A Peptide Found by Phage Display Discriminates a Specific Structure of a Trisaccharide in Heparin*

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A number of recent studies have shown that heparan sulfate can control several important biological events on the cell surface through changes in sulfation pattern. The in vivo modification of sugar chains with sulfates, however, is complicated, and the discrimination of different sulfation patterns is difficult. Heparin, which is primarily produced by mast cells, is closely approximated by the structural analog heparan sulfate. Screening of heparin-associating peptides using phage display and antithrombin-bound affinity chromatography identified a peptide, heparin-associating peptide Y (HappY), that acts as a target of immobilized heparin. The peptide consists of 12 amino acid residues with characteristic three arginines and exclusively binds to heparin and heparan sulfate but does not associate with other glycosaminoglycans. HappY recognizes three consecutive monosaccharide residues in heparin through its three arginine residues. HappY should be a useful probe to detect heparin and heparan sulfate in studies of glyobiology.

Heparin, which is comprised of heterogeneous mixtures of complex, linear, sulfated polysaccharides consisting of one to four linked uronic acid and glucosamine residues, is well known for its potent anticoagulant activity (1–3). The antithrombin-binding (ATB)2 domain mainly exerts its activity by binding to antithrombin (AT), a 55-kDa single-chain plasma glycoprotein (4, 5), and has a unique pentasaccharide structure (6). This pentasaccharide sequence is randomly distributed along the heparin chains and is present in only about one-third of the heparins currently used in the treatment of thrombosis (7). For a review of the prevention of thrombosis, see Ref. 8.

Heparan sulfate (HS), which is a structural analog of heparin, is ubiquitously distributed on the cell surface and in the extracellular matrix, whereas heparin is usually sequestered in mast cells. HS plays important roles in a number of biological phenomena such as blood coagulation, viral infection, tumor metastasis, and various developmental processes (9). HS and heparin are composed of the same disaccharide units, although the proportions of sulfated monosaccharide residues vary. HS is less sulfated and more heterogeneous than heparin. The biosynthetic process of HS generates enormous structural diversity and leads to various physiological functions through different affinities with a variety of proteins such as growth factors, enzymes, and extracellular matrix components. Numerous studies of the critical role of HS and heparin in biological events have stimulated interest in elucidating the detailed structure of the polysaccharides. HS and heparin, however, are structurally heterogeneous. Further, it is difficult to raise useful antibodies against HS or heparin because these polysaccharides are ubiquitous on the cell surfaces of all animals normally used for antibody production, such as chickens, mice, and rabbits. To gain insights into the functions of HS and/or heparin, it is necessary to obtain specific probes against each sulfation pattern of HS or heparin.

Thus, we decided to use phage display to obtain ligands that specifically target HS and/or heparin structures. Phage display has become a powerful method for identifying polypeptides since it was described by Smith (10). In this method, a peptide is displayed on the N-terminal domain of the phage coat protein (11). To ensure monovalent display of objective genes, phagemid vectors to express peptides on pIII were used in this study (12, 13). This monovalent display allows selection based purely on affinity to the tightest binding variants from a cDNA library.

In this study, we selected heparin because of its characterized structure for further elucidating the structure-function relationship between polysaccharides and proteins. We report the identification of a novel peptide through phage display and its characteristics for discriminating heparin in a structure-specific manner.

EXPERIMENTAL PROCEDURES

Materials—All materials were purchased from Nacalai Tesque (Kyoto, Japan). HappY and its derivative peptides were synthesized by Medical & Biological Laboratories (Nagoya, Japan). The ready-to-use phage display cDNA library of mouse brain was purchased from Spring Bioscience (Fremont, CA). The insert size for all phage display cDNA libraries ranged from 200 to 2000 bp. The insert percentage for all phage display libraries was >70%. The colony-forming units for all phage display libraries were >106 different clones. Heparan sulfate (from bovine kidney), chondroitin sulfate A (from whale cartilage), chondroitin sulfate C (from shark cartilage), and keratan sulfate
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(from bovine cornea) were purchased from Seikagaku Biusiness (Tokyo, Japan). Hyaluronic acid (from rooster comb) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Low-molecular-weight heparin (LMWH) sodium salts were purchased from MP Biomedicals (Irvine, CA) (LMWH<sub>3000</sub>) and Santa Cruz Biotechnology (Santa Cruz, CA) (LMWH<sub>5000</sub>). Alcian blue 8GX was purchased from Fluka (St. Louis, MO).

