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Functional analysis of the carbohydrate recognition domains and a linker peptide of galectin-9 as to eosinophil chemoattractant activity

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Human galectin-9 is a β-galactoside-binding protein consisting of two carbohydrate recognition domains (CRDs) and a linker peptide. We have shown that galectin-9 represents a novel class of eosinophil chemoattractants (ECAs) produced by activated T cells. A previous study demonstrated that the carbohydrate binding activity of galectin-9 is indispensable for eosinophil chemoattraction and that the N- and C-terminal CRDs exhibit comparable ECA activity, which is substantially lower than that of full-length galectin-9. In this study, we investigated the roles of the two CRDs in ECA activity in conjunction with the sugar-binding properties of the CRDs. In addition, to address the significance of the linker peptide structure, we compare the three isoforms of galectin-9, which only differ in the linker peptide region, in terms of ECA activity. Recombinant proteins consisting of two N-terminal CRDs (galectin-9NN), two C-terminal CRDs (galectin-9CC), and three isoforms of galectin-9 (galectin-9S, -9M, and -9L) were generated. All the recombinant proteins had hemagglutination activity comparable to that of the predominant wild-type galectin-9 (galectin-9M). Galectin-9NN and galectin-9CC induced eosinophil chemotaxis in a manner indistinguishable from the case of galectin-9M. Although the isoform of galectin-9 with the longest linker peptide, galectin-9L, exhibited limited solubility, the three isoforms showed comparable ECA activity over the concentration range tested. The interactions between N- and C-terminal CRDs and glycoprotein glycans and glycolipid glycans were examined using frontal affinity chromatography. Both CRDs exhibited high affinity for branched complex type sugar chain, especially for tri- and tetraantennary N-linked glycans with N-acetyllactosamine units, and the oligosaccharides inhibited the ECA activity at low concentrations. These results suggest that the N- and C-terminal CRDs of galectin-9 interact with the same or a closely related ligand on the eosinophil membrane when acting as an ECA and that ECA activity does not depend on a specific structure of the linker peptide.

Key words: chemoattractant/eosinophil/frontal affinity chromatography/galectin

Introduction

Galectins constitute a family of animal lectins that show binding specificity for β-galactoside. Nine members of the human galectin family (galectin-1–4, galectin-7–10, and galectin-12) have been identified to date. The members of the family can be classified into three subtypes according to their structures (Hirabayashi and Kasai, 1993). Galectins-1, -2, -7, and -10 have a single carbohydrate recognition domain (CRD) consisting of about 130–140 amino acid residues and belong to the prototype. Galectin-3, which is composed of a carboxy-terminal CRD and a different type of amino-terminal domain, is the sole member of the chimera type. Galectins-4, -8, -9, and -12 have two CRDs joined by a linker peptide and are classified as the tandem repeat type. Although the members of prototype and chimera type have a single CRD, galectins-1, -2, and -3 (Cho and Cummings, 1996; Gitt *et al.*, 1992, 1995; Hsu *et al.*, 1992) and possibly galectins-7 and -10 are known to form dimer/multimers, resulting in multivalent carbohydrate binding capacity. Some nonmammalian galectins are also known to form noncovalent dimer/multimers (Pfeifer *et al.*, 1993). Hence, most if not all galectins are functionally multivalent, making it possible to cross-link glycoconjugate ligands.

Human galectin-9 was first identified as a putative autoantigen in patients with Hodgkin's disease and was suggested to play an important role in the regulation of cellular interactions of the immune system (Tureci *et al.*, 1997). Mouse galectin-9 has been cloned and characterized by Wada and Kanwar (1997). They showed that galectin-9 was capable of inducing apoptosis of thymocytes *in vitro* (Wada *et al.*, 1997). These reports, together with our previous finding that human ecalectin/galectin-9 has potent eosinophil chemoattractant (ECA) activity (Matsumoto *et al.*, 1998), suggest that galectin-9, in addition to other members of the galectin family (especially galectins-1 and -3), plays multiple roles in the immune system. To understand the mechanism underlying the immune-modulating effects of galectins, it is important to elucidate the function of each CRD in relation to its carbohydrate binding specificity.

