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Development of highly stable galectins: Truncation of the linker peptide confers protease-resistance on tandem-repeat type galectins

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Abstract Galectin-9 and galectin-8, members of β-galactoside-binding animal lectin family, are promising agents for the treatment of immune-related and neoplastic diseases. The proteins consist of two carbohydrate recognition domains joined by a linker peptide, which is highly susceptible to proteolysis. To increase protease resistance, we prepared mutant proteins by serial truncation of the linker peptide. As a result, mutant forms lacking the entire linker peptide were found to be highly stable against proteolysis and retained their biological activities. These mutant proteins might be useful tools for analyzing the biological functions and evaluating the therapeutic potential of galectin-9 and galectin-8.

Keywords: Galectin; Linker peptide; Proteolysis; Protein engineering; Therapeutic agent

1. Introduction

Galectins constitute a family of soluble animal lectins that are defined based on their affinity for β-galactosides and their conserved sequence elements. To date, 10 members of the human galectin family are known. The members can be classified into three subtypes according to their structures. The prototype (galectin-1, -2, -7, -10 and -13) and chimera-type (galectin-3) galectins have a single carbohydrate recognition domain (CRD), and they usually form a non-covalent homodimer resulting in homobifunctional sugar-binding activity. While tandem-repeat-type galectins (galectin-4, -8, -9, and -12) have two CRDs, which generally show different sugar-binding specificities, joined by a linker peptide. This heterobifunctional property makes them capable of crosslinking a wide variety and combinations of glycoconjugates. Tandem-repeat-type galectins, however, are more susceptible to proteolysis than other galectins due to the presence of the relatively long linker peptide.

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Abbreviations: CRD, carbohydrate recognition domain; GST, glutathione *S*-transferase; MMP-3, matrix metalloproteinase-3; ECA, eosinophil chemoattractant

Human galectin-9 was first identified as a novel tumor antigen of unknown function in patients with Hodgkin's disease [1]. Recent studies suggested that galectin-9 is a novel type of modulator of immune functions: galectin-9 has been shown to induce chemotaxis of eosinophils [2] and apoptosis of activated but not resting T lymphocytes [3,4]. Up-regulation of galectin-9 gene expression by interferon-γ [5] and synthetic double-stranded RNA (poly IC) [6] in human endothelial cells is consistent with this idea. In addition, galectin-9 induces the apoptosis of a wide variety of tumor cells much more efficiently than other family members [3]. Although these findings indicate that galectin-9 is a promising agent for the treatment of immune-related and neoplastic diseases, the protease susceptibility of the protein makes it difficult to efficiently carry out in vivo experiments with recombinant proteins. Here, we report development of protease resistant galectin-9 and galectin-8, a modulator of neutrophil function [7], by modification of their linker peptides.

2. Materials and methods

2.1. Construction of expression vectors

The following forward (F) and reverse (R) primers (Fig. 1) were used to amplify cDNAs for the wild-type and mutant galectins from plasmids containing the respective cDNA [8]:
G9NF1e (5′-CGTCCTCATATGGCCTTCAGCGGTTCCCAG-3′),
G9NF1g (5′-CGTCCTGAATTCCCATGGCCTTCAGCGGTTCCCAG-3′),
G9NR1e (5′-CGACCGCATATGCTGGAAGCTGATGTAGGACAG-3′),
G9NR1g (5′-CGACCGCTCGAGCTACTGGAAGCTGATGTAGGACAG-3′),
G9NR2 (5′-CGACCGCATATGGTGGATGACTGTCTGGGTCTG-3′),
G9NR3 (5′-CGACCGCTCGAGCTAAGAGCCATTGACGGAGATGGT-3′),
G9CF1e (5′-CGTCCTCATATGACTCCCGCCATCCCACCTATG-3′),
G9CF1g (5′-CGTCCTGAATTCCCACTCCCGCCATCCCACCTATG-3′),
G9CF2e (5′-CGTCCTCATATGATGATGTACCCCCACCCCGCC-3′),
G9CF2g (5′-CGTCCTGAATTCCCATGATGTACCCCCACCCCGCC-3′),
G9CF3e (5′-CGTCCTCATATGGCCTATCCGATGCCTTTCATC-3′),
G9CF3g (5′-CGTCCTGAATTCCCGCCTATCCGATGCCTTTCATC-3′),