Purification of Heparin by Affinity Chromatography with Antithrombin III—AT-III was coupled to HiTrap NHS-activated HP as described in the manufacturer’s instructions. Briefly, bovine AT-III (HYPHEN BioMed, Neuville-sur-Oise, France) was dissolved in coupling buffer (0.2 M NaHCO<sub>3</sub>, 0.5 M NaCl) and applied to a HiTrap NHS-activated HP column (GE Healthcare). After incubation at 25°C for 30 min, unbound proteins were eluted by coupling buffer.

Commercial heparin from pig small intestines (ACROS, Geel, Belgium) was incubated together with 4 M ammonium formate and NaBH₄CN at 70°C for 2 days to introduce an amino base at the reducing terminal, and then NaBH₄CN was added and incubated at 70°C for 2 more days. After desalting treatment, reductively aminated heparin was dissolved in binding buffer (10 mM Tris-HCl, 150 mM NaCl, 0.0004% TX-100, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 1 mM MnCl<sub>2</sub> (pH 7.4)) and applied to an AT-immobilized affinity column. After incubation at 25°C for 1 h, unbound heparin, which did not contain the pentasaccharide structure, was eluted by binding buffer. The AT-binding heparin was eluted by elution buffer (10 mM Tris-HCl, 0.0004% TX-100, 0.02% NaN<sub>3</sub> (pH 7.4)) containing 1 M NaCl. A biotin tag was introduced to the primary amino group generated at the reducing end of heparin molecules by EZ-link sulfo-NHS-LC-Biotin (Pierce). Biotinylated heparin was purified by UNosphere Q anion exchange support (Bio-Rad) and then checked by Reacti-Bind streptavidin high binding capacity coated plates (Pierce) to ensure that biotin was covalently bound to heparin.

Immobilization of AT-binding Heparin on Microtiter Plates—Biotinylated heparin was coupled to Reacti-Bind streptavidin high binding capacity coated plates (Pierce) as described in the manufacturer’s instructions. Briefly, each well of the plate was washed with 200 μl of PBS-T (0.025% of Tween 20 in PBS) three times. Biotinylated heparin diluted with 100 μl of PBS-T was added in each well and incubated at 4°C overnight. Then, each well of the plate was washed with 200 μl of PBS-T three times, followed by 200 μl of blocking solution (1% BSA in PBS) being added in each well, and incubated at room temperature for 30 min with shaking. To confirm the immobilization efficiency of heparin onto the plate, each well was stained by Alcian blue 8GX (Fluka) dye solution (5% Alcian blue, 0.018 M H<sub>2</sub>SO<sub>4</sub>, 0.02 M guanidine-HCl, 0.25% Triton X-100), and the absorbance at 620 nm was measured by Immuno Mini NJ-2300 (Nalge Nunc International K. K., Tokyo, Japan).

Screening of Positive Phage Clones by Panning and Evaluation of Their Specificity by ELISA—A mouse brain cDNA phage display library was purchased from Spring Bioscience. The fusion genes generated from mouse brain cDNA and the gene III coat protein of the M13 phage were cloned into the phagemid vector pHD9. Phagemid DNA was packaged in M13 phage particles by superinfection with helper phage and transduced in a single reaction into Escherichia coli strain TG1 (supE hsdS30 thi Δ(lac-proAB) F' [traD36 proAB+ lacIq lacZΔM15]) cells grown in 2X YT supplemented with ampicillin, 0.2% glucose, and 10 mM MgCl<sub>2</sub>. The library was subjected to three rounds of biopanning against immobilized ATB heparin. In the first selection round, 10<sup>13</sup> colony-forming units of phage diluted with 1% BSA in PBS were allowed to react with an ATB heparin-coated plate at 37°C for 1 h. After five washing steps with PBS-T and five washing steps with PBS, captured phage were recovered by the infection of E. coli TG1 cells. In further rounds, 1–5 × 10<sup>11</sup> amplified phages were biopanned against the same heparin. Selected clones were characterized by automated fluorescent DNA sequencing on an ABI PRISM 3100 sequencer using BigDye Terminator ver. 3.1 chemistry (Applied Biosystems, Carlsbad, CA). Primary binding specificity was checked by phage ELISA. Streptavidin-coated microtiter plates were immobilized with ATB heparin and incubated with candidate phages diluted in 1% BSA in PBS at 37°C for 1 h. After five washes with PBS, bound phages were incubated with anti-pVIII antibodies (Spring Bioscience) diluted in 1% BSA in PBS at 4°C overnight. The antibodies were incubated with secondary horseradish peroxidase-conjugated anti-mouse-IgG antibodies (Spring Bioscience) for 1 h and stained with 3,3′,5′,5′-tetramethylbenzidine solution (Spring Bioscience) for 15 min. After the addition of 0.5 M H<sub>2</sub>SO<sub>4</sub>, absorbance at 450 nm was observed as the ELISA signal.

