Generation of monoclonal antibodies against the Galβ1-4Gal epitope: A key tool in studies of species-specific glycans expressed in fish, amphibians and birds

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Whereas the Galβ1-4Gal epitope is rarely found in mammalian glycans, it has been found in glycans of various species of non-mammalian vertebrates, such as fish, amphibians and birds. Although glycans containing Galβ1-4Gal in these vertebrates were detected by precise structural analysis of the glycans using mass spectrometry and/or NMR spectrometry, there are no convenient methods to detect Galβ1-4Gal from various samples. To analyze systematically the distribution of Galβ1-4Gal in nature, we generated mouse monoclonal antibodies (mAbs) specific for Galβ1-4Gal using extracts of medaka eggs as an immunogen. Four mAbs (two immunoglobulin (Ig)Ms and two IgG1s) were obtained by enzyme-linked immunosorbent assay-based screening. The specificities of these mAbs were evaluated by frontal affinity chromatography using 142 kinds of 2-aminopyridine (PA)-derivatized oligosaccharides. While all mAbs interacted with (Galβ1-4Gal)-containing oligosaccharides at their non-reducing termini with dissociation constants (Kd) ranging from 1.0 × 10⁻⁵ to 2.8 × 10⁻⁴ M, no apparent interaction was observed with any other glycans. The number of branches containing Galβ1-4Gal on N-glycans did not significantly affect Kd of mAbs of IgG1 subclasses, but those of IgM mAbs were decreased by ≏1 order of magnitude, in increments of the number of branches present. Using the mAbs, we established that Galβ1-4Gal is also expressed on glycoproteins in various tissues from the African clawed frog. Immunohistochemical staining of medaka sections revealed that Galβ1-4Gal epitopes were expressed in the endothelium, epithelium and epidermis, which directly contact the external environment or invading organisms. Thus, these mAbs are useful for systematically investigating the species-specific expression of glycans, which may act as a barrier against infection.

Keywords: frontal affinity chromatography / galactosyltransferase / glycan diversity / medaka / vertebrates

Introduction

A great variety of glycan structures exists in biological systems. Glycan diversity is generated by numerous factors, including variations in the glycosidic linkages that form among monosaccharides. Since each monosaccharide can theoretically form either an α- or a β-linkage at the anomeric carbon with one of several possible hydroxy groups on another monosaccharide in a chain, various isomers with different linkages can be formed. The isomers generally possess different three-dimensional structures and are often distinguished by specific recognitions of glycan-binding proteins. Glycans with different glycosidic linkages also have different sensitivities to specific glycosidases and different reactivity with glycosyltransferases. Thus, even a subtle change in glycan structure often influences the biological activities of glycans.

Inter- and intraspecies variations in glycans exist in nature. Because cell surfaces covered with a dense coating of glycans are often utilized as targets for host recognition by pathogens and symbiotic microbes, it is speculated that the generation of species/individual-specific glycans has been driven by evolutionary pressures involving pathogen evasion and maintenance of appropriate interactions with symbiotic microbes (Gagneux and Varki 1999; Hooper and Gordon 2001). For instance, Norwalk viruses bind to human ABH(O) histo-blood group antigen receptors (Marionneau et al. 2002) and people who lack the functional α1,2-fucosyltransferase gene (FUT2) are resistant to infection by the virus (Lindesmith et al. 2003). Another example is Galβ1-3Gal, which is present in most mammals except humans, apes and Old World monkeys (Galili et al. 1992).
High-titered circulating anti-(Galβ1-3Gal) antibodies are present in animals lacking this epitope. It is suggested that these antibodies exert protective effects causing complement-mediated lysis of enveloped viruses, which are generated in other host cells expressing Galβ1-3Gal epitopes (Rother et al. 1995; Takeuchi et al. 1996).

The evolutionarily differentiated glycan structures seem to be the consequence of the gain and loss of enzymes that mediated the biosynthesis of glycans during the course of evolution (Koike et al. 2007; Turecot-Dabois et al. 2007). However, there is limited information available of glycan diversity in nature, and there are few comprehensive data sets of glycan expression in a wide range of organisms (Varki et al. 2008). To decipher the mechanism that produces glycan differentiation and to determine the biological significance of glycan diversity in nature, it is necessary to investigate the presence and the absence of particular glycan structures in various organisms by systematic comparisons.

Glycans with the Galβ1-4Gal epitopes are one of the representative structures exhibiting evolutionarily differentiated expression profiles among vertebrates. The Galβ1-4Gal epitopes on either glycoproteins or glycolipids have not been found in humans, mice and other species of mammals, except for hogs, which are reported to express glycolipids that contain the Galβ1-4Galβ1-4Glc-Cer structure in their stomachs (Slomiany et al. 1974). Since human sera show high anti-(Galβ1-4Gal) antibody titers (Bouhours et al. 2000), it is speculated that these antibodies are protective against enveloped viruses and/or microbes expressing Galβ1-4Gal, as is the case for the Galα1-3Gal epitope. In contrast, the Galβ1-4Gal epitopes on glycoproteins in birds was initially found in O-glycans from salivary gland mucin of the Chinese swiftlet (Wieruszewski et al. 1987) and in N-glycans of pigeon immunoglobulin (Ig)G (Suzuki et al. 2003). A glycolipid with a pentasaccharide structure, Galβ1-4Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer, was found in the ostrich liver (Bouhours et al. 2000). We previously detected the enzyme activities of UDP-Gal:β-D-galactosyltransferase (βGalT1-Gal)β, which directs the production of the Galβ1-4Gal epitopes in various tissues of pigeon and ostrich (Suzuki et al. 2011). The Galβ1-4Gal epitopes are also reported to be present on glycoproteins found in several fishes, such as Japanese medaka (Oryzias latipes; Iwaski et al. 1992; Taguchi et al. 1994), Indian medaka (Oryzias melastigma; Taguchi et al. 1993), zebrafish (Danio rerio; Guerardel et al. 2006; Moriguchi et al. 2007), dace (Tribolodon hakonensis; Inoue et al. 1989; Taguchi et al. 1996) and salmoid fishes (e.g. rainbow trout, Oncorhynchus mykiss; Shimamura et al. 1983; Iwaski et al. 1984; Iwaski and Inoue 1985), and on those found in the egg jelly coats of amphibians, such as the common frog (Rana temporaria; Coppin et al. 1999; Maes et al. 1997), march frog (R. ridibunda; Mourad et al. 2001), agile frog (R. dalmatina; Florea et al. 2002), fire-bellied toad (Bombina bombina; Coppin et al. 2003) and yellow-bellied toad (B. variegata; Coppin et al. 2003). In most cases, the presence of glycans containing the Galβ1-4Gal epitopes was discovered rather coincidentally by exhaustive structural analysis using mass spectrometry (MS) and/or NMR spectrometry. Although the Galβ1-4Gal epitopes could be distributed in a wider range of vertebrates than suspected previously, extensive studies were not performed due to the lack of convenient tools to detect this glycan epitope rapidly in samples from various sources.