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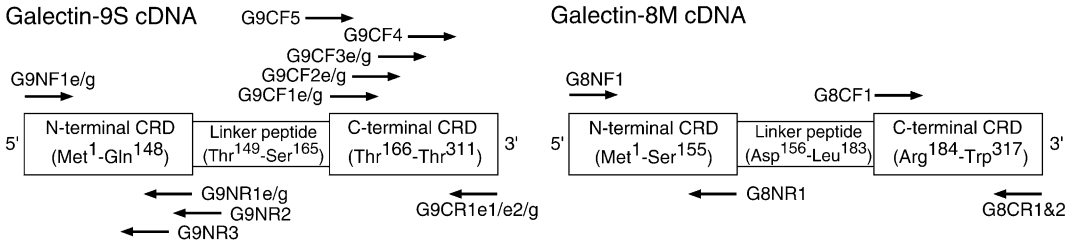

Fig. 1. Schematic representation of the primer sites used for amplification of galectin-9 and galectin-8 cDNAs. As it is not easy to unequivocally determine the linker peptide region without three-dimensional structural data, the assignment should be considered as provisional (see footnote of Table 1). Galectin-9S, a wild-type isoform of galectin-9 with the shortest linker peptide. Galectin-8M, a wild-type isoform of galectin-8 with a short linker peptide.

G9CF4 (5′-CGTCCTGAATTCCCCTGGGAGGGCTGTACCCA-TCC-3′),
G9CF5 (5′-CGTCCTCATATGCCTGGACAGATGTTCTCTACT-3′),
G9CR1e1 (5′-CGACCGAGATCTCTATGTCTGCACATGGGTC-AG-3′),
G9CR1e2 (5′-CGACCGGGATCCCTATGTCTGCACATGGGTC-AG-3′),
G9CR1g (5′-CGACCGCTCGAGCTATGTCTGCACATGGGTC-AG-3′),
G8NF1 (5′-CGTCCTCATATGATGTTGTCCTTAAACAACCTA-3′),
G8NR1 (5′-CGACCGCATATGCGAGCTGAAGCTAAAACCA-AT-3′),
G8CF1 (5′-CGTCCTCATATGAGGCTGCCATTCGCTGCAAGG-3′),
G8CR1 (5′-CGACCGAGATCTCTACCAGCTCCTTACTTCCAG-3′),
G8CR2 (5′-CGACCGGGATCCCTACCAGCTCCTTACTTCCAG-3′).

Expression vectors for tag-free wild-type galectins were prepared as follows: full-length cDNAs were amplified using G9NF1e + G9CR1e1 (galectin-9) and G8NF1 + G8CR1 (galectin-8). Amplified cDNAs were digested with *Nde*I and *Bgl*II, and then inserted into the *Nde*I–*Bam*HI site of pET-11a (Stratagene). In the case of mutant galectins, cDNAs coding for different types of C-terminal CRDs were amplified using G9CF1e/G9CF2e/G9CF3e/G9CF5 + G9CR1e2 and G8CF1 + G8CR2, digested with *Nde*I and *Bam*HI, and then inserted into the *Nde*I–*Bam*HI site of pET-11a. The resulting plasmids were designated as pET-G9C/-G9C(-6)/-G9C(-12)/-G9C(+6) and pET-G8C. The plasmids were digested with *Nde*I and then dephosphorylated. A cDNA coding for the N-terminal CRD was amplified using G9NF1e + G9NR1e and G8NF1 + G8NR1, digested with *Nde*I, and then inserted into the dephosphorylated plasmids. The resulting plasmids, coding for galectin-9 mutants consisting of a wild-type N-terminal CRD and different types of C-terminal CRDs, were designated as pET-G9Null/-G9 Null(-C6)/-G9 Null(-C12)/-G9 Null(LC6). The cDNA amplified using G9NF1e + G9NR2 was digested with *Nde*I, and then inserted into dephosphorylated pET-G9C. The resulting plasmid was designated as pET-G9Null (LN6).

Expression vectors for glutathione *S*-transferase (GST)-fusion proteins were prepared as follows: cDNAs coding for different types of N- and C-terminal CRDs were amplified using G9NF1g + G9NR1g/G9NR3 and G9CF1g/G9CF2g/G9CF3g/G9CF4 + G9CR1g, digested with *Eco*RI and *Xho*I, and then inserted into the *Eco*RI–*Xho*I site of pGEX-4T-2 (Amersham Biosciences). The DNA sequences of all the expression vectors were confirmed by automated sequencing.

