

COMMUNICATION

Galectin-8 and galectin-9 are novel substrates for thrombin

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Galectin-8 and galectin-9, which each consist of two carbohydrate recognition domains (CRDs) joined by a linker peptide, belong to the tandem-repeat-type subclass of the galectin family. Alternative splicing leads to the formation of at least two and three distinct splice variants (isoforms) of galectin-8 and galectin-9, respectively, with tandem-repeat-type structures. The isoforms share identical CRDs and differ only in the linker region. In a search for differences in biological activity among the isoforms, we found that their isoforms with the longest linker peptide, that is, galectin-8L and galectin-9L (G8L and G9L), are highly susceptible to thrombin cleavage, whereas the predominant isoforms, galectin-8M and galectin-9M (G8M and G9M), and other members of human galectin family so far examined were resistant to thrombin. Amino acid sequence analysis of proteolytic fragments and site-directed mutagenesis showed that the thrombin cleavage sites (-IAPRT- and -PRPRG- for G8L and G9L, respectively) resided within the linker peptides. Although intact G8L stimulated neutrophil adhesion to substrate more efficiently than G8M, the activity of G8L but not that of G8M decreased on thrombin digestion. Similarly, thrombin treatment almost completely abolished eosinophil chemoattractant (ECA) activity of G9L. These observations suggest that G8L and G9L play unique roles in relation to coagulation and inflammation.

Key words: galectin/linker peptide/proteolysis/thrombin

Introduction

Galectins comprise a family of animal lectins that preferentially bind β -galactosides through a carbohydrate recognition domain (CRD) consisting of ~130–140 amino acids. Several lines of evidence indicate that most, if not all, extracellular activities of galectins result from cross-linking of their glycoconjugate receptors. Galectins can be classified into three sub-

types, that is, proto, chimera, and tandem-repeat types. Although the proto-type and chimera-type galectins each have a single CRD, they usually form a homodimer/multimer, making it possible for them to act as cross-linkers for glycoconjugates. On the other hand, the tandem-repeat-type galectins (galectin-4, -8, -9, and -12 of the human galectin family) have two homologous but distinct CRDs joined by a linker peptide. N-terminal and C-terminal CRDs of the tandem-repeat-type galectins generally have different sugar-binding specificities (Hirabayashi *et al.*, 2002). This intrinsic heterobifunctional cross-linking ability makes them unique among the members of the galectin family. The presence of a linker peptide confers not only cross-linking ability without the formation of a dimer/multimer but also protease susceptibility to the tandem-repeat-type galectins (Nishi *et al.*, 2005). In addition, the generation of variants through alternative splicing of mRNA precursors is known only for tandem-repeat-type galectins, and for galectin-3 (Bidon, Brichory, Bourguet *et al.*, 2001; Hotta *et al.*, 2001; Gorski *et al.*, 2002; Sato *et al.*, 2002; Wooters *et al.*, 2005). Alternative splicing leads to the formation of at least two and three distinct splice variants (isoforms) of galectin-8 and galectin-9, respectively, with tandem-repeat-type structures. In the case of galectin-8, alternative splicing also generates mRNAs coding for isoforms with proto-type structures (Bidon, Brichory, Hanash *et al.*, 2001). The isoforms with tandem-repeat-type structures share identical CRDs and differ only in the linker region (Figure 1).

In a previous study, we examined the differences among three isoforms of galectin-9 as to eosinophil chemoattractant (ECA) activity (Sato *et al.*, 2002). Although the isoform of galectin-9 with the longest linker peptide, galectin-9L (G9L), exhibited limited solubility, the three isoforms showed comparable ECA activity over the concentration range tested, that is, ECA activity does not depend on a specific structure of the linker peptide. In addition, an artificial mutant of galectin-9 lacking almost the entire linker peptide retained the biological activities of the wild-type protein so far examined (Nishi *et al.*, 2005). On the basis of these observations, it appears reasonable to assume that the isoforms are functionally interchangeable *in vivo*. However, we recently found a significant difference between two isoforms of galectin-8 (G8M and G8L) in neutrophil adhesion-inducing activity. Moreover, G8L and G9L, but not other isoforms, were found to be highly susceptible to thrombin cleavage. This report describes the presence of novel thrombin recognition sites in the insertion sequences (linker regions) of G8L and G9L and the effects of thrombin digestion on the biological activities of the isoforms.

