Carbohydrate-recognition domains of galectin-9 are involved in intermolecular interaction with galectin-9 itself and other members of the galectin family

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Galectin-9 (Gal-9) is a tandem-repeat-type member of the galectin family associated with diverse biological processes, such as apoptosis, cell aggregation, and eosinophil chemotraction. Although the detailed sugar-binding specificity of Gal-9 has been elucidated, molecular mechanisms that underlie these functions remain to be investigated. During the course of our binding study by affinity chromatography and surface plasmon resonance (SPR) analysis, we found that human Gal-9 interacts with immobilized Gal-9 in the protein–protein interaction mode. Interestingly, this intermolecular interaction strongly depends on the activity of the carbohydrate recognition domain (CRD), because the addition of potent saccharide inhibitors abolished the binding. The presence of multimers was also confirmed by Ferguson plot analysis of result of polyacrylamide gel electrophoresis and matrix-assisted laser-desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). Moreover, this intermolecular interaction was observed between Gal-9 and other galectin members, such as Gal-3 and Gal-8, but not Gal-1. Because such properties have not been reported yet, they may explain an unidentified mechanism underlying the diverse functions of Gal-9.

Key words: galectin/interaction/multimer/CRD/cross-interaction

Introduction

Galectins form a large family of β-galactoside-binding lectins (Gitt and Barondes 1986; Caron et al. 1990; Barondes, Castronovo, et al. 1994; Cooper 2002; Leffler et al. 2004), which share significant sequence similarity in the carbohydrate recognition domain (CRD) (Hirabayashi and Kasai 1991; Barondes, Cooper, et al. 1994). To date, 15 members have been identified in mammals and named in the order of their identification. On the basis of structural architecture, they are classified into three types, namely, the prototype, chimera type, and tandem-repeat type. Prototype galectins (e.g., Gal-1, Gal-2, Gal-5, Gal-7, Gal-10, Gal-11, Gal-13, Gal-14, and Gal-15) comprise a single CRD, typically forming a non-covalent homodimer that has the ability to cross-link to various animal cells (Cho and Cummings 1996; Dunphy et al. 2002; Almkvist and Karlsson 2004). Gal-1 induces T-cell apoptosis in a sugar-dependent manner (Hahn et al. 2004), whereas its oxidized form without sugar-binding ability promotes the functional recovery of injured peripheral neurons (Kadoya et al. 2005). On the other hand, chimera type galectins represented by Gal-3 consist of N-terminal nonCRD and C-terminal CRD. Although Gal-3 appears to form multimers through for consistency N-terminal nonCRD (Ahmad et al. 2004), the molecular mechanism underlying its reported functions is only poorly understood (Nakahara et al. 2005; Ortega et al. 2005; Ruebel et al. 2005). Tandem-repeat type galectins (e.g., Gal-4, Gal-6, Gal-8, Gal-9, and Gal-12) consist of two homologous CRDs connected by a linker peptide. They are also involved in various biological processes, typically in immunological events (Kashio et al. 2003; Wooters et al. 2005; Zhu et al. 2005). Among them, however, Gal-9 exhibits distinguishably diverse biological functions, such as the chemotraction of eosinophils (Hirashima 2000; Hirashima et al. 2004) and the apoptosis of murine thymocytes (Wada et al. 1997), T cells (Hirashima 1999), and human melanoma cells (Kageshita et al. 2002). Most of these physiological activities of galectins are believed to be triggered by the recognition of specific counterpart oligosaccharide ligands expressed on target cells, although it has been argued that galectin functions cannot simply be explained by such sugar-binding properties. Recent analysis of the sugar-binding properties of Gal-9 has clearly demonstrated that the individual CRDs of Gal-9 have both common and distinct features of specificity: both N-terminal and C-terminal CRDs have an increased affinity to highly branched or extended N-acetyllactosamine structures, whereas only N-terminal CRD recognizes the Forssman pentasaccharide with a markedly high affinity (Hirabayashi et al. 2002). Although such information is essential to understand the basic mechanism underlying Gal-9 functions, other factors that attenuate or enhance this initial recognition event should also be
considered. In this context, Gal-9 may modulate its multivalency by oligomer formation with itself, or with other galectin or nongalectin partners. This is a novel idea, and as yet no substantial studies clarifying the oligomeric properties of Gal-9 have been carried out. Another notable feature is that Gal-9 is susceptible to proteolysis in its linker region that combines the N-terminal and C-terminal CRDs. This may also be associated with the regulation of biological functions (Nishi et al. 2005).