Computer Modeling of the Associated Structure between Happy and the AT-III Binding Motif in Heparin—Molecular modeling with the Spartan modeling software (Wavefunction, Irvine, CA) was carried out to validate the potential association between the peptide and the AT-III binding motif in heparin (GlcN<sub>6</sub>-SO<sub>3</sub>α<sub>1</sub>–4 GlcAβ1–4 GlcN<sub>3</sub>,6-SO<sub>3</sub>α<sub>1</sub>–4 IdoA<sub>2</sub>-SO<sub>3</sub>α1–4 GlcN<sub>6</sub>-SO<sub>3</sub>; structural data from PDB code 2GD4) (14). The peptide was built in silico as β-sheet forms and arbitrarily located around the pentasaccharide heparin structure. A spatial position of the peptide was changed around the pentasaccharide by the Spartan program in order to find the optimal condition that a distance between the nitrogen atom of the peptide and the oxygen atom of the heparin was about 2.5 Å.

Analysis of Molecular Interactions Using SPR—Real-time analysis of the interactions of peptides with ATB heparin was performed with an SPR instrument, Biacore J (GE Healthcare). For binding experiments, the Biacore J was operated at 25°C and HBS-EP running buffer (GE Healthcare) was used for all assays. The running buffer was filtered through a 0.22-μm filter and degassed. The contact time of analytes and ligands was limited to 120 s. The Biacore J was set for a dual-channel flow and detection system so that one channel was used as an in-line reference for sample analysis to enhance the specificity of analyte detection. For the SPR biosensor analysis, 70 μl of peptide solution and/or glycosaminoglycans was injected with a flow rate of “medium.” To make sure that complete dissociation of analytes had occurred, 2 M NaCl was injected at the end of each cycle. Each experiment was repeated at least four times in random order. The K<sub>d</sub> value was calculated using BIAevaluation in the Biacore J program (GE Healthcare).
Degradation of Heparin by Nitrous Acid and Collection of Fragments by Gel Filtration Chromatography—Heparin was cleaved by nitrous acid as reported previously (15). Briefly, 0.25 g of the sodium salt of heparin (Nacalai Tesque) was dissolved in 5 ml of water. Sodium nitrite (19 mg) was added, and the pH of the solution was reduced to 2.5 with concentrated hydrochloric acid. The deaminative cleavage was allowed to proceed for 60 min. The pH of the solution was then raised to 10 with 5 M of sodium hydroxide, and 8 mg of sodium borohydride was added. After stirring overnight, the pH of the solution was reduced to about 3 with concentrated hydrochloric acid to remove the remaining sodium borohydride. After adjusting the pH to 7, the resulting oligosaccharide mixture was purified by dialysis and fractionated by gel filtration with Bio-Gel P-10 (Bio-Rad) tandem-column chromatography (100 × 1.5 cm × 2). The oligosaccharides were eluted with 0.5 M of ammonium carbonate at a flow rate of 0.25 ml/min, and 4 ml of eluate was collected per tube. Concentrations of the oligosaccharides were measured by the carbazole-sulfate method as an uronic acid (16). Fractionated oligosaccharides up to the size of octasaccharide were pooled, desalted, and lyophilized. The oligosaccharide fragments were analyzed by HPLC on a YMC-Pack Diol-60 (YMC Co., Ltd., Kyoto, Japan) column (0.8 × 30 cm) to confirm their size by using an unsaturated HS/heparin-disaccharide mixture (Seikagaku Corp., Tokyo, Japan) and heparin-derived decasaccharide (molecular weight, 3350; Funakoshi Co., Ltd., Tokyo, Japan) as standards. This analysis demonstrated that the oligosaccharides obtained corresponded to the ones expected for di-, tetra-, hexa-, and octasaccharides.

Neutralization of the Anticoagulant Effect of Heparin by Happy—A 25-μl aliquot of heparin solution (0.001 IU/ml) or dilution buffer (25 mM Tris-HCl (pH 7.6), 125 mM NaCl, 0.20% BSA) and 25 μl of Happy or dilution buffer were incubated at 37 °C for 3 min, followed by thorough mixing with 25 μl of AT-III (0.1 μl) or dilution buffer and incubation at 37 °C for 3 min. Then, 25 μl of thrombin (0.75 units/ml, Calbiochem) was mixed and incubated at 37 °C for 3 min. Twenty-five microliters of thrombin substrate II (0.45 mM, Calbiochem) was mixed and incubated at 37 °C for 30 min, followed by addition of 25 μl of glacial acetic acid. Neutralization of the anticoagulant effect of heparin was determined by measuring absorbance at 405 nm with a Tecan Infinite® M200 (Tecan Group Ltd., Männedorf, Switzerland).

