Galectin-9, a tandem-repeat-type β-galactoside-specific animal lectin with two carbohydrate recognition domains (CRDs) at the N- and C-terminal ends, is involved in chemoattraction, apoptosis, and the regulation of cell differentiation and has anti-allergic effects. Its ability to recognize carbohydrates is essential for its biological functions. Human galectin-9 (hG9) has high affinity for branched N-glycan-type oligosaccharides (dissociation constants of 0.16–0.70 μM) and linear β1–3-linked poly-N-acetyllactosamines (0.09–8.3 μM) and significant affinity for the α2–3-sialylated oligosaccharides (17–34 μM). Further, its N-terminal CRD (hG9N) and C-terminal CRD (hG9C) differ in specificity. To elucidate this unique feature of hG9, x-ray structures of hG9C in the free form and in complexes with N-acetyllactosamine, the biantennary pyridylaminated oligosaccharide, and α2–3-sialyllactose were determined. They are the first x-ray structural analysis of C-terminal CRD of the tandem-repeat-type galectin. The results clearly reveal the mechanism by which branched and α2–3-sialylated oligosaccharides are recognized and explained the difference in specificity between hG9N and hG9C. Based on structural comparisons with other galectins, we propose that the wide entrance for ligand binding and the shallow binding site of hG9C are favorable for branched oligosaccharides and that Arg221 is responsible for recognizing sialylated oligosaccharides.

The galectins are a family of β-galactoside-specific animal lectins that contain conserved elements for carbohydrate recognition (1, 2) and have attracted much attention as novel regulators of the immune system (3). Recently, galectins have been shown to bind glycans on the surface of potentially pathogenic microorganisms and function as recognition and effect factors in innate immunity (4, 5). Currently, there are 14 members of the mammalian galectin family, classified into three subtypes in innate immunity (4, 5). Currently, there are 14 members of microorganisms and function as recognition and effect factors shown to bind glycans on the surface of potentially pathogenic microorganisms.

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‡ The abbreviations used are: CRD, carbohydrate recognition domain; TIM3, T-cell immunoglobulin and mucin-containing-protein 3; Th1, T-helper type 1; hG9, human galectin-9; hG9N, hG9 N-terminal CRD; hG9C, hG9 C-terminal CRD; BG1, bovine galectin-1; HG1, human galectin-1; hG8N, human galectin-8 N-terminal CRD; LacNAc, N-acetyllactosamine; LN3, LacNAc trimer; SiaLac, α2–3-sialyllactose; BIPA, biantennary pyridylaminated oligosaccharide; BIOS, biantennary octasaccharide.

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** The atomic coordinates and structure factors (codes 3NV1, 3NV2, 3NV3, and 3NV4) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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oligosaccharides. Frontal affinity chromatography also revealed that hG9, as well as galectin-8 and galectin-3, have significant affinity for H92512–3-sialylated oligosaccharides (17–34 H9262M). The unique specificity of hG9 would be closely related to its diverse biological functions.

Structural studies on galectins alone and in complexes with oligosaccharides have provided important clues to their structure-function relationships (21–30). Nagae et al. (29, 30) reported the x-ray structures of hG9N in complexes with the Forssman pentasaccharide and oligolactosamines, clearly revealing the mechanism of recognition by hG9N. However, x-ray structures of hG9C have not been reported yet. The structure determinations of both CRDs should be important to understand the biological functions of hG9. Also, structural information on very few galectins in complexes with branched and/or sialylated oligosaccharides is available (23). We report here x-ray structures of hG9C in its free form and in complexes with N-acetyllactosamine (LacNAc), the biantennary pyridylaminated oligosaccharide (BIPA), and H92512–3-sialyllactose (SiaLac), providing new insights into how hG9C recognizes these oligosaccharides (Fig. 1). The presented results are the first x-ray structural analysis of C-terminal CRD of the tandem-repeat-type galectin.