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Results and discussion

Presence of a thrombin recognition site in the linker peptide of human G8L

To compare the biological activities of galectin-8 isoforms (Figure 1), G8M and G8L were expressed as glutathione S-transferase (GST)-fusion proteins and then purified by lactose-affinity chromatography. The purified fusion proteins were treated with thrombin to prepare GST-free proteins. GST-G8M was separated into GST and G8M moieties as expected (Figure 2A). On the contrary, two distinct bands (bands A and B, Figure 2A) in addition to a band corresponding to GST were detectable after thrombin digestion of GST-G8L. The N-terminal amino acid sequences of the two fragments were determined to be GSMMLSLN and TVYTKSKD for band A and band B, respectively. The former sequence coincides with the expected N-terminal sequence of G8L after cleavage of GST-G8L with thrombin, whereas the latter sequence resides in the insertion sequence (a 42-amino-acid-long sequence specific to the G8L isoform, Figure 1) of G8L. There are two possible thrombin recognition sites in the insertion sequence, that is, -PSNRG- (possible cleavage

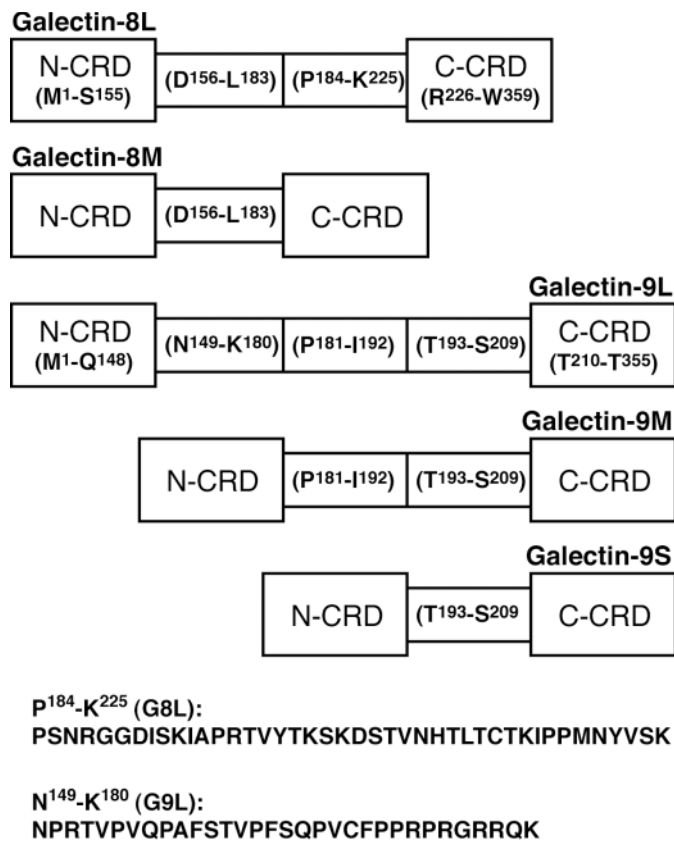


Fig. 1. Schematic representation of human galectin-8 and galectin-9 isoforms. Superscript numbers indicate amino acid positions in G8L and G9L. In this study, the N- and C-terminal CRDs (N-CRD and C-CRD) and a linker peptide region were tentatively assigned based on intron-exon structures. However, X-ray crystallographic studies and phylogenetic analyses (Houzelstein *et al.*, 2004) predict that Ile17-Ser155 (Val15-Gln148) and Leu227-Trp359 (Met225-Thr355) form stably folded cores of N-CRD and C-CRD of G8L (G9L), respectively.