During the course of our binding study by affinity chromatography and surface plasmon resonance (SPR) analysis, we found that human Gal-9 interacts with immobilized Gal-9 only in the absence of lactose, even though a recombinant protein produced in bacteria, and therefore lacking carbohydrate, was used. Moreover, Gal-9 was also observed to interact with Gal-3 and Gal-8, but not with Gal-1. Although very recently, a similar observation has been reported for the N-terminal domain of mouse Gal-9 (Nagae et al. 2006), these lines of evidence that Gal-9 can oligomerize into homo- and hetero-complexes in a CRD-dependent manner have never been presented from a systematic viewpoint. Therefore, our novel finding elucidates various molecular functions of Gal-9.

Results and discussion

Affinity chromatography on Gal-9Null-agarose

Recently, we have developed a stable Gal-9 mutant designated Gal-9Null, which lacks a cleavable linker region, but retains its complete biochemical and physiological activities (Nishi et al. 2005). During the course of our ligand-binding experiment using a Gal-9Null column, we happened to observe a phenomenon that suggests Gal-9/Gal-9 interaction. Almost one-half of the amount of free Gal-9Null (25 μg) applied firmly bound to the Gal-9Null column (0.3 mg/mL Sepharose; column volume, 1.0 mL), and was eluted with 0.2 M lactose (Figure 1). When a similar experiment was carried out using free Gal-1, it completely passed through the Gal-9Null column. This observation suggests that Gal-9Null interacts with immobilized Gal-9Null in a CRD-dependent manner. There was no sign of posttranslational modifications such as glycosylation with the recombinant Gal-9 protein used, which was produced in bacteria.

Frontal affinity chromatography

As a more quantitative approach to confirm the observation in Affinity chromatography on Gal-9Null-agarose section, a series of binding experiments was carried out by FAC. For this purpose, Gal-9Null immobilized gel was packed into a miniature column as described in Materials and methods. To evaluate the column, PA-LNFP-III and PA-LNnT were eluted as positive controls, whereas PA-rhamnose was used as a negative control. The elution was monitored by measuring the fluorescence of the PA residue (excitation and emission wavelengths, 310 and 380 nm, respectively). From their retardations ($V - V_0$; 5.3 and 18.1 μL for LNFP-III and LNnT, respectively) and the $K_d$ values (4.1 x $10^{-5}$ M and 1.0 x $10^{-5}$ M, respectively) of Gal-9 reported previously (Hirabayashi et al. 2002), the $B_0$ of Gal-9Null in agarose gel packed into the column was determined to be 0.2 nmol, which corresponds to 67% of the immobilized Gal-9Null. When free Gal-9Null was eluted instead of PA-oligosaccharides, a significant retardation was observed, that is, $V - V_0 = 53.7$ μL (Figure 2). Hence, the dissociation constant ($K_d$) of the Gal-9Null/Gal-9Null interaction was determined to be 3.3 x $10^{-6}$ M using the basic equation of FAC (equation 1). The determined $K_d$ of the Gal-9Null/Gal-9Null intermolecular interaction is six-fold smaller than that of the mouse Gal-9N that has been reported recently by Nagae et al. (2006) (2.0 x $10^{-5}$ M), who examined the interaction by SPR analysis, whereas it is within the same range as that reported for Gal-1/Gal-1 dimerization (7 x $10^{-6}$ M) determined by gel-filtration analysis (Cho and Cummings 1996), although there was some disagreement on the latter report. Giudicelli et al. (1997) claimed that $K_d$ value was less than 2 μM.