In a previous study (Matsushita *et al.*, 2000) we demonstrated that ECA activity appears to be unique to galectin-9. Among the four human galectins studied, galectins-1 and -3 had

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negligible ECA activity. Galectin-8 showed low ECA activity, that is, less than 10% of that of galectin-9. We also demonstrated that the carbohydrate binding activity of galectin-9 is indispensable for ECA activity. In addition, experiments involving the N- and C-terminal CRDs of galectin-9 showed that these CRDs may exist at least in part as dimers and/or multimers and exert comparable ECA activity that is substantially lower than that of full-length galectin-9. Based on these results, we postulated that galectin-9 exerts its ECA activity via cross-linking of galactoside-containing glycoconjugates on the surface of eosinophils. There are three possibilities for the interaction between the two CRDs and cell surface glycoconjugates: (1) the two domains bind to two distinct glycoconjugates; (2) the two domains bind to two identical glycoconjugates, and thus galectin-9 functions by inducing homo dimer/multimer formation of the molecules; and (3) the two domains bind to two different oligosaccharide moieties on the same glycoconjugates. In the present study we examined these possibilities by using mutant galectin-9 molecules consisting of two N- terminal CRDs (galectin-9NN) and two C-terminal CRDs (galectin-9CC) and by determining the carbohydrate binding specificities of the two CRDs. Moreover, three isoforms of galectin-9 with different linker peptides were used to examine the significance of the linker peptide structure as to ECA activity.

Results

Purification of recombinant proteins

To examine the three possible modes of interaction between galectin-9 and eosinophil surface glycoconjugates, we produced recombinant proteins composed of two tandemly linked N-terminal CRDs and C-terminal CRDs of galectin-9 (galectin-9NN and galectin-9CC, Figure 1). Three isoforms of galectin-9 (galectin-9S, -9M, and -9L), which are expressed in Jurkat cells and differ only in the linker peptide, were also produced to determine the effect of the linker peptide structure on ECA activity. All the recombinant proteins were expressed in *Escherichia coli* and efficiently purified by lactose affinity chromatography to apparent homogeneity (Figure 2). In the case of galectin-9L, the isoform with the longest linker peptide, the overall yield was significantly lower than those of the other isoforms probably due to the limited solubility of galectin-9L.

Hemagglutination activity toward rabbit erythrocytes

Hemagglutination activity is one of the typical characteristics of multivalent lectins. We showed that the minimum concentration of galectin-9 required for hemagglutination was comparable with that of galectin-1 (Matsushita *et al.*, 2000), a prototypic galectin known to exist in monomer-dimer equilibrium (Cho and Cummings, 1996). To assess the functionality of the CRDs of both galectin-9NN and galectin-9CC, the hemagglutination activity of these mutant proteins was compared with that of wild-type galectin-9. All the recombinant proteins, including galectin-9S and galectin-9L, showed comparable hemagglutination activity (Table I). The results show that the CRDs of both galectin-9NN and –9CC are capable of interacting with erythrocyte cell surface glycoconjugate(s).

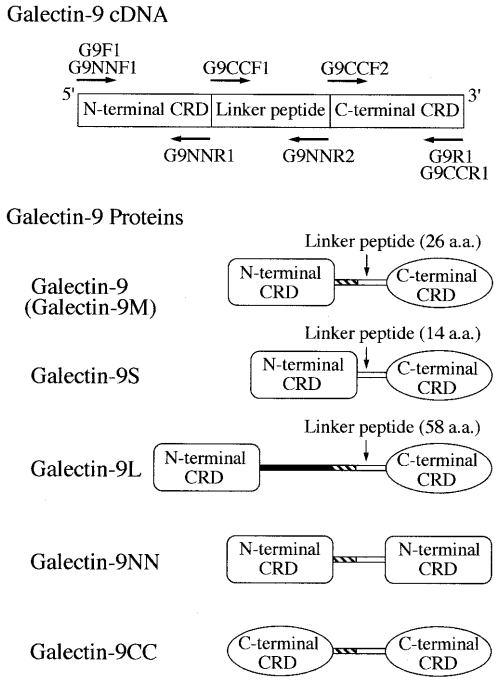

Fig. 1. Schematic representation of the primer sites used for amplification of galectin-9 cDNAs and the structures of recombinant proteins. Because it is not easy to determine unequivocally the carboxy-terminal end of a linker peptide without 3D structural data, the lengths of the linker peptides should be considered to be provisional. Recently, Lipkowitz *et al.* (2001) reported the genomic structure of human galectin-9/urate transporter. Their results suggest that the carboxy-terminal end of a linker peptide is Ser^{177} in the case of galectin-9M.