**EXPERIMENTAL PROCEDURES**

**Purification and Crystallization**—The expression and purification of hG9C have been reported previously (31). Briefly, hG9C was expressed as a glutathione S-transferase fusion protein in *Escherichia coli* BL21 cells and purified by affinity chromatography using glutathione-Sepharose 4B (GE Healthcare UK Ltd.). The bound protein (5 ml) was washed with 10 bed volumes of TBS buffer containing 0.03% CHAPS and 3 bed volumes of PBS in an Econo-Pac column (Bio-Rad), and incubated with 60 units of thrombin (GE Healthcare UK Ltd.) at 293 K overnight. The cleaved target hG9C was eluted from the gel with 2 bed volumes of PBS, and the protein solution was dialyzed against a buffer solution (10 mM Tris-HCl, pH 7.5, 100 mM NaCl) overnight. The purified protein solution was concentrated to 3.9 mg/ml using an Amicon Ultra-4 10-kDa Ultracel (Millipore).

Initial crystallization screening was performed using Crystal Screen kits 1 and 2 and PEG/Ion Screen (Hampton Research Corp.) and Emerald BioSystems Wizard I, II, and III (Emerald BioSystems, Inc.) by the sitting drop method with 96-well plates (Corning Inc.) at 293 K. Crystals of 0.1 x 0.1 x 0.1 mm using diffraction experiments were grown in a droplet containing 1.2 l of protein solution (3.9 mg/ml in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl) and 0.8 l of reservoir solution (0.01 M NiCl2, 20% (w/v) PEG monomethyl ether 2000, and 0.1 M Tris-HCl, pH 8.5) against 500 l of the reservoir solution by the hanging drop method using a 24-well plate (TPP AG).

**X-ray Structure Determination**—Data were collected at Photon Factory AR-NW12A and BL5A (Tsukuba, Japan). A crystal mounted in a loop was soaked in crystallization solution containing 20% (v/v) ethylene glycol and flash-cooled in a stream of evaporating nitrogen. The diffraction data were collected using an Area Detector Systems Corp. 210r or 315 CCD detector with a wavelength of 1.0 Å at 100 K. All data were processed using the HKL2000 system (32). Crystals of complexes with oligosaccharides were obtained by a soaking method. Aqueous solutions of LacNAc (1.0 M), SiaLac (400 mM), and BIPA (30 mM) were added to droplets of the crystals (0.2–0.4 µl) and incubated for 6 h (LacNAc), 2 days (SiaLac), and 4 weeks (BIPA). LacNAc and SiaLac were pur-
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Table 1

Data collection and refinement statistics

|                          | Free hG9C | h9GC-LacNAc | hG9C-BIPA | hG9C-SiaLac |
|--------------------------|-----------|-------------|-----------|-------------|
| Data collection          |           |             |           |             |
| Beamline                 | PF-AR NW12A | PF-AR NW12A | PF-BL5A  | PF-AR NW12A |
| Temperature (K)          | 100       | 100         | 100       | 100         |
| Wavelength (Å)           | 1.5       | 1.5         | 1.5       | 1.5         |
| Resolution range (Å)     | 50.0–1.50 (1.55–1.50) | 50.0–2.34 (2.38–2.34) | 50.0–1.57 (1.60–1.57) | 50.0–1.99 (2.02–1.99) |
| No. of unique refinements| 22,811    | 6,135       | 19,985    | 9,959       |
| Redundancy               | 4.6 (4.5) | 11.2 (11.2) | 4.8 (3.9) | 5.5 (5.1)   |
| Completeness (%)         | 99.4 (99.5) | 100.0 (100.0) | 99.5 (99.9) | 99.7 (99.5) |
| Mean |
| r.m.s.d., root mean square deviation. |

Further model building was performed with the programs Coot (35) and X-fit (36), and refinement was performed with the programs Refmac5 (37) and CNS (38). Water molecules were gradually introduced if the structure was refined using the programs Refmac5 (37) and CNS (38). The data collection statistics are summarized in Table 1.