SPR analysis

We further analyzed the Gal-9/Gal-9 interaction using another biosensor technique, SPR. The interaction analysis was performed using immobilized Gal-9Null (ligand, 5000 RU) and free Gal-9Null (analyte) at various concentrations. As a result, dose-dependent sensorgrams were obtained (Figure 3A). A very similar result was obtained, when Gal-9S, a native form of human Gal-9 with a “short” linker region, was used for immobilization (Figure 3B). Association (on) and dissociation (off) rate constants were calculated by a direct nonlinear curve fitting method. A global fitting of mono-exponential rate equations on the assumption of a bivalent binding model produced reasonable fits to the obtained data. The on-rate and off-rate constants of the Gal-9Null/Gal-9Null interaction were determined to be 1.35 x $10^4$ M$^{-1}$ s$^{-1}$ and 1.39 x $10^{-2}$ s$^{-1}$, whereas those for the Gal-9S/Gal-9S interaction were 8.48 x $10^5$ M$^{-1}$ s$^{-1}$ and 1.10 x $10^{-2}$ s$^{-1}$, respectively. Hence, the dissociation constants of the Gal-9Null/Gal-9Null and Gal-9S/Gal-9S interaction were calculated to be 1.0 x $10^{-6}$ M and 1.3 x $10^{-6}$ M, respectively. $K_d$ of Gal-9Null obtained by SPR analysis was 3.3-fold lower (i.e., higher affinity), than that determined by FAC (3.3 x $10^{-6}$ M). For this discrepancy, the clustering of free Gal-9Null (analyte) occurring on the sensor chip caused by the high-density immobilization of Gal-9Null is considered. Alternatively, the presence of CM5 on the chip should amplify the response.

Effect of temperature and contribution of individual domains

As described earlier, Gal-9 comprises two tandemly repeated CRDs, namely, Gal-9N and Gal-9C. We next investigated in more detail their binding features. For this, three recombinant

Fig. 1. Elution profiles of Gal-9Null and Gal-1 in affinity chromatography on Gal-9Null-agarose column. Gal-9Null was immobilized to Sepharose 4 Fast Flow resin, and was packed into the column (i.d. 7 mm x 25 mm) equilibrated with DPBS(−) at a flow rate of 5 mL/hr. Closed circles (Gal-9Null); closed triangles (Gal-1).
proteins Gal-9Null, Gal-9N, and Gal-9C were immobilized on the sensor chip to obtain sufficient (5000) RU values. On this chip, Gal-9Null solution (16.5 mg/mL) was allowed to flow at various temperatures (4, 10, 20, 25, 30, and 40 °C) at a flow rate of 10 μL/min for 60 s. When the entire Gal-9Null molecule was analyzed, a substantial enhancement of the interaction was observed with the increase in temperature, and the maximum interaction was attained at 30 °C (Figure 4A). The association was enhanced until the temperature reached 30 °C, whereas the dissociation was much enhanced at 40 °C. Interestingly, both the domains of Gal-9Null, namely, Gal-9N and Gal-9C, were found to be involved in the interaction. When Gal-9N or Gal-9C was immobilized instead of Gal-9Null, its interaction with Gal-9Null became apparently weaker (Figure 4B and C). Notably, however, Gal-9Null/Gal-9C interaction was less significant than Gal-9Null/Gal-9N interaction. These observations suggest that the N-terminal domain plays a dominant role in the intermolecular Gal-9 interaction.