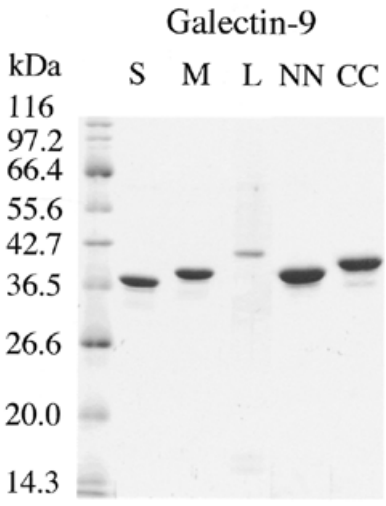

Fig. 2. Sodium dodecyl sulfate–polyacrylamide gel electrophoretic analysis of purified recombinant proteins. Recombinant proteins purified by lactose-agarose affinity chromatography were electrophoretically separated in a SDS/12% polyacrylamide gel under reducing conditions. Recombinant proteins of galectins-9S (38.7 kDa), -9M (39.9 kDa), -9L (43.5 kDa), -9NN (39.5 kDa), and -9CC (40.6 kDa) were visualized by staining with Coomassie brilliant blue R-250. The molecular masses of the recombinant proteins include an about 4-kDa tag sequence.

Table I. Hemagglutination activity of the three isoforms of galectin-9, galectin-9NN, and galectin-9CC (the minimum concentrations necessary for hemagglutination activity of His-tag containing recombinant proteins are shown)

Recombinant protein	Minimum concentration for hemagglutination (nM)
Galectin-9M	12.5
Galectin-9S	12.5
Galectin-9L	25.0
Galectin-9NN	25.0
Galectin-9CC	25.0

ECA activity of galectin-9 mutants and isoforms

In a previous study we produced recombinant galectins as glutathione S-transferase (GST) fusion proteins. The presence of the GST moiety did not affect the ECA activity of galectin-9. In this study recombinant proteins carrying a tag sequence (which has a molecular weight of about 4,000 and includes a hexa-histidine sequence) at the N-terminal were used. GST-galectin-9 and galectin-9 carrying the his-tag sequence showed indistinguishable ECA activity (data not shown).

The ECA activity of galectin-9CC at 0.1 μM was similar to that of eotaxin-1 (100 ng/ml), a typical ECA. Galectin-9NN showed somewhat lower activity than that of galectin-9CC at this concentration. The overall dose-response data, however, were closely similar among the wild type (galectin-9M) and mutant galectins (Figure 3). In a separate experiment, we compared the ECA activity among the three isoforms of galectin-9 (Figure 4). The dose-response curve for galectin-9S, the isoform with the shortest linker peptide, was comparable with that for galectin-9M. As it was difficult to obtain a high concentration preparation, the ECA activity of galectin-9L at concentrations lower than 0.14 μM was measured. Galectin-9L showed similar ECA activity to that of the other isoforms over the concentration range tested.

Oligosaccharide-binding specificity of N- and C-terminal CRDs of galectin-9

We used recently reinforced frontal affinity chromatography (Hirabayashi *et al.*, 2000; Arata *et al.*, 2001) to determine the binding affinity of pyridylamino (PA) derivatives of oligosaccharides toward immobilized GST-galectin-9NT and -9CT. Twelve glycoprotein N-glycans and 21 glycolipid glycans were tested. Both galectin-9NT and -CT showed relatively high affinity (K_d < 1 μM) for N-linked tri- and tetraantennary complex-type glycans with N-acetyllactosamine units (Table II and Figure 5). The ECA activity of galectin-9 was inhibited by 87% by the tetraantennary type glycan at 2 μM. On the other hand, the oligosaccharide showed a negligible effect (less than 10% inhibition) on the activity of eotaxin. Fucosylation at the N-acetyllactosamine unit and sialylation of the glycans resulted in decreased binding strength (Table II; compare 6 and 9, 10 and 12, 1 and 3–5). In addition to the glycoprotein N-glycans, galectin-9NT exhibited high affinity for two glycolipid glycans, Forssman pentasaccharide and A-hexasaccharide. However, the binding affinity of galectin-9CT for glycolipid glycans was generally lower than that of galectin-9NT. Forssman pentasaccharide and A-hexasaccharide (2 μM) inhibited the ECA activity of galectin-9 by 55% and 52%, respectively.