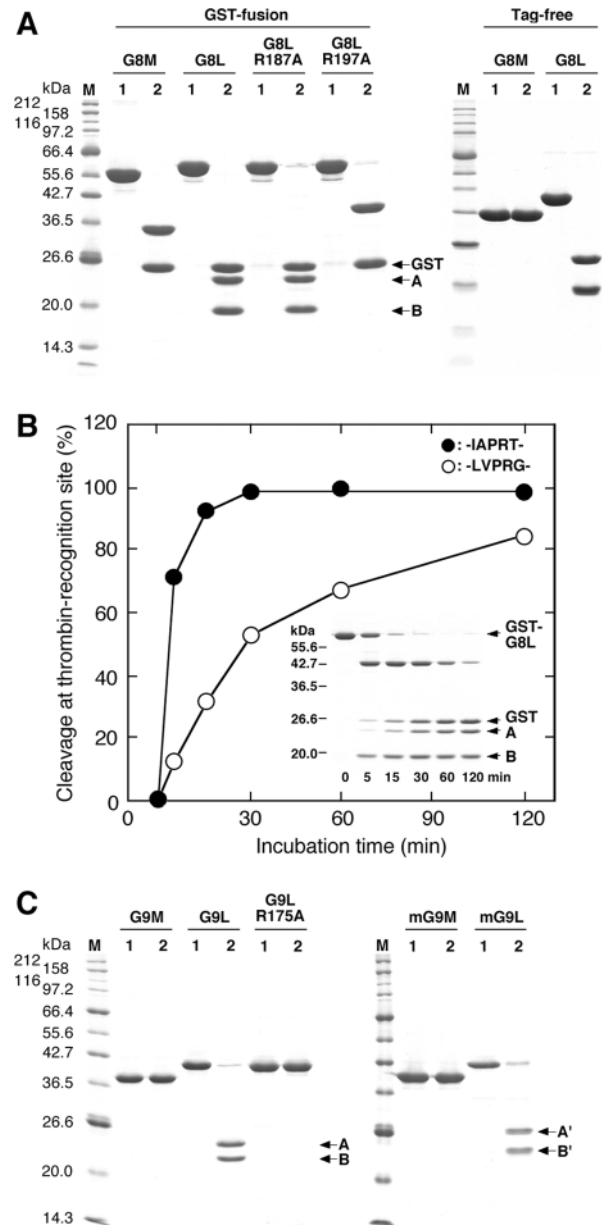


Fig. 2. Effects of thrombin treatment on the wild-type and mutant forms of galectin-8 and galectin-9 expressed in *Escherichia coli*. (A) Purified recombinant proteins were dissolved in 0.05 M Tris-HCl (pH 8.0), 0.15 M NaCl, and 1 mM CaCl₂ and then treated with thrombin at an enzyme to substrate weight ratio of 1:300 for 4 h at 37°C. (B) In a time-course experiment, purified GST-G8L was dissolved in 0.05 M Tris-HCl (pH 8.0), 0.15 M NaCl, and 1 mM CaCl₂ and then treated with thrombin at an enzyme to substrate weight ratio of 1:300 at 37°C. Aliquots were withdrawn from the reaction mixture at different times and then subjected to SDS-PAGE (inset). The integrated intensities of GST (a protein band corresponding to the released GST moiety) and the band B material (C-terminal fragment of G8L) were obtained by densitometric scanning. The integrated intensities of GST and the band B material after 4-h incubation (data not shown) were taken as 100%. (C) In the case of human and mouse galectin-9, recombinant proteins were eluted from lactose-agarose gel with 0.05 M Tris-HCl (pH 8.0), 0.15 M NaCl, 1 mM CaCl₂, and 0.2 M lactose. The eluted proteins were subjected to thrombin digestion (4 h at 37°C; enzyme to substrate weight ratio, 1:300) without removal of the lactose. Samples (2 µg/lane) were electrophoretically separated in a SDS/12.5% polyacrylamide gel under reducing conditions and then stained with Coomassie brilliant blue R-250. M, molecular weight marker proteins; lane 1, untreated recombinant proteins; lane 2, recombinant proteins treated with thrombin.