Fig. 2. Elution profiles of PA-oligosaccharides and Gal-9Null in FAC. PA-oligosaccharides, LNFP-III (A) and LNnT (B) were dissolved in DPBS(−) at a concentration of 10 pM, and 300 μL of each solution was applied to a miniature column at a flow rate of 0.125 mL/min at 20 °C; their profiles are indicated by a solid line. PA-rhamnose (A, B) and BSA (C, D) were used as a negative control; their profiles are indicated by a dotted line. Gal-9Null was dissolved in DPBS(−) at concentrations of 0.25 μM (C) and 0.5 μM (D), and 300 μL of each solution was applied to the same column at a flow rate of 0.125 mL/min at 20 °C that described with a solid line. BSA was used as a negative control to determine V-V₀ that described with a dotted line.

Fig. 3. SPR analysis of intermolecular interaction of Gal-9S and Gal-9Null. Sensorgrams obtained for sensor chips with immobilized Gal-9S (A) or Gal-9Null (B) are shown. The y-axis represents the amount of bound analyte in terms of RU, and the x-axis represents time after injection (seconds). Gal-9S or Gal-9Null at various concentrations was injected to the CM5 sensor chips, on which Gal-9S or Gal-9Null was immobilized, at a flow rate of 10 μL/min at 10 °C. The analyte concentrations were 500 μM (a), 250 μM (b), 125 μM (c), 62.5 μM (d), 31.3 μM (e), 15.6 μM (f), and 7.8 μM (g).
Effect of sugars
In the first experiment using affinity chromatography (Figure 1), the adsorbed Gal-9 was eluted with lactose, although the latter experiments strongly suggested the protein–protein interaction of Gal-9. To examine this point, the effect of sugar on the intermolecular interaction was investigated using mono- and disaccharides with the SPR assay system. Gal-9Null was immobilized on the sensor chip, to which Gal-9Null (analyte) was injected over a period of 60 s. After washing (250 s), the sensor chip was treated with 0.2 M saccharide solution for 60 s, and the residual amount of Gal-9Null was measured in RU. The Gal-9Null/Gal-9Null interaction was completely abolished by the elution with 0.2 M lactose, the most potent saccharide inhibitor among the inhibitor tested (Figure 5A), whereas galactose (Figure 5B), N-acetylgalactosamine (sensorgram not shown) and melibiose (Figure 5C), which are less potent inhibitors of galectins, only partially inhibited the elution. On the other hand, glucose (Figure 5D), mannose, and maltose (sensorgram not shown) had no ability to elute Gal-9Null from the sensor chip (Table I). It is known that Gal-9 has high affinity to β-galactosides, while it has much lower affinity to free galactose, N-acetylgalactosamine and melibiose (α-galactoside). These findings indicate that the interaction strongly depends on the carbohydrate-binding ability of Gal-9Null. Lactose, galactose, N-acetylgalactosamine, and melibiose inhibited the CRD interactions equally. Interestingly, though the affinity of Gal-9C toward lactose was more than eight times higher than that of Gal-9N (Sato et al. 2002), there was no difference in the inhibitory power of lactose between Gal9-N and Gal-9C (Table I).

Further evidence of intermolecular interaction of Gal-9Null
As described in the Effect of sugars section, the intermolecular interaction of Gal-9Null largely depended on N-terminal CRD (Figure 4). To confirm this point, we further analyzed the oligomer formation of Gal-9Null by Ferguson plot analysis of result of polyacrylamide gel electrophoresis. BSA, known to form a dimer as well as a monomer, was used as a standard. Plots of log $R_m$ (relative mobility) versus gel concentration for the two molecular species of BSA yielded two nonparallel lines typical of oligomeric proteins (Figure 6A). Similar results were obtained for Gal-9N, which formed both a monomer and a dimer (Figure 6B). On the other hand, only one species was observed for Gal-9Null (data not shown). This should correspond to a stable dimer, considering its stronger intermolecular interaction ($K_d$, $3.3 \times 10^{-6}$ M). Unfortunately, no detectable band was observed for Gal-9C even under several experimental conditions in electrophoresis. As a most probable reason, it is considered that...
Gal-9C is a more basic protein compared with Gal-9N (pI values estimated for Gal-9N and Gal-9C are 7.30 and 8.43, respectively).

