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Crosslinking of low-affinity glycoprotein ligands to galectin LEC-1 using a photoactivatable sulfhydryl reagent

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Abstract

The N-terminal lectin domain (Nh) of the tandem repeat-type nematode galectin LEC-1 has a lower affinity for sugars than the C-terminal lectin domain. To confirm that LEC-1 forms a complex with *N*-acetyllactosamine-containing glycoproteins, we used several mutants of LEC-1 in which a unique cysteine residue was introduced into the Nh domain and examined their binding to bovine asialofetuin with a photoactivatable sulfhydryl crosslinking reagent. A crosslinked product was formed with the Q38C mutant, strongly suggesting the low-affinity interaction of Nh with the glycoprotein could be detected with this system.

Keywords: Galectin; Lectin; Ligand; Crosslink; Maleimide; Benzophenone

Galectins are a group of animal lectins characterized by their specificity for β-galactosides and an evolutionarily conserved sequence motif in the carbohydrate-binding site. Galectins are involved in a wide variety of biological phenomena, including development, cell differentiation, tumor metastasis, apoptosis, RNA splicing, and regulation of immune function [1–6]. Galectins can be classified into three types based on their molecular architecture, namely, proto-type, chimera-type, and tandem-repeat type. LEC-1, the 32-kDa galectin of the nematode *Caenorhabditis elegans*, was the first example of a tandem repeat-type of galectin composed of two homologous regions [7].

Frontal affinity chromatography [8] revealed that the two lectin domains of LEC-1 have different sugar-binding properties [9]. Both the N- and C-terminal lectin domains (Nh and Ch domains, respectively) have an affinity for *N*-acetyllactosamine-containing, N-linked, complex-type sugar chains, and the binding strength is higher for more branched complex-type sugar chains. This suggests independent sugar binding by the two domains, although the Ch domain displays 2- to 5-fold stronger binding than the Nh domain toward every complex-type sugar chain so far examined. Because this interaction was demonstrated using independently expressed Nh and Ch domains, we wanted to confirm that the lower affinity Nh domain interacts with *N*-acetyllactosamine-containing glycoconjugates within the context of the whole LEC-1 protein.

We have employed site-directed mutants of LEC-1 containing a single introduced cysteine residue combined with chemical crosslinking using the photoactivatable sulfhydryl reagent benzophenone-4-maleimide (BPM) [10] to identify *N*-acetyllactosamine-containing glycoproteins that interact with the Nh domain in LEC-1. As a model *N*-acetyllactosamine-containing glycoprotein, we used bovine asialofetuin because its sugar structure has well been described [11] and because it is commonly used to isolate galectins.

In the current studies, we first allowed BPM to react with the sulfhydryl group of the introduced unique cysteine residue in the Nh domain. Next, asialofetuin was added, and the reaction mixture was irradiated with ultraviolet light to initiate a photoreaction. Western blotting with anti-LEC-1 and anti-fetuin antisera showed a covalent complex between one of the mutants (Q38C) and asialofetuin. The

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product was not formed when a competing sugar, lactose, was added to the reaction mixture or using β-galactosidase-treated asialofetuin. These results confirm that the Nh domain is also functional in the whole LEC-1 molecule and indicate that it is possible to isolate and identify its counterpart glycoconjugates. Furthermore, the present method should be useful for isolating and identifying physiological lectin ligands, especially those that bind with low affinity.

Material and methods

Materials. Rabbit anti-bovine fetuin polyclonal antibody was purchased from Chemicon International. BPM and bovine fetuin were purchased from Sigma. Sialidase and β-galactosidase were purchased from Seikagaku Co.