site: R-G bond) and -IAPRT- (possible cleavage site: R-T bond). The N-terminal amino acid sequence of the band B material suggests that thrombin recognizes the latter site (-IAPRT-). To exclude the possibility that G8L was cleaved by thrombin at both sites, we generated two site-directed mutant fusion proteins, GST-G8L·R187A and GST-G8L·R197A, and examined their susceptibility to thrombin. Treatment of GST-G8L·R187A with thrombin resulted in the generation of three distinct bands as in the case of the wild-type fusion protein (GST-G8L) (Figure 2A). On the contrary, GST-G8L·R197A was separated into the GST moiety and a 40-kDa band material, which was identified as GST-free G8L·R197A on N-terminal amino acid sequence analysis and mass spectrometry (data not shown). These results indicate that G8L was digested by thrombin at the C-terminal side of Arg197. A time-course experiment demonstrated that the appearance of band B was much faster than that of the GST moiety, indicating that the thrombin recognition site of G8L is more sensitive than the site coded by the pGEX-4T-2 vector (-LVPRG-) to thrombin digestion (Figure 2B). The time-course experiment was carried out with different enzyme/substrate ratios. Thrombin was capable of cleaving G8L at a ratio as low as 1/1000 with a reduced rate (~25% of that shown in Figure 2B).

Presence of a thrombin recognition site in the linker peptides of human and mouse G9L

The above results led us to investigate whether other tandem-repeat-type galectins have thrombin recognition site(s) in their linker peptides. Human and mouse galectin-9 (G9M, G9L, mG9M, and mG9L) were produced using a prokaryotic expression vector, pTrcHisB, because the use of the pGEX-4T-2 vector resulted in low yields of G9L and mG9L. The recombinant proteins expressed in the pTrcHisB expression system have a tag sequence of ~4 kDa at the N-terminus, which cannot be removed on thrombin digestion. G9L and mG9L were separated into two fragments after treatment with thrombin, whereas G9M and mG9M were almost completely resistant to thrombin digestion under the conditions used (Figure 2C). There are two and three possible thrombin recognition sites in the insertion sequences of G9L and mG9L, respectively, that is, -PRPRGRRQ- (possible cleavage sites: R-G and R-R bonds) of G9L and -QFPRTP-KGRK- (possible cleavage sites: R-T, K-G and R-K bonds) of mG9L. The N-terminal amino acid sequence of the band B material (GRRQKPPG) and the thrombin resistance of the G9L·R175A mutant (Figure 2C) indicate that G9L was digested by thrombin at the C-terminal side of Arg175. A small amount of G9L·R175A was digested at the second site (R-R bond) when a large amount of thrombin (enzyme : substrate ratio, 1:10) was used, indicating that the bond exhibits very weak sensitivity to thrombin (data not shown). The N-terminal amino acid sequence of the band B' material, TPKGRKQT, suggests that mG9L was digested at the C-terminal side of Arg170, although we did not confirm this on site-directed mutagenesis.

Other members of human galectin family so far examined (galectin-1, -2, -3, -4, -7, -10, and -13) did not show evidence for thrombin susceptibility, although they contain at least one possible cleavage site for thrombin in their CRDs

(data not shown). In the case of human galectin-4, the presence of isoforms (splice variant forms) has not been reported. Recently, Wooters and others (2005) identified two galectin-4 isoforms that differed in the length of their linker region in porcine small intestine. Contrary to human galectin-8 and galectin-9, their linker peptides do not contain a possible cleavage site for thrombin.