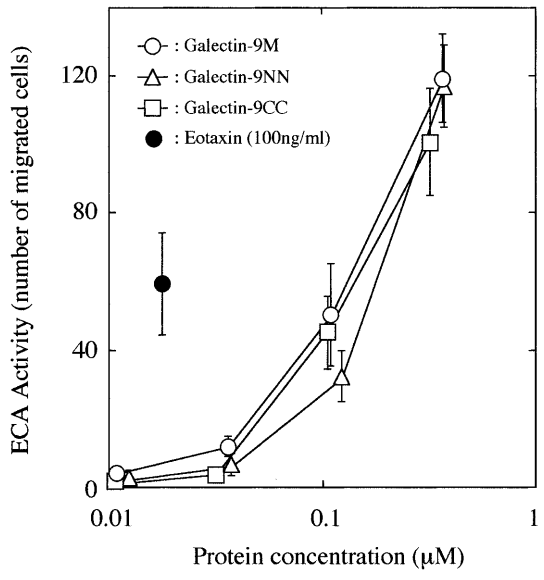

Fig. 3. Comparison of ECA activity of galectins-9M, -9NN, and -9CC. In the *in vitro* ECA activity assay, a porous membrane separated the top chamber, containing human eosinophils, from the bottom chamber, containing medium with or without an ECA. After the incubation, the membrane was removed and stained with Diff-Quick. The number of eosinophils that had migrated through the membrane is indicated. Artificial galectin-9 consisting of two N-terminal CRDs (galectin-9NN) and two C-terminal CRDs (galectin-9CC) and wild-type galectin-9 (galectin-9M) were produced in *E. coli* and purified by lactose-agarose affinity chromatography. The results represent the means ± SD for two experiments performed in triplicate.

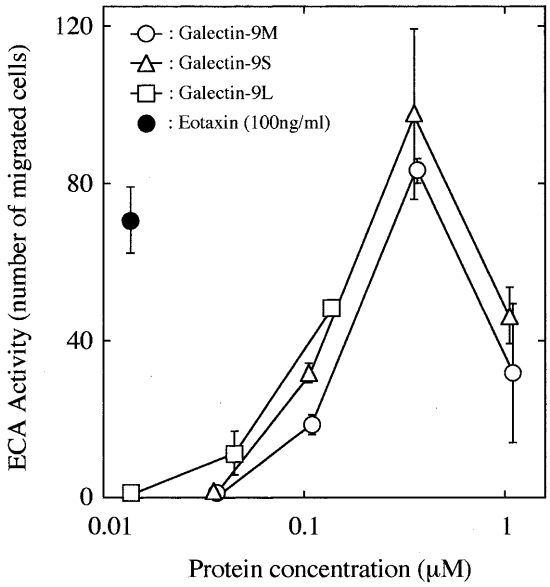

Fig. 4. Comparison of ECA activity of galectins-9S, -9M, and -9L. Three isoforms of galectin-9 with different linker peptides were produced in *E. coli* and purified by lactose-agarose affinity chromatography. In the case of the isoform with the longest linker peptide, galectin-9L, the ECA activity at concentrations lower than 0.14 μM was determined. The results represent the means ± SD for two experiments performed in triplicate.

Table II. Profiling of the sugar binding specificities of the N- and C-terminal CRDs of human galectin-9 (the binding affinity of PA oligosaccharides toward immobilized GST-galectins-9NT and -9CT was determined by frontal affinity chromatography)