More direct evidence of the multimeric formation of Gal-9 was obtained by MALDI-TOF MS. Figure 7 shows MS spectra of Gal-9Null both in the absence and presence of 25 mM lactose. Five evident peaks were observed; that is, \( m/z = 32799, 49209, 65847, 98741, \) and \( 131800 \), apparently corresponding to the Gal-9Null monomer, trimer (2+ charge), dimer, trimer, and tetramer, respectively [theoretical molecular weight of Gal-9Null, 33143.2; under the assumption that no posttranslational modification occurs on Gal-9Null; Figure 7A]. Importantly, the latter four peaks disappeared almost completely when Gal-9Null was dissolved in buffer containing 25 mM lactose (Figure 7B). A slight amount of the peak (corresponding to dimer, MW \( = 65847 \)) remained possibly because of partial effect of the sugar. These findings provide strong evidence that Gal-9 forms a stable dimer as well as multimers.

**Evidence of cross-interaction of Gal-9Null with other galectin members**

To date, 15 mammalian galectins have been characterized. However, there have been no reports on different members of the galectin family interacting with each other except for two chicken isolectins previously designated C14 and C16 (Hirabayashi et al. 1987). Regarding the intermolecular interaction of Gal-9Null, we further examined the possibility of the cross-interaction of Gal-9Null with other galectin...
members (all from the human). For this purpose, Gal-9Null was immobilized on a sensor chip, and its interactions with recombinant Gal-1, Gal-3, and Gal-8 were analyzed. As a result, a significant intermolecular interaction was observed for Gal-3 and Gal-8 in addition to Gal-9Null (positive control), but not for Gal-1, which forms a homodimer (Figure 8A). Again, the interaction was abolished by the presence of 0.2 M lactose (data not shown). However, their binding kinetics ($k_{\text{on}}$, $k_{\text{off}}$) were apparently different from those of the self-interaction of Gal-9Null. In particular, the Gal-3/Gal-9Null interaction has a lower association rate ($k_{\text{on}}$, $3.35 \times 10^3$ M$^{-1}$s$^{-1}$) than the Gal-9Null/Gal-9Null interaction (1.35 $\times 10^4$ M$^{-1}$ s$^{-1}$). We also investigated the cross-intermolecular interactions of Gal-9N and Gal-9C. Unexpectedly, Gal-9N showed no detectable affinity to Gal-3, whereas it retained a significantly high affinity to Gal-8 (Figure 8B). On the other hand, the overall features of Gal-9C clearly resembled those of Gal-9Null (Figure 8C), although the maximum binding of Gal-9C was much reduced in terms of RU (approximately 60 RU) compared with that of Gal-9Null (300 RU).

**Immunohistochemical localization**

As described in (Evidence of cross-interaction of Gal-9Null with other galectin members), evidence of the cross-intermolecular interactions of Gal-9 with Gal-3 and Gal-8 was obtained by in vitro experiments. To examine the possibility that these galectins actually cross-interact in vivo, a preliminary immunohistochemical experiment was carried out using serial sections of the ascending colon tissue, where Gal-9 has been found (Lahm et al. 2001, 2004). The sections were fixed and stained with one of the polyclonal antibodies raised against human Gal-1, Gal-3, Gal-8, and Gal-9 (Figure 9). It was shown that Gal-1 was distributed on the lamina propria mucosae, but it hardly existed in the glandular system, whereas they were hardly detected in the lamina propria mucosae where Gal-1 exists.