To overcome this problem, we have raised the specific monoclonal antibodies (mAbs) that recognize the Galβ1-4Gal epitopes using extracts of medaka eggs as an immunogen. The fine specificities of four antibodies were evaluated by frontal affinity chromatography (FAC) using various structures of oligosaccharides. Moreover, using western blotting analysis, we demonstrated for the first time that the Galβ1-4Gal epitopes on glycoproteins is also expressed in various tissues of the African clawed frog (Xenopus laevis). Immunohistochemical staining of medaka tissue sections revealed that Galβ1-4Gal epitopes were expressed in various tissues including the ovary, intestine, brain, liver and heart. The highest levels of expression were observed in the endothelium, epithelium and epidermis throughout the entire body, which come into direct contact with the external environment or invading organisms.

### Results

**Enzyme-linked immunosorbent assay of mAb binding to Galβ1-4Galβ1-4Glc bovine serum albumin**

We used extracts of medaka eggs as the immunogen to obtain anti-(Galβ1-4Gal) antibodies, because some N-glycans from medaka eggs were reported to contain the Galβ1-4Gal epitopes (Iwaski et al. 1992). Prior to immunization, the extracts were treated under mild acid conditions to remove sialic acids on non-reducing termini of glycans. Twelve days after the second immunization, antisera were obtained from the immunized mice and tested with enzyme-linked immunosorbent assay (ELISA). To detect the titer of antibodies, ELISA was performed using plates coated with Galβ1-4Galβ1-4Glc bovine serum albumin (BSA), Lac-BSA or BSA. An increased reactivity of antisera to Galβ1-4Galβ1-4Glc-BSA was observed compared with those of preimmune sera (data not shown), whereas no interaction with BSA was observed in either the antisera or the preimmune sera. Mice were immunized with extracts of medaka eggs four times in total, and 3 days after the last immunization, the splenocytes were fused with mouse myeloma cell PAI. Using ELISA-based screening, we successfully obtained four hybridoma cell lines (designated clones 27, 44, 67 and 68) that produced mAbs that were reactive with Galβ1-4Galβ1-4Glc-BSA but not with Lac-BSA or BSA. Two of the four clones were IgG1s (clones 27 and 68) and the other two clones were IgMs (clones 44 and 67). After purifying each antibody from culture supernatant, dose-dependent bindings of purified antibody to Galβ1-4Galβ1-4Glc-BSA were demonstrated by ELISA (Figure 1A).

**βGal-oligo** is the mixture of β-D-galactosyloligosaccharides produced from lactose reacted with β4-transgalactosidase from

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1In this paper, GalTs are conveniently abbreviated as linkageGalT (acceptor substrate) to distinguish their acceptor substrate specificities from each other, e.g., UDP-galactose-β-D-galactoside β1,4-galactosyltransferase is designated as β4GalT1(Gal).
Fig. 1. Binding of mAbs to Galβ1-4Galβ1-4Glc-BSA immobilized on microplates. (A) Serially diluted mAbs (27, 44, 67 and 68) were incubated with immobilized Galβ1-4Galβ1-4Glc-BSA, Galβ1-4Glc-BSA (Lac-BSA) or BSA and binding activity was analyzed with ELISA using AP-labeled goat anti-mouse IgG (for 27 and 68) or AP-labeled goat anti-mouse IgM (for 44 and 67) as second antibodies. (B) Inhibition of the binding between immobilized Galβ1-4Galβ1-4Glc BSA and mAbs by oligosaccharides. After anti-(Galβ1-4Gal) mAbs (27, 44, 67 and 68) were preincubated with serially diluted βGal-oligo, lactose or maltose, binding of mAbs to immobilized Galβ1-4Galβ1-4Glc-BSA was analyzed with ELISA. The values represent the mean ± SD of duplicate samples.
βGal-oligo consists mainly of Galβ1-(4Galβ1-4)Glc and Galβ1-(4Galβ1-4)Gal (n = 1–4), and 4′-galactosylactose (Galβ1-4Galβ1-4Glc) is the most abundant component (more than 50%). As shown in Figure 1B, 1–10 mM βGal-oligo (calculated as Galβ1-4Galβ1-4Glc) inhibited over 90% of the binding of each antibody to Galβ1-4Galβ1-4Glc-BSA. In contrast, 10–100 mM lactose (Galβ1-4Glc) only partially inhibited the binding of two antibodies (27 and 68) and had no significant inhibitory effect on those of the other two antibodies (44 and 67). Maltose (Glcα1-4Glc) had no significant inhibitory effect on the binding of any of the four antibodies, suggesting that the inhibitory effect of oligosaccharides depended on their structure.

Detection of (Galβ1-4Gal)-containing N-glycans on pigeon egg yolk IgG by western blot analysis

The binding activities of mAbs for native glycoproteins were demonstrated by western blot analysis using pigeon IgG, which is known to possess the Galβ1-4Galβ1-4GlcNAc sequences on N-glycans (Suzuki et al. 2003). As shown in Figure 2, none of the mAbs bound to untreated pigeon IgG, but they all bound to α-galactosidase-treated pigeon IgG, which possessed the Galβ1-4Gal epitopes at the non-reducing termini. The binding of mAbs was abrogated when α-galactosidase-treated pigeon IgG was further treated with β4-galactosidase. These results indicate that the mAbs recognize Galβ1-4Gal epitopes at non-reducing ends, but not at internal positions masked by α-galactosylation. We also demonstrated that the mAbs did not react with glycoproteins such as human transferrin, bovine fetuin, human α1-acid glycoprotein and their asialo-derivatives, which are known to possess oligosaccharides with Galβ1-4GlcNAc but not Galβ1-4Gal (data not shown).