Construction of mutant lectin genes. A unique cysteine residue was introduced to each mutant by site-directed mutagenesis of LEC-1 cDNA (LEC-1 lacks endogenous cysteine residues). Residues subjected to substitution were selected on the basis of the X-ray crystal structure of LEC-1 in complex with galactose [12]. To facilitate the formation of a covalently crosslinked heterodimer, hydrophilic amino acid residues with side-chains that extend from the surface of the protein towards galactose but not contacting the galactose were selected for replacement by cysteine. Site-directed mutants were constructed using the Altered Sites II *in vitro* mutagenesis system (Promega) according to the manufacturer's protocol. A cDNA fragment of LEC-1 in the pET21a expression vector [13] was cloned into pALTER-1 using the *Xba*I and *Hin*dIII sites. The following oligonucleotide primers were used for mutagenesis (substitution sites are underlined):

R14C, 5'-CCAGTACCATACTGCTCAGTACTCCAGGAGAAGT-3'; Q38C, 5'-CGATCGACGAGTCCTGCAGATTCACCATCAACCTT CACTCG-3'; T41C, 5'-TCGCAACGCTTCTGCATTAATCTTCACTC GAAGACCGCG-3'; D50C, 5'-CACTCGAAGACCGCATGCTTCTC CGGAAACGATGTG-3'; and G53C, 5'-GACCGCGGATTTCAGCT GTAACGATGTGCCACTCCACGTCTCTGTC-3'. After the mutagenesis reaction, the *Xba*I/*Hin*dIII fragments were ligated to generate mutant forms of LEC-1 cDNA encoding single cysteine-containing LEC-1 proteins in the pET21a expression vector. The sequences of all mutants were confirmed by DNA sequencing.

Purification of recombinant galectins. Expression and purification of recombinant proteins (LEC-1 or single cysteine-introduced mutants of LEC-1) were performed as described previously [9,13].

Preparation of asialofetuin for crosslinking. Bovine fetuin was treated with sialidase to remove the sialic acid attached to the nonreducing end of the sugar chain. There was contamination of the commercially available fetuin with higher molecular weight proteins. These proteins appeared to also contain *N*-acetyllactosamine structure because they bound to LEC-1-Sepharose and were eluted with lactose. The contaminating proteins were therefore removed by gel filtration on a Sephadex G-75 column, after which the collected fractions were loaded on an immobilized LEC-1 affinity column, and adsorbed protein was eluted with lactose.

β-galactosidase treatment of asialofetuin. To remove the galactose moiety from the nonreducing end of the sugar chains attached to asialofetuin, Sephadex G-75-purified asialofetuin was treated with β-galactosidase from *Streptococcus 6646K* according to the manufacturer's protocol.

Covalent crosslinking of mutated LEC-1 proteins to asialofetuin. Purified recombinant LEC-1 or the single cysteine-containing mutant of LEC-1 was dialyzed against phosphate-buffered saline containing 1 mM EDTA (pH 7.2) (EDTA-PBS). BPM was dissolved in dimethylformamide at 100 μM and 0.2 μl of the BPM solution was added to 2.5 μg of the recombinant protein in 10 μl EDTA-PBS (final concentration of 1 μM BPM). For negative controls, 0.2 μl of dimethylformamide was added instead of the BPM solution. After incubation in the dark for 30 min at 23 °C, unreacted BPM was quenched by addition of 0.2 μl of 1 M dithiothreitol (final concentration 20 mM). Asialofetuin in EDTA-PBS (5 μg in 10 μl) was added, and the reaction mixture was irradiated with a long-wavelength ultraviolet lamp for 3 min at 4 °C.

Electrophoresis and Western blotting. For analysis of crosslinked products, proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE) using 12.5% acrylamide gels. After electrophoresis, the samples were electrophoretically transferred to nitrocellulose membranes. The membranes were then cut into strips and analyzed by Western blotting using a rabbit polyclonal antibody raised against LEC-1 [7] and a rabbit polyclonal antibody against fetuin.

