Crosslinking of Cys-Mutated Human Galectin-1 to the Model Glycoprotein Ligands Asialofetuin and Laminin by Using a Photoactivatable Bifunctional Reagent

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Galectins are a group of animal lectins characterized by their specificity for β-galactosides. In our previous study, we showed that a human galectin-1 (hGal-1) mutant, in which a cysteine residue was introduced at Lys 28, forms a covalently cross-linked complex with the model glycoprotein ligands asialofetuin and laminin by using the photoactivatable sulfhydryl reagent benzophenone-4-maleimide (BPM). In the present study, we used several hGal-1 mutants in which single cysteine residues were introduced at different positions and examined their ability to form a covalent complex with asialofetuin or laminin by using BPM. We found that the efficiency of formation of the cross-linked products differed depending on the positions of the cysteine introduced and also on the ligand used for crosslinking. Therefore, by using different cysteine hGal-1 mutants, the chances of isolating different ligands for hGal-1 should increase depending on the systems and cells used.

Key words lectin; gallactose; crosslink; maleimide; benzophenone

Galectins comprise a group of animal lectins characterized by their specificity for β-galactosides and are characterized by an evolutionarily conserved sequence motif at their carbohydrate-binding site. 1–3) Galectins are involved in a wide variety of biological phenomena, including development, cell differentiation, tumor metastasis, apoptosis, RNA splicing, and regulation of immune function. 4,5) Interactions between lectins and their carbohydrate ligands play important roles in various biological systems. Although the basic sugar structure recognized by mammalian galectins, such as human galectin-1 (hGal-1), is the N-acetyllactosamine disaccharide unit (Gal|/1→4GlcNAc), the sugar-binding specificity of each galectin could be enhanced by branched, repeated, or substituted glycans, 6,7) suggesting that galectins could interact with a variety of endogenous ligands possessing different carbohydrate structures. However, because the binding ability of galectins is insufficient for some sugar structures, it may not be possible to isolate some endogenous ligand glycoconjugates by conventional methods such as affinity chromatography.

We previously prepared several mutants of LEC-1 (a galectin from the nematode Caenorhabditis elegans), which has two lectin domains tandemly repeated in a single polypeptide chain, 8,9) in which a cysteine residue was introduced near the sugar-binding site. 10,11) Cross-linked products were formed using a photoactivatable bifunctional reagent, benzophenone-4-maleimide (BPM). 12–14) when the mutant Q38C was incubated with a model glycoprotein ligand, asialofetuin. 9) BPM 12 reacts with the sulfhydryl groups of cysteine residues via its maleimide moiety. Upon ultraviolet irradiation, a reactive species that can form covalent bonds with neighboring groups is generated. We used the same method to form a cross-linked product between hGal-1 and N-acetyllactosamine-containing glycoproteins via BPM. 15) Lys 28 in hGal-1, at a location that corresponds to Gln 18 of LEC-1, was selected for cysteine mutagenesis of the Cys-less form of hGal-1, and BPM was reacted with the sulfhydryl group of this unique cysteine residue. Asialofetuin or laminin was added to the reaction mixture followed by irradiation with ultraviolet light to initiate photo-crosslinking. Western blotting with anti-hGal-1 and anti-fetuin or anti-laminin antisera showed the formation of a covalently cross-linked product between the hGal-1 mutant K28C and asialofetuin or laminin.

In this study, we generated several single-Cys mutants of the Cys-less form of hGal-1. To facilitate the formation of a covalently crosslinked heterodimer via BPM, hydrophilic amino acid residues near the sugar binding site with side-chains extending from the surface of the protein toward galactose but not contacting it were selected for replacement by Cys. We then examined the ability of these mutants to form covalent complexes with asialofetuin or laminin using BPM.

MATERIALS AND METHODS

Chemicals Rabbit anti-bovine fetuin and anti-human laminin polyclonal antibodies were purchased from Chemicon International (Temecula, CA, U.S.A.). BPM, bovine fetuin from fetal calf serum, and human laminin from placenta were purchased from Sigma (St. Louis, MO, U.S.A.). Antiserum against human Gal-1 was prepared as described previously. 16) Construction of Cys-Less and K28C Mutants of hGal-1 The recombinant Cys-less and K28C mutants of hGal-1 were constructed as described previously. 15) Construction of Other Mutants of hGal-1 A unique cysteine residue was introduced by site-directed mutagenesis of the Cys-less form of hGal-1 DNA. Point mutants of hGal-1 were generated by polymerase chain reaction (PCR) using the pET21a-hGal-1 Cys-less plasmid and the following primers (substitution sites are underlined), as previously described 17): S7C, 5′-TGTAAC  CTG  AAT  CTC  AAA  CCT  G-3′.
and 5'-GGC GACCAG ACC AGA AGC-3' A51C, 5'-CAC GGC GAG GCC AAC ACA-3' and 5'-ACAGTT GAAGCG AGG GTT GAA GTG C-3' K63C, 5'-GAC GCC GG GCC TG GGG GGG-3' and 5'-ACAGCT GTT GCT CAC AAT TGT GTT-3' A121C, 5'-TGTTGCT GAC GGG GTT GAC TTT AAA ATC-3' and 5'-CAT GTA GTT GAT GCG AGG GTT GAA GTG C-3' A121C, 5'-TGTTGCT GAC TTT AAA ATC-3' and 5'-AGC TG C CAT GTA GTT GAT G-3'.