2.2. Expression and purification of recombinant proteins

Expression of GST-fusion proteins in *Escherichia coli* (*E. coli*) BL21 cells was carried out as described previously [8]. Recombinant proteins were purified by affinity chromatography on a lactose–agarose column (Seikagaku Corp., Tokyo, Japan) and/or a glutathione–sepharose column (Amersham Biosciences). 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. Tag-free proteins were purified by affinity chromatography on a lactose–agarose column. The protein concentration was determined using BCA protein assay reagent (Pierce) and bovine serum albumin as a standard.

2.3. Protease treatment

Tag-free recombinant proteins were first incubated at 37 °C for 5 min in digestion buffer. After the addition of a protease, the reaction mixture was incubated for 120 min at 37 °C. The total reaction mixture, 600 μl, contained 36 μg of protein sample with an enzyme to substrate ratio of 1:100 (weight ratio). Aliquots were withdrawn from the reaction mixture at different times for sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). The following digestion buffers were used for trypsin/elastase and matrix metalloproteinase-3 (MMP-3), respectively: 0.1 M Tris–HCl (pH 8.0), 0.15 M NaCl, 1 mM $CaCl_2$, and 0.05 M Tris–HCl (pH 7.5), 0.15 M NaCl, 10 mM $CaCl_2$, 1 μM $ZnCl_2$. Activated MMP-3 was prepared as described previously [7].

2.4. In vitro biological activities

2.4.1. Chemotaxis. Eosinophil chemoattractant (ECA) activity was evaluated in vitro [2]. 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.). Human eosinophils ($0.5–1 \times 10^6$/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, the membrane separating the two chambers was removed and placed in Diff-Quick stain (Baxter Healthcare Corp.). Stained eosinophils were counted under a microscope. Human eotaxin-1 (Seikagaku Corp.) was used as a control.

2.4.2. Apoptosis. Apoptosis-inducing activity was assessed using MOLT-4 cells [3]. Cultured cells were incubated with an assay sample for 24 h. The cells were recovered by centrifugation, and then resuspended in 300 μl of PBS and 700 μl of 100% ethanol. The cells were washed with PBS, and then incubated with 50 μg/ml ribonuclease A for 30 min at 37 °C, followed by with 50 μg/ml of propidium iodide for 10 min. Stained cells were analyzed by flow cytometry.

2.4.3. Cell proliferation. The antiproliferative effect on a human prostatic cancer cell line (PC-3) was determined by means of the WST-8 assay. PC-3 cells (3×10^3 cells in 100 μl) were plated in 96-well plates and then cultured for 48 h. Test samples were added at various concentrations, and the culture was continued for 24 h. WST-8 reagent (Cell counting kit-8; Dojin Laboratories, Kumamoto, Japan) was added to the cells (10 μl/well), followed by incubation for 2 h. Each assay was performed in triplicate. Using an enzyme-linked immunoadsorbent assay autoreader, the viable cell number was determined by measuring the difference between the absorbance at 450 and that of 620 nm.

2.4.4. Neutrophil adhesion. The neutrophil adhesion assay was carried out as described previously [7]. 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 μl 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.

3. Results and discussion

3.1. Assignment of the linker peptide

In the present study, the N- and C-terminal CRDs and a linker peptide region were tentatively assigned (Fig. 1) based on the intron–exon structures of the galectin-9 and -8 genes [9,10]. However, X-ray crystallographic data for proto-type and chimera-type galectins predict that sequences flanking the linker peptide region may not constitute tightly folded CRDs [11]. To identify the sequences that are dispensable for the sugar-binding activity of galectin-9 CRDs, several truncation mutants were expressed and their affinity for lactose was tested. GST-fusion proteins of the N- and C-terminal CRDs (GST-G9NCRD and GST-G9CCRD) were expressed in *E. coli* and could be affinity purified with lactose–agarose gel, although significant parts of these fusion proteins were not retained by the gel (Fig. 2, lane 3 vs. lane 4). The solubility of GST-G9NCRD decreased on truncation of the C-terminal 9 amino acids, which may constitute the most C-terminal β-sheet of G9NCRD [12,13]; only a negligible amount of the fusion protein, GST-G9NCRD(-9), could be solubilized and the solubilized fraction did not show any affinity for lactose. On the other hand, truncation of the N-terminal 6 and 12 amino acids hardly affected the lactose-binding activity of G9CCRD (GST-G9CCRD(-6) and GST-G9CCRD(-12)). Further truncation of 10 amino acids, which may constitute the most N-terminal β-sheet of G9CCRD, resulted in almost complete inhibition of expression of the protein, GST-G9CCRD(-22). These results suggest that the removal of at least 12 amino acids of the N-terminal portion of G9CCRD in addition to the linker peptide may not affect the sugar-binding activity of galectin-9, although quantitative data are lacking.