FAC analysis for determination of the fine specificities of mAbs for 2-aminopyridine oligosaccharides

To determine the binding specificities of the four mAbs for carbohydrate epitopes, FAC analysis was performed. Each mAb was immobilized on NHS-activated Sepharose by amine coupling. To evaluate the column capacity (B), mixtures of 0.3 μM Galβ1-4Galβ1-4Glc-pNP and unlabeled 1–200 μM Galβ1-4Galβ1-4Glc were used, based on the assumption that Galβ1-4Galβ1-4Glc-pNP and Galβ1-4Galβ1-4Glc are equally able to bind to the immobilized antibodies. By concentration-dependence analysis using these sugars (Figure 3), B and Kd values were determined to be 0.59 nmol and 2.1 × 10^−7 M for mAb 27, 1.20 nmol and 4.4 × 10^−5 M for mAb 44, 4.29 nmol and 2.2 × 10^−4 M for mAb 67 and 0.78 nmol and 2.5 × 10^−5 M for mAb 68, respectively. Based on the limitation of the V−F0 value (5 μL), limits of detection (calculated as Kd values) on the columns were 1.2 × 10^−4 M for mAb 27, 2.4 × 10^−4 M for mAb 44, 8.6 × 10^−4 M for mAb 67 and 1.6 × 10^−4 M for mAb 68 [see Equation (3) in Materials and methods].

To compare the reactivity of those antibodies with oligosaccharides containing Galβ1-4Glc, Galβ1-4GlcNAc, Galβ1-4Galβ1-4GlcNAc, Galβ1-4Galβ1-4GlcNAc and Galα1-4Galβ1-4GlcNAc on their non-reducing termini, we prepared 24 oligosaccharides (Figure 4) as described in Materials and methods. We also analyzed the 118 oligosaccharides shown in Supplementary data, Figure S1, which were prepared previously (Itakura et al. 2007). The FAC analysis revealed that no detectable interactions were observed between each of the four mAbs and oligosaccharides lacking Galβ1-4Gal on their non-reducing termini (data not shown). In contrast, all four mAbs interacted with β4-Lac-2-aminopyridine (PA) (Galβ1-4Galβ1-4Glc-PA), although the affinities of mAbs 27, 44 and 67 to β4-LacNAc-PA (Galβ1-4Galβ1-4GlcNAc-PA) were below the detectable range (Table 1, Figure 5). The results indicated that the Galβ1-4Gal sequence is the minimum essential residue to be recognized by all of the mAbs, but that the internal Glc/GlcNAc structures also influence the affinities. The antibodies are presumed to be more resistant to the opened circular form of Glc than to that of GlcNAc on β4-LacNAc-PA, since these mAbs were screened with Galβ1-4Galβ1-4Glc conjugated with BSA, which also contains the opened circular form of Glc due to reductive amination. Four antibodies did not bind to Galα1-4Galβ1-4GlcNAc (Figure 5), indicating that α4-galactosylation of Galβ1-4Gal masked the epitope. This result is consistent with the observation that the mAbs bound to pigeon IgG only after α-galactosidase treatment (Figure 2). It is noteworthy that the apparent Kd values were not significantly affected by the number of branches containing Galβ1-4Gal on N-glycan.
in the case of mAbs 27 and 68, which belong to the IgG1 subclass (Table I, Figure 5). In contrast, the binding of mAbs 44 and 67 (IgM subclass) to the biantennary \(N\)-glycans was beneath the detection limits, and the apparent binding constants increased \(\approx 6\)-fold, in increments of the number of branches from tri- to tetra-antennary. It should also be emphasized that the FAC analysis indicated negligible cross-reactivity between the mAbs and \(PA\) glycans containing \(\text{Gal}^\alpha_1-\text{Gal}^\beta_1-\text{Gal}^\beta_1-\text{Glc(NAc)}, \text{Gal}^\beta_1-\text{GlcNAc}, \text{GalNAc}^\beta_1-\text{Gal}^\beta_1-\text{Glc}\) or \(\text{Gal}^\beta_1-\text{Man},\) suggesting that these mAbs are highly specific for \(\text{Gal}^\beta_1-\text{Gal}\) containing oligosaccharides.

We also performed inhibitory experiments using FAC analysis to estimate the strength of the interaction between mAb 68 and \(\text{Gal}^\beta_1-\text{Gal}\), which is found in 6-gala-series glycosphingolipids in invertebrates (Matsubara and Hayashi 1986; Persat et al. 1992). Several other free di- or trisaccharides (Figure 6A and B) were also examined in these inhibitory experiments to compare the affinity of mAb 68 for these different sugars. We choose mAb 68 for the following experiments, because it showed detectable binding to both linear and branched \(PA\) glycans with \(\text{Gal}^\beta_1-\text{Gal}\) on non-reducing termini by FAC analysis (Figure 5, Table I). A \(PA\)-derivatized triantennary \(N\)-glycan with \(\text{Gal}^\beta_1-\text{Gal}^\beta_1-\text{Glc(NAc)}\) termini (\(\beta\text{Tri-PA}\) in Figure 4) was used as the analyte. When \(\beta\text{Tri-PA}\) was loaded onto an mAb 68-immobilized column in the absence of inhibitors, the elution volume of the analyte \((V)\) was increased comparing with that of the reference oligosaccharide \((V_0)\) due to the interaction of the analyte with the antibody. This increment of elution volume \((V - V_0)\) was reduced in the presence of \(\text{Gal}^\beta_1-\text{Gal}\) disaccharides \((V_i - V_0)\) in a dose-dependent manner (Figure 6A). Even at 100 \(\mu\text{M}\) \(\text{Gal}^\beta_1-\text{Gal}\), the level of inhibition was calculated to be 76.2%, and the interaction was almost completely inhibited (93.5%) in the presence of 1000 \(\mu\text{M}\) \(\text{Gal}^\beta_1-\text{Gal}\). In contrast, both \(\text{Gal}^\beta_1-\text{Glc}\) (lactose; 14.5% at 100 \(\mu\text{M}\); 28.3% at 1000 \(\mu\text{M}\)) and \(\text{Gal}^\beta_1-\text{Gal}\) partially inhibited the interaction between mAb 68 and the analyte. Similarly, 100 \(\mu\text{M}\) of \(\text{Gal}^\alpha_1-\text{Gal}\) (53.8%) and \(\text{Gal}^\beta_1-\text{GlcNAc}\) (46.1%) partially inhibited the interaction (Figure 6B), whereas the mAb did not recognize glycoproteins carrying \(\text{Gal}^\alpha_1-\text{Gal}\) or \(\text{Gal}^\beta_1-\text{GlcNAc}\) at the non-reducing termini. These results indicated that the affinity of mAb 68 for \(\text{Gal}^\beta_1-\text{Gal}\) was lower than that for \(\text{Gal}^\beta_1-\text{Gal}\), and it is unlikely that the mAb cross-reacts with \(\text{Gal}^\beta_1-\text{Gal}\).