Purification of Recombinant Galectins Recombinant proteins were expressed and purified as described previously. In brief, *Escherichia coli* cells were grown at 37°C in 250 mL of 2×YT medium containing 125 µg/mL ampicillin and subjected to protein expression at 20°C overnight by adding isopropyl-β-thiogalactopyranoside to give a final concentration of 0.4 mM. The cells were harvested, suspended in 10 mL of ethylenediamine tetraacetic acid (EDTA)-2-mercaptoethanol (ME)-phosphate buffered saline (PBS), and disrupted by sonication. The debris was removed by centrifugation and the extracts were applied to asialofetuin-Sepharose 4B columns. After extensive washing of the column with EDTA-ME-PBS, adsorbed mutant galectins were eluted with EDTA-ME-PBS containing 0.1 M lactose.
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. Asialofetuin was purified as described previously. The contaminating proteins were removed by gel filtration on a Sephadex G-75 column, after which the collected fractions were loaded on an immobilized human Gal-1 C2S affinity column (3.95 mg of hGal-1 C2S immobilized on a 1-mL column bed), and the adsorbed protein was eluted with lactose.

Cova lent Crosslinking of Mutated hGal-1 Proteins to Asialofetuin or Laminin Cys-mutated hGal-1 was crosslinked to the model glycoprotein ligand asialofetuin or laminin as described previously, with minor modifications. Briefly, purified recombinant Cys-less or single-cysteine mutants of hGal-1 were dissolved in PBS containing 1 mM EDTA (pH 7.2) (EDTA-PBS). BPM was dissolved in dimethylformamide, and the BPM solution was added to the recombinant protein in EDTA-PBS (final concentration of BPM, 4 µM). For negative controls, dimethylformamide was added rather than the BPM solution. After incubation in the dark for 30 min at 23°C, untreated BPM was quenched by addition of dithiothreitol (final concentration, 20 mM). After addition of asialofetuin or laminin in EDTA-PBS, the reaction mixture was irradiated with a long-wavelength ultraviolet lamp for 30 min at 4°C.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting For analysis of the crosslinked products, the proteins were separated by SDS-PAGE and subjected to Western blotting using a rabbit polyclonal antibody raised against hGal-1 and anti-bovine fetuin or anti-human laminin antibodies.

RESULTS

Construction of Gal-1 Mutants Five Cys mutants of the

![Fig. 3. Crosslinking of hGal-1 Mutants and Asialofetuin](image)

Photoactivated crosslinking of recombinant hGal-1 to asialofetuin was performed in the presence (+) or absence (−) of BPM in the dark, followed by irradiation with ultraviolet light in the presence of purified asialofetuin. The proteins were then separated using SDS-PAGE and examined by Western blotting with antiserum against either hGal-1 or fetuin. An additional band indicated by a white arrowhead was detected for the S7C, A51C, K63C, A121C, and D123C hGal-1 mutants along with the K28C mutant, as previously reported, using both anti-hGal-1 and anti-fetuin antibodies. This band was not detected for the Cys-less mutant. The black arrowhead shows the position of hGal-1, whereas the positions of asialofetuin are shown by the bracket on the right side of the panel. Molecular weight markers are indicated on the left in each panel.
Cys-less form of hGal-1 were generated; these mutants contained unique cysteine residues at Ser7 (S7C), Ala21 (A51C), Lys63 (K63C), Ala121 (A121C), or Asp123 (D123C). The mutation positions are described in Fig. 1 based on the structure of hGal-1.19

As shown in Fig. 2, each of the single-cysteine mutants of hGal-1 was adsorbed onto the asialofetuin-Sepharose resin and could be eluted with 0.1 M lactose, confirming that the mutants retained the ability to interact with asialofetuin. The yield of the recombinant proteins was 4.3, 3.4, 2.8, 1.8, and 1.7 mg for S7C, A51C, K63C, A121C, and D123C, respectively. The purity of each recombinant protein was checked by SDS-PAGE, and each of the recombinant proteins showed a single major band approximately at the position of the deduced molecular weight of 15 kDa (data not shown).

