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Development of a photoreactive probe-based system for detecting heparin

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ABSTRACT

We previously identified a peptide heparin-associated peptide Y (HappY) that binds specifically to heparin. In this article, we report a novel heparin detection system using chemically modified HappY as a probe. The photoreactive HappY probe was serially diluted and dispensed into a 96-well plate coated with biotinylated heparin. After ultraviolet irradiation, the HappY probe crosslinked to the heparin on the plate was detected with fluorescein isothiocyanate-conjugated streptavidin. Furthermore, the photoreactive HappY probe was used to stain cutaneous tissue sections obtained from dermatitis-affected or mastocytoma-affected cats and dogs. The photoreactive HappY probe stained limited resident mast cells in the connective tissue of skin compared with the anti-heparan sulfate monoclonal antibody 10E4, suggesting that the probe can be used to distinguish the structure of heparin in tissues. The interactions between glycosaminoglycans and proteins in vivo tend to be weak. Therefore, our method for enhancing such weak interactions may be a promising tool for intermolecular interaction studies in glycobiology research.

Higher organisms ubiquitously express heparan sulfate (HS)[1] proteoglycans on the cell surface and in the extracellular matrix. HS has been implicated in many biological phenomena such as blood coagulation, viral infection, tumor metastasis, and various developmental processes [1]. The biosynthetic processing of HS chains comprising alternating 1,4-linked glucosamine and hexuronic acid residues produces enormous structural diversity from the relatively simple structure through differences in the extent and position of N- and O-sulfation as well as by uronic acid epimerization [2]. The HS structure is also spatiotemporally altered during development [3]. Structurally distinct HS variants exhibit differing affinities for various proteins, such as growth factors, enzymes, and extracellular matrix components, regulating the above-mentioned biological processes [4,5].

Numerous studies examining the critical roles of glycosaminoglycans, including HS, in biological events have increased interest in elucidating the detailed structure of polysaccharides. However, glycosaminoglycans are structurally heterogeneous. Furthermore, it is difficult to raise useful antibodies against glycosaminoglycans because polysaccharides are ubiquitous on the cell surfaces in all animals used for antibody production (e.g., chickens, mice, rabbits). To gain further insights into the functions of glycosaminoglycans, specific probes against each sulfation pattern are required. Several attempts have been made to design specific antibodies against glycosaminoglycans [6–10]. Some of these antibodies are suitable for practical use, whereas others have low binding affinity or low specificity. Recently, a phage display method was shown to be useful to avoid such difficulties [11,12].

Our previous study focused on heparin, which is primarily produced by mast cells and whose structure is closely approximated by the structural analog HS; using the phage display technique, a novel peptide, heparin-associated peptide Y (HappY), was identified as a specific probe for heparin/HS [13]. HappY binds strongly to heparin and weakly to HS, but it does not bind to other glycosaminoglycans. HappY consists of 12 amino acid residues, including 4 arginines (RTRGSTREFRTG), and recognizes three consecutive monosaccharide residues in heparin (GlcN,3,6-SO_3α1-4IdoA,

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[1] *Abbreviations used:* HS, heparan sulfate; HappY, heparin-associated peptide Y; Mts-Atf-LC-biotin, methanethiosulfonate-azidotetrafluoro-long-chain-biotin; UV, ultraviolet; sulfo-NHS-LC-biotin, biotinamidohexanoic acid 3-sulfo-*N*-hydroxy-succinimide ester; HBC, high binding capacity; BSA, bovine serum albumin; HRP, horseradish peroxidase; IgM, immunoglobulin M; PBS, phosphate-buffered saline; PBS-T, 0.025% Tween 20 in PBS; DMSO, dimethyl sulfoxide; DAB, diaminobenzidine; RFU, relative fluorescence units.

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2-SO_3α1-4GlcN,6-SO_3) through its first three arginine residues [13].

The methanethiosulfonate-azidotetrafluoro-long-chain-biotin (Mts-Atf-LC-biotin) label transfer reagent was used to detect heparin in this study. To generate a probe, the photoactivatable crosslinker Mts-Atf-LC-biotin was used to label peptides at the sulfhydryl group of a cysteine residue through the Mts moiety. The Atf moiety is activated by ultraviolet (UV) irradiation, and crosslinking between the probe and target molecules can lead to nonspecific carbon–hydrogen bond insertion [14]. Due to the perfluorinated phenyl azide group (Atf moiety), the reactive intermediate nitrene can survive long enough to react with target molecules and become inserted more efficiently into carbon–hydrogen bonds than hydroxyphenyl azide [14].

Although HappY has high specificity for epitopes, its relatively low binding affinity makes it challenging to use in conventional biochemical methods. Weak binding generally tends to occur between glycosaminoglycans and proteins in vivo. Therefore, a novel method for enhancing these weak interactions could lead to novel tools for intermolecular interaction analysis in glycobiology research. Here, we established a new assay system to specifically detect heparin using the HappY peptide as a probe; the results suggested that UV crosslinking between a weak probe and polysaccharides improves heparin detection in biological specimens.

