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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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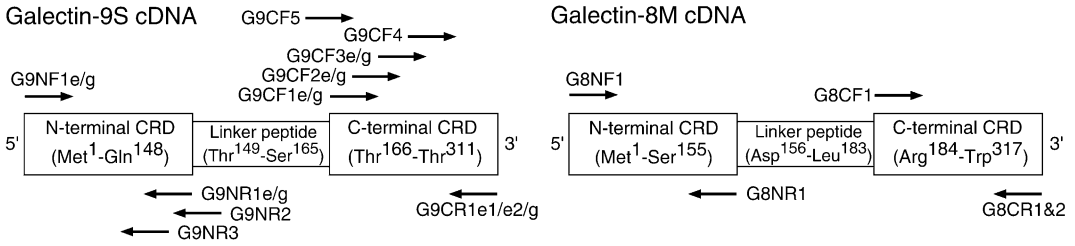

Fig. 1. Schematic representation of the primer sites used for amplification of galectin-9 and galectin-8 cDNAs. As it is not easy to unequivocally determine the linker peptide region without three-dimensional structural data, the assignment should be considered as provisional (see footnote of Table 1).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