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COMMUNICATION

Structural analysis of the recognition mechanism of poly-*N*-acetyllactosamine by the human galectin-9 N-terminal carbohydrate recognition domain

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Galectins are a family of β-galactoside-specific lectins bearing a conserved carbohydrate recognition domain. Interactions between galectins and poly-*N*-acetyllactosamine sequences are critical in a variety of biological processes. Galectin-9, a member of the galectin family, has two carbohydrate recognition domains at both the N- and C-terminal regions. Here we report the crystal structure of the human galectin-9 N-terminal carbohydrate recognition domain in complex with *N*-acetyllactosamine dimers and trimers. These complex structures revealed that the galectin-9 N-terminal carbohydrate recognition domain can recognize internal *N*-acetyllactosamine units within poly-*N*-acetyllactosamine chains. Based on these complex structures, we propose two putative recognition modes for poly-*N*-acetyllactosamine binding by galectins.

Keywords: carbohydrate recognition/galectin/poly-*N*-acetyllactosamine/X-ray structure

Introduction

Galectins, widely distributed animal lectins, share a conserved carbohydrate recognition domain (CRD) of approximately 130 amino acids; this sequence is tightly folded into a sandwich structure of 5- to 6-stranded β-sheets (Barondes et al. 1994). These proteins also share specificity for β-galactoside-containing glycoconjugates. Evolutionarily, this family is highly conserved with homologs even in lower organisms, such as nematodes and sponges (Kasai and Hirabayashi 1996). The mammalian galectin family currently has 14 members (Cooper 2002), which have been classified into three subfamilies, the proto, chimera, and tandem-repeat types, based on their domain organization (Hirabayashi and Kasai 1993). The prototype galectins (galectins 1, 2, 5, 7, 10, 13, 14, and 15) consist of a single CRD with a short N-terminal sequence, while the tandem-repeat-type galectins (galectins 4, 6, 8, 9, and 12) contain two nonidentical CRDs joined by a short peptide linker. The single chimera-type galectin (galectin-3) has a CRD and an additional N-terminal nonlectin domain.

The tandem-repeat-type galectin human galectin-9 was originally isolated from tumor cells in Hodgkin's disease (Tureci et al. 1997). This protein was determined to be identical to the potent eosinophil chemoattractant ecalectin, originally cloned from a human T-cell line (Matsumoto et al. 1998; Matsushita et al. 2000). The chemoattractant activity of galectin-9 depends on its carbohydrate-binding activity and requires both CRDs (Matsushita et al. 2000). Although the exact structure of the target carbohydrates recognized by human galectin-9 is unclear, biochemical analyses have revealed that both the N-terminal CRD (NCRD) and C-terminal CRD (CCRD) of human galectin-9 have a high affinity for repeating β1-3-linked *N*-acetyllactosamine (Galβ1-4GlcNAc; LacNAc) residues (poly-*N*-acetyllactosamine) (Hirabayashi et al. 2002; Sato et al. 2002). The dissociation constants of human galectin-9 NCRD for LacNAc dimers (LN2), trimers (LN3), and pentamers (LN5) are 3.0, 0.81, and 0.12 μM, respectively, and those of CCRD are 17, 2.7, and 0.41 μM, respectively (Hirabayashi et al. 2002). This enhanced affinity for larger repeating LacNAc sequences was shared by both CRDs, but was more prominent for the NCRD. The particular preference for poly-*N*-acetyllactosamines should help elucidate the molecular mechanisms by which human galectin-9 exerts its varied biological functions.

Poly-*N*-acetyllactosamine chains are widely distributed in mammalian cells in multiple species (Fukuda 1985). These motifs are preferentially bound by other galectins with high avidity, although the affinities for single LacNAc residues are relatively low (Barondes et al. 1994; Di Virgilio et al. 1999; Hirabayashi et al. 2002). The molecular mechanism governing the interaction between galectins and glycoconjugates containing repeating LacNAc motifs is still unresolved. In this report, we determined the crystal structures of the human galectin-9 NCRD in complex with LN2 and LN3. We discuss the possible interactions between the galectin-9 NCRD and poly-*N*-acetyllactosamine residues.