Effects of thrombin digestion on the biological activities of G8L and G9L

The tag-free recombinant proteins of G8M and G8L were expressed by using the pET-11a vector to assess the effects of thrombin digestion on their biological activities. The tag-free proteins were efficiently expressed in *Escherichia coli* and purified to apparent homogeneity by affinity chromatography using lactose-agarose (Figure 2A). As in the case of the GST-fusion proteins, tag-free G8L but not G8M was sensitive to thrombin. The GST-fusion proteins (GST-G8M and GST-G8L) and tag-free proteins were examined as to their ability to induce the adhesion of peripheral blood neutrophils to a plastic surface. The addition of GST-G8M to the culture medium resulted in rapid and almost complete adhesion of neutrophils (Figure 3), confirming our previous results (Nishi *et al.*, 2003). The dose-response curves for GST-G8M and GST-G8L were almost indistinguishable. Unexpectedly, tag-free G8M exhibited low activity as to neutrophil adhesion (about 1/10 that of GST-G8M) (Figure 3). A decrease in the specific activity was also observed when the GST-tag was removed from GST-G8M by thrombin digestion (data not shown). A pos-

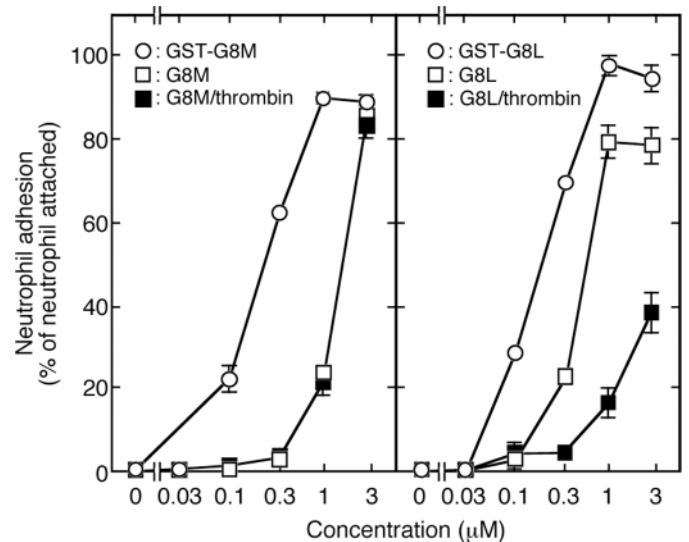


Fig. 3. Comparison of galectin-8 preparations as to the promotion of neutrophil adhesion. Purified recombinant proteins were dissolved in 0.05 M Tris-HCl (pH 8.0), 0.15 M NaCl, and 1 mM CaCl₂ and then incubated in the absence or presence of thrombin (enzyme to substrate weight ratio, 1:300) for 4 h at 37°C. The recombinant proteins were dialyzed against phosphate-buffered saline before use. Purified human neutrophils suspended in serum-containing medium were added to 24-well tissue culture plates. After the addition of various concentrations of control recombinant proteins or recombinant proteins treated with thrombin (G8M/thrombin and G8L/thrombin), the cells were allowed to adhere for 60 min at 37°C. Cell adhesion was determined as described under *Materials and methods*. Data represent means ± SD of triplicate measurements.

sible mechanism for the effect of GST-tag could be that the GST moiety facilitates dimer formation by GST-G8M, resulting in higher avidity for oligosaccharide chains. Tag-free G8L also exhibited low activity as to neutrophil adhesion (about 1/3 that of GST-G8L). However, the activity of G8L was 2–3 times higher than that of G8M. Thrombin treatment of tag-free G8L significantly suppressed its adhesion-inducing activity (~80% suppression) (Figure 3). The C-terminal fragment of G8L may contribute to the residual activity of thrombin-treated G8L, because our previous study demonstrated that a mutant form of G8M with an inactivated N-terminal CRD possessed reduced but apparent adhesion-inducing activity (Nishi *et al.*, 2003). The activity of tag-free G8M was not affected by thrombin treatment. Although there has been no other report showing differences in biological activity among naturally occurring isoforms of tandem-repeat-type galectins, these results suggest that G8L may play a physiological role distinct from that of G8M.