PA-Sugar chain	Binding affinity (*Kd*, μM) N-terminal CRD	C-terminal CRD
Glycoprotein N-glycans (N-acetyllactosamine type)		
1 Biantennary Galβ1-4GlcNAcβ1-2Manα1-6 Manβ1-4R Galβ1-4GlcNAcβ1-2Manα1-3	3.1	1.2
2 Biantennary (core-fucosylated) Galβ1-4GlcNAcβ1-2Manα1-6 Manβ1-4R' Galβ1-4GlcNAcβ1-2Manα1-3	3.5	1.6
3 Biantennary (monosialylated) Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6 Manβ1-4R Galβ1-4GlcNAcβ1-2Manα1-3	26	31
4 Biantennary (monosialylated) Galβ1-4GlcNAcβ1-2Manα1-6 Manβ1-4R Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3	44	44
5 Biantennary (disialylated) Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6 Manβ1-4R Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3	*	*
6 Triantennary Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4GlcNAcβ1-4 Manβ1-4R Manα1-3 Galβ1-4GlcNAcβ1-2	0.38	0.43
7 Triantennary Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-3GlcNAcβ1-4 Manβ1-4R Manα1-3 Galβ1-4GlcNAcβ1-2	0.59	0.39
8 Triantennary (core-fucosylated) Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4GlcNAcβ1-4 Manβ1-4R' Manα1-3 Galβ1-4GlcNAcβ1-2	0.45	0.50
9 Triantennary (fucosylated at N-acetyllactosamine unit) Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4GlcNAcβ1-4 Manβ1-4R Fucα1/3 Manα1-3 Galβ1-4GlcNAcβ1-2	9.3	2.0
10 Tetraantennary Galβ1-4GlcNAcβ1-6 Manα1-6 Galβ1-4GlcNAcβ1-2 Manβ1-4R Galβ1-4GlcNAcβ1-4 Manα1-3 Galβ1-4GlcNAcβ1-2	0.18	0.35
11 Tetraantennary (core-fucosylated) Galβ1-4GlcNAcβ1-6 Manα1-6 Galβ1-4GlcNAcβ1-2 Manβ1-4R' Galβ1-4GlcNAcβ1-4 Manα1-3 Galβ1-4GlcNAcβ1-2	0.23	0.44
12 Tetraantennary (fucosylated at N-acetyllactosamine unit) Galβ1-4GlcNAcβ1-6 Manα1-6 Galβ1-4GlcNAcβ1-2 Manβ1-4R Galβ1-4GlcNAcβ1-4 Fucα1/3 Manα1-3 Galβ1-4GlcNAcβ1-2	0.47	0.81
Glycolipid glycans		
13 Lactose Galβ1-4Glc-PA	100	12
14 asialo GM2-trisaccharide GalNAcβ1-4Galβ1-4Glc-PA	*	120
15 asialo GM1-tetrasaccharide Galβ1-3GalNAcβ1-4Galβ1-4Glc-PA	5.4	44
16 GM3-Neu5Ac-trisaccharide Neu5Acα2-3Galβ1-4Glc-PA	310	*
17 GM2-tetrasaccharide GalNAcβ1-4Galβ1-4Glc-PA Neu5Acα2/3	*	*
18 GM1-pentasaccharide Galβ1-3GalNAcβ1-4Galβ1-4Glc-PA Neu5Acα2/3	3.8	39
19 GD1a-hexasacchride Galβ1-3GalNAcβ1-4Galβ1-4Glc-PA Neu5Acα2/3 Neu5Acα2/3	4.2	84
20 GD1b-hexasaccharide Galβ1-3GalNAcβ1-4Galβ1-4Glc-PA Neu5Acα2-8Neu5Acα2/3	7.4	79
21 Globotriose Galα1-4Galβ1-4Glc-PA	*	200
22 Globo-N-tetraose GalNAcβ1-3Galα1-4Galβ1-4Glc-PA	9.7	59
23 Forssman pentasaccharide GalNAcα1-3GalNACβ1-3Galα1-4Galβ1-4Glc-PA	0.09	59
24 Lacto-N-neotetraose Galβ1-4GlcNAcβ1-3Galβ1-4Glc-PA	4.1	15
25 Lacto-N-tetraose Galβ1-3GlcNAcβ1-3Galβ1-4Glc-PA	4.8	12
26 Lacto-N-fucopentaose-I Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc-PA	3.8	9.0
27 Lacto-N-fucopentaose-II Galβ1-3GlcNAcβ1-3Galβ1-4Glc-PA Fucα1/4	3.9	34
28 Lacto-N-fucopentaose-III Galβ1-4GlcNAcβ1-3Galβ1-4Glc-PA Fucα1/3	9.4	38
29 Le^b-hexasaccharide Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc-PA Fucα1/4	2.6	27
30 A-hexasaccharide GalNAcα1-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc-PA Fucα1/2	0.26	7.7
31 A-heptasaccharide GalNAcα1-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc-PA Fucα1/2 Fucα1/4	3.2	26
32 2'-Fucosyllactose Fucα1-2Galβ1-4Glc-PA	*	150
33 A-tetrasaccharide GalNAcα1-3Galβ1-4Glc-PA Fucα1/2	6.4	47