Results

Crystal structure of galectin-9 N-terminal CRD in complex with LN2

To investigate the mechanism by which the human galectin-9 NCRD and poly-*N*-acetyllactosamine interact, we determined

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the crystal structures of the human galectin-9 NCRD in the presence of LN2 or LN3. The crystal structure of human galectin-9 NCRD in complex with LN2 (called LN2-bound crystal 1 throughout this manuscript) was refined to a 1.8 Å resolution. The crystal structure demonstrated that no obvious structural difference was found between the human galectin-9 NCRD-lactose complex structure reported recently by our group (Nagae et al. 2008).

In LN2-bound crystal 1, the protein structures of the two complexes in the asymmetric unit (referred to as complexes A and B) are essentially identical with a root mean square deviation (rmsd) of 0.37 Å for all Cα atoms (Figure 1A). The interaction modes with the carbohydrates, however, are quite different between the complexes. The protein molecule in complex A interacts closely with the reducing end of the LacNAc moiety (Figure 1B, left), while the protein molecule in complex B interacts tightly with the nonreducing end (Figure 1B, right). Thus, the carbohydrate molecules bound in these complexes are shifted by one complete LacNAc unit. The amino acid residues that interact with the LN2 molecules in both complexes are identical, with the exception of Asn48 and Asn137. These two asparagine residues form additional interaction sites for the ligand in complex A.

We obtained another crystal which also belongs to an orthorhombic space group with different cell constants (referred to as LN2-bound crystal 2). The asymmetric unit of LN2-bound crystal 2 contains four galectin-9 NCRD-LN2-bound complexes. The interaction modes of these complexes were also divided into two classes. Protein–ligand interactions for three of the molecules, conformed to that seen in complex A of the LN2-bound crystal 1 where the interaction is with the reducing end of the LN2, whilst one of the protein molecules interacts with the nonreducing end. The backbones of these four protein molecules superimpose well on each other with rmsd values within 0.30 Å. The *c*-axis of crystal 1 is half that of the *b*-axis of crystal 2 and the former has lost the screw axis (Table I). We reprocessed the crystal 2 data to interchange the *b*- and *c*-axes and confirmed that the crystal packings of the protein molecules in LN2-bound crystal 2 are almost identical with those of LN2-bound crystal 1. The main difference in the packing is derived from positional differences of the carbohydrates.

Crystal structure of galectin-9 N-terminal CRD in complex with LN3

Crystals of the human galectin-9 NCRD-LN3-bound complex were found to adopt two crystal forms, one of which exhibited $P2_12_12_1$ space group symmetry (LN3-bound crystal 1) while the other belonged to the $P4_12_12$ group (LN3-bound crystal 2). The cell constants of LN3-bound crystal 1 are almost identical to those of LN2-bound crystal 2 (Table I). The asymmetric unit of the LN3-bound crystal 1 also contains four complexes, designated complexes A–D. In these four complexes, the positions of the carbohydrate molecules could also be divided into two classes. The protein molecules in both complexes A and B primarily recognize the third LacNAc unit (fifth and sixth carbohydrate residues) from the nonreducing end, while complexes C and D interact with the second LacNAc unit (third and fourth carbohydrate residues) from the nonreducing end (Table II). The carbohydrate recognition mode of complex B is similar with that of complex A, with the exception of the interactions of the main chain oxygen atom of Ser54. The recognition modes of complexes C and D are identical to each other. The four protein molecules in the asymmetric unit are superimposable with rmsd values within 0.30 Å (Figure 2A).

In LN3-bound crystal 2, only one complex existed in the asymmetric unit. The protein recognizes the second LacNAc unit from the nonreducing end (Figure 2B). The carbohydrate interaction in LN3-bound crystal 2 is identical to that seen in complexes C and D of LN3-bound crystal 1.