The effect of thrombin digestion on G9M and G9L was assessed by measuring ECA activity. G9M and G9L exhibited comparable ECA activity in accordance with our previous results (Sato *et al.*, 2002). The ECA activity of G9M was hardly affected by thrombin treatment, whereas that of G9L decreased to <10% of the original value on the treatment (Table I). G9M is the predominant isoform in most cell and tissue types so far examined. However, the content of G9L exceeds that of G9M in Jurkat T cells depending on the culture conditions (unpublished results). Inactivation of G9L may be of physiological significance when G9L becomes predominant under specific conditions *in vivo*.

In this study, we accidentally found the presence of a thrombin-sensitive site in the insertion sequence of G8L. Similar sites were also found in G9L and mG9L. These sites were highly susceptible to thrombin digestion in the native proteins, suggesting possible physiological relevance of the structure. Most galectins, including human galectin-8 and galectin-9, have characteristics typical of cytoplasmic proteins and are detected primarily intracellularly in most cells and tissues studied. However, it is also widely accepted that galectins are secreted via nonclassical pathway and localize to the surface of a variety of cells. We demonstrated that Jurkat cells produce three isoforms of galectin-9 (G9S/M/L), and they are detectable on

surface of the cells, although we could not distinguish galectin-9 isoforms by FACS analysis (Chabot *et al.*, 2002). The data suggest that G9L (and probably G8L) localized to the cell surface can serve as a substrate for thrombin.

Although G8L and G9L (mG9L) are minor isoforms in most cells and tissues, inactivation of the L-type isoform may become important when the content of the isoform increases relative to those of other isoforms under specific physiological conditions. In addition, when the L-type isoforms exhibit biological activities qualitatively and/or quantitatively distinct from those of other isoforms, degradation of even a small amount of L-type isoform may affect cellular functions.

Thrombin is known as a key enzyme in the blood coagulation system (Huntington, 2005). Thrombin acts as a procoagulant when it converts fibrinogen into an insoluble fibrin clot. Digestion of fibrinogen by thrombin is not simple degradation but reveals a novel function of the protein. In contrast, thrombin acts as an anticoagulant when it activates protein C upon binding to thrombomodulin. In this case, protein C, an inactive proenzyme, is converted to an active serine protease through thrombin digestion. In addition to these soluble substrates, thrombin stimulates cells involved in inflammatory processes and tissue repair. The effects of thrombin on cells are mainly mediated by membrane-bound receptors, that is, protease-activated receptors (PAR-1, PAR-3, and PAR-4) (Cirino *et al.*, 2000; Strukova, 2001). Thrombin cleaves a specific site in the extracellular domains of PARs, and the new N-terminal site (the so-called tethered ligand) interacts with the cleaved receptor and thereby activates it. In this study, we demonstrated only inactivation (loss of function) of G8L and G9L after thrombin digestion. However, a common feature of the thrombin action described above, that is, the unveiling of a hidden function of its substrates, indicates the possibility that the fragments of G8L and G9L generated on thrombin digestion possess unknown biological function(s).