The observation that Gal-3, Gal-8, and Gal-9 existed in the same area in the ascending colon tissue suggests that Gal-9 interacts with not only Gal-9 itself but also other galectin members in vivo to exert their functions (Figure 9). Even though more direct evidence is necessary to prove this hypothesis, the observation that galectin members excluding Gal-1 associate via intermolecular interaction is important, particularly for the elucidation of molecular mechanisms by which Gal-9 exerts its functions in various contexts.

**Conclusions**

In this study, we demonstrated for the first time that human Gal-9 interacts with itself and other members of the galectin family via CRDs. For elucidation of Gal-9 functions, identification of Gal-9 counterpart ligands in various contexts is necessary. In the observed intermolecular interaction of Gal-9, the presence of CRD was required, while that of carbohydrate ligands rather inhibited the interaction. Supporting evidence from X-ray crystallography has recently been obtained, which showed that mouse Gal-9N forms a dimer in both crystals and solution (Nagae et al. 2006). We speculate that Gal-9 forms a series of multimers, until it reaches a target receptor. If this is the case, multimerized Gal-9 should have an extremely high affinity for endogenous glycan ligands at the moment it binds, and the multimers dissociate immediately after the ligand binding has occurred. This seems contradictory; however, it is plausible when one considers a flexible membrane structure similar to a raft, which is formed by rearrangement processes between glycolipids and glycoproteins and possibly galectins (Braccia et al. 2003). In this context, the possibility of cross-galectin interactions is intriguing considering a wide variety of galectin functions, that is, the “galectin network”.

**Fig. 6.** Fergusson plot analysis of results of polyacrylamide gel electrophoresis of Gal-9N. After electrophoresis of Gal-9N in polyacrylamide gel at different concentrations (7.5, 10, 12.5 and 15%, w/v), Fergusson plot analysis was carried out. The results of plot for BSA [control, (A), and Gal-9N (B)] are shown. Open and closed circles represent the monomer and dimer, respectively.
Materials and methods


Human N-terminal CRD of Gal-9 (Gal-9N) and C-terminal CRD of Gal-9 (Gal-9C) fused to glutathione S-transferase (GST) were produced in *Escherichia coli* BL21 cells as described previously (Matsushita et al. 2000; Nishi et al. 2005), and purified using glutathione-sepharose in accordance with the manufacturer’s instruction (GE Healthcare Bio-science, Edison, NJ). The affinity-purified fusion proteins were digested with thrombin, and the GST moiety was removed by glutathione-sepharose. Intact (tag-free) recombinant proteins of human Gal-1, Gal-3, Gal-8, and Gal-9 having a short linker region [galectin-9S (Gal-9S)], and Gal-9 having no linker region [galectin-9Null (Gal-9Null)] were purified by affinity chromatography on a lactose–agarose column (Seikagaku Corporation, Tokyo, Japan), as described previously (Nishi et al. 2003, 2005). Protein concentration was determined by the Bradford method with bovine serum albumin (BSA) as a standard (Bradford 1976).

**Affinity chromatography on Gal-9Null-agarose**

Purified Gal-9Null was immobilized on a Hi-Trap N-hydroxysuccinimide (NHS)-activated cartridge (1 mL, Amersham Biosciences) with slight modifications of the manufacturer’s procedure. Briefly, after the cartridge was washed with 1 mM HCl, 0.3 mg of Gal-9Null (dissolved in 1 mL of Dulbecco’s phosphate-buffered saline without calcium and magnesium [DPBS(–); Dulbecco and Vogt 1954] was gradually injected into the cartridge over a period of at least 1 min. The coupling reaction was allowed to proceed for 30 min at 20°C. The residual NHS group was blocked with an excess amount of ethanolamine (0.5 M, 0.2 mL, pH 8.3) for 30 min at 20°C, and the cartridge was washed extensively with DPBS(–)(acid wash was omitted).