Detection of glycoproteins containing \(\text{Gal}^\beta_1-\text{Gal}\) in tissues from medaka, zebrafish and African clawed frogs by western blot analysis

To demonstrate that the antibody is also useful to explore the expression of \(\text{Gal}^\beta_1-\text{Gal}\) in nature, we examined the expression of glycoproteins with \(\text{Gal}^\beta_1-\text{Gal}\) epitopes in egg extracts from medaka, zebrafish and African clawed frogs. Because we used egg extracts from medaka as an immunogen.
to obtain the anti-(Galβ1-4Gal) mAb, the egg glycoproteins were expected to be stained with the antibody. We found that several glycoproteins from medaka eggs were indeed stained with anti-(Galβ1-4Gal) mAb 68 (Figure 7A). Zebrafish embryos had been reported to express the Galβ1-4Gal, (±Neu5Gc/Neu5Ac)α2-3Galβ1-4(Fucα1-3)GlcNAc sequences on the non-reducing termini of N-glycans (Guerardel et al. 2006; Moriguchi et al. 2007). The mAb 68 also stained several glycoproteins from zebrafish eggs, although the intensities of the stained bands were not as high as those found in medaka (Figure 7A). In contrast, the presence of Galβ1-4Gal in the African clawed frog had not hitherto been reported. However,
when we analyzed the frog egg proteins by western blotting, the results suggested the presence of this glycan epitope. We further analyzed several tissues from the African clawed frog by western blotting, and the presence of Gal\(\beta_1-4\)Gal on glycans was also detected with the antibody (Figure 7B). Most of the bands vanished when the mAb was pre-incubated with \(\beta\)Gal-oligo, supporting the specificity of the interaction (Figure 7A and B). The activity of \(\beta 4\)GalT(Gal) in the frog tissues was analyzed by the same high performance liquid chromatography (HPLC) method (Suzuki et al. 2011), and enzyme activity was detected in several tissues such as the liver, stomach and small intestine (data not shown). Thus, we have demonstrated for the first time that the African clawed frog also expresses Gal\(\beta_1-4\)Gal on glycoproteins.

**Immunohistochemical staining of whole medaka tissue sections with the anti-(Gal\(\beta_1-4\)Gal) mAb**

To explore the biological features of the Gal\(\beta_1-4\)Gal epitope, we examined glycan localization in medaka sections using anti-(Gal\(\beta_1-4\)Gal) mAbs. Since a typical adult Japanese medaka is <30 mm in length, it is possible to stain different tissues and organs in a single head-to-tail histological section (Figure 8). Medaka tissue sections were incubated with anti-(Gal\(\beta_1-4\)Gal) mAb 68 and visualized with 3,3′-diaminobenzidine tetrahydrochloride (DAB). Immunohistochemical staining revealed that mAb 68 bound to the vascular endothelium, epithelium and epidermis throughout the entire body (Figure 8A). Notably, the cell surface and the inside of intestinal epithelial cells showed strong staining (Figure 8A, intestine). The antibody also stained epithelial cells in the oviduct, the epithelial layer lining the mouth to the esophagus, cells in gills and the epidermis of skin (Figure 8A, whole body), all of which are likely to come into direct contact with bacteria or other invasive microbes. The vascular endothelium in the brain [Figure 8A, brain (a and b)], the sinusoidal endothelium and hepatocytes (Figure 8A, liver) and the endocardial endothelium in the atra of the heart (Figure 8A, heart) were also

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**Table 1.** Dissociation constants for binding of PA-derivatized oligosaccharides containing Gal\(\beta_1-4\)Gal at their non-reducing termini to immobilized antibodies by FAC

<table>
<thead>
<tr>
<th>Oligosaccharides</th>
<th>(K_d) (M)</th>
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<tr>
<td></td>
<td>27 (IgG1)</td>
</tr>
<tr>
<td>(\beta)Lac-PA</td>
<td>(4.5 \times 10^{-5})</td>
</tr>
<tr>
<td>(\beta)LacNAc-PA</td>
<td>ND</td>
</tr>
<tr>
<td>(\beta)BiF-PA</td>
<td>(1.5 \times 10^{-5})</td>
</tr>
<tr>
<td>(\beta)Bi-PA</td>
<td>(1.4 \times 10^{-5})</td>
</tr>
<tr>
<td>(\beta)Tri-PA</td>
<td>(1.6 \times 10^{-5})</td>
</tr>
<tr>
<td>(\beta)Tetra-PA</td>
<td>(1.5 \times 10^{-5})</td>
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\(aND,\) not detected.

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**Fig. 5.** Bar graph representation of the affinity constants (\(K_a\) values) of the four anti-(Gal\(\beta_1-4\)Gal) mAbs for PA-oligosaccharides. The structures of the PA-oligosaccharides are shown in Figure 4.
stained by mAb 68. Secretory and endothelial cells in islets of Langerhans and in the pituitary gland [arrow in Figure 8A, brain (a)] were also clearly stained. In contrast, the bones and nerve fibers were not stained. Some goblet cells in the intestinal epithelium (arrows in Figure 8A, intestine) showed nonspecific staining, even in the absence of the primary antibody (data not shown). Except these goblet cells, the immunostaining of cells and tissues was completely abrogated or markedly reduced by preincubating the mAb with βGal-oligo.

Stage-dependent immunoreactivity against mAb 68 was observed in the ovaries in medaka sections. During the spawning season, medaka ovaries contain oocytes at all stages of oogenesis (Stages I–X; Iwamatsu et al. 1988). mAb 68 showed specific reactivity to the cytosol and yolk droplets in oocytes at the vitellogenic stages [later than Stage V; Figure 8A, ovary (a,c)]. In immature oocytes (Stage II), mAb 68 recognized both fine granules and a mass of the granules in the perinuclear region [Figure 8A, ovary (b)]. Immunoreactivity in the cytosol was higher in Stage III oocytes than in Stage II oocytes [Figure 8A, ovary (b)]. In contrast, the cytosol and granules of more immature oocytes [Stage I, Figure 8A ovary (b)] showed no immunoreactivity. This suggests that the expression of Galβ1-4Gal epitopes occurs in oocytes after Stage II of oogenesis.