Molecular replacement was applied with the program MOLREP (34). The initial phase was determined using human galectin-3 CRD (Protein Data Bank [PDB] code: 1KJL), which has 42% sequence identity with hG9C. Further model building was performed with the programs Coot (35) and X-fit (36), and the structure was refined using the programs Refmac5 (37) and CNS (38). Water molecules were gradually introduced if the peaks above 3.5 σ in the (Fo − Fc) electron density map were in the range of a hydrogen bond. In a Ramachandran plot (39), the number of residues in the most favored regions was determined by the program PROCHECK (40). Refinement statistics are listed in Table 1. Figs. 2, 3, and 5 were drawn by the program PyMOL (41).

RESULTS

Overall Structure—We successfully determined the x-ray structures of free hG9C, hG9C-LacNAc, hG9C-BIPA, and hG9C-SiaLac complexes, all of which are isomorphous. There is one molecule of hG9C in an asymmetric unit. Almost all the atoms of the protein molecules, each of which comprises 138 amino acid residues, the bound oligosaccharides, and solvent molecules, fitted nicely to the final (2Fo − Fc) electron density map, except for the N-terminal residues (His188 and Pro189) and terminal sugar units of the oligosaccharides.

The hG9C adopts a β-sandwich structure formed by two anti-parallel β-sheets consisting of six (S1–S6) and five (F1–F5) β-strands, with a short α-helix, as shown in Fig. 2a. The nomenclature for β-strands follows that for hG9N (29). The β-strands align in the following sequence: S1, F2, S3, S4, S5, S6a/S6b, F3, F4, F5, S2, and F1, from the N to the C terminus, and an additional short α-helix is located between F5 and S2. Oligosaccharides bind to the concave surface formed by S3, S4, S5, and S6. In overall structure, free hG9C is almost identical to the hG9C in complexes with oligosaccharides, with the root mean square deviations for main chain atoms being 0.14–0.33 Å, suggesting that the binding of oligosaccharides was unlikely to induce a large structural change in hG9C. However, slight structural differences are found in the conformation of Arg221 and the loop region between S5 and S6a, depending on the bound oligosaccharides, as described later.

In a crystal, three molecules related by a crystallographic three-fold symmetry associate to give a trimer with an intermolecular contact area of 998 Å² for each molecule (14% of the total surface area of the molecule) as calculated with the program AREAIMOL in the CCP4 program suite (42) (Fig. 2b). There is a Ni²⁺ on the three-fold axis, coordinated by three His320 residues from the three molecules, stabilizing the trimeric structure. Thr198 and Tyr204 form intermolecular hydrogen bonds with Tyr191 and Gin322 of one of the other molecules, respectively, and there are intermolecular hydrophobic interactions among the three molecules, also contributing to a stable trimer, as shown in Fig. 2c.

Structure of the Carbohydrate-binding Site—Oligosaccharides bind to hG9C via non-reducing and reducing ends located at S3 to S6. For clarity, sugar units are numbered −1, +1, +2, and +3, from the non-reducing end to reducing end. Because
Gal residues of three oligosaccharides occupy the same positions in each complex, the position of Gal is defined as subsite /H11001 1 (Gal /H11001 1), as shown in Figs. 2 and 3. The bound LacNAc in hG9C-LacNAc has almost the same conformation as the LacNAc moiety in hG9C-BIPA, except that GlcNAc /H11001 2 in LacNAc adopts an /H9251 -anomer different from that in BIPA where the GlcNAc residue forms a /H9252 1–4-glucoside bond with Man /H11001 3 (supplemental Fig. S1a). Therefore, the description here concentrates on hG9C-BIPA and hG9C-SiaLac.