Photoactivated Crosslinking of Mutant hGal-1s to Asialofetuin or Laminin

Photoactivated cross-linking was performed using BPM.12) The Cys hGal-1 mutants were reacted with BPM in the dark, mixed with asialofetuin, irradiated with ultraviolet light, and analyzed by Western blotting. As shown in Fig. 3, when the Cys-less form of hGal-1 was used for crosslinking, only the bands of hGal-1 or asialofetuin were detected, and no crosslinked product was observed. Therefore, if any bands with a higher molecular weight were detected in the Western blots of the mutant proteins, those bands could only be derived from the introduction of the unique Cys residue to the hGal-1 protein. As shown in Fig. 3, similar to the K28C mutant reported previously, Western blotting with antibodies against both hGal-1 and fetuin detected additional bands for the five (S7C, A51C, K63C, A121C, and D123C) mutants only when BPM was added to the reaction mixture. In contrast, when laminin was used as a model glycoprotein ligand for hGal-1, potential crosslinked product formation via BPM was detected for only S7C, K28C, and A51C with

![Fig. 4. Crosslinking of hGal-1 Mutants and Laminin](image-url)

Photoactivated crosslinking of the recombinant hGal-1 to laminin was performed in the presence (+) or absence (−) of BPM in the dark, followed by irradiation with ultraviolet light in the presence of laminin. The proteins were then separated by SDS-PAGE and examined by Western blotting with antiserum against either hGal-1 or laminin. An additional band indicated by a white arrowhead was detected for the S7C and A51C hGal-1 mutants, along with the K28C mutant as previously reported.13) Using both anti-hGal-1 and anti-laminin antibodies. This band was not detected for the other hGal-1 mutants. The black arrowhead shows the position of hGal-1, whereas the positions of laminin are shown by the bracket on the right side of the panel. Molecular weight markers are indicated on the left in each panel.
antibodies against hGal-1 and laminin (Fig. 4). Therefore, although the Cys mutants of hGal-1 preserved their binding ability to the N-acetyllactosamine structure, the efficiency of crosslinking with its potential ligands differed depending on the positions of the introduced cysteine residue. Furthermore, as previously described for K28C,\textsuperscript{15} the addition of 0.1 M lactose to the reaction mixture induced a significant reduction in the amount of crosslinked products that formed between the five (S7C, A51C, K63C, A121C, and D123C) mutants and asialofetuin (Fig. 5a) or between the S7C and A51C mutants and laminin (Fig. 5b). These results suggest that crosslinking occurs only when specific lectin–sugar interactions are present between the mutants and asialofetuin or laminin.

**DISCUSSION**

In the present study, five hGal-1 mutants were generated by introduction of a cysteine residue. These mutants, along with the previously generated K28C mutant (six mutants in total), were subjected to BPM-mediated crosslinking with either of the two model galectin ligands, asialofetuin and laminin. All of the Cys mutants were adsorbed onto the asialofetuin-immobilized affinity column and were eluted with lactose, suggesting that they retained their ability to bind to the N-acetyllactosamine structure abundant in immobilized asialofetuin. Furthermore, the expressed mutants were subjected to frontal affinity chromatography\textsuperscript{6,9,20} to determine whether they maintained a sugar-binding profile similar to that of wild-type hGal-1. All five (S7C, A51C, K63C, A121C, and D123C) mutant recombinant proteins showed sugar binding profiles similar to those of wild-type hGal-1 (data not shown).

When the Cys mutants were subjected to BPM-mediated crosslinking to asialofetuin, formation of a crosslinked product with asialofetuin via BPM was observed for all six mutants with Cys at different positions (i.e., S7C, K28C, A51C, K63C, A121C, and D123C). In the case of S7C, A51C, K63C, and D123C, multiple bands were detected. These results suggest that crosslinking between mutated hGal-1 and asialofetuin may have occurred at different orientations. Asialofetuin was generated by desialylation of fetuin at the non-reducing end of the sugar chains attached to the polypeptide chain. Fetuin has been reported to have three N-linked carbohydrate chains and three O-linked carbohydrate chains.\textsuperscript{21,22} Therefore, it is possible that the SDS-PAGE mobility differed depend-

![Fig. 5. Inhibition of Crosslinking between the Cys-Introduced Mutants and Asialofetuin or Laminin by Lactose](image-url)
ing on which N-acetyllactosamine unit was crosslinked with the mutated hGal-1. Another possibility is that two (or more) Cys-mutated hGal-1 molecules formed a crosslinked product with asialofetuin at the same time, thus resulting in a larger crosslinked product.

In contrast, only the S7C, K28C, and A51C mutants generated BPM-mediated crosslinked products with laminin, possibly because of the differing efficiency of crosslinking depending on the accessibility of the benzophenone moity of BPM to the laminin molecule, given that the number of sugar chains, the structure of the sugars, and the structure of the folded polypeptide chain differ between these two model glycoproteins.

Various glycoproteins, such as the T-cell surface glycoproteins CD3, CD7, CD43, and CD45, have been reported as ligand candidates for Gal-1. These potential galectin targets were isolated by conventional affinity chromatography performed using columns of immobilized galectins, and the isolation was probably possible because of the potential formation of a lattice between the galectin and the candidate glycoproteins. Considering that galectin-1 is variably expressed by immune cells and endothelial cells and is suggested to play significant roles in immune responses, and that galectins have a variety of functions, additional ligands may exist that have not yet been identified. The use of multiple mutants with a single cysteine residue introduced at different positions together with the cross linker BPM may result in the formation of new crosslinked products, thereby enabling the isolation and identification of new ligands.

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