To compare the expression profiles of Galβ1-4Gal epitopes with those of several other glycan epitopes expressed on non-reducing termini, we stained serial medaka whole body sections with five different plant lectins: Maackia amurensis hemagglutinin (MAH, Siaα2-3Galβ1-3 GalNAc-specific), M. amurensis leukoagglutinin (MAL, Siaα2-3Galβ1-4GlcNAc-specific), Sambucus seiboldiana agglutinin [Sambucus seiboldiana agglutinin (SSA), Siaα2-6Gal-specific], Erythrina cristagalli agglutinin [Erythrina cristagalli agglutinin (ECA), Galβ1-4GlcNAc-specific] and peanut agglutinin [peanut agglutinin (PNA), Galβ1-3GlcNAc-specific]. Of these, only MAL also stained the endothelium (data not shown). Unlike mAb 68, however, MAL did not stain the epidermis. As exemplified by the lectin staining pattern observed in the ovary (Figure 8B), the different lectins showed staining patterns that were clearly distinct from that of mAb 68 [Figure 8A, ovary (a)]. Both MAH and MAL stained egg shells (chorion) but not egg yolks or egg cytosol. SSA did not stain egg shells, but did stain egg yolk.

![Fig. 6. Inhibitory effects of di- or trisaccharides on the interaction between anti-(Galβ1-4Gal) mAb 68 and PA-derivatized triantennary N-glycans (βTri-PA) as assessed by FAC. (A) Serially diluted disaccharides were mixed with 10 nM βTri-PA and loaded onto a column containing immobilized mAb 68. (B) Various di- or tri-saccharides (100 μM) were mixed with βTri-PA and then loaded onto the column. Inhibition (%) was calculated as described in Materials and methods.](https://academic.oup.com/glycob/article-abstract/23/1/91/1988306)
granules in maturing oocytes (Stage V and later). In contrast, ECA stained the cytosol in early stage immature oocytes (Stages I and II), but not the cytosol in more mature oocytes (Stage III and later). In the oocytes at Stage III and later, ECA stained only the egg shells. PNA stained yolk drops and the most inner layer of the egg shell in oocytes at Stage III or later. Thus, the lectin staining and the immunostaining with mAb 68 showed that the distribution of Gal\(\beta_1\)4Gal epitopes in the medaka tissues is different from that of glycans containing sialic acids or \(\beta\)-galactosides in other structures such as Gal\(\beta_1\)4GlcNAc or Gal\(\beta_1\)3GlcNAc at the non-reducing termini.

Discussion

Lectins and antibodies recognizing specific glycan structures are powerful tools for studying glycans expressed in various samples. Although detection by lectins and/or antibodies cannot reveal the precise glycan structures, they are useful to
investigate the presence of glycan epitopes expressed on cell surfaces, tissues and other biological materials. For instance, we have previously succeeded in examining the expression profiles of Galβ1-4Gal on glycoproteins in egg white from 181 species of birds, as probed by western blot with *Griffonia simplicifolia*-I lectin (terminal α-Gal/GalNAc-specific) and anti-P₁ mAb (Galβ1-4Galβ1-4GlcNAc-specific) (Suzuki et al. 2004, 2006). Lectins and anti-glycan antibodies are also used for purification of glycans and isolation of cells expressing particular glycan structures. Currently, many lecins and anti-glycan mAbs are commercially available, but most of them are specific for glycans expressed in mammals. Since relatively few studies have focused on glycans rarely expressed in mammals, such as the Galβ1-4Gal epitopes, a convenient method for detecting these structures was hitherto not available. To overcome this problem, we generated specific antibodies that recognize Galβ1-4Gal epitopes.

In this study, we obtained four hybridoma cell lines producing anti-(Galβ1-4Gal) mAbs by immunizing mice with phenol extracts of medaka eggs. To define the specific epitopes recognized by these mAbs, we used the FAC system with various PA-derivatized oligosaccharides as analytes (Tateno et al. 2007). FAC analysis is advantageous for the detection of relatively weak interactions, such as those between lectins and carbohydrate ligands and is used to determine the specificities of various kinds of lectins (Kasai et al. 1986; Hirabayashi 2004; Tateno et al. 2007). In addition to lecins, we revealed that the same procedures are also applicable for determining the precise specificities of the anti-carbohydrate mAbs. The other advantage of the FAC system is that the number of analytes can conveniently be expanded if necessary, since PA-oligosaccharides are easily prepared and isolated by HPLC (Tomiya et al. 1988; Hase 1994). We recently succeeded in preparing PA-N-glycans containing Galβ1-4Gal, Galα1-4Gal or Galα1-4Galβ1-4Gal sequences at the non-reducing termini using β4GalT(Gal) and α4GalT(Gal) (Suzuki and Yamamoto 2010; Suzuki et al. 2011). By the same procedures, PA-glycans with a different number of branches were prepared as shown in Figure 4, and the affinity to the mAbs was compared by FAC analysis. The apparent affinities of IgM mAbs increased in increments of the number of branches, while the number of branches did not significantly affect the apparent affinities of IgG mAbs. The pentameric structures of IgM may have a good chance of meeting epitopes on multiple branches, whereas the monovalent binding between one of the antigen-binding sites if IgM and one epitope is relatively weak. Since no one has ever reported such a precise analysis for anti-carbohydrate mAbs, the information will be useful to engineer some artificial lecins or antibodies for studies of glycans. The FAC analysis also revealed that the obtained mAbs have an affinity for oligosaccharides with Galβ1-4Gal at their non-reducing termini, but not for Galβ1-4GlcNAc, which is commonly found in various animals, suggesting that the mAbs are useful for distinguishing between these structures.

The specificity of mAb 68 was also examined in inhibition experiments using di- or trisaccharides on FAC analysis. The advantage of this method is that free oligosaccharides without any modification of the reducing termini can be analyzed using the same FAC system by comparing the elution volume of the analyte mixed with inhibitors. When free oligosaccharides inhibit the interaction between an immobilized mAb and a PA-derivatized analyte, decreased elution volumes of the analyte can be observed. Thus, this method is useful as it reduces the influence of structural changes induced by reductive amination and derivatization of the reducing termini of oligosaccharides. The present study examined the interaction between mAb 68 and Galβ1-6Gal, which is expressed on 6-gala-series glycosphingolipids in invertebrates. Although Galβ1-6Gal partially inhibited the interaction between immobilized mAb 68 and PA-derivatized triantennary N-glycan with Galβ1-4Galβ1-4GlcNAc-terminal (βTri-PA) analyte (Figure 6A), the inhibition (%) was still lower than that observed for Galα1-4Gal or Galβ1-4GlcNAc (Figure 6B). Because small glycans, such as mono- or disaccharides, sometimes interact with binding sites on lecins/antibodies if they are present at high concentrations, the observed partial inhibition is probably due to a degree of similarity between the structures of the free disaccharides and the analyte. As we demonstrated that mAb 68 does not recognize glycoproteins containing Galα1-4Gal or Galβ1-4GlcNAc at the non-reducing termini, it is less likely that the mAb recognizes Galβ1-6Gal sequences.