Results

Construction of LEC-1 mutants containing unique cysteine residues

In the current studies, we investigated whether the Nh domain, which is one of the two lectin domains in LEC-1 and which has only a low affinity for small oligosaccharides (pyridylaminated sugars [14]), can interact with *N*-acetyllactosamine-containing glycoproteins in the context of the whole LEC-1 molecule. To investigate the interaction between lectins with multiple carbohydrate-binding domains such as galectin LEC-1 and their glycoprotein ligands, we used site-directed mutagenesis to introduce a unique cysteine into the galectin LEC-1 and analyzed their interaction with asialofetuin using a photoactivated crosslinker. We used the X-ray crystal structure of LEC-1 in complex with galactose [12] to select the amino acids for mutation to cysteine. All selected amino acids were hydrophilic surface residues in the Nh domain of LEC-1 with side-chains that extend toward the bound ligand. Furthermore, to avoid inhibiting the interaction with the carbohydrate structure, amino acid residues close to or within the carbohydrate-binding site were excluded.

Each of the single-cysteine mutants of LEC-1 was adsorbed to the asialofetuin–Sepharose and could be eluted with 0.1 M lactose, confirming that they retained the ability to interact with asialofetuin. The purity of each recombinant protein was checked by SDS–PAGE (Fig. 1A). Each of the recombinant proteins showed a single major band (indicated by black arrowhead) approximately at the position of the deduced molecular weight (32 kDa). The recombinant proteins were also detected by Western blotting using an antibody raised against purified native LEC-1 protein (black arrowhead in Fig. 1B) [7]. Because no other bands with higher molecular weights were detected, the appearance of higher molecular weight bands after the crosslinking reaction indicated the formation of crosslinked complexes containing asialofetuin.

Photoactivated crosslinking of mutant LEC-1s to asialofetuin

Photoactivated crosslinking was carried out using BPM [10]. BPM, also known as 4-(*N*-maleimido)benzophenone, reacts with the sulfhydryl groups of cysteine residues via a maleimide moiety. Upon ultraviolet irradiation, a

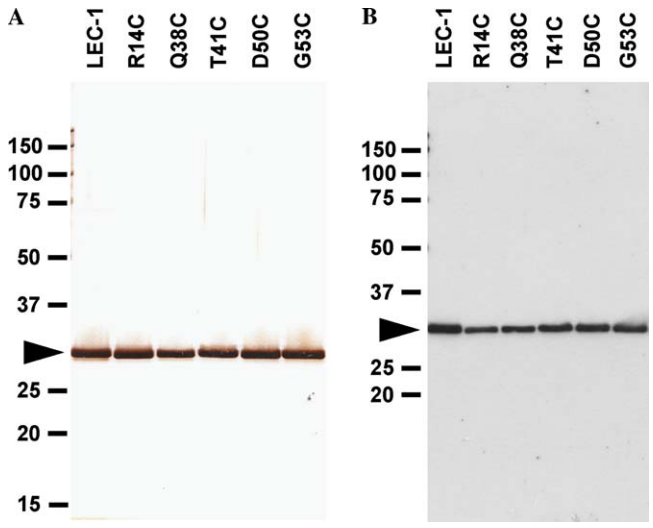

Fig. 1. Purification of cysteine-introduced mutant LEC-1s by affinity chromatography on asialofetuin–Sepharose 4B. (A) Analysis of purified proteins by reducing SDS–PAGE. Proteins were visualized by silver staining. The molecular masses of marker proteins are indicated to the left of the panel. The black arrowhead indicates the position of the recombinant proteins LEC-1 or cysteine-introduced mutants (32 kDa). (B) Purified recombinant proteins were analyzed by Western blotting using the antiserum raised against LEC-1 [7]. The black arrowhead indicates the position of the recombinant protein LEC-1 or cysteine-introduced mutants (32 kDa).

reactive species is generated that can form covalent bonds with nearby groups. The linker arm connecting the two reactive species is approximately 10 Å.

