galactoside-immobilized column and specifically eluted with the addition of lactose. Furthermore, the recombinant form of Gal-2-GFP, which presumably has a molecular weight of 42 kDa, was detected in the lactose-eluted fraction (data not shown) by SDS-PAGE and Coomassie brilliant blue staining. Therefore, Gal-2-GFP appears to maintain the same β-galactoside-binding ability as the wild-type Gal-2 (without the GFP-tag). This Gal-2-GFP was used to detect the interaction between Gal-2 and mucin in a 96-well plate. When the amount of Gal-2-GFP was increased up to 100 ng for each well, the intensity of the fluorescence at 505 nm increased depending on the amount of Gal-2-GFP added to the well (data not shown). To study whether this interaction was β-galactoside-dependent, we added 50 μL of 0.25 mg/mL Gal-2-GFP to wells with 50 μg immobilized mucin, also in-

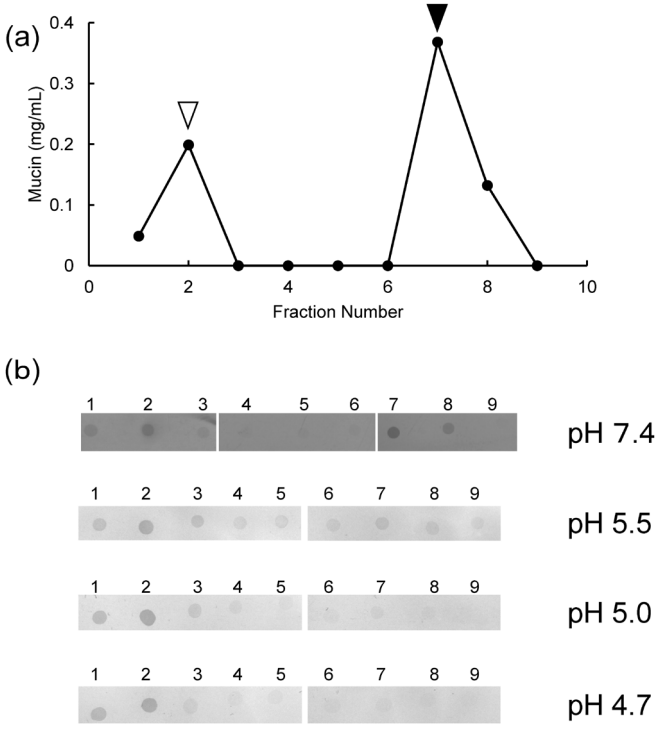

Fig. 1. Adsorption of Mucin to a Gal-2-Immobilized Column and Elution with Lactose in Different pH Conditions

Porcine mucin in EDTA-PBS (pH 7.4) or in acetic acid–sodium acetate buffer at pH 5.5, 5.0, and 4.7 was applied to a HiTrap column (bed volume 1 mL) with immobilized recombinant Gal-2 and fractions (1 mL each) were collected. (a) At pH 7.4, mucin concentration in each fraction was measured by the method described in Materials and Methods. Excess mucin that was not retained in the column was eluted during the wash phase (white triangle). The peak of adsorbed mucin eluted with 0.1 M lactose is shown with a black triangle. (b) Mucin concentrations were determined in each fraction obtained at pH 7.4, 5.5, 5.0, and 4.7 by alcian blue staining of the PVDF membrane.

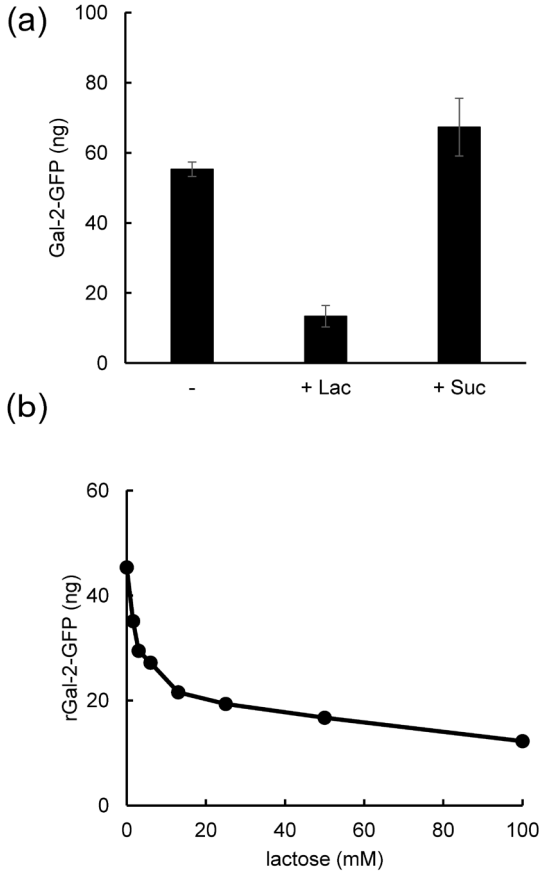

Fig. 2. Inhibition of Gal-2-GFP Binding to Immobilized Mucin by the Addition of Lactose