For binding experiments, 0.2 mL of Gal-9Null solution (0.25 mg/mL) was applied to the cartridge column equilibrated with DPBS(–). After the column was washed with 4.5 mL of the same buffer, elution was performed with 0.2 M lactose dissolved in DPBS(–). Fractions (0.1 mL each) were collected at a flow rate of 5 mL/h. Each fraction

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Fig. 7. MALDI-TOF MS spectra of Gal-9Null. MALDI-TOF MS spectra were measured in the positive reflectron mode with sinapinic acid as the matrix in both the absence (A) and presence of 25 mM lactose (B).
Fig. 8. Observation of cross-intermolecular interaction between Gal-9Null and other galectins. Immobilized-Gal-9Null, -Gal-9N, and -Gal-9C sensor chips were docked to the SPR sensor system, and various galectin solutions were injected into the sensor chip. The analytical conditions of the SPR sensor were the same as those described in Figure 3. Gal-9Null (A), Gal-9N (B), and Gal-9C (C) were immobilized on sensor chips. The analytes were Gal-1 (a), Gal-3 (b), Gal-8 (c), and Gal-9Null (d).

Fig. 9. Immunohistochemical staining of the ascending colon tissue for Gal-1, Gal-3, Gal-8, and Gal-9 expression. Sections of the ascending colon tissue were fixed and reacted with one of the polyclonal antibodies raised against Gal-1, Gal-3, Gal-8, and Gal-9. The bound antibodies were visualized by staining with the horseradish-peroxidase-conjugated secondary antibody and 3,3'-diaminobenzidine tetrahydrochloride. Magnification, 400×. Polyclonal antibodies are control (A), and antibodies to Gal-1 (B), Gal-3 (C), Gal-8 (D), and Gal-9 (E), hematoxylin–eosin staining is shown in (F).
was monitored by measuring the absorbance at 280 nm. Essentially the same procedure was performed for Gal-1 (0.5 mg/mL, 0.2 mL) as the control experiment.

**Frontal affinity chromatography**

As mentioned earlier in Affinity chromatography on Gal-9Null-agarose section, cartridge column packed with agarose gel (0.3 mg of Gal-9Null/mL of gel) was disrupted, and the gel was repacked into a stainless miniature column (i.d. 2 × 10 mm; volume, 31.4 μL). The resultant column was set on a manual system for frontal affinity chromatography (FAC) consisting of a Shimadzu LC-10AD pump, an RF-10AXL fluorescence detector, and an SCL-10A system controller. Sample solutions were injected through a Rheodyne 7125 injector equipped with a 300-μL PEEK sample loop, as described previously (Hirabayashi et al. 2003). The column and sample loop were wrapped in a water cooling apparatus set at 20 °C. After the manual injection of the sample, protein elution was monitored by measuring the fluorescence of tryptophan (excitation and emission wavelengths, 285 and 350 nm, respectively). The retardation of the elution front was compared with that of BSA, in terms of V−V0 (microliter). Dissociation constant (Kd) was obtained using the basic equation of FAC (equation 1), as described previously (Kasai et al. 1986).

\[ K_d = B_t/(V - V_0) - [A]_0 \] (1)

Here, [A]0 is the initial concentration (molar) of analyte A, and Bt is the effective content (molar) of the immobilized ligand B. In this study, Bt was obtained using the reported Kd values of pyridylaminated (PA)-lacto-N-fucopentaose III (LNFP-III) and PA-lacto-N-neotetraose (LNTnT) (Hirabayashi et al. 2002), from which V−V0 was experimentally determined.