R = GlcNAcβ1-4GlcNAc-PA
R' = GlcNAcβ1-4GlcNAc-PA
Fucα1/6
*, > 2000

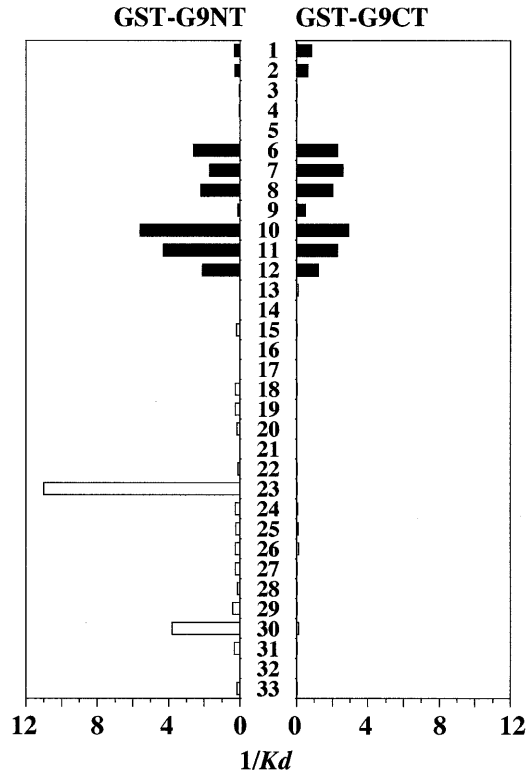

Fig. 5. Graphic representation of the sugar binding specificities of the N-and C-terminal CRDs of human galectin-9 shown in Table II. Glycoprotein N-glycans and glycolipid glycans are shown as closed and open bars, respectively.

Discussion

In the first report concerning the molecular characterization of a T cell–derived factor that has eosinophil-specific chemotactic activity, we showed that ecalectin, a variant form of human galectin-9, is a novel and potent ECA (Matsumoto *et al.*, 1998). In the following paper (Matsushita *et al.*, 2000), we concluded that ecalectin and human galectin-9 are one and the same protein based on the results of DNA sequencing of Jurkat cell–derived galectin-9 cDNA clones and computational analysis of EST sequence database resources. The latter study also revealed fundamental properties of the ECA activity of galectin-9. (1) Other members of the human galectin family show negligible (galectins-1 and -3) or low (galectin-8) ECA activity. (2) The N- and C-terminal CRDs of galectin-9 retained low but significant levels of ECA activity compared to full-length galectin-9. (3) The ECA activity of galectin-9 is inhibited by lactose and mutated CRDs lacking carbohydrate binding activity lose ECA activity, showing that the interaction between the two CRDs of galectin-9 and cell surface glycoconjugate(s) is an essential step in eosinophil chemoattraction. These results, especially the results involving N- and C-terminal CRDs, prompted us to produce and examine ECA activity of mutant galectin-9 molecules consisting of two N-terminal CRDs and two C-terminal CRDs.

The finding in this study that artificial tandem repeat–type galectins, galectins-9NN and -9CC, showed ECA activity indistinguishable from that of wild-type galectin-9 is consistent with previous results concerning galectins-9NT and -CT. Although galectin-8 has low ECA activity, the tandem repeat–type structure is not a sufficient condition for acquisition of the activity, because the two galectin-1 molecules linked by a linker peptide of galectin-8 were completely inactive in the ECA activity assay (unpublished data). These observations indicate that the ECA activity depends on the specific sugar-binding character of an individual CRD and that the two CRDs of galectin-9 interact with the same or a closely related sugar chain when it acts as an ECA.