Discussion

Our determination of the crystal structures of the human galectin-9 NCRD in complex with varying numbers of LacNAc repeats provides the first structural insights into the mechanisms of interaction between the galectin CRD and poly-*N*-acetyllactosamine. The structures of LN2- and LN3-bound complexes clearly indicate that the human galectin-9 NCRD could recognize three continuous carbohydrate residues (GlcNAcβ1-3Galβ1-4GlcNAc) irrespective of the positions of the LacNAc unit (Table II). The amino acid residues involved in β-galactoside recognition are identical among human galectin-1, -3, and galectin-9 NCRD (Leonidas et al. 1998; Seetharaman et al. 1998; Lopez-Lucendo et al. 2004; Nagae et al. 2008). We have previously demonstrated that nonconserved amino acid residues on the concave surface (Ala46 and Asn137 in human galectin-9 NCRD) were important for target carbohydrate specificity (Nagae et al. 2008). In this report, two asparagine residues, Asn48 and Asn137, appear to contribute to the enhanced affinity observed over that of the single LacNAc unit (Nagae et al. 2006). Human galectin-9 CCRD is fairy homologous to NCRD (amino acid sequence identity of 37%) but exhibits 3.3 to 5.7 times lowered affinities for LN2, 3, and 5 than NCRD (Hirabayashi et al. 2002). A sequence comparison between two CRDs reveals that Asn137 is replaced by glycine in CCRD (data not shown). Moreover, our recent results revealed that mouse galectin-9 NCRD also shows the lower affinities for LN2, 3, and 5 than human one (Nagae et al. 2008). Asn137 in human galectin-9 NCRD is also replaced by serine in mouse galectin-9 NCRD. Thus, Asn137 would be an important residue for the tight interaction with poly-*N*-acetyllactosamine in human galectin-9 NCRD.

Although previous frontal affinity chromatography analyses revealed that the longer carbohydrates show increasing affinity for human galectin-9 NCRD (LN2, 3.0 μM of K_d value; LN3, 0.81 μM of K_d value; LN5, 0.12 μM of K_d value) (Hirabayashi et al. 2002), no additional interaction was observed between LN2- and LN-3 complexes. In frontal affinity chromatography analyses, retardation of elution of carbohydrate ligand molecules from lectin immobilized column is measured, and an association constant is calculated from the retardation (Hirabayashi et al. 2002). Therefore, apparent affinity may increase by the multivalent binding effect of human galectin-9 NCRD to longer carbohydrates. Or else, two CRDs may simultaneously bind to one carbohydrate molecule.

Structural comparison among other galectins gives the potential effect of the local structural differences on the various interaction modes between galectins and poly-*N*-acetyllactosamine. Superposition of human galectin-1 CRD (PDB code: 1GZW, Lopez-Lucendo et al. 2004) on the LN3-bound complexes suggests that there are structural conflicts between LN3 and