Cell Culture—PC-12 cells were obtained from the RIKEN Cell Bank (Tsukuba, Japan). Cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 5% heat-inactivated FBS (PAA Laboratories GmbH, Pasching, Austria) and 10% heat-inactivated horse serum (PAA Laboratories GmbH) in 5% CO2 at 37 °C. Cells were plated onto transparent 96-well microwells precoated with poly-L-lysine (Nacalai Tesque).

PC-12 Differentiation with Growth Factors—The transient 96-well microwells were seeded with 1 × 104 cells per well, and then cells were cultured with the RPMI medium containing both sera. After 24 h, the medium was replaced by RPMI medium with low sera (0.05% heat-inactivated FBS and 0.1% heat-inactivated horse serum), which was supplemented with recombinant human FGF-1 (R&D Systems Inc., Minneapolis, MN) or recombinant human FGF-2 (ReproCELL, Inc., Yokohama, Japan) at 5 or 10 ng/ml to induce differentiation. After 3 days, the culture medium was replaced with new low-serum medium with 5 or 10 ng/ml of growth factors, followed by culture of the cells for more than 2 days. HappyY (10, 100, or 1000 nM) or control peptide was added to the low-serum culture medium, with 5 ng/ml of growth factors for neurite outgrowth evaluation.

Neurite Outgrowth Evaluation—Cells were fixed for 30 min using 4% paraformaldehyde in PBS at room temperature and then were stained with toluidine blue O (Sigma-Aldrich). Morphological changes were observed with an inverted microscope (IX70, Olympus, Tokyo, Japan) with a CCD camera (DP71, Olympus). For the analysis of morphological changes, three random areas per well were selected and photographed. The lengths of neurite and cell body in the image were quantified by ImageJ software (National Institutes of Health, Bethesda, MD). Measurements were performed in triplicate. A sufficient number of parameters were acquired for the analysis of at least 90 cells. The sum of the neurite lengths was divided by the cell numbers, and then the average neurite length per cell was calculated.

RESULTS

ATB heparin and Its Biotinylation—Heparin is well known to be heterogeneous. To obtain a characterized heparin target structure in this study, commercial heparin was applied to an AT-immobilized affinity column. AT-III associates with a specific pentasaccharide structure including 3-O-sulfated glucosamine modified by HS 3-O-sulfotransferase 1 (17). Thus, the heparin molecules extracted by affinity chromatography are expected to contain the 3-O-sulfated pentasaccharide structure. The yield of ATB heparin was determined to be 7.38% by Alcian blue 8GX staining. Furthermore, the biotinylation of ATB heparin prior to purification could also be confirmed by Alcian blue 8GX staining.

Screening of the Heparin-associating Peptide through Phage Display—We previously reported that the structure of HS is spatiotemporally altered in the regulation of various biological activities in the developing brain, including the proliferation of neuronal progenitors, extension of axons, and formation of dendrites (18). Our findings suggest that some proteins in the mouse brain bind to HS in a structure-specific manner. A mouse brain cDNA phage library was thus biopanned against
immobilized ATB heparin on microtiter plates. After three rounds of panning, 39 clones were isolated as positive. We focused on three unique positive clones. Unfortunately, all three did not encode natural proteins expressed in the mouse brain, and in fact they were expressed with reverse orientation in the vector. The resulting two peptides, however, had a consensus region consisting of 12 amino acid residues characterized by four arginines, RTRGSTREFRTG (Fig. 1, A and B). Molecular modeling based on the Spartan modeling software implied that the first three arginines of the peptide participate in binding to pentasaccharide (Fig. 1C) and that the critical interactions involve 6-O-sulfate of the first glucosamine, 3-O-sulfate of the middle glucosamine, and 6-O-sulfate of the third glucosamine in pentasaccharide. This peptide has a novel amino acid sequence and is a heparin-binding ligand candidate. We designated this peptide heparin-associating peptide Y, or HappY.