We performed detailed analysis of the sugar-binding specificity of galectins-9NT and -9CT using a panel of 33 oligosaccharides and frontal affinity chromatography. Galectins-9NT and -9CT exhibited comparable binding affinity for most glycoprotein N-glycans, the highest affinity being for N-linked tetraantennary complex-type glycans with N-acetyllactosamine units. Fucosylation and sialylation reduced the affinity of the oligosaccharides for both CRDs. On the other hand, the binding affinity for nearly half of the glycolipid glycans tested varied by about one order of magnitude between the two CRDs. In addition, a tetraantennary glycan strongly inhibited the ECA activity of galectin-9 but not that of eotaxin. Collectively, these results suggest that galectin-9 binds to eosinophil surface glycoprotein(s) via tri- and/or tetraantennary N-linked glycans with N-acetyllactosamine units. This interaction may result in dimerization/multimerization of the cell surface glycoproteins/receptors and thereby initiate intracellular signaling pathways linked to chemotactic responses.

It is noteworthy that galectin-9NT but not galectin-9CT shows exceptionally high affinity for Forssman pentasaccharide among the glycolipid glycans examined. It is postulated that Forssman antigen is involved in the pathogenesis of autoimmune diseases such as Graves' disease and Hashimoto's thyroiditis (Ariga *et al.*, 1991). It is possible that recognition of Forssman antigen by the N-terminal CRD of galectin-9 is an important step for some unknown immune-modulating activity of galectin-9.

Wada and Kanwar (1997) reported the presence of an isoform (intestine isoform) of mouse galectin-9 with a 31-amino-acid insertion in the linker peptide region. When analyzing galectin-9 cDNA clones expressed in Jurkat cells, we found three isoforms of human galectin-9 (galectins-9S, -9M, and -9L): galectin-9M corresponds to authentic galectin-9, and galectins-9L and -9S have a 32-amino-acid insertion and a 12-amino-acid deletion, respectively, in the linker peptide. The expression of these isoforms was detected at the protein level in Jurkat cells (data not shown). Although the linker peptide of galectin-9L is about four times larger than that of galectin-9S, the three isoforms showed comparable ECA activity. The amino acid insertions of galectins-9L and -9M, compared to galectin-9S, are characterized by high proline contents, that is, 13 proline residues out of 44 amino acid residues (galectin-9L) and 5 out of 12 (galectin-9M). Because glycine and proline residues are common within the beta-turn, it is not likely that the linker peptides can form an extended structure. The Chou-Fasman prediction algorithm supports this premise. Hence, in spite of the large difference in linker peptide size, there may be little difference among the isoforms in configuration between N- and C-terminal CRDs. We cannot, however, exclude the possibility that the three isoforms play different roles in yet unknown function(s) of galectin-9, that is, other than ECA activity.

Materials and methods

Construction of expression vectors for galectins-9, -9NN, and -9CC

The primers used to amplify the cDNAs for galectin-9 isoforms and galectin-9 mutants are summarized in Table III. The pTrcHisB prokaryotic expression vector (Invitrogen) was used throughout the present study except for the production of the N- and C- terminal CRDs of galectin-9. Expression vectors for wild-type galectin-9 (galectins-9S, -9M, and -9L) were constructed by inserting cDNAs amplified with G9F1 + G9R1 into the *Xho*I/*Eco*RI sites of pTrcHisB. To construct an expression vector for galectin-9NN, cDNAs corresponding to the N-terminal CRD plus linker peptide (amplified with G9F1 + G9NNR2) and the N-terminal CRD carrying an extra stop codon at the 3′ end (amplified with G9NNF1 + G9NNR1) were inserted into the *Xho*I/*Kpn*I and *Kpn*I/*Eco*RI sites, respectively, of the same vector. To construct an expression vector for galectin-9CC, cDNAs corresponding to the C-terminal CRD without the stop codon (amplified with G9CCF2 + G9CCR1), and the linker peptide plus the C-terminal CRD (amplified with G9CCF1 + G9R1) were inserted into the *Xho*I/*Kpn*I and *Kpn*I/*Eco*RI sites, respectively, of the same vector.

Expression vectors for GST-fusion proteins of the N-terminal and C-terminal CRDs of galectin-9 (GST-galectin-9NT and GST-galectin-9CT) were prepared as described previously (Matsushita *et al.*, 2000).