3.2. Protease susceptibility of the wild type and mutant forms of galectin-9

We prepared a series of mutant forms of galectin-9 lacking different parts of the linker peptide and the N-terminal region of G9CCRD based on the data given in the previous section (Table 1). These proteins were successfully expressed as tag-free forms and purified with lactose–agarose, and then their susceptibilities to extracellular proteases were compared with that of wild-type galectin-9. Two isoforms of wild-type galectin-9, G9S and G9M, were almost completely degraded by MMP-3/stromelysin within 2 h at 37 °C and an enzyme to

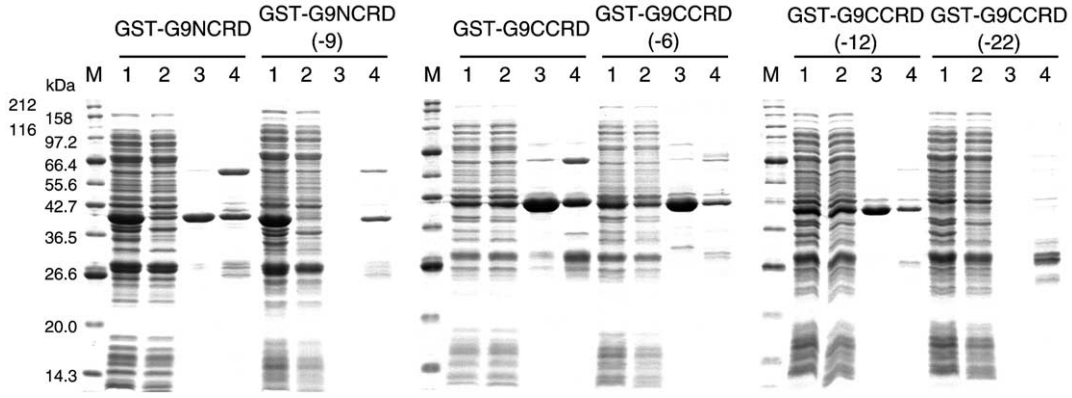

Fig. 2. Purification profiles of GST-fusion proteins on SDS–PAGE. The wild-type and mutant forms of galectin-9 CRDs were expressed as GST-fusion proteins, and then purified by lactose-affinity chromatography and glutathione-affinity chromatography. Samples at each purification step were electrophoretically separated in a SDS/12% polyacrylamide gel under reducing conditions and then stained with Coomassie brilliant blue R-250. M, molecular weight markers; lane 1, *E. coli* BL21 cell lysate; lane 2, *E. coli* BL21 crude extract (soluble fraction); lane 3, eluate from lactose–agarose; lane 4, eluate from glutathione–sepharose (the flow-through fraction from lactose–agarose was subjected to glutathione-affinity chromatography).

Table 1
Structures of the wild-type and mutant forms of galectin-9

Protein name	N-terminal CRD	Linker region	C-terminal CRD
G9M	Met^{1}–Gln^{148}	Pro^{149}–Ser^{177}	Thr^{178}–Thr^{323}
G9S	Met^{1}–Gln^{148}	Thr^{149}–Ser^{165}	Thr^{166}–Thr^{311}
(S-type linker peptide		TQTVIHTVQSAPGQMFS)	
G9Null	Met^{1}–Gln^{148}	HM	Thr^{166}–Thr^{311}
G9Null(LN6)	Met^{1}–Gln^{148}	TQTVIHHM	Thr^{166}–Thr^{311}
G9Null(LC6)	Met^{1}–Gln^{148}	HMPGQMFS	Thr^{166}–Thr^{311}
G9Null(-C6)	Met^{1}–Gln^{148}	HM	Met^{172}–Thr^{311}
G9Null(-C12)	Met^{1}–Gln^{148}	HM	Ala^{178}–Thr^{311}