Materials and methods

Materials

Biotinamidohexanoic acid 3-sulfo-*N*-hydroxysuccinimide ester (sulfo-NHS-LC-biotin), Mts-Atf-LC-biotin label transfer reagent, and streptavidin-coated high binding capacity (HBC) black 96-well plates were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The HappY–Cys peptide (RTRGSTREFRTGC) was synthesized by Medical & Biological Laboratories (Nagoya, Japan), Vivapure S Mini M spin columns were obtained from Sartorius Stedim Biotech (Goettingen, Germany), and toluidine blue O and Alcian blue 8GX were obtained from Sigma–Aldrich (St. Louis, MO, USA). Heparin (pig small intestine), bovine serum albumin (BSA, EIA/RIA grade, globulin free), and Peroxidase Stain DAB kits (Brown Stain) were purchased from Nacalai Tesque (Kyoto, Japan), streptavidin/biotin blocking kit and fluorescein-conjugated streptavidin were obtained from Vector Laboratories (Burlingame, CA, USA), and streptavidin–horseradish peroxidase (HRP) conjugate was obtained from Invitrogen (Carlsbad, CA, USA). Chondroitin sulfate A, chondroitin sulfate C, heparinase (from *Flavobacterium heparinum,* EC 4.2.2.7), heparitinase I (from *F. heparinum,* EC 4.2.2.8), heparitinase II (from *F. heparinum,* no EC number), and anti-HS monoclonal antibody (10E4) were purchased from Seikagaku (Tokyo, Japan), and HRP-conjugated goat anti-mouse immunoglobulin M (IgM) antibody was obtained from Bethyl Laboratories (Montgomery, TX, USA). Paraffin sections of dermatitis and mastocytoma tissues were obtained from dermatitis-affected animals (cats and dogs) and skin mastocytoma-affected animals (cats and dogs), respectively. All specimens were obtained from animals diagnosed at the Animal Medical Center at Gifu University (Japan). All experiments were performed according to the guidelines for the care and use of laboratory animals approved by the animal care and use committee of Gifu University.

Introduction of biotin tag in heparin molecules

Commercial heparin from pig small intestine was incubated with 2 M ammonium chloride and $NaBH_3CN$ at 70 °C for 2 days to introduce an amino group at the reducing terminal. Then, $NaBH_3CN$ was added to the mixture, which was incubated for an additional 2 days. After desalting on a HiTrap desalting column (GE Healthcare, Little Chalfont, UK), reductively aminated heparin was applied to a UNO Q column (Bio-Rad, Hercules, CA, USA) for further purification. Fractions eluted with 2 M NaCl were dialyzed against Milli-Q water. The purified reductively aminated heparin was biotinylated by incubation with sulfo-NHS-LC-biotin at 4 °C overnight. Biotinylated heparin was purified using a HiTrap desalting column and UNO Q column as mentioned above. The 2 M NaCl-eluted fractions were dialyzed against Milli-Q water, lyophilized, and reconstituted in phosphate-buffered saline (PBS).

Immobilization of biotinylated heparin on microtiter plates

Biotinylated heparin was coupled to streptavidin-coated black 96-well plates as reported previously [13]. Briefly, each well was washed with 200 µl of PBS-T (0.025% Tween 20 in PBS) three times. Biotinylated heparin was diluted with 100 µl of PBS and added to each well, followed by incubation at 4 °C overnight. Then, each well of the plate was washed three times with 200 µl of PBS-T and twice with PBS. The amount of immobilized heparin was 0.4 µg/well, as determined colorimetrically as follows. Each well was stained with Alcian blue 8GX dye solution (5% Alcian blue, 0.018 M H_2SO_4, 0.02 M guanidine–HCl, and 0.25% Triton X-100), and the absorbed dye was dissolved in 8 M guanidine–HCl after a brief wash with Milli-Q water. The absorbance was measured at 620 nm.

Photoreactive biotin-labeled HappY probe

All of the procedures described below were performed in a dark room under a red safelight. Mts-Atf-LC-biotin label transfer reagent was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1 mg in a volume of 25 µl. HappY–Cys peptide (1.045 mg, 685 nmol) was dissolved in 317.5 µl of PBS. Mts-Atf-LC-biotin label transfer reagent solution (32.5 µl, 2-fold molar excess over the peptide) was added to the peptide and gently mixed. The reaction mixture was incubated at room temperature for 1 h. The reactant was diluted 10-fold with Milli-Q water and then loaded onto a Vivapure S Mini M spin column, which was prewashed with 20 mM potassium phosphate buffer (pH 7.0) for purification. The columns were centrifuged at 500*g* for 5 min, and the flow-through fraction was collected. The columns were then washed four times by loading with 200 µl of 20 mM potassium phosphate buffer (pH 7.0) and centrifugation at 500*g* for 5 min to remove unbound substances. The flow-through fraction was collected as wash fractions. Membrane-bound substances were eluted by loading 200 µl of 20 mM potassium phosphate buffer (pH 7.0) containing 1 M KCl onto the columns, which were then centrifuged at 500*g* for 5 min. The elution step was repeated four times, and all eluates were pooled together and referred to as the elution fraction. Purification of the Mts-Atf-LC-biotin label transfer reagent-bound HappY–Cys (HappY probe) was confirmed by bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific) and enhanced chemiluminescent (ECL) dot blotting (GE Healthcare).

Fluorometric detection of heparin by a competitive method involving HappY probe

Biotinylated heparin was immobilized onto a streptavidin-coated HBC black 96-well plate as described above. To block excess biotin, 100 µl of streptavidin solution (Vector Laboratories) diluted with 5% BSA in PBS-T (1:5) was added to the wells, which were then incubated at room temperature for 15 min. After washing three times with 200 µl/well of PBS-T, excess streptavidin was