Once the specific antibodies against Galβ1-4Gal are successfully established, they can be used to accelerate studies of the expression of Galβ1-4Gal in nature. For instance, we have previously demonstrated that Galβ1-4Gal on glycoproteins is expressed in ostrich using the anti-(Galβ1-4Gal) mAb 68 (Suzuki et al. 2011). The presence of Galβ1-4Gal in the African clawed frog has not been reported, but we have demonstrated in this study for the first time that this frog also expresses the glycans containing the Galβ1-4Gal in various tissues (Figure 7). The result is consistent with the observation that genes encoding putative proteins similar to pigeon β4GalT(Gal) are found in DNA/protein databases of *X. laevis* (Suzuki and Yamamoto 2010). It should be noted that the anti-(Galβ1-4Gal) mAb was also useful for the expression cloning of pigeon β4GalT(Gal) (Suzuki and Yamamoto 2010). The obtained information of DNA and amino acid sequences of the enzyme will further accelerate investigations of the species-specific expression of Galβ1-4Gal.

We also showed that the anti-(Galβ1-4Gal) mAb can be used for immunohistochemistry. Staining of medaka tissue sections revealed characteristic expression patterns for (Galβ1-4Gal)-containing glycans: (i) Galβ1-4Gal epitopes were distributed throughout various tissues in the body, including the ovary, intestine, liver, brain and heart; (ii) the unique distribution of Galβ1-4Gal epitopes was mainly due to their expression in the vascular endothelium, epithelium and epidermis, which are exposed to the external environment and (iii) the tissue distribution of Galβ1-4Gal epitopes was different from that of sialic acids or β-galactosides in other structures such as Galβ1-4GlcNAc or Galβ1-3GlcNAc at non-reducing termini. It is notable that Galβ1-4Gal epitopes are expressed in tissues exposed to the external environment and/or invading organisms in medaka, which is a teleost, one of the large taxa of vertebrates. Teleosts possess unique non-specific defense systems that exclude foreign bodies. For
instance, cardiac endothelial cells function as phagocytic cells (Nakamura and Shimozawa 1994). The skin of the fish, which is in direct contact with water, comprises non-keratinized stratified squamous epithelium, unlike that of terrestrial vertebrates such as mammals. This surface tissue of medaka is also important for protecting the body and for the elimination of exogenous materials (Nakamura et al. 1992). Since the fish possess such unique defense systems, it is possible that unique non-mammalian glycans are somehow implicated in the interaction with foreign organisms. For example, the expression of Galβ1-4Gal epitopes could lead to masking or decreased the expression of Galβ1-4GlcNAc or sialic acids at non-reducing termini, which are often the targets of infectious microbes, viruses or toxins. Alternatively, Galβ1-4Gal epitopes can be used to target and absorb foreign organisms. Although further extensive studies are needed to explore the biological functions of Galβ1-4Gal epitopes in non-mammalian vertebrates, our findings promote the investigation of Galβ1-4Gal epitopes as a species-specific barrier against foreign organisms in primitive vertebrates.

Immunohistochemical and lectin staining also revealed distinct expression profiles for glycan epitopes during oocyte maturation. Among the mAb and lectins used for tissue staining in this study, only mAb 68 and ECA stained the cytosol of oocytes in the ovary (Figure 8). Immature oocytes at Stage I were stained only with ECA, which recognizes Galβ1-4GlcNAc at non-reducing termini. In contrast, the expression of Galβ1-4Gal epitopes in oocytes was initiated from Stage II onward. It seems that the non-reducing termini of glycans expressed in oocytes were converted from Galβ1-4GlcNAc to Galβ1-4Galβ1-4GlcNAc by β4GalT(Gal) along with the oocyte maturation. Alternatively, (Galβ1-4Gal)-containing glycans biosynthesized in follicle cells or other tissues might be transported into the oocytes as they matured. The mechanism underlying the accumulation of epitopes in the cytosol remains to be elucidated. Isolation and characterization of glycans from the cytosol will further investigations into the biological implications of the glycans carrying Galβ1-4Gal epitopes. Anti-(Galβ1-4Gal) mAbs will be a useful tool for isolating and investigating such cytosolic glycans.

Highly heterogeneous structures and species-specific variations are found in glycans from eggs of several fishes and amphibians. Although the number of species analyzed for glycan structures are limited, some of the fishes and amphibians possess Galβ1-4Gal (Shimamura et al. 1983; Iwasaki and Inoue 1985; Iwasaki et al. 1984, 1992; Inoue et al. 1989; Taguchi et al. 1993, 1994, 1996; Maes et al. 1997; Coppin et al. 1999, 2003; Mourad et al. 2001; Florea et al. 2002; Guerardel et al. 2006; Moriguchi et al. 2007). However, it is unclear whether the expression abilities of the glycan epitope in fishes and amphibians were directly inherited by birds or generated independently. The current data of glycan structures expressed in reptiles are extremely limited, and it is not possible to compare the presence of species-specific glycans in reptiles with that in other animals. Since birds are believed to be the close relatives of the extinct lineages of reptiles, i.e. dinosaurs, systematic investigation of the presence of Galβ1-4Gal in reptiles will be helpful in understanding the origin of these glycans in vertebrates and the relationship between them. As we have demonstrated the presence of Galβ1-4Gal in ostrich and in the African clawed frog, the anti-(Galβ1-4Gal) mAb will be useful in investigating the presence of Galβ1-4Gal in a wide range of vertebrates and in many other organisms in nature, including invertebrates, microbes and parasites. Because glycan variation plays an important role in determining host-pathogen/symbiont interactions, comprehensive surveillance of the unique distribution of Galβ1-4Gal epitopes will increase the understanding of diseases, particularly epidemics or zoonotics. For instance, because fish and birds are utilized as foodstuffs or kept as pets, studies on the molecular basis of the infectious diseases and pathogens they harbor will be required for industrial and environmental purposes.
Fertilized eggs from zebrafish (*D. rerio*) were gifts from Dr Shoji Kawamura (Department of Integrated Biosciences, University of Tokyo). Unfertilized eggs and various tissues from the African clawed frog (*X. laevis*) were kind gifts from Dr Kazushige Touhara (Department of Integrated Biosciences, University of Tokyo). DAB was from Dojindo Laboratories (Kumamoto, Japan). Vectastain Elite ABC kit (mouse IgG) was purchased from Vector Laboratories Inc. (Burlingame, CA). Biotinylated SSA, ECA and PNA were purchased from Seikagaku Corp. (Tokyo, Japan). MAH and MAL were purified (Yamamoto et al. 1997) and biotinylated using sulfon-NHS-LC-biotin (from Thermo Scientific, Rockford IL).