Superscript numbers for mutant forms indicate amino acid positions in G9S. The amino acid sequences of the S-type linker peptide and the linker peptide regions of mutant forms are shown in a one-letter code. His–Met/HM residues in the linker regions of mutant forms are derived from the *Nde*I sites (CATATG) of the respective expression vectors. In the present study, the N- and C-terminal CRDs and a linker peptide region were tentatively assigned as shown in Fig. 1. However, X-ray crystallographic studies and phylogenetic analyses [16] predict that Val^{15}–Gln^{148} and Met^{181}–Thr^{311} form stably folded cores of N-CRD and C-CRD, respectively. Because this prediction agrees closely with the results shown in Fig. 2, residues Thr^{149}–Pro^{177}/Pro^{180} (TQTVIHTVQSAPGQMFSTPAIPPMMYPHP[AYP]) most likely constitute functional linker region of G9S.

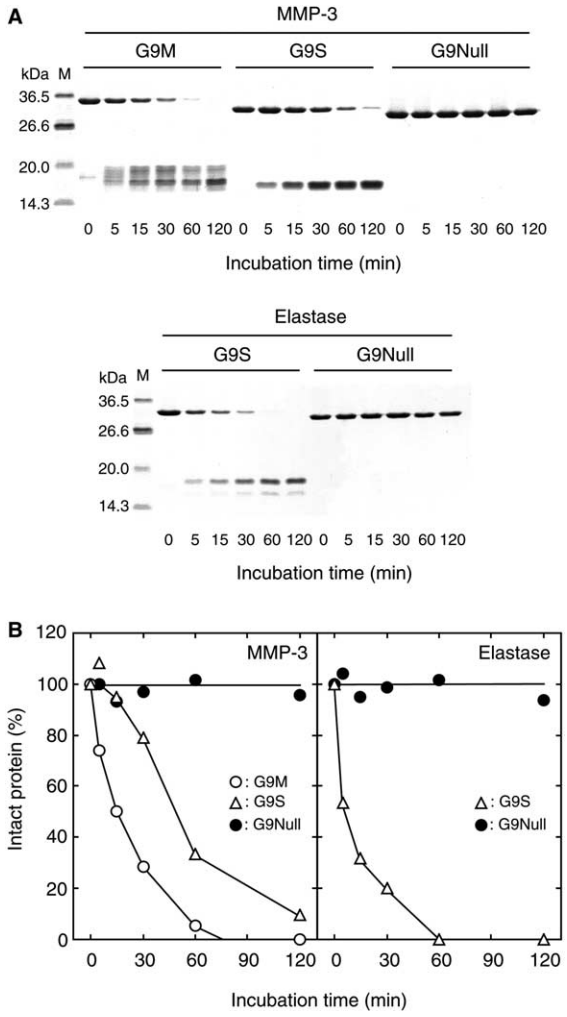

Fig. 3. Protease susceptibility of the wild-type and a mutant form of galectin-9. (A) Two isoforms of wild-type galectin-9 (G9S and G9M) and a mutant form lacking the entire linker region (G9Null) were incubated with MMP-3 or elastase at an enzyme to substrate ratio of 1:100 at 37 °C. Aliquots were withdrawn from the reaction mixture at different times and then subjected to SDS–PAGE. The molecular masses of G9S, G9M and G9Null are 34.7, 35.9 and 33.1 kDa, respectively. M, molecular weight markers. (B) Integrated intensities of intact protein bands were determined by densitometric scanning. Data represent the mean values for two independent experiments.

substrate ratio of 1:100 (Fig. 3). G9M was degraded more rapidly than G9S, which has the shortest linker peptide among the wild-type isoforms [14]. Elastase was more potent than MMP-3 as to degradation of G9S and G9M (data for G9M not shown). The isoforms were hardly degraded by trypsin under the above conditions, probably due to the absence of arginine and/or lysine residues in the linker peptide (data not shown). A mutant form lacking the entire linker region, G9Null, was completely resistant to both proteases and trypsin during 2 h incubation. Detailed time course analysis showed that G9Null is at least 100 times more stable than G9M against MMP-3 and elastase. G9Null(LN6) and G9Null(LC6), having the most N-terminal and C-terminal 6 amino acids of the S-type linker peptide, respectively, showed different susceptibilities (Fig. 4). G9Null(LN6) exhibited sensitivity to elastase comparable to that of G9S, but was resistant to MMP-3. While G9Null(LC6) was degraded by both proteases with lower sensitivity than in the case of G9S. Truncation of 6 and 12 amino acids of the N-terminal region of G9CCRD (G9Null(-C6) and G9Null(-C12)) did not change the sensitivity of G9Null to MMP-3, but unexpectedly resulted in slightly enhanced sensitivity to elastase (Fig. 4). These results indicate that different regions of the linker peptide exhibit different susceptibilities to proteases and that removal of the entire linker region of galectin-9 greatly improved the stability against proteases. In addition, it is possible that the N-terminal 12 amino acids of G9CCRD cause to the structural stability of G9CCRD, although, the sequence may not be essential for the sugar-binding activity.