The structure of the carbohydrate-binding site with the bound BIPA is shown in Fig. 3a. Gal /H11001 1 nicely forms stacking interactions with Trp 255 and six hydrogen bonds with the following amino acid residues: O4-His 235, O4-Asn237, O4-Arg239, O5-Arg239, O6-Asn248, and O6-Glu258. The axial conformation of the O4 of Gal /H9252 1–4 is strictly recognized by three hydrogen bonds with His235, Asn237, and Arg239. The hydroxyl of the O2 and O3 of Gal /H11001 1 are directed toward the solvent-accessible surface and form hydrogen bonds with water molecules, which are linked to the amino acid residues, His223, Asn225, and Trp255, by a water-mediated hydrogen bond network. The O3 of GlcNAc /H11001 2 forms direct hydrogen bonds with the following amino acid residues: O3-Arg239 and O3-Glu258. Because Arg239 and Glu258 form bifurcated hydrogen bonds with both Gal /H11001 1 and GlcNAc /H11001 2, they could efficiently recognize the Gal (β1–4) GlcNAc moiety of BIPA. These hydrogen bonds by Arg and Glu residues are strictly conserved in galectin structures. The O6 of Gal /H11001 1 and O6 of GlcNAc /H11001 2 are also connected by a water-mediated hydrogen bond. An N-acetyl group of GlcNAc /H11001 2 forms a water-mediated hydrogen bond with Glu242. Although the electron density for Man /H11001 3 of BIPA is relatively poor when compared with that for Gal /H11001 1 and GlcNAc /H11001 2, the orientation of the pyranose ring of Man /H11001 3 could be determined (supplemental Fig. S1b). A water-mediated hydrogen bond between the O3 of Man /H11001 3 and the N-acetyl group of GlcNAc /H11001 2 was found, but the electron density for other water molecules in hydrogen bonds with Man /H11001 3 could not be determined. The electron density for additional sugar residues at the reducing end (Man /H11001 4, GlcNAc /H11001 5, and GlcNAc /H11001 6) could not be determined either, maybe due to the highly disordered structure.

The structure of the carbohydrate-binding site with the bound SiaLac is shown in Fig. 3b. As in hG9C-BIPA, Gal /H11001 1 forms stacking interactions with Trp255 and forms six hydrogen bonds with His235, Asn237, Arg239, Asn248, and Glu258. Gal /H11001 1 and Glc /H11001 2 are connected by bifurcated hydrogen bonds from Arg239, Glu258, and a water molecule, and the O2 of Glc /H11001 1 forms a water-mediated hydrogen bond with Glu242. The electron density for Sia /H11002 1 allowed us to determine the orientation of the pyranose ring and the conformation of the carboxyl

FIGURE 2. Crystal structure of hG9C. a, the overall structure of hG9C is shown with the bound LacNAc (blue), BIPA (yellow), and SiaLac (green). Secondary elements and subsite numbers are also indicated. b, a trimer of hG9C with the three molecules (pink, yellow, and green) in a crystal is shown with a three-fold axis and 63 screw axes. Ni2+/H11001 on the three-fold axis, three coordinated His residues, and the bound LacNAc are also shown. c, area of contact between two molecules (pink and green) with intermolecular hydrogen bonds and metal coordination shown by dotted lines.
group at the 2-position but not the conformation of an N-acetyl group at the 5-position or a glycerol part at the 6-position (supplemental Fig. S1c). The α2–3-glucoside bond between Sia\(^{-1}\) and Gal\(^{-1}\) is bent extensively, placing Sia\(^{-1}\) beyond the concave surface of the carbohydrate-binding site. Interestingly, Arg221 changes its side chain conformation to form hydrogen bonds with the carboxyl group and the glucoside oxygen atom of Sia\(^{-1}\). The Arg221 found in hG9C-BIPA is denoted as “conformer-1,” and that newly found in hG9C-SiaLac is denoted as “conformer-2.” The electron density of Arg221 in hG9C-SiaLac indicates both conformers (1 and 2), each with an occupancy of 0.5, suggesting that Arg221 could not completely fix the position and orientation of Sia\(^{-1}\) in motion with a large B-factor of 54.2 Å\(^2\) (supplemental Fig. S1d). Nevertheless, Arg221 is thought to be responsible for the recognition of sialylated oligosaccharides because two such conformers of Arg221 have not been observed in the structures of free hG9C and hG9C complexes with LacNAc and BIPA.