**Standard procedures**

Protein concentrations were measured by the BCA assay using the BCA Protein Assay Reagent Kit (Pierce). BSA was used as a standard. Procedures of GalT assays and protein extractions from tissues (Suzuki et al. 2009, 2011).

**Electrophoresis and western blotting**

Electrophoresis was performed under reducing conditions in 12.5% SDS–polyacrylamide gel. The separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes and then detected with Coomassie Brilliant Blue R-250 (CBB) or by antibody staining. For antibody staining, the membranes were soaked in blocking solution, which consisted of Tris-buffered saline (TBS; 20 mM Tris–HCl, pH 7.5, with 150 mM NaCl) containing 3% (w/v) BSA. The blot membranes were first incubated with the anti-(Galβ1-4Gal) mAb. After washing with 0.1% Tween-20 in TBS (TBST), the membranes were incubated with AP-labeled secondary antibodies for antibody staining. For inhibitory experiments on antibody staining, mAb 68 was preincubated with 100 mM β-Gal-oligo (calculated as Galβ1-4Galβ1-4Glc) at room temperature for 30 min, prior to the incubation with blotted membranes.

**Preparation of neoglycoproteins**

4′-Galactosyllactose (Galβ1-4Galβ1-4Glc) or lactose (Galβ1-4Glc) was coupled with BSA by reductive amination (Yamaji et al. 2003). Briefly, the free tri- or disaccharides (10 mg) were mixed with BSA (10 mg) in 822 μl of 0.2 M sodium borate (pH 9.0) and incubated at 50°C for 15 min. After adding 126 μl of pyridine–borane complex in methanol (methanol: pyridine–borane = 5:2), the mixtures were incubated at 50°C for 2 days and then dialyzed against water. The prepared neoglycoproteins coupled with Galβ1-4Galβ1-4Glc and Galβ1-4Glc were designated Galβ1-4Galβ1-4Glc-BSA and Lac-BSA, respectively. The products were analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS (Suzuki and Lee 2004). The detected molecular masses indicated that 11–19 oligosaccharides/protein in Galβ1-4Galβ1-4Glc-BSA and 14–22 oligosaccharides/protein in Lac-BSA were coupled to BSA.

**Immunization, hybridoma preparation and antibody selection**

A cluster of fertilized eggs were collected from spawned females of medaka (Hd-rf) and were rubbed between two pieces of paper towel to remove filaments on egg shell and to isolate each egg. Phenol extracts of medaka eggs were prepared (Kitajima et al. 1989). To remove sialic acids, the phenol extracts were dissolved in 50 mM HCl and heated at 80°C for 30 min. After neutralizing with NaOH, the sample was dissolved with phosphate-buffered saline (PBS) and used as an immunogen. BALB/c mice were injected intraperitoneally with 200 μg per mouse of the extract emulsified in complete Freund’s adjuvant on day 0. The same mice were injected intraperitoneally with the same immunogens emulsified in incomplete Freund’s adjuvant on days 14, 26 and 38. The splenocytes were obtained 3 days after the last injection and fused with murine myeloma cell line PAI using PEG 1500. Screening of hybridomas for anti-(Galβ1-4Gal) production was performed by ELISA. The anti-(Galβ1-4Gal)-positive hybridoma cells were cloned by limiting dilution, and the antibody isotype was determined using the Mouse MonoAb ID/SP Kit. The mAbs were purified from supernatants of the culture media using an ImmunoAssist MG-PP column and a Superdex 200 26/60 column according to the manufacturers’ procedures.

**Enzyme-linked immunosorbent assay**

Microplates were coated with BSA, Galβ1-4Galβ1-4Glc-BSA or Lac-BSA (10 μg/mL and 100 μL/well) in 50 mM Tris–HCl (pH 8.0) and left overnight at 4°C. Unbound antigen was washed out of the wells with TBST, and a blocking solution of TBST containing 3% BSA was added (200 μL/well) to the wells. The plates were incubated for 3 h at 4°C, and 100 μL/well of supernatants of hybridoma cells or purified mAbs diluted with TBST were added. After incubating the plate for 1.5 h at room temperature and washing again with TBST, secondary antibodies (AP-goat anti-mouse IgG and/or anti-mouse IgM) were added (100 μL/well). For further incubation for 30 min at room temperature, plates were washed thoroughly and antibody binding was detected by the addition of p-nitrophenyl phosphate.

For inhibition assays, mAbs were preincubated with serially diluted β-Gal-oligo, lactose or maltose for 1 h at room temperature and then added to the microplates coated with Galβ1-4Galβ1-4Glc-BSA.

**Preparation of PA-oligosaccharides**

Bi-, tri- and tetraantennary complex-type *N*-glycans were prepared from porcine thyroglobulin, bovine fetuin and pigeon ovalbumin, respectively. N-Glycans were released by hydrazinolysis and re-N-acetylated and then PA-derivatized (Suzuki et al. 2011). The major PA-N-glycans from each glycoprotein were isolated with reversed-phase (CLC-ODS column) and normal-phase (Amide-80 column) HPLC (Suzuki et al. 2011). The isolated PA-oligosaccharides were treated with neuraminidase, α-galactosidase or α-fucosidase, if necessary, to obtain PA-N-glycans containing the Galβ1-4GlcNAc sequence at the non-reducing termini of all branches (Bi-PA, BiF-PA, Tri-PA and Tetra-PA in Figure 4).
Disaccharides (Galβ1-4Glc and Galβ1-4GlcNAc) and trisaccharides (Galβ1-4Galβ1-4Glc) were PA-derivatized by the same procedures. After uncoupled PA was removed using Dowex 50W-X8 (the NH₄⁺ form), PA-oligosaccharides were isolated with normal-phase HPLC using an Amide-80 column, (Fan et al. 1995).