The linker peptides of human tandem-repeat-type galectins so far identified comprise at least about 20 amino acid residues. Whereas some *Caenorhabditis elegans* galectins (LEC-1–LEC-4) have a very short linker peptide (less than 5 amino acid residues). These galectins may be more resistant to proteolysis than other *C. elegans* galectins with the tandem-repeat type structure. It is possible that they have relatively long in vivo half-lives and play distinct physiological roles.

Lactose-affinity chromatography yields galectin-9 preparations, both wild type and mutant forms, of high purity. However, affinity-purified G9M was degraded during storage at 4 °C, possibly due to contaminating bacterial proteases. The

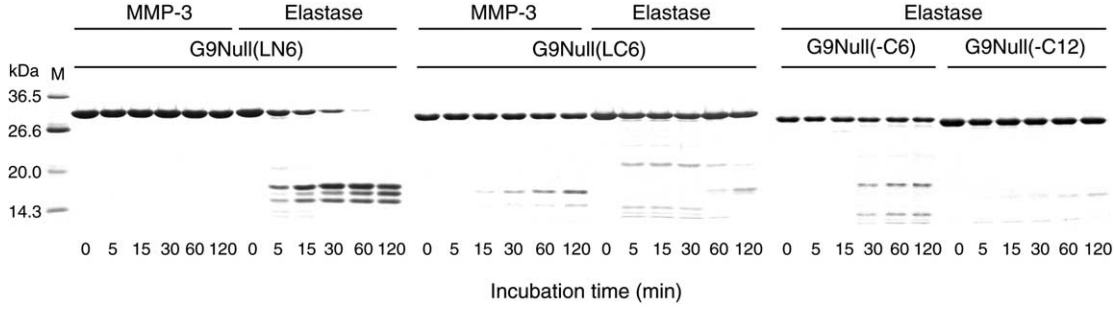

Fig. 4. Effects of linker peptide and CRD structures on the protease susceptibility of galectin-9. Mutant forms of galectin-9 lacking different parts of the linker peptide, G9Null(LN6) and G9Null(LC6), and ones lacking the entire linker region and different parts of the C-terminal CRD, G9Null(-C6) and G9Null(-C12), were incubated with MMP-3 or elastase at an enzyme to substrate ratio of 1:100 at 37 °C. Aliquots were withdrawn from the reaction mixture at different times and subjected to SDS–PAGE. M, molecular weight markers.

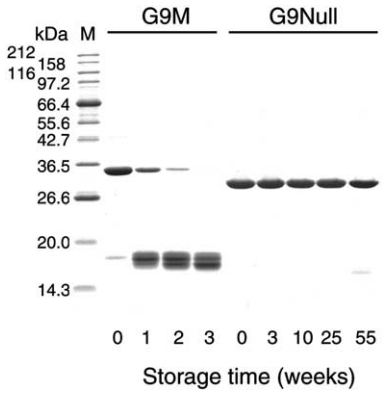

Fig. 5. Comparison of long-term stability during storage between the wild-type and a mutant form of galectin-9. Purified G9M and G9Null (phosphate buffered saline solution) were stored at 4 °C under sterile conditions. Aliquots were withdrawn at different times and then subjected to SDS–PAGE. M, molecular weight markers.

intact G9M molecule completely disappeared within three weeks of storage at 4 °C under sterile conditions (Fig. 5). While G9Null remained intact for at least 6 months and only a small amount of degradation products was detectable even after one year of storage. Because galectin-9 has a tendency to aggregate upon freeze-thaw treatment, the long-term stability of G9Null in solution could solve the problems regarding storage of recombinant protein.