Materials and methods

Construction of expression vectors

The following forward (F) and reverse (R) primers were used to amplify the cDNAs for the wild-type and site-directed mutants of galectin-8 and galectin-9:

- G8-F1 (5'-CGTCCTAGATCTATGATGTTGTCCT-TAAACAAC-3'),
- G8-R1 (5'-CGACCGGTCGACCTACCAGCTCCT-TACTTCCAG-3'),
- G8-F2 (5'-CGTCCTCATATGATGTTGTCCTTAAACAACCTA-3'),
- G8-R2 (5'-CGACCGAGATCTCTACCAGCTCCT-TACTTCCAG-3'),
- mG9-F1 (5'-CGTCCTGGATCCATGGCTCTCTTCA GTGCCAG-3'),
- mG9-R1 (5'-CGACCGGAATTCCTATGTCTGCAG TGGGTCAG-3'),
- G8LR187A-F1 (5'-CTTCTAGTAATGCAGGAG-GAGACATT-3'),
- G8LR187A-R1 (5'-AATGTCTCCTCCTGCATTAC-TAGGAAG-3'),

Table I. Effects of thrombin digestion on ECA activity of G9M and G9L

	ECA activity (number of migrated cells)
Phosphate-buffered saline	3.3 ± 1.5
Eotaxin (100 ng/mL)	71.6 ± 16.5
G9M (0.1 μM)	58.3 ± 11.4
G9M/thrombin (0.1 μM)	65.7 ± 6.2
G9M (0.3 μM)	107.7 ± 18.0
G9M/thrombin (0.3 μM)	97.3 ± 11.7
G9L (0.1 μM)	66.0 ± 11.6
G9L/thrombin (0.1 μM)	5.5 ± 1.2

Data represent means of three independent experiments. Recombinant proteins were treated with thrombin at an enzyme to substrate weight ratio of 1:300 for 4 h at 37°C. The recombinant proteins were dialyzed against phosphate-buffered saline before use.

- G8LR197A-F1 (5'-AAAATCGCACCCCGCAACTGTC-TACACC-3'),
- G8LR197A-R1 (5'-GGTGTAGACAGTTGCGGGTGC-GATTTT-3'),
- G9LR175A-F1 (5'-CCACCCAGGCCCGCGGGGCG-CAGACAA-3'),
- G9LR175A-R1 (5'-TTGTCTGCGCCCCGCGGGC-CTGGGTGG-3').

Expression vectors for GST-fusion proteins were prepared as follows: human galectin-8 cDNAs encoding two isoforms (G8M and G8L) were amplified from first-strand cDNAs prepared from the poly(A)+RNA fraction of a human epidermoid carcinoma cell line (A431) using forward and reverse primers tagged with extra 5' BglII (G8-F1) and SalI (G8-R1) sequences, respectively. The amplified cDNAs were digested with BglII and SalI, and the resulting cDNA fragments were inserted into the BamHI-XhoI site of pGEX-4T-2 (Amersham Biosciences, Piscataway, NJ). Alternatively, the galectin-8 cDNAs were amplified using G8-F2 + G8-R2 to construct expression vectors for tag-free proteins. The amplified cDNAs were digested with NdeI and BglII and then inserted into the NdeI-BamHI site of pET-11a (Stratagene, La Jolla, CA). Expression vectors for human galectin-9 (G9M and G9L) were prepared using the pTrcHisB vector (Invitrogen, Carlsbad, CA) as described previously (Sato *et al.*, 2002). Expression vectors for mouse galectin-9 (mG9M and mG9L) were prepared as follows: mouse galectin-9 cDNAs were amplified from first-strand cDNAs prepared from the poly(A)+RNA fraction of mouse intestine using mG9-F1 + mG9-R1. The amplified cDNAs were digested with BamHI and EcoRI and then inserted into the BglII-EcoRI site of pTrcHisB. The DNA sequences of all the expression vectors were confirmed by automated sequencing.