As shown in Fig. 2A, higher molecular mass species containing the original LEC-1 were not observed upon reaction with BPM, suggesting that, although asialofetuin contains other cysteine residues that can react with BPM, it cannot be crosslinked to wild-type LEC-1. Thus, any crosslinked products observed for mutant LEC-1 should be due to reaction of BPM with the introduced cysteine residues.

Five LEC-1 mutants containing unique cysteine residues at Arg^{14} (R14C), Gln^{38} (Q38C), Thr^{41} (T41C), Asp^{50} (D50C), or Gly^{53} (G53C) were reacted with BPM in the dark, mixed with asialofetuin, irradiated with ultraviolet light, and analyzed by Western blotting. As shown in Fig. 2B, Western blotting with the antibody against LEC-1 detected an additional major band with a molecular mass of approximately 90 kDa (white arrowhead in Fig. 2B) for the Q38C mutant in the presence of BPM. In contrast, higher molecular mass bands were not detected for the R14C, T41C, D50C, or G53C mutants. The observed molecular mass of approximately 90 kDa is consistent with the formation of a covalently crosslinked complex of LEC-1 and asialofetuin. The additional minor band of higher molecular mass (also indicated by white arrowheads) may be due to crosslinking with asialofetuin species that have different mobilities on SDS–PAGE due to different sugar structures.

Effect of lactose on photoactivated crosslinking of Q38C

As shown in Fig. 3A, in the presence of 0.1 M lactose, crosslinking between the Q38C mutant and asialofetuin was significantly reduced. This suggests that crosslinking occurs only when there is a specific lectin–sugar interaction.

Effect of β-galactosidase treatment of asialofetuin on photoactivated crosslinking

As shown in Fig. 3B, crosslinked product was not observed when *Streptococcus 6646K* β-galactosidase-treated asialofetuin was used. This is not surprising because β-galactosidase treatment removes the galactose moiety in the reducing end of the carbohydrate structure, which should eliminate the interaction between galectin and asialofetuin. These results support the idea that specific lectin–carbohydrate interaction between Q38C and asialofetuin is necessary for the crosslinking.

Discussion

Interaction between recombinant single carbohydrate-binding domains of the multi-domain lectin LEC-1 and pyridylaminated oligosaccharides has been studied mainly by frontal affinity chromatography [8,9]. Unfortunately, it is difficult to confirm that a particular carbohydrate-recognition domain within the whole lectin molecule interacts with sugars, especially when the interaction is not very strong. The Nh domain of the galectin LEC-1 has affinity for *N*-acetyllactosamine, but the affinity is lower than for the Ch domain, and the affinity of recombinant Nh protein is too low to allow purification of Nh on an asialofetuin–Sepharose column [13]. The situation for mannose receptors [15] is somewhat similar to that for the tandem repeat-type galectin LEC-1 in that multiple carbohydrate-recognition domains exist within a single polypeptide chain. Because high-affinity ligand binding is achieved only when at least three domains are clustered [16], it is difficult to identify ligands for each domain or detect the interaction for each carbohydrate-binding domain.

We employed a new approach in the current studies: we introduced unique cysteine residues near the carbohydrate-binding site to allow attachment of the photoactivatable crosslinking reagent BPM [10]. BPM has been used to crosslink unique cysteine residues introduced into cysteine-less form of subunit B of yeast vacuolar H^+-ATPase to other subunits in the complex [17,18] and to obtain information about the proximity of tropomyosin and caldesmon in muscle thin filaments [19].