3.3. Biological activities of G9Null

Galectin-9 has several distinct biological activities, which depend on the sugar-binding specificities of the CRDs and the tandem-repeat type structure of the protein [8,15]. To determine the effects of removal of the linker peptide on its biological activities, we compared ECA activity and apoptosis-inducing activity between wild-type galectin-9 and G9Null. G9M, G9S and G9Null showed maximum ECA activities that were comparable at 0.3 μM (Fig. 6A). This finding is consistent with our previous report showing that the ECA activity of galectin-9 does not depend on a specific structure of the linker peptide [14]. However, G9Null showed higher activity than that of wild-type galectin-9 at concentrations lower than 0.3 μM. Apoptosis-inducing activity was assessed using MOLT-4 human lymphoblastic leukemia cells and PC-3 human prostate tumor cells, although the mechanism underlying the antiproliferative effect of galectin-9 on PC-3 cells remains obscure (apoptosis or necrosis). G9Null induced apoptosis of MOLT-4 cells and growth inhibition of PC-3 cells in dose-dependent manners (Fig. 6B and C). The effects of G9Null were higher than those of G9M and G9S at all concentrations tested. G9Null was about two times more potent than G9M in inducing the apoptosis of MOLT-4 cells and growth inhibition of PC-3 cells. Western blot analysis revealed that not only G9Null but also G9M and G9S remained intact during the assay period (data not shown). Therefore, inactivation of wild-type isoforms by proteolysis in the cell culture medium is not the reason for these increased biological activities of G9Null. It is possible that glycoconjugates crosslinked with G9Null can interact more efficiently with each other than those crosslinked with wild-type galectin-9 depending on the spatial arrangement of two CRDs, which results in the increased biological activities of G9Null.

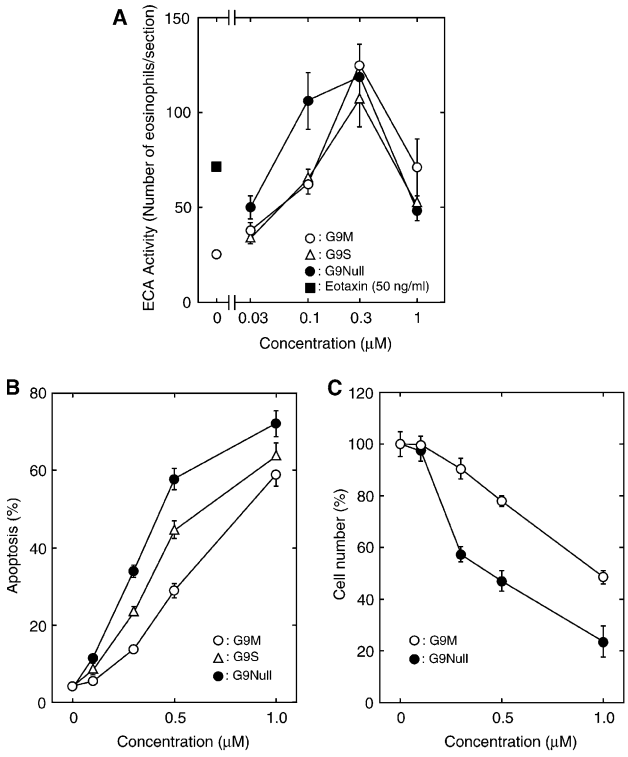

Fig. 6. Comparison of the biological activities of the wild-type and a mutant form of galectin-9. (A) ECA activity was assessed in vitro by the chamber method. In the assay, a porous membrane separated the top chamber containing purified human eosinophils from the bottom chamber containing the assay sample. After 1- to 2-h incubation at 37 °C, the membrane was removed and stained with Diff-Quick. The numbers of eosinophils that had migrated through the membrane are indicated. The results represent the means ± S.D. for three experiments performed in triplicate. (B) Proapoptotic activity was assessed by propidium iodide labeling. MOLT-4 cells cultured in the presence of an assay sample for 24 h were recovered by centrifugation, fixed and then treated with ribonuclease A. The cells were stained with propidium iodide and then analyzed by flow cytometry. The results were expressed as % of total cells. The results represent the means ± S.D. for three experiments. (C) The antiproliferative effect on PC-3 cells was determined by means of the WST-8 assay. PC-3 cells were cultured in the presence of an assay sample for 24 h. After the addition of WST-8 reagent, the culture was continued for 2 h. The viable cell number was determined with an automated plate reader. The viable cell number of untreated control culture was taken as 100%. The results represent the means ± S.D. of triplicate measurements.