Site-directed mutagenesis

Site-directed mutagenesis of G8L residue Arg187 (and Arg197) to Ala was carried out as follows. In two separate reactions, the cDNA fragments upstream and downstream of the point mutation were amplified using G8-F1 + G8LR187A-R1 (G8LR197A-R1) and G8LR187A-F1 (G8LR197A-F1) + G8-R1. The amplified fragments were then mixed and subjected to a second round of polymerase chain reaction (PCR) using G8-F1 + G8-R1 to generate full-length cDNAs. The resultant cDNAs each carrying a point-mutation were digested with BglII and SalI and then inserted into the BamHI-XhoI site of pGEX-4T-2. Site-directed mutagenesis of G9L residue Arg175 to Ala was carried out by the same method using G9LR175A-F1 and G9LR175A-R1. The resultant mutations were confirmed by DNA sequencing.

Expression and purification of recombinant proteins

Expression of GST-fusion proteins (pGEX vector) and His-tag proteins (pTrcHis vector) in *E. coli* BL21 cells was carried out as described previously (Matsushita *et al.*, 2000; Sato *et al.*, 2002). Tag-free proteins (pET vector) were expressed by essentially the same method as that for GST-fusion proteins except that *E. coli* BL21(DE3) cells were used. Recombinant proteins were purified by affinity chromatography on a lactose-agarose column (Seikagaku

Corp., Tokyo, Japan). The purified proteins were dialyzed against 0.05 M Tris-HCl (pH 8.0), 0.15 M NaCl unless otherwise specified. Protein concentrations were determined using BCA protein assay reagent (Pierce, Rockford, IL) and bovine serum albumin as a standard.

Thrombin treatment

Recombinant proteins were first incubated at 37°C for 5 min in digestion buffer. After the addition of thrombin, the reaction mixture was further incubated at 37°C for the indicated times. The total reaction mixture volume, 300 μ L, contained 80 μ g of protein sample (G8M or G8L), the enzyme to substrate weight ratio being 1:300. Aliquots were withdrawn from the reaction mixture at different times for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The following digestion buffer was used: 0.05 M Tris-HCl (pH 8.0), 0.15 M NaCl, and 1 mM CaCl₂. In the case of human and mouse galectin-9 (G9M, G9L, mG9M, and mG9L), recombinant proteins were eluted from lactose-agarose gel with 0.05 M Tris-HCl (pH 8.0), 0.15 M NaCl, 1 mM CaCl₂, and 0.2 M lactose. The eluted proteins were subjected to protease digestion without removal of the lactose.

Chemotaxis

ECA activity was evaluated *in vitro* as described previously (Matsumoto *et al.*, 1998). Briefly, eosinophils were enriched by applying peripheral blood leukocytes to a discontinuous density gradient of Percoll (Amersham Biosciences). ECA activity was evaluated using a 48-well chamber (Neuro Probe Inc., Gaithersburg, MD). Human eosinophils ($0.5\text{--}1 \times 10^6/\text{mL}$) and various concentrations of a test sample were placed in the top and bottom chambers, respectively. Each assay was performed in triplicate. After 1- to 2-h incubation at 37°C under a humidified atmosphere of 5% CO₂, the membrane separating the two chambers was removed and placed in Diff-Quick stain (Baxter Healthcare Corp., Deerfield, IL). Stained eosinophils were counted under a microscope. Human eotaxin-1 (Seikagaku Corp.) was used as a control.

Neutrophil adhesion

The neutrophil adhesion assay was carried out as described previously (Nishi *et al.*, 2003). Isolated cells were added to 24-well tissue culture plates (2.5×10^5 cells in 0.45 mL of medium/well) in triplicate. After the addition of 50 μ L of the assay sample, the cells were allowed to adhere for 60 min at 37°C. At the end of the incubation period, loosely attached cells were removed by pipetting. The attached cells were recovered by treatment with trypsin/EDTA, and then sonicated. The DNA content of the sonicate was determined.

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Conflict of interest statement

None declared.

Abbreviations

CRD, carbohydrate recognition domain; ECA, eosinophil chemoattractant; FACS, fluorescence activated cell sorting; G8L, gelatin-8L; G8M, gelatin-8M; G9L, gelatin-9L; G9M, gelatin-9M; GST, glutathione S-transferase.

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