**SPR analysis**

The binding properties of galectins were characterized by SPR analysis using a Biacore 2000 instrument with a multichannel integrated microfluidic cartridge (Biacore AB, Uppsala, Sweden). Gal-9Null, Gal-9N, and Gal-9C were immobilized on research-grade CM5 (carboxymethyl group) sensor chips in 10 mM sodium acetate (pH 4.5) at 30 °C using an NHS/1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide coupling chemistry in accordance with the manufacturer’s instruction. Immediately after the immobilization treatment, the temperature of the flow cell was set at 10 °C. The protein amount required for each immobilization was set at 5000 resonance units (RU) (note that 1000 RU correspond to a surface concentration of 1 ng/mm²). All SPR analyses were carried out at a flow rate of 10 μL/min in 10 mM 4-(2-hydroxyethyl)-1-piperazinmethanesulfonic acid (pH 7.4), 150 mM NaCl, 3 mM ethylenediaminetetraacetic acid, and 0.005% surfactant P20 filtered and degassed at 10 °C, unless otherwise mentioned. Each sample (analyte) was kept in contact with the immobilized protein on the sensor chip for 60 s, and the subsequent dissociation was monitored for 120 s. To determine kinetic parameters, namely, association (k_on) and dissociation (k_off) rate constants, the analysis software package BIAevaluation 4.1 (Biacore AB) was used. From the obtained rate constants, Kd was determined using the following relation: \[ K_d = k_{off}/k_{on}. \]

To investigate the effect of saccharides used in the SPR experiments, Gal-9Null was injected into a Gal-9Null chip and was kept in contact with them for 60 s. After the chip was washed with the running buffer as described earlier for 250 s, it was treated with 0.2 M saccharide solution dissolved in the running buffer for 60 min to elute Gal-9Null. The amount of residual Gal-9Null was measured as %RU after further washing with the running buffer.

**Ferguson plot analysis of results of polyacrylamide gel electrophoresis**

Ferguson plot analysis was carried out for Gal-9N after its separation by polyacrylamide gel electrophoresis under nondenaturing conditions. For this purpose, polyacrylamide gels of different concentrations (7.5, 10, 12.5, and 15%) were prepared, and the electrophoretic mobilities of the observed molecular species were analyzed as described by Hedrick and Smith (1968). BSA was used as a standard oligomeric protein.

**Matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry**

Gal-9Null (8.6 μM) was dissolved in 1 μL of saturated sinapinic acid containing 0.1% trifluoroacetic acid. The solution was then loaded onto a 100-well target plate (Applied Biosystems, Framingham, MA) and air-dried. The molecular weight and oligomeric features of the galectin were analyzed by matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) using Voyager System 4314 (Applied Biosystems).

**Immunohistochemistry**

The immunohistochemical staining of sections of formalin-fixed, paraffin-embedded tissue was carried out using antibodies to Gal-9 and an EnVision+ peroxidase rabbit system (Dako, Kyoto, Japan). In brief, the sections (thickness, 4 μm) were heated at 100 °C for 16 min in 10 mM sodium citrate buffer (pH 6.0), subjected to paraffin removal, and rehydrated. After quenching endogenous peroxidase activity with 0.3% hydrogen peroxide, the sections were treated for 2 h at room temperature with 5% BSA to block nonspecific staining. They were incubated first with a primary antibody at room temperature overnight (5 μg/mL) and then with EnVision+ solution containing a horseradish peroxidase-conjugated secondary antibody for 1 h. A chromogen, 3,3′-diaminobenzidine tetrahydrochloride was used. An immunoglobulin fraction isolated from normal rabbit serum (Dako) was used as a negative control. All the sections were counterstained with Mayer’s hematoxylin solution. Gal-1, Gal-3, and Gal-8 were stained similarly. Anti-Gal-1, -Gal-3, -Gal-8, and -Gal-9 antisera were raised in Japanese white rabbits as described previously (Irie et al. 2005).

**Abbreviations**

BSA, bovine serum albumin; CRD, carbohydrate recognition domain; DPBS(−), Dulbecco’s phosphate-buffered saline without calcium and magnesium; FAC, frontal affinity chromatography; Gal-9, galectin-9; Gal-9C, galectin-9C; Gal-9N, galectin-9Null; Gal-9S, galectin-9S; Gal-9Null, galectin-9Null; GST, glutathione S-transferase; LNFP-III, lacto-N-fucopentaose
References