Porcine mucin in EDTA-PBS was immobilized to the bottom of the 96-well plastic plates and recombinant Gal-2 with a GFP-tag on its C-terminus (Gal-2-GFP) in EDTA-PBS was added to the well. The amount of Gal-2-GFP bound to the immobilized mucin was detected by measuring the fluorescence using a microplate reader (Ex: 475 nm, Em: 505 nm). Data are shown as the mean±S.D. (n=3). (a) Bound Gal-2-GFP was measured without addition of sugar (−) or addition of 0.1 M lactose (+Lac) or 0.1 M sucrose (+Suc). (b) Bound Gal-2-GFP was measured with different lactose concentrations in each well.

cluding the addition of lactose or sucrose as inhibition sugars. The binding of Gal-2-GFP to the wells was markedly inhibited with the addition of 0.1 M lactose but not with the addition of 0.1 M sucrose (Fig. 2(a)). Although 0.1 M was the highest concentration that could be obtained owing to the solubility of lactose, the results shown in Fig. 2(b) suggest that 0.1 M lactose has almost reached a plateau. The results also suggest that Gal-2 and mucin interact *via* carbohydrate-containing β-galactoside structure(s).

Formation of a High Molecular Weight Lattice in Solution by Gal-2 and Mucin As we noted the formation of some insoluble precipitate when the solutions of Gal-2-GFP and mucin were mixed, we hypothesized that Gal-2-GFP formed a dimer in solution through crosslinking high-molecular-weight mucin molecules by binding to the carbohydrate structures on the mucin protein, resulting in an insoluble lattice. Therefore, we tested whether this formation of crosslinked lattice depended on the recognition of the carbohydrate structures on mucin.

Gal-2-GFP and mucin were mixed in the wells of a 96-well plate and the formation of insoluble high molecular weight lattice was analyzed by measuring the turbidity at 600 nm.

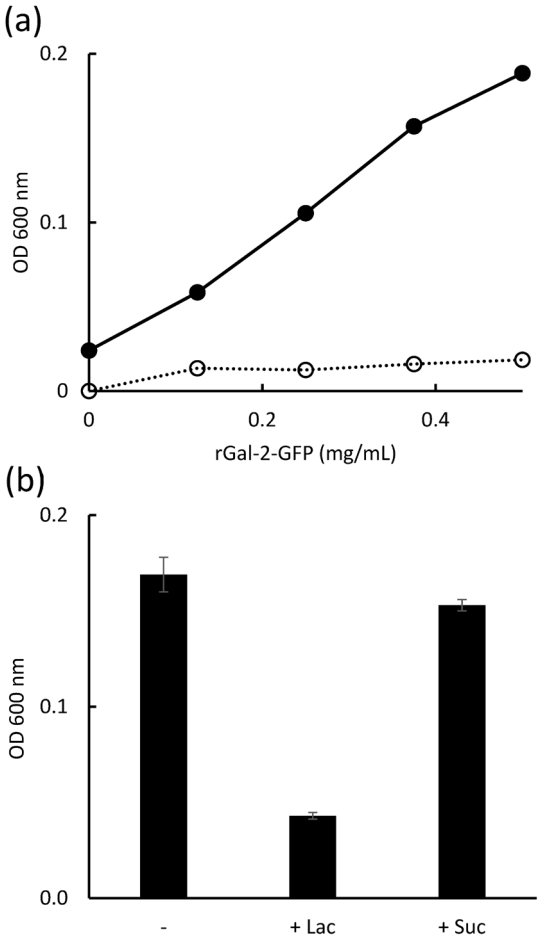

Fig. 3. Detecting the Formation of a High Molecular Weight Insoluble Lattice between Gal-2-GFP and Mucin

A total of 50 μg porcine mucin in EDTA-PBS was added to the wells of a 96-well plate followed by the addition of Gal-2-GFP to yield a final concentration of 0 to 0.5 mg/mL. After an incubation of 2 h in the dark at room temperature, the turbidity at 600 nm was measured. (a) The formation of insoluble lattice measured by turbidity at 600 nm in the absence (open circles) or presence (closed circles) of mucin in the well. (b) Inhibition of lattice formation by Gal-2-GFP and mucin *via* 0.1 M lactose or sucrose. Data are shown as the mean±S.D. (n=3).