**Interaction Analysis between HappY and ATB Heparin by SPR**—To gain insights into the specificity of the interaction between HappY and ATB heparin, both the original HappY and its derivative peptides were synthesized, and interactions with ATB heparin were investigated with Biacore, a surface plasmon resonance (SPR)-based instrument. First, ATB heparin was immobilized on the surface of a streptavidin-coated sensor chip. To confirm immobilization of heparin on the sensor chip, AT-III was applied as a positive control analyte. As a result, we confirmed that AT-III was strongly bound to the heparin (data not shown), and the $K_D$ value was calculated to be 7.66 nM. Muñoz et al. (19) reported that the $K_D$ value of interaction between AT-III and heparin is 8.05 nM, suggesting that ATB heparin was certainly immobilized onto the sensor chip.

Concentration-dependent HappY peptide binding was confirmed by assaying four different concentrations of peptide (44, 110, 220, and 440 μM) with the ATB heparin-immobilized sensor chip (Fig. 2A). The response gradually strengthened with increasing peptide concentration, indicating that HappY bound to the ATB heparin immobilized on the chip surface in a concentration-dependent manner. Based on molecular graphics modeling, the first three arginines within the peptide should participate in the binding to the AT-III-associated pentasaccharide in heparin (Fig. 1C). Hence, we prepared a peptide in which all the arginines of HappY were substituted with alanines and applied the resulting peptide to ATB heparin on the chip. There was no interaction between the substituted peptide and ATB heparin in the same concentration range as for HappY (Fig. 2B). When only the fourth arginine from the N terminus of HappY was substituted with alanine (Arg4Ala), the injection of 110 μM of this peptide resulted in a small decrease in response compared with the original HappY, but it resisted dissociation from the chip as well as HappY (Fig. 2C). In contrast, biotinylation of the N-terminal arginine of HappY (N-Biot) resulted in no interaction with ATB heparin. The N-Biot peptide com-
weights for the other glycosaminoglycans were also variable, the same weights (196 μg/ml) of other glycosaminoglycans were used for the structural specificity test.

The structure of HS is similar to that of heparin, although the number of sulfated residues is much less than that in heparin, suggesting that HS contains fewer AT-III-association sites. Thus, HS was less competitive than heparin (Fig. 3B). The other glycosaminoglycans, chondroitin sulfate A, chondroitin sulfate C, and keratan sulfate, were not competitive with the HappY peptide (Fig. 3C). To confirm these data, the HappY peptide was immobilized on sensor chip CM5 (GE Healthcare). The immobilization level of the HappY peptide onto the surface of the flow cell was 2134 resonance units, corresponding to 2.67 ng of the HappY peptide bound to the surface of FC1. Then, glycosaminoglycans were applied to the chip as an analyte (data not shown). Heparin was specifically associated onto the peptide chip and saturated at a concentration of 50 μg/ml. Furthermore, HS was also specifically associated onto the chip, although the sensitivity was 10 times weaker than heparin. In contrast, keratan sulfate, chondroitin sulfate A, and chondroitin sulfate C did not associate onto the chip at all. Taken together, these results suggested that HappY bound to a specific structure that is characteristic in heparin.

The HappY Recognition Domain of Heparin—AT-III binds to pentasaccharide in heparin, which has the characteristic structure GlcN,6-SO₃α₁—4GlcAβ₁—4GlcN,3,6-SO₃α₁—4idoA₂-SO₃α₁—4GlcN,6-SO₃. Because HappY was found to be a peptide for binding to ATB heparin, we hypothesized that the HappY peptide also recognized the same pentasaccharide motif in heparin. To reveal which region in heparin is recognized by HappY, heparin was digested by nitrous acid and then fractionated by gel filtration chromatography (Fig. 4A). The oligosaccharides fractionated by HPLC were confirmed to be di-, tetra-, hexa-, and octasaccharides. HappY (155 μg/ml, 110 μM) and the oligosaccharides (19.6 μg/ml corresponding to 11 μM of ATB heparin) were applied to the ATB heparin chip competitively. As a result, disaccharide was not competitive with the HappY peptide, whereas tetra-, hexa-, and octasaccharides competed efficiently for binding, depending on the length of the saccharide chains (Fig. 4B). Nitrous acid treatment results in conversion of a GlcN-SO₃ residue at the reducing ends of products into an artificial structure, 2,5-anhydro-β-mannose. Thus, the fraction of the tetrasaccharide produced by nitrous acid contains three native saccharide residues. Taken together, these results indicated that HappY recognized trisaccharide in heparin as the minimum binding partner, in contrast to AT-III.