Here, photoactivation resulted in crosslinking of the unique sulfhydryl group in mutant galectin Q38C with asialofetuin. The crosslinking was inhibited by lactose or by the removal of the terminal galactose residues of asialofetuin with β-galactosidase. This suggests that the BPM-crosslinked Q38C-asialofetuin heterodimer was mediated by a specific lectin–sugar interaction. Therefore, our results

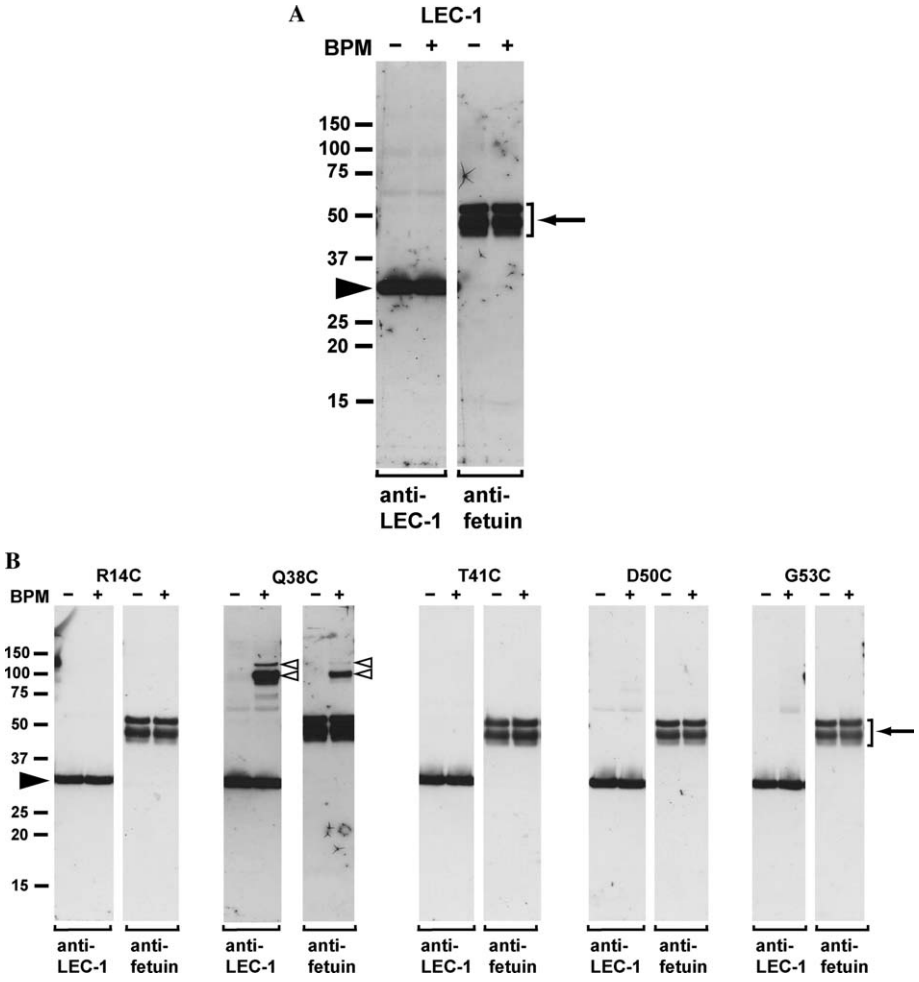

Fig. 2. Crosslinking of LEC-1 mutants and asialofetuin. Photoactivated crosslinking was carried out using BPM. (A) To determine whether BPM could crosslink LEC-1 to asialofetuin, recombinant LEC-1 was incubated in the presence (+) or absence (−) of BPM in the dark and then irradiated with ultraviolet light in the presence of asialofetuin. The proteins were then separated by SDS–PAGE and examined by Western blotting with an antiserum against either LEC-1 or fetuin. (B) Recombinant single cysteine-introduced LEC-1 mutants were reacted with BPM as described in panel A. A new band with a molecular mass of 90 kDa (and an additional minor band) was detected with the antisera against LEC-1 for the Q38C mutant but not for the remaining mutants. This 90-kDa band (and the additional minor band) was also detected with anti-fetuin antiserum. The molecular masses of marker proteins are indicated to the left of the panel. The black arrowhead shows the positions of LEC-1 or single cysteine-containing LEC-1 mutants, and the positions of asialofetuin (arrow) and the predicted migration position for the Q38C-asialofetuin heterodimer (white arrowheads) are shown to the right.

confirm the interaction between the glycoprotein and the Nh domain within the context of whole LEC-1 molecule.