When only Gal-2-GFP was added to the well with no mucin, turbidity at 600 nm did not increase when the amount of added Gal-2-GFP increased (Fig. 3(a), open circle). Conversely, when mucin was present in the well, turbidity at 600 nm increased depending on the amount of Gal-2-GFP added to the well (Fig. 3(a), closed circle). The formation of the lattice detected by the turbidity at 600 nm was markedly inhibited when 0.1 M lactose was also added to the well (Fig. 3(b) "+Lac"). This inhibition was not observed when sucrose was added (Fig. 3(b) "+Suc"). Therefore, we suggest that Gal-2-GFP crosslinked mucin and formed a high molecular weight lattice that became insoluble *via* the carbohydrate-containing β-galactoside structure(s) on mucins. Furthermore, we determined whether the insoluble precipitate could also form at lower pH. As can be seen in Fig. 4, a substantial amount of insoluble precipitate formed at pH 4.7 when Gal-2 and mucin were present in the same solution, suggesting that Gal-2–mucin lattice formation is possible even under acidic conditions.

DISCUSSION

Multiple reports suggest that Gal-2 plays an important role in protecting the gastrointestinal tract and may help prevent ulcers or gastric cancer. However, the molecular mechanism underlying how Gal-2 contributes to the mucosal barrier is not well understood. In the current study, we found that Gal-2 was able to interact with mucin in a β-galactoside-dependent manner and may form a high molecular weight lattice. As Gal-2 forms a homodimer and mucin has multiple sugar chains attached to its backbone polypeptide chain, it is possible that dimeric Gal-2 crosslinks mucins *via* its carbohydrates, with the resultant β-galactoside structure likely forming an insoluble precipitate. In the stomach, mucin constitutes one of the main components of the mucus. In addition, membrane-attached forms of mucin also exist that are known to play an important role in protecting the surface of the gastric mucosa. Because surface mucous cells and mucous neck cells, which produce mucus, show strong Gal-2 immunoreactivity in mice, but no reaction was observed in parietal cells and chief cells,[7] Gal-2 may form a complex with mucin upon secretion from these cells. Crosslinking mucin by Gal-2 may therefore result in an even tighter barrier of mucins on the mucosal surface. We are planning to investigate further, *via* affinity purification or immunoprecipitation, if Gal-2 is actually forming a complex with mucin in the stomach mucus.

It has been proposed that Gal-3 interacts with the *O*-glycans of mucins and forms a highly organized and protective cell surface lattice barrier on the apical glycocalyx of ocular surface epithelial cells.[15–17] The mucins involved in this lattice barrier are considered to comprise MUC1 and MUC16, which are membrane-associated mucins.[16] The mucin used in the current study is commercially available porcine mucin, which is obtained by the digestion of hog stomach with pepsin, followed by precipitation and partial purification.[27] Therefore, not only membrane-associated mucins but also secreted mucins, such as MUC5AC and MUC6, may also be included. We are currently investigating which mucins are included in the identified insoluble complex containing Gal-2.

Although the main recognition structure that is bound by galectins constitutes the Galβ1→4GlcNAc (*N*-acetyl-lactosamine) structure, each galectin possesses different binding

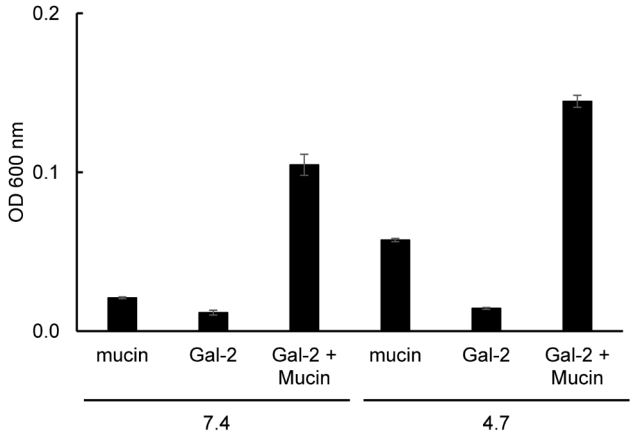

Fig. 4. Detecting the Formation of a High Molecular Weight Insoluble Lattice between Gal-2 and Mucin

A total of 50 μg porcine mucin in EDTA-PBS (pH 7.4) or acetic acid–sodium acetate buffer (pH 4.7) were added to the wells of a 96-well plate followed by the addition of 50 μL Gal-2 at 1.0 mg/mL in EDTA-PBS (pH 7.4) or acetic acid–sodium acetate buffer (pH 4.7). After incubation for 2 h in the dark at room temperature, the turbidity at 600 nm was measured in each well. Wells containing only mucin or Gal-2 were also measured. Data are shown as the mean±S.D. (n=3).