Specific Recognition of HappY for ATB Heparin—To demonstrate that the ATB heparin specifically binds the HappY peptide, the competitive interaction analysis between a mixture of no-affinity fraction (ATU heparin), isolated from AT affinity chromatography, and HappY was performed by SPR (Fig. 5A). HappY (155 μg/ml, 110 μM) and the ATU heparin or unfractionated heparin were applied to the ATB heparin chip competitively. As a result, ATU heparin was less competitive than unfractionated heparin. In contrast, HappY and ATB heparin were much more competitive than unfractionated heparin, suggesting that ATB heparin specifically binds the HappY peptide. Furthermore, two kinds of LMWH were investigated for

### FIGURE 3. Specific recognition of HappY for heparin and HS

A, mixtures of HappY (110 μM) and various concentrations of heparin were injected onto an ATB heparin-immobilized sensor chip. B, mixtures of HappY (110 μM) and 196 μg/ml of HS or heparin were injected onto the chip. C, mixtures of HappY (110 μM) and 196 μg/ml of chondroitin sulfate A (CS-A), keratan sulfate (KS), or chondroitin sulfate C (CS-C) were injected onto the chip. The samples were injected for 120 s, and then the dissociation phase began.

**A**

- HappY (110 μM)
- Heparin 0, 0.0196, 0.196, 1.96, 19.6, and 196 μg/ml

**B**

- HappY (110 μM)
- HS 196 μg/ml
- Heparin 196 μg/ml

**C**

- HappY (110 μM)
- CS-A 196 μg/ml
- CS-C 196 μg/ml

**Specific Recognition of HappY**—Because the molecular weight of ATB heparin is variable, we considered it as a population of pentasaccharides with the AT-III binding motif and calculated the molarity of ATB heparin. The specific recognition of HappY for a structure of heparin and/or other glycosaminoglycans was competitively investigated against immobilized ATB heparin on a chip using the Biacore system. HappY competitively bound to ATB heparin against applied heparin in a concentration-dependent manner (Fig. 3A). We then found that 196 μg/ml of heparin (corresponding approximately to 110 μM of AT-III binding pentasaccharide) was efficiently competitive for 155 μg/ml of HappY, corresponding to 110 μM against immobilized ATB heparin (Fig. 3A). Because the molecular
The competitive binding test (Fig. 5B). Each of the LMWH was fractionated by a different size: LMWH₃₀₀₀, average molecular weights of 3000; LMWH₅₀₀₀, average molecular weights of 5000. LMWH₅₀₀₀ was more binding to HappY than LMWH₃₀₀₀ against immobilized ATB heparin on a chip using the Biacore system.

Neutralization of the Anticoagulant Effect of Heparin—If the trisaccharide in heparin recognized by the HappY peptide is a part of the pentasaccharide in the AT-binding domain, the peptide should affect the anticoagulant activity of heparin. AT is a serine protease inhibitor that inactivates several members of the blood coagulation cascade, the most important of which are thrombin and factor Xa. AT is not an effective inhibitor of the protease unless it first binds to heparin. HappY caused a dose-dependent increase in thrombin activity, probably by hampering the formation of the heparin-AT complex (Fig. 6). HappY thus discriminates the trisaccharide in the AT-binding domain of heparin.

Inhibition of Neurite Outgrowth Induced by the Fibroblast Growth Factor in PC-12 Cells—To consider the possibilities of other biological functions for the HappY peptide, inhibition of neurite outgrowth induced by fibroblast growth factors was investigated (Figs. 7 and 8). Acidic (FGF-1) and basic (FGF-2) fibroblast growth factors promote stable neurite outgrowth in cultures of PC-12 cells (20). Effect of FGF-1 on neurite outgrowth in PC-12 cells was increased by the presence of heparin (Fig. 7A). As shown in Fig. 7B, the biological function by FGF-1 was not affected by any concentrations of HappY, even if heparin was added to the culture medium. In contrast, the neurite outgrowth in PC-12 cells by FGF-2 was significantly inhibited by HappY (Figs. 7C and 8D). On the other hand, the control peptide, in which all the arginines of HappY were substituted with alanines and there was no binding activity to ATB heparin.
did not affect the neurite outgrowth in PC-12 cells by FGF-2 (Figs. 7C and 8C). Thus, HappY would play a role in several biological functions through the interaction with heparin and HS.