According to the X-ray crystallographic structure of bovine galectin-1 in complex with biantennary oligosaccharide [20], the polypeptide portion of the glycoprotein appears to be relatively far from the galectin molecule compared with the carbohydrate portion bound to the lectin. This suggests that the benzophenone portion of BPM reacted with the carbohydrate portion of asialofetuin and not with its polypeptide chain. Because several sugar chains exist on the asialofetuin molecule [11], the observation of multiple crosslinked products with different mobilities on SDS–PAGE may be due to the crosslinking of Q38C to different carbohydrate chains on asialofetuin.

Lectin–sugar interactions play important roles in various biological systems. Such interactions may be different than those between receptor and ligand or antibody and antigen in that relatively weak interactions allow more frequent binding and dissociation. For instance, leukocyte rolling is the result of a repetition of binding and dissociation between selectins and their ligands [21]. Furthermore, the relatively weak interaction makes it possible for lectins to check their counterparts much more frequently.

On the other hand, these weak interactions can make it difficult to identify physiological ligands, especially in the case of multi-functional lectins. In fact, the low affinity of the Nh domain made it difficult to identify its ligands by conventional methods such as affinity purification. In the current studies, we were able to detect the interaction of a specific domain (Nh) in a multi-sugar-binding domain lectin (LEC-1) with a ligand even though the binding strength was low.

The present procedure using introduction of a unique cysteine and a photoactivatable sulfhydryl crosslinker

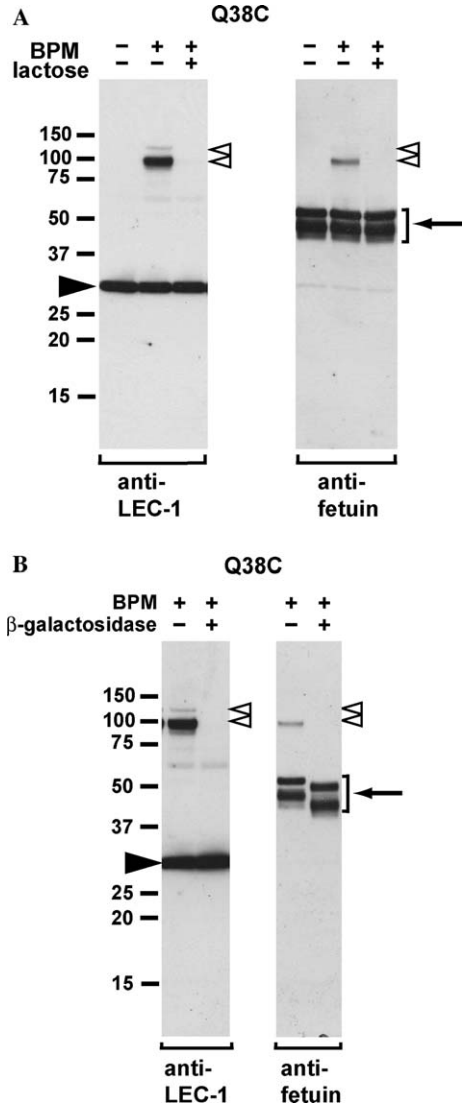

Fig. 3. Inhibition of crosslinking between the Q38C mutant and asialofetuin by lactose or β-galactosidase treatment of asialofetuin. (A) Crosslinking of Q38C and asialofetuin was significantly reduced when 0.1 M lactose was present in the crosslinking reaction mixture. (B) Asialofetuin was treated with β-galactosidase and used for crosslinking. The crosslinked product was not detected when the β-galactosidase-treated asialofetuin was used.

should be useful in a variety of fields where weak interactions play important roles. Given the advancements in the identification of unknown proteins in recent years, especially using mass spectrometry, it should be easy to identify binding proteins present in complex cellular and tissue samples.

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