To obtain oligosaccharides containing Galα1-4Gal or Galβ1-4Gal sequences as shown in Figure 4, PA-oligosaccharides were treated with recombinant pigeon αGalT(Gal) (Suzuki and Yamamoto 2010) or βGalT(Gal) in ostrich plasma (Suzuki et al. 2011), and the products were isolated with normal-phase HPLC on an Amide-80 column. Similarly, Galβ1-4Galβ1-4Glcβ1-pNP was prepared from Lac-pNP by the action of ostrich β4GalT(Gal). The molecular masses of the products were confirmed by MALDI-TOF MS analysis (Suzuki and Yamamoto 2010).

The PA-oligosaccharides shown in Supplementary data, Figure S1, were obtained previously (Itakura et al. 2007), and the code numbers and sources of the oligosaccharides are the same as those described in the literature.

Frontal affinity chromatography
Each antibody was immobilized on NHS-activated Sepharose by amine coupling and was packed into a column (31.4 μL bed volume) (Tateno et al. 2007). FAC was performed using an automated system for FAC (FAC-1, Shimadzu Co.; Hirabayashi 2004; Tateno et al. 2007). The flow rate and the column temperature were kept at 0.125 mL/min and 25°C, respectively. An excess volume (0.5–0.8 mL) of each pNP-glycan (0.3 μM) and PA-glycan (1.5–5 nM) in 10 mM Tris–HCl (pH 7.4) and 133 mM NaCl was successively injected into the column by an autosampling system. Elution of pNP- and PA-glycans was monitored with detectors for UV (280 nm) and fluorescence (ex/em = 310/380 nm), respectively. The elution volumes of the analytes (V) and the reference oligosaccharide (Man₃GlcNAc₂-PA, Code No. 014 in Supplementary data, Figure S1) (V₀) were determined from elution profiles. The total amount of the functional immobilized ligand in the column, i.e. effective ligand content (Bₐ), was determined by analysis of concentration dependency (Kasai et al. 1986; Hirabayashi et al. 2003), using mixtures of 0.3 μM Galβ1-4Galβ1-4Glc-pNP and various concentrations (1–200 μM) of Galβ1-4Galβ1-4Glc. Woolf–Hofstee-type plots were obtained by plotting (V–V₀) and (V–V₀)[A]₀ on x- and y-axes, respectively, where [A]₀ is the initial concentration of the analyte. The dissociation constant (Kd) toward Galβ1-4Galβ1-4Glc-pNP and the Bₐ value of the column were determined from the slope of the linear plots and the y-intercept, respectively, according to Equation (2), which is derived from Equation (1) as follows:

\[ K_d = \frac{B_t}{(V - V_0) - [A]_0} \]  

\[ [A]_0(V - V_0) = -K_d(V - V_0) + B_t \]  

Once the Bₐ value of the column was determined, values of Kₐ and the association constant (Kₐ) toward PA-glycans were determined based on Equations (3) and (4).

\[ K_d = \frac{B_t}{(V - V_0)} \text{, when } K_d >> [A]_0 \]  

\[ K_a = \frac{1}{K_d} \]  

For the inhibitory experiments, 10 nM PA-derivatized triantennary N-glycan (βTri-PA in Figure 4) was mixed with each di- or trisaccharide in 10 mM Tris–HCl (pH 7.4), 133 mM NaCl and injected onto the column containing the immobilized anti-(Galβ1-4Gal) mAb. The elution volume of the analyte in the presence (V) or the absence (V₀) of different inhibitors or a reference oligosaccharide (Man₃GlcNAc₂-PA, Code No. 001 in Supplementary data, Figure S1) (V₀) were determined from the elution profiles. Inhibition (%) was calculated as follows:

\[ \text{Inhibition} (\%) = \left( 1 - \frac{(V - V_0)}{(V - V_0)} \right) \times 100 \]

Immunohistochemical staining and lectin staining of medaka tissue sections
Adult female medaka (wild-type) were fixed in Davidson’s fixative (33% ethanol, 8% formalin and 11% acetic acid) for 5 days and embedded in paraffin. Sections (5-μm thick) were mounted on glass slides. These sections were subsequently deparaffinized in xylene, followed by sequential rehydration in ethanol and water. For antigen retrieval, the slides were soaked in 10 mM citric acid buffer (pH 6.0) and autoclaved at 120°C for 10 min. After cooling, the slides were placed in PBS. To quench endogenous peroxidase activity, the slides were soaked in 3% H₂O₂ in PBS for 15 min and then washed with PBS. The sections were blocked by incubation in PBS containing normal horse serum at room temperature for 30 min and then incubated with diluted primary antibodies (15 μg/mL) in PBS containing 0.5% BSA, 0.5% Triton X-100 and 0.1% NaN₃, at room temperature for 3 h. After washing the slides with PBS, the sections were incubated with secondary antibodies (biotinylated horse anti-mouse IgG) at room temperature for 3 h. After washing the slides with PBS, the sections were incubated with avidin–horseradish peroxidase complex according to the manufacturer’s instructions. After washing again, the slides were incubated with DAB (1 mM in 50 mM Tris–HCl, pH 7.4) activated with 1% H₂O₂. The developed slides were washed with PBS and water and counterstained with hematoxylin. After washing again with water, the sections were dehydrated and mounted. Samples were examined under a microscope (BX50, Olympus Corporation, Shinjuku, Japan) equipped with a digital camera (DP70, Olympus). For the antibody absorption test, the primary antibody was preincubated with βGal-oligo (100 mM as Galβ1-4Galβ1-4Glc) at 4°C for 3 h prior to incubation with the tissue sections. For lectin staining, sections were incubated with each of the biotinylated lectins (5 μg/mL) at room
temperature for 3 h, washed with PBS and then incubated with an avidin–biotin–horseradish peroxidase complex.

**Supplementary data**

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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**Conflict of interest**

None declared.

**Abbreviations**

AP, alkaline phosphatase; βGal-oligo, β-galactosyloligosaccharides; BSA, bovine serum albumin; CBB, Coomassie Brilliant Blue R-250; Cer, ceramide; DAB, 3,3′-diaminobenzidine tetrahydrochloride; ECA, *Erythrina cristagalli* agglutinin; FAC, frontal affinity chromatography; GalT, galactosyltransferase; HPLC, high performance liquid chromatography; Ig, immunoglobulin; mAb, monoclonal antibody; MAH, *Maackia amurensis* hemagglutinin; MALDI-TOF, mass spectrometry; ODS, octadecylsilica; PA, 2-aminoypyridine; PBS, phosphate-buffered saline; PNA, peanut agglutinin; PVDF, polyvinylidene difluoride; SSA, *Sambucus sieboldiana* agglutinin.

**References**