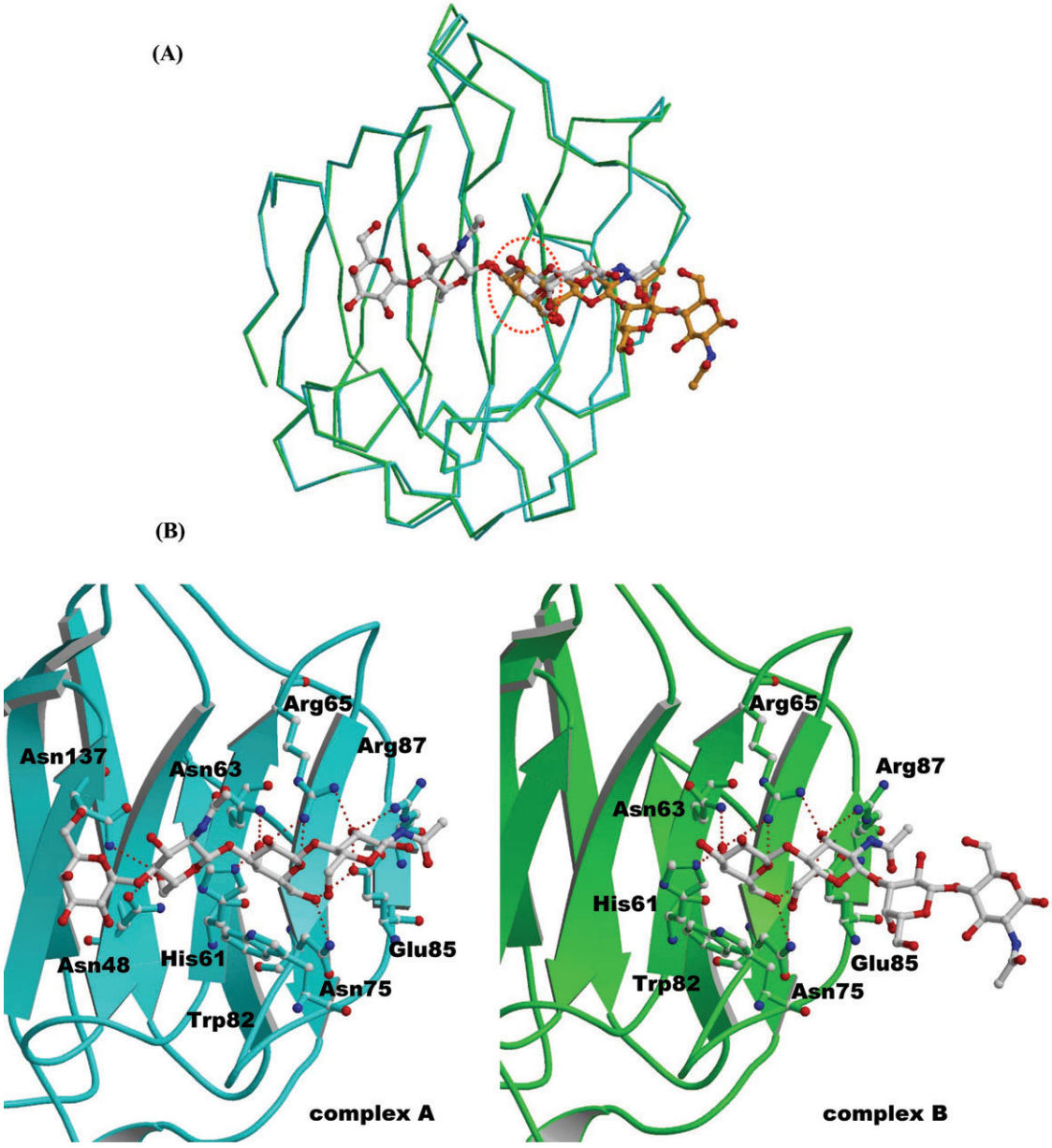

Fig. 1. Crystal structure of the human galectin-9 N-terminal CRD-LN2-bound complex. (**A**) The two complexes in the asymmetric unit of crystal 1 are superimposed. The main chains of complexes A (cyan) and B (green) and the carbohydrates of complexes A (white) and B (orange) are shown as wire and ball-and-stick models, respectively. The oxygen and nitrogen atoms of the carbohydrates are shown in red and blue, respectively. The positions of the galactose residues that interacted tightly with the protein molecules are shown in an orange dotted circle. (**B**) Magnified views of the carbohydrate-binding sites of complexes A (left) and B (right). The carbohydrate molecules and amino acid residues involved in carbohydrate recognition are shown as a ball-and-stick model. Hydrogen bonds are depicted by red dotted lines.

N-terminus of human galectin-1. Galectin-1 is thought to bind to nonreducing terminal LacNAc units rather than mid-chain LacNAc residues within a poly-*N*-acetyllactosamine chain (Solomon et al. 1991; Di Virgilio et al. 1999; Stowell et al. 2004). Thus, these conflicts would affect the interaction with poly-*N*-acetyllactosamine. Both the galectin-3 and -7 CRDs (Leonidas et al. 1998; Seetharaman et al. 1998) display close structural similarities to the human galectin-9 NCRD. In the case of human galectin-3 (PDB code: 1A3K), replacement of Asn137 in the human galectin-9 NCRD with Ser237 may affect the interaction with poly-*N*-acetyllacotsamine chains. When human galaectin-3 CRD is structurally superimposed to the human galectin-9 NCRD-LN2 or -LN3 complex, there are no structural conflicts between human galectin-3 and each oligosaccharide. In contrast, superposition of human galectin-7 (PDB code: 1BKZ) on human galectin-9 NCRD-LN3 complex suggests that the complexes A and B in LN3-bound crystal 1 revealed heavy steric conflicts between the LN3 molecule and the side chain of Gln42, which is located in close proximity of the carbohydrate binding site. These structural comparison explains well the previous observations that the affinities of the human galectin-3 CRD for repeating LacNAc chains increased with the increasing