3.4. Protease susceptibility and biological activity of galectin-8 lacking the linker peptide

G8Null, a mutant form of galectin-8 lacking the entire linker region, was prepared by a method analogous to that used for galectin-9 and was examined as to its susceptibility to proteases. Wild-type galectin-8, G8M, was almost completely degraded by trypsin and elastase within 15 min at 37 °C (Fig. 7A), but was resistant to MMP-3 (data not shown). As in the case of galectin-9, removal of the linker peptide greatly increased the protease resistance of galectin-8.

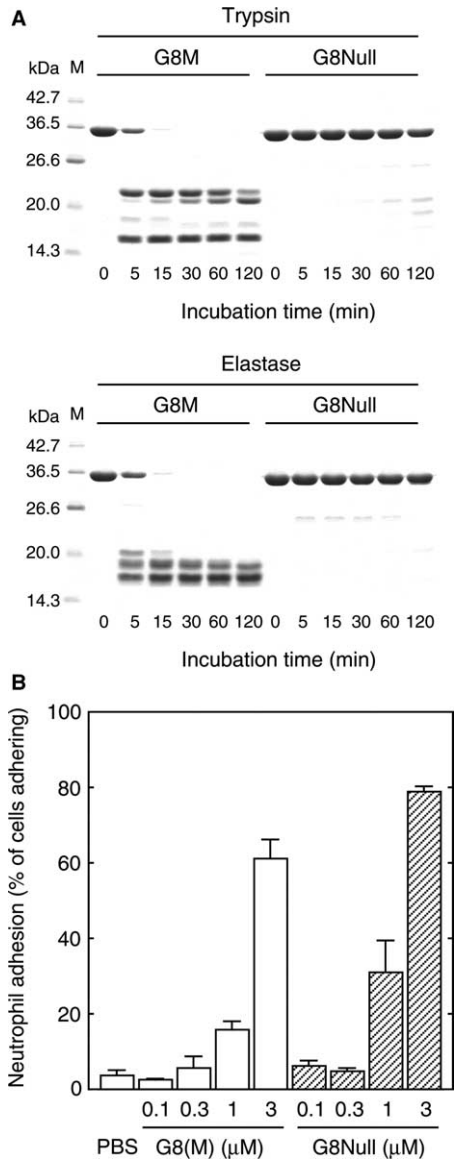

Fig. 7. Protease susceptibility and neutrophil adhesion-inducing activity of the wild-type and a mutant form of galectin-8. (A) The wild-type galectin-8 (G8M) and a mutant form lacking the entire linker region (G8Null) were incubated with trypsin or elastase at an enzyme to substrate ratio of 1:100 at 37 °C. Aliquots were withdrawn from the reaction mixture at different times and then subjected to SDS–PAGE. The molecular masses of G8M and G8Null are 35.8 and 33.1 kDa, respectively. M, molecular weight markers. (B) Neutrophil adhesion-inducing activity was assessed in vitro. Purified human neutrophils were plated on tissue culture plates. After the addition of an assay sample, the cells were allowed to adhere for 60 min at 37 °C. The DNA content of the attached cells was determined. The results represent the means ± S.D. of triplicate measurements.

About 72% and 94% of G8Null remained intact after 2-h incubation with trypsin and elastase, respectively. We have previously reported that galectin-8 modulates the neutrophil function (cell adhesion and superoxide production) via interaction with integrin αM [7]. G8Null induced neutrophil adhesion in a manner comparable to that in the case of G8M (Fig. 7B).

4. Conclusions

In the present study, we aimed to produce protease-resistant forms of tandem-repeat-type galectins by modifying the linker peptide structure. This strategy was based on our previous observation that three isoforms of galectin-9, which only differ in the linker peptide structure, exhibit comparable ECA activity. As expected, removal of the entire linker peptide region of galectin-9 and galectin-8 greatly improved their stability against proteolysis without negative effects on their biological activities. The increased specific activity of G9Null as to induction of apoptosis/growth inhibition of tumor cells was an unexpected outcome of the modification. The mutant forms of tandem-repeat-type galectins, i.e., G9Null and G8Null, might be useful agents for evaluating the therapeutic potential of these proteins. In vivo experiments with several disease models are currently in progress.

In addition to human galectin-9 and -8, we have prepared a mutant form of mouse galectin-9 and found that this method works equally well for the mouse protein (data not shown). Therefore, this simple and readily applicable method is expected to be useful for producing protease-resistant forms of other tandem-repeat-type galectins, namely galectin-4 and galectin-12 in the case of human galectin family.

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