properties for linking isomers and other compounds that have additional sugar modifications to the basic Galβ1→4GlcNAc unit.[28,29] Furthermore, there are a variety of sugar structures attached to the mucin backbone polypeptides.[13] As it has been reported that recombinant Gal-1 recognizes mucin and epithelial cell surface glycocalyces of the gastrointestinal tract,[30] Gal-2, which possesses certain common sugar-binding specificities with Gal-1, may also recognize mucins of the gastrointestinal tract. Because Gal-2 (and most galectins) maintains its binding affinity to β-galactoside structures by forming hydrogen bonds with the hydroxyl moieties at positions 4 and 6 of the terminal β-galactose,[1,28] sialic acids attached to the terminal galactose with an α2–6 bond should inhibit galectin binding but sialic acid attached with an α2–3 bond should not. Therefore, we assume that formation of the Gal-2 and mucin complex occurs *via* the terminal galactose with Siaα2–3 or a terminal galactose without sialylation and not through terminal galactose with Siaα2–6. In addition, it has been reported that *O*-glycans released from mouse MUC5AC, one of the main mucins in the stomach, contain elongated lactosamine structures devoid of sialic acid, which should be recognized by Gal-2.[31] We are planning to compare the insoluble-complex-forming-ability of asialomucin (by treating mucins with sialidase) with non-treated mucins. After collecting the fraction (fraction 2 in Fig. 1) containing mucin not adsorbed to the column and subjecting this fraction to binding to the Gal-2-immobilized column, we found that these mucins are adsorbed and eluted by lactose, which indicates that most of the non-adsorbed fraction in the first column binding round was due to over-flow (data not shown). Furthermore, the flow-through and lactose-eluted fractions were both subjected to lectin-blotting using SSA lectin (lectin from *Sambucus sieboldiana*), which recognizes Siaα2–6. Both fractions showed a positive reaction to the lectin (data not shown). Although we assume that Gal-2 immobilized to the column bound to the sugar structures with terminal galactose without attached Sia or terminal galactose with Siaα2–3 on mucin, because mucin contains a very high number of sugar chains attached to its core polypeptide backbone and the structures are very variable, it is difficult to distinguish between bound and unbound mucins. We are planning to determine the mucin structure in the near future. Furthermore, the carbohydrate structure that is responsible for the recognition by Gal-2 to form the insoluble complex should therefore be identified, which represents a research aim of our laboratory in the near future.

Adsorption of mucin to a Gal-2-immobilized column and elution with lactose was determined in different pH conditions. At a lower pH, the adsorption of mucin to the immobilized Gal-2 was substantially reduced (pH 4.7). However, insoluble particles precipitated even under acidic conditions (as shown in Fig. 4) when Gal-2 and mucin were mixed. Therefore, these results suggest that, although the 1 : 1 interaction between mucin and the monomeric form of Gal-2 immobilized to the column resin in acidic condition could be weak, the Gal-2–mucin complex could be generated when Gal-2 forms dimers and the affinity increases at higher concentrations. These results may suggest that Gal-2 and mucin form a complex right after secretion (or even before secretion), presumably at surface mucous cells and/or mucous neck cells before being exposed to the acidic condition, and resists exposure as it moves toward the outer layer of the mucus barrier. We are also planning to test the monomer-dimer ratio of Gal-2 at a different pH.

The two well-known proto-type galectins, Gal-1 and Gal-2, have multiple endogenous cysteine (Cys) residues in their polypeptide chain and lose their ability to bind to carbohydrates through oxidative inactivation.[23,24,32] Therefore, for Gal-2 to remain active in an oxidative environment such as in the stomach may appear difficult. However, it is notable that Gal-2 was identified in a screen of mouse gastric mucosal proteins that are uniquely sensitive to *S*-nitrosylation,[33] which involves the coupling of an nitric oxide (NO) group to the reactive thiol of a Cys residue in the polypeptide.[34] Furthermore, we have found that although *S*-nitrosylation does not alter the carbohydrate-binding properties of the Gal-2 molecule, *S*-nitrosylation prevents the oxidative inactivation of Gal-2.[23,24] Large quantities of NO are known to be generated in the stomach by the non-enzymatic acid reduction of salivary nitrite, in addition to the NO generated enzymatically by NO synthase from L-arginine, and have been proposed to exert various physiological functions in the gastrointestinal tract[35–38] including as a barrier in the stomach.[39–41] Therefore, it is possible that the NO generated in the stomach *S*-nitrosylates Gal-2 to sustain a stronger barrier through the crosslinking of mucins and to prevent the oxidative inactivation of Gal-2.

In conclusion, we found that Gal-2 was able to interact with gastric mucin in a β-galactoside-dependent manner and possibly form a lattice by crosslinking with mucins. The results suggest that Gal-2 plays an important role in the gastric mucosa by strengthening the barrier structure of the mucosal surface. We believe that our findings could contribute to the prevention of gastric ulcers and cancers by establishing a new way to strengthen the barrier *via* Gal-2–mucin interaction.

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Conflict of Interest The authors declare no conflict of

interest.

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