Table I. Data collection and refinement statistics

Data collection statistics				
Crystal	LN2-crystal 1	LN2-crystal 2	LN3-crystal 1	LN3-crystal 2
Space group	$P2_12_12$	$P2_12_12_1$	$P2_12_12_1$	$P4_12_12$
Unit cell (Å)	$a = 35.4$, $b = 221.2$, $c = 33.1$	$a = 35.4$, $b = 67.7$, $c = 220.1$	$a = 35.2$, $b = 68.0$, $c = 220.0$	$a = b = 52.2$, $c = 113.8$
Beam line	BL-5A PF	BL-5A PF	NW12A PF-AR	NW12A PF-AR
Wavelength (Å)	1.0000	1.0000	1.0000	1.0000
Resolution (Å)[a]	50–1.80 (1.86–1.80)	50–1.75 (1.81–1.75)	100–1.85 (1.92–1.85)	50–1.30 (1.35–1.30)
Total reflections	289,033	454,995	501,830	502,613
Unique reflections	24,663	54,161	46,066	39,630
Completeness (%)[a]	97.7 (90.8)	98.9 (91.8)	98.9 (99.5)	100 (100)
R_{merge} (%)[a]	9.6 (24.2)	7.0 (25.3)	7.6 (40.1)	9.5 (50.6)
$\langle I/\sigma I \rangle$[a]	26.6 (7.9)	42.3 (4.1)	28.5 (6.6)	22.2 (5.1)
Refinement statistics				
Resolution range (Å)	223.6–1.80	218.2–1.75	218.2–1.85	114.0–1.30
No. of reflections	23,403	51,410	43,720	37,638
No. of nonhydrogen atoms				
Protein	2,221	4,460	4,469	1,141
Water	182	490	507	167
Carbohydrate	106	212	294	88
R_{work} (%)	20.1	19.5	18.4	18.3
R_{free} (%)	23.8	23.8	23.7	19.2
r.m.s. deviations				
Bond length (Å)	0.012	0.014	0.012	0.007
Bond angle (°)	1.529	1.487	1.407	1.221
Mean *B*-factors (Å^2)	25.6	21.3	21.3	8.4

[a]Values in parentheses are for the highest resolution shell.

number of carbohydrate repeats, while human galectin-7 exhibited comparable affinities irrespective of ligand length (Hirabayashi et al. 2002).

Galectin-1 and -2 form noncovalent homodimers in solution (Morris et al. 2004); galectin-3 forms oligomers via interactions of their N-terminal domains in the presence of multivalent carbohydrate (Ahmad et al. 2004). The tandem-repeat-type galectins are bivalent, possessing two CRDs. The interaction mode between human galectin-9 CCRD and poly-*N*-acetyllactosamine still remains unclear. To presume the relative positions of two

Table II. The LN3 recognition by human galectin-9 N-terminal CRD (LN3-bound crystal 1)