DISCUSSION

We describe a novel peptide, HappY, which binds to a specific structure of trisaccharide in heparin isolated via phage display. Our computer modeling showed that the peptide binds to ATB heparin through three arginine residues. In particular, the N-terminal arginine residue of the peptide is critical for binding to ATB heparin because the biotinylation of the N-terminal arginine residue, which was mainly labeled at the α-amino group, abolished association with the polysaccharide completely (Fig. 2C). On the basis of a binding experiment for the binding domain of ATB heparin to HappY using fractionated oligosaccharides (Fig. 4B) and for the two LMWH to HappY (Fig. 5B), we concluded that a trisaccharide in heparin was recognized by the peptide as the minimal region. For the neutralization of the anticoagulant effect of heparin by the HappY peptide, the peptide may bind to the unique pentasaccharide region of heparin required for specific binding to AT-III, or the region of heparin that is recognized by the heparin-binding site of thrombin, also known as TABE2 (thrombin anion binding exosite 2) (21). To distinguish the two possibilities for binding, the binding between LMWH and HappY was investigated (Fig. 5B). LMWH has less of an anticoagulant effect on thrombin compared with heparin but maintains the same effect on factor Xa, which means that LMWH contains the unique pentasaccharide region (22). Our results showed that LMWH competitively bound to HappY, suggesting that the target of HappY is the pentasaccharide region of heparin. In addition, the fact that LMWH_{5000} competitively bound to HappY more than LMWH_{3000} (Fig. 5B) would indicate that the recognition domain in heparin by HappY should be consecutive monosaccharide residues. Furthermore, the fact that HappY exclusively bound to heparin or HS (Fig. 3, B and C), competitively bound to heparin than LMWH_{5000} (Fig. 5B), did not affect the neurite outgrowth in PC-12 cells by FGF-2 (Figs. 7C and 8C). Thus, HappY would play a role in several biological functions through the interaction with heparin and HS.
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tively bound to AT heparin unlike ATU heparin (Fig. 5A), and neutralized the anticoagulant effect of heparin (Fig. 6) also indicates that it recognizes the trisaccharide, including an iduronic acid residue, that exists in both heparin and HS. Therefore, these results strongly support the hypothesis that the trisaccharide GlcN,3,6-SO₃,α1→4IdoA,2-SO₃,α1→4GlcN,6-SO₃, in heparin and HS is the target domain for HappY.

Cardin and Weintraub (23) reported potential heparin-binding sites based on the sequence organizations of basic and non-basis residues from 49 regions in 21 proteins. Consensus sequences for heparin recognition were determined as X-B-B-X-B-X-B and X-B-B-B-X-B-X-B, where B is the probability of a basic residue and X is a hydrophobic residue. The sequence of the heparin-binding peptide obtained in this study was RTRG-X, resulting in B-heparin-binding peptide obtained in this study was RTRG-X-B.

Verrecchio et al. (24) reported that heparin-binding peptides were designed based on consensus sequences, (AKKARA)_n and (ARKKAAKA)_n (n = 1–6), and then higher Mₚ peptides (2000–3500) exhibited significant affinities (K_D ≈ 50–150 nM), which increased with peptide Mₚ. Meanwhile, HappY is a low Mₚ peptide (Mₚ = 1423) but showed significant affinities. Furthermore, Tashiro et al. (25) also reported that IKLLI-containing peptides were found to mediate heparin binding. Unlabeled heparin and HS inhibited the peptide-mediated [³H]heparin binding competitively in a dose-dependent manner. The IC₅₀ for inhibition of heparin-peptide binding was observed with 9 μM heparin/HS, and chondroitin sulfate did not inhibit it. In addition, Schuksz et al. (26) reported that the small molecule antagonist of heparan sulfate, surfen, bound to glycosaminoglycans, and the extent of binding increased according to charge density in the order heparin > dermatan sulfate > heparan sulfate > chondroitin sulfate. Surfen also inhibited FGF-2-stimulated sprouting by endothelial cells with an IC₅₀ of ≈ 5 μM. On the other hand, HappY-mediated AT heparin binding was inhibited by 196 μg/ml of heparin in almost 50% inhibition (Fig. 3A). This concentration corresponds approximately to 16 μM (average mass of heparin, 12 kDa). HappY also showed an approximately 50% inhibition of neurite outgrowth induced by FGF-2 in PC-12 cells with ≈ 1 μM (Fig. 7C). Taken together, our data demonstrate that HappY has a high potency comparable with the known materials.

As shown in Fig. 1C, three arginine residues in HappY seem to lie in a plane and face the sulfate groups of heparin when interacting with the AT-binding domain. Both the specific heparin pentasaccharide sequence and the AT polypeptide sequence that participate in the interaction were reported previously (27, 28), and docking of the pentasaccharide between two peptide segments of AT has been demonstrated. This binding interaction is electrostatic, arising from the negatively charged sulfate and carboxyl groups of the pentasaccharide and the positively charged lysine and arginine side chains of the protein. It has been revealed that Arg-47, Lys-114, Lys-125, and Arg-129 are the most important residues in the heparin-binding site of AT, using a chemically modified, naturally occurring mutant and recombinant AT (29–32). In addition, the Ala-124-Arg145 sequence, found in the D helix of AT, is rich in basic amino acids, including lysines (125, 133, and 136) and arginines (129 and 132) that face the heparin pentasaccharide (27, 28). Furthermore, Onoue et al. (33) reported that human AT-III-derived heparin-binding peptide is a novel heparin antagonist in which lysines are substituted with arginines. We have deduced that HappY interacts with heparin through an electrostatic association between the positively charged arginines and negatively charged saccharides. Therefore, HappY associates with its target molecule in a structure-dependent manner, resulting in specific binding to heparin and/or HS.