Depending on the binding of BIPA and SiaLac, a slight but significant structural difference is found in the loop region between S5 and S6a, as shown in Fig. 3, a and b in red. This loop contains a β-turn consisting of Ile251, Asp252, Asn253, and Ser254. The electron density showed that the β-turn is of type II in hG9C-BIPA and type I in hG9C-SiaLac with deviations of Ca atoms by 0.6, 2.6, 1.3, and 0.4 Å, for Ile251, Asp252, Asn253, and Ser254, respectively. The binding of a sugar unit to subsite \(-1\) seems to affect the conformation of the β-turn because Sia\(^{-1}\) directs toward the β-turn. However, no direct interaction between Sia\(^{-1}\) and the β-turn could be found, and the correlation between the β-turn and the binding of a sugar unit to subsite \(-1\) is still unclear.

**Structural Comparison with hG9N**—Fig. 3c shows the superimposition of hG9C (pink) and hG9N (cyan) with the bound oligosaccharides, BIPA (yellow) and SiaLac (green) in hG9C, and LacNAc trimer (LN3, blue), which is three LacNAc moieties linked by α1–3-glucoside bond, in hG9N (PDB code: 2ZHN). Structural differences between hG9C and hG9N are found in the loop regions. Apart from the N-terminal loop, the loop regions between F2 and S3 (F2–S3 loop), between S3 and S4, between S4 and S5, between S6b and F3, and between F5 and S6b deviate greatly. These structural differences are due to the insertion or deletion of amino acid residues (Fig. 4). The F2–S3 and S3–S4 loops of hG9N with additional residues protrude to cover the β-sheet of S2–S4, giving a favorable carbohydrate-binding site for LN3. The S4–S5 and S6b–F3 loops construct the entrance for ligand binding, which is relatively widely opened in hG9C, where sugar units at subsites \(+2\) and \(+3\) move to the backside; the deviations of the center of mass of pyranose rings at subsites \(+2\) and \(+3\) between BIPA and LN3 are 1.1 and 3.7 Å, respectively. The α-helix of hG9C also shifts to the outside of the protein by 2.8 Å due to an additional Arg302.

The bound SiaLac in hG9C is bent at a glucoside bond between Sia\(^{-1}\) and Gal\(^{-1}\), placing Sia\(^{-1}\) out of the carbohy-
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**FIGURE 4.** Amino acid alignment among hG9N, hG9C, bG1, hG1, and hG8N, with the linker peptide sequence of hG9 short isoform (National Center for Biotechnology Information (NCBI) reference: NP_002299). Secondary elements are shown by arrows (β-strands) and a rectangle (α-helix). The loops with large deviations found between hG9N and hG9C are indicated by boxes. The positions of Arg⁺1⁻ and Asp⁺1⁻ in hG9C and of Arg⁺6 in hG8N are highlighted in black.

**DISCUSSION**

In a crystal, hG9C associates around a three-fold axis to give a trimer. Because Ni²⁺ from the crystallization solution is thought to be one of the major driving forces of molecular association, the trimer seems to be artificially constructed. However, 14% of the total surface area was buried by the formation of a trimer, and the significant intermolecular hydrogen bonds and hydrophobic interactions suggest another possibility. Also, the mouse galectin-9 N-terminal CRD has been reported to associate as a trimer in solution, although it is unclear whether the formation of a trimer is related to the biological function of hG9Cs or not.

As indicated in Fig. 2b, the carbohydrate-binding site of hG9C is exposed to a 6₃-screw axis in a crystal. Three Man⁺³ residues in hG9C-BIPA around a three-fold symmetry resulting from the 6₃-screw axis approach one another with a distance of 4.9 Å between O1 atoms (supplemental Fig. S2). It is likely that two of the three Gal⁺¹⁻GlcNAc⁺²⁻Man⁺³ moieties binding in hG9Cs belong to the same BIPA molecule, forming a cross-linking structure. If this is the case, Man⁺⁴ and GlcNAc⁺⁵ are almost on the 6₃-screw axis, and their electron density cannot be observed due to the highly disordered structure. In fact, there is a large solvent channel along the 6₃-screw axis, allowing enough space for the passing of BIPA molecules (supplemental Fig. S2).

The x-ray structure of bovine galectin-1 (bG1) in a complex with the biantennary octasaccharide (BIOS), which has exactly the same chemical structure as BIPA at subsites +1 to +5, has been reported in three crystal forms, hexagonal, trigonal, and monoclinic (23). Careful inspection revealed that BIPA in hG9C adopts a similar conformation to that in bG1-BIOS in the hexagonal form (PDB code: 1SLA), where the distance between O1 atoms of Man⁺³ to 4.9 Å equal to that in hG9C-BIPA (Fig. 5a). Using the structure of bG1-BIOS in the hexagonal form, a model of the cross-linking of hG9C-BIPA was built, as shown in Fig. 5b. The procedure of model building is given in supplemental Fig. S3. Although the model is possibly dependent on the protein-protein interactions in the crystals, the plausible cross-linking structure of hG9C was obtained. The deviation in the torsion angles of glucoside bonds of Man⁺³ to Man⁺⁴ is 2–17° between bG1-BIPA (crystal structure, PDB code: 1SLA) and hG9C-BIPA (model), showing that the model is chemically reliable. The α₁–6-glucoside bond adopts a low energy gauche-gauche conformation in both structures. In bG1-BIOS, two Gal⁺¹⁻GlcNAc⁺²⁻Man⁺³ moieties make an angle of 150°, and the cross-linked bG1 molecules are close together on the side of GlcNAc⁺⁵ with a distance of 8.3 Å (Gly⁺³⁻Gly⁺⁵) and far apart on the opposite side with a distance of 28.6 Å (Trp⁺⁶⁻Trp⁺⁶). The S₄–S₅ loop of bG1, including His⁺² and Gly⁺⁵, overlaps BIOS, giving a deep carbohydrate-binding site. His⁺² and Trp⁺⁶ sandwich Gal⁺¹ and GlcNAc⁺² to fix their positions, and Gly⁺⁵ efficiently forms van der Waals contacts with Man⁺³ and Man⁺⁴. In hG9C-BIPA, two Gal⁺¹⁻GlcNAc⁺²⁻Man⁺³ moieties make an angle of 118°, and the cross-linked hG9C molecules are located almost parallel to each other at a distance of 18.0 Å (Asp⁺²⁴⁻Asp⁺²⁴) and 19.5 Å (Trp⁺²⁵⁻Trp⁺²⁵). The S₄–S₅ loop of hG9C is not directed toward the Gal⁺¹ and
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FIGURE 5. The cross-linking of bG1 (PDB code: 15LA, green) (a) and hG9C (pink) (b) by the biantennary oligosaccharides. Subsite numbers and selected amino acid residues are given. In b, the modeled sugar units at subites +4 and +5 are shown in blue.

According to the frontal affinity chromatography analysis, human galectin-1 (hG1) has significant affinity for branched oligosaccharides ($K_d$ values of 4.5–7.7 μm) but less affinity than hG9N and/or hG9C. Because bG1 has 87% sequence identity to hG1 (Fig. 4), it should have comparable affinity. Supposing that a branched oligosaccharide binds to bG1 as a ligand, bG1, having a deep carbohydrate-binding site, can form a stable protein-ligand complex of low structural energy with many attractive direct interactions. In practice, the Arg221 of hG8N adopts conformer-2 to avoid short contacts with Lys78.

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