Sugar atom	Complex A Protein atom distance (Å)		Complex B Protein atom distance (Å)		Complex C Protein atom distance (Å)		Complex D Protein atom distance (Å)	
GlcNAc(2)N2			O(Ser54)	3.1				
GlcNAc(2)O6					ND2(Asn48)	2.9	ND2(Asn48)	3.0
GlcNAc(2)O6					ND2(Asn137)	3.2	ND2(Asn137)	3.2
Gal(3)O2			O(Ser54)	3.4				
Gal(3)O4					NE2(His61)	2.8	NE2(His61)	2.8
Gal(3)O4					ND2(Asn63)	3.0	ND2(Asn63)	3.0
Gal(3)O4					NH2(Arg65)	3.0	NH2(Arg65)	2.9
Gal(3)O5					NH2(Arg65)	2.9	NH2(Arg65)	3.0
Gal(3)O6					ND2(Asn75)	3.0	ND2(Asn75)	2.8
Gal(3)O6					OE2(Glu85)	2.7	OE2(Glu85)	2.8
GlcNAc(4)O3					NH1(Arg65)	2.9	NH1(Arg65)	3.0
GlcNAc(4)O3					OE2(Glu85)	2.4	OE2(Glu85)	2.4
GlcNAc(4)O3					NH2(Arg87)	3.4	NH2(Arg87)	3.4
GlcNAc(4)O6	ND2(Asn48)	3.2	ND2(Asn48)	3.3				
GlcNAc(4)O6	ND2(Asn137)	3.4	ND2(Asn137)	3.0				
Gal(5)O4	NE2(His61)	2.8	NE2(His61)	2.8				
Gal(5)O4	ND2(Asn63)	2.9	ND2(Asn63)	3.0				
Gal(5)O4	NH2(Arg65)	3.0	NH2(Arg65)	3.0				
Gal(5)O5	NH2(Arg65)	3.0	NH2(Arg65)	2.9				
Gal(5)O6	ND2(Asn75)	3.0	ND2(Asn75)	3.0				
Gal(5)O6	OE2(Glu85)	2.7	OE2(Glu85)	2.8				
GlcNAc(6)O3	NH1(Arg65)	2.9	NH1(Arg65)	3.0				
GlcNAc(6)O3	OE2(Glu85)	2.5	OE2(Glu85)	2.5				
GlcNAc(6)O3	NH2(Arg87)	3.1	NH2(Arg87)	3.2				

Hydrogen bonds between LN3 and human galectin-9 N-terminal CRD are listed. The residue number of the LN3 molecule is counted from the nonreducing end.

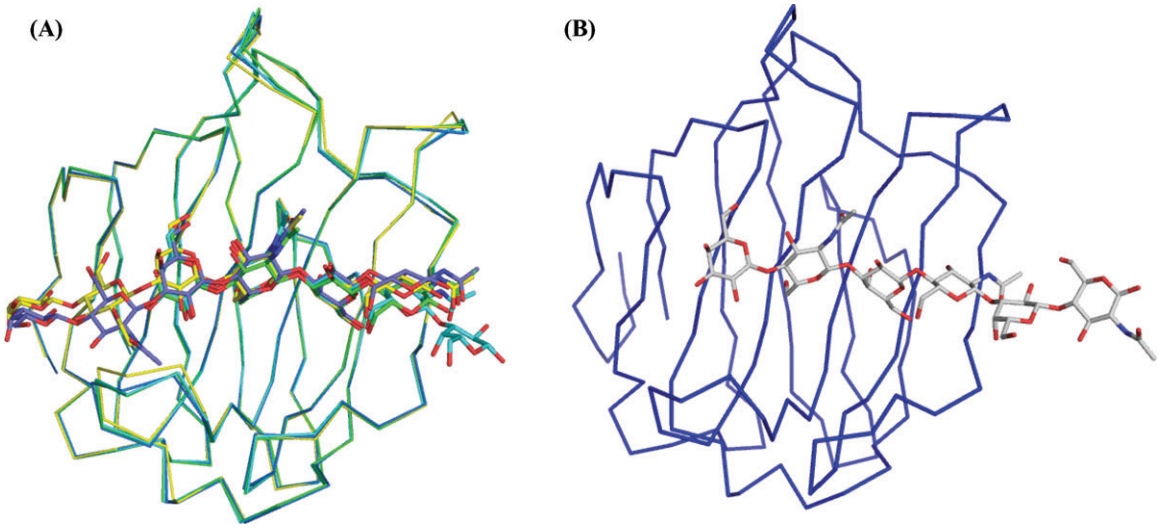

Fig. 2. (**A**) Superimposition of the four human galectin-9 N-terminal CRD-LN3-bound complexes in an asymmetric unit (crystal 1). Protein molecules and carbohydrates are depicted as wire and rod models, respectively. Complexes A–D are shown in blue, yellow, cyan, and green, respectively. The oxygen and nitrogen atoms of the carbohydrates are colored in red and blue, respectively. (**B**) The crystal structure of the human galectin-9 NCRD complexed with LN3 (crystal 2). The protein and LN3 molecules are shown as wire and rod models, respectively.