AT associates with a specific pentasaccharide structure that has 3-O-sulfated glucosamine modified by HS 3-O-sulfotransferase 1 (17). In this study, HappY associated with the trisaccharide structure, including the 3-O-sulfated glucosamine residue. 3-O-sulfation of α-glucosamine residues is the rarest modification in HS/heparin biosynthesis and is a key regulator for generating HS-unique sequences that are believed to define specific protein interaction patterns. Thus, the activities of several effectors associated with HS are influenced by selective binding to 3-O-sulfated HS motifs. Other than AT, the envelope glycoprotein D of the herpes simplex virus type 1 has also been found to bind to the 3-O-sulfated HS on target cells (34). Furthermore, functions that are accelerated by 3-O-sulfated HS have recently been reported, such as binding to fibroblast growth factor 7 (34) and to a receptor for fibroblast growth factors (36), the predominant restriction of daytime pineal glands (37), regulation of Notch signaling (38), and interaction with cyclophilin B binding to responsive cells (39). This modification of HS is very difficult to detect in vivo because it is the rarest modification for HS biosynthesis. HappY has the potential to detect this functional moiety of HS in the near future.

Heparin has also shown strong anti-inflammatory effects apart from its anticoagulant activity, including the inhibition of complement activation (40, 41) and inhibition of neutrophil-derived elastase (42). Although heparin has great potential as an anti-inflammatory agent, its clinical use is impaired by its strong anticoagulant activity and hemorrhagic complications. To overcome this problem, some chemical modifications from mammalian heparin have been developed (43, 44), and a heparin-like compound from shrimp was reported to be a better alternative than mammalian heparin as a possible anti-inflammatory drug (45). The fact that HappY neutralizes the anticoagulant effect of heparin (Fig. 6) makes it potentially useful for facilitating the anti-inflammatory effects of heparin without the anticoagulant effects. HappY may contribute to the development of treatments for chronic and acute inflammatory diseases.

In PC-12 cells, agrin, HS proteoglycan, increases the efficacy of FGF-2 stimulation of neurite outgrowth mediated by the FGF receptor (46). The possible mechanisms by which agrin may modulate neurite outgrowth had been investigated, analyzing ERK phosphorylation and c-fos phosphorylation. HappY inhibited the neurite outgrowth in PC-12 cells by FGF-2 (Fig. 7). This result suggests that HappY binds to HS attached at the agrin and affects neurite outgrowth.

A major advantage of the phage display system is that amino acid sequences of exogenous peptides expressed on the phage
envelope are easily available after screening. Because almost no glycosaminoglycans are immunoreactive and there are few antibodies against them, this advantage is extremely useful for generating probes that bind to glycosaminoglycans. Thus, phage display technology is increasingly important for studying the functions of glycosaminoglycans (47–52). The generation of type-specific anti-HS antibodies using phage display technology was initially applied by van Kuppevelt et al. (53) for investigating HS heterogeneity in the kidney. In their study, three different phage clones expressing anti-HS single-chain variable fragment antibodies were obtained by a human semi-synthetic phage library instead of single-chain variable fragment antibodies in our phage display method. Thus, peptides of various sizes that bound to heparin were expected through our method. Recently, some physiological functions regulated by interactions between proteins and glycosaminoglycans have been reported (for reviews, see Refs. 54–56), and phage display will also be useful for finding novel proteins with these features. Furthermore, if peptides that discriminate the different structures of heparin from HappY or the structures of other glycosaminoglycans are generated through this technique, they should facilitate clinical and basic studies of glycosaminoglycans. In the near future, such peptide tools will make a contribution to studying diseases that are caused by alterations of the structure of glycosaminoglycans in tissues and will potentially be used in diagnosing such diseases.

In conclusion, the HappY peptide, which discriminates the specific structure of the trisaccharide GlcN,3,6-SO₄α1–4IdoA,2-SO₄α1–4GlcN,6-SO₄ in heparin and HS, can be used as a probe to detect the structure of heparin and/or HS. Further studies on synthetic trisaccharides of similar structure