Expression and purification of recombinant proteins

E. coli BL21 cells carrying each expression plasmid were grown in 2× YT medium supplemented with 2% (w/v) glucose and 100 μg/ml ampicillin to an optical density of 0.7 at 600 nm. The expression of fusion proteins was induced by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside, and the cultures were continued for 2 h at 37°C (galectins-9S, -9M, -9NN and -9CC) or for 3 h at 30°C (galectin-9L). The cell pellet obtained from 500-ml culture was suspended in 90 ml of 10 mM Tris–HCl (pH 7.5) containing 0.5 M NaCl, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride, and then sonicated for 10 min. The sonicate was supplemented with 10 ml of 10% (w/v) Triton X-100 and then stirred for 30 min at 4°C, followed by centrifugation at 15,000 × *g* for 30 min. The resulting supernatant was subjected to lactose-agarose (Seikagaku, Tokyo) affinity chromatography. Protein concentrations were determined with BCA protein assay reagent (Pierce) and bovine serum albumin as a standard.

Table III. Nucleotide sequences of oligonucleotide primers used for construction of expression vectors

Primer	Nucleotide sequence (5′-3′)
G9F1	CGTCCTCTCGAGAATGGCCTTCAGCGGTTCCCAG
G9R1	CGACCGGAATTCCTATGTCTGCACATGGGTCAG
G9NNF1	CGTCCTGGTACCATGGCCTTCAGCGGTTCCCAG
G9NNR1	CGACCGGAATTCCTACTGGAAGCTGATGTAGGACAG
G9NNR2	CGACCGGGTACCCTGTCCAGGGGCGCTCTGCAC
G9CCF1	CGTCCGGGTACCCCTCCCGGCGTGTGGCCTGCC
G9CCF2	CGTCCTCTCGAGAATGTTCTCTACTCCCGCCATC
G9CCR1	CGACCGGGTACCTGTCTGCACATGGGTCAGCTG

In vitro chemotaxis

ECA activity was evaluated *in vitro* as described previously (Matsumoto *et al.*, 1998; Hirashima *et al.*, 1992). Briefly, CD16-negative-eosinophils were enriched by applying peripheral blood leukocytes from healthy volunteers to a discontinuous density gradient of Percoll (Amersham Pharmacia Biotech), followed by immunomagnetic treatment of the cells with anti-CD16 immunoglobulin (Dako S., Glostrup, Denmark). The purity and viability of the purified eosinophils were >97% and >95%, respectively. ECA activity was evaluated using a 48-well chamber (Neuro Probe) containing a polyvinylpyrrolidone-free membrane of 5 μm pore size. Human eosinophils (0.5–1 × 10^6/ml) and various concentrations of a test chemoattractant were placed in the top and bottom chambers, respectively. Each assay was performed in triplicate. After 1–2-h incubation at 37°C under a humidified atmosphere of 5% CO_2, the membrane separating the two chambers was removed and placed in Diff-Quick stain (Baxter Healthcare). Stained eosinophils were counted under a microscope. Human eotaxin-1 (Seikagaku) was used as a control.

Hemagglutination assays

Hemagglutination activity was assessed by the method of Nowak *et al.* (1976). Briefly, assay samples were prepared by serial twofold dilution of recombinant proteins in a 96-well microtiter plate. After the addition of bovine serum albumin and glutaraldehyde-fixed trypsin-treated rabbit erythrocytes to final concentrations of 0.25% (w/v) and 1% (v/v), respectively, the reaction mixture was incubated for 1 h at room temperature. The minimum concentration required for hemagglutination was visually determined.

Frontal affinity chromatography

The interactions between the N- and C-terminal CRDs of galectin-9 and synthetic oligosaccharides were studied by frontal affinity chromatography. The recombinant proteins dissolved in 0.1 M $NaHCO_3$ (pH 8.3) containing 0.25 M NaCl and 0.1 M lactose were coupled to HiTrap NHS-activated column following the manufacturer's instructions. The galectin-immobilized agarose beads were taken out from the cartridge and then packed into a stainless steel column (4 × 10 mm). Determination of the elution volume of the analyte (PA-oligosaccharides) and the calculation of K_d value were carried out as described previously (Kasai and Oda, 1986; Hirabayashi *et al.*, 2000; Arata *et al.*, 2001).

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Abbreviations

CRD, carbohydrate recognition domain; ECA, eosinophil chemoattractant; GST, glutathione S-transferase; PA, pyridylamino.

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