CRDs, we generated a hypothetical model structure for the human galectin-9 NCRD in complex with LN6 based on the LN3-bound complexes (data not shown). The model structure implies that two human galectin-9 CRD molecules can bind simultaneously to a single LN6 molecule, and the distance between two CRDs is about 45 Å. The long linker peptide (61 amino acids) of human galectin-9 might connect both CRDs, which bind poly-*N*-acetyllactosamine chains by intra- or intermolecular manner. These differences in the quaternary structure may account for the formation of a crosslinking network of multivalent carbohydrates (Brewer 2002). Molecular modeling of the low-energy conformers of poly-*N*-acetyllactosamine revealed generally extended structures (Atrih et al. 2005), which may make galectins able to access each LacNAc unit externally. Our results suggest the possibility of a variety of interaction modes between galectins and poly-*N*-acetyllactosamine. These differences may be responsible for the physiological variety among the galectins and related biological systems.

Material and methods

Protein purification and crystallization

The recombinant human galectin-9 NCRD was expressed and purified as described previously (Nagae et al. 2008). LN2 and LN3 were synthesized as previously reported (Murata et al. 2005; Nagae et al. 2006). Initial crystallization trials were performed using an automated protein crystallization and monitoring system (Hiraki et al. 2006). The crystals containing the LN2 or LN3 complexes were prepared by cocrystallization in the presence of 10 mM ligand at 293 K. LN2-bound crystal 1 appeared under conditions of 200 mM potassium fluoride and 20% (w/v) polyethylene glycol (PEG) 3350. To obtain LN2-bound crystal 2, the protein solution was mixed with 200 mM imidazole malate and 22.5% (w/v) PEG 10000. LN3-bound crystal 1 was obtained in the presence of 200 mM ammonium fluoride and 20% (w/v) PEG 3350. LN3-bound crystal 2 was obtained using a reservoir solution containing 1.0 M monoammonium dehydrate phosphate and 0.1 M trisodium citrate (pH 5.6).

Data collection, structure determination, and refinement

Synchrotron data were collected at beamlines BL-5A and AR-NW12A at the Photon Factory (Tsukuba, Japan). All datasets were processed and scaled using the HKL2000 program software (Otwinowski and Minor 1997). The phase of the LN2-bound crystal 1 was determined by molecular replacement using Morlep software from the CCP4 program suite (CCP4 1994). We used the crystal structure of the human galectin-9 NCRD–lactose complex (PDB code: 2EAK; Nagae et al. 2008) without lactose and solvent molecules as a search model. The phases of the other carbohydrate complex structures were determined utilizing the LN2-bound crystal 1 structure as a search model. Model reconstructions were performed manually using Xfit software from XtalView (McRee 1999). Crystallographic refinement was performed using REFMAC5 software from the CCP4 suite (CCP4 1994). Quality of the protein models was assessed with the PROCHECK software (Laskowski et al. 1993). Data collection and refinement statistics are summarized in Table I. Figures were created with the Molscript (Kraulis 1991), Raster3D (Merrit and Murphy 1994), and PyMOL (DeLano Scienctific LLC) programs.

Coordinates for the galectin-9 NCRDs have been deposited in the Protein Data Bank under the following accession numbers: 2ZHK (LN2-bound crystal 1), 2ZHL (LN2-bound crystal 2), 2ZHM (LN3-bound crystal 1), and 2ZHN (LN3-bound crystal 2).

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Conflict of interest statement

None declared.

Abbreviations

CCRD, C-terminal CRD; CRD, carbohydrate recognition domain; GlcNAc, *N*-acetylglucosamine; LacNAc, *N*-acetyllact osamine; LN2, *N*-acetyllactosamine dimer; LN3, *N*-acetyllactosamine trimer; LN6, *N*-acetyllactosamine hexamer; NCRD, N-terminal CRD; PEG, polyethylene glycol; rmsd, root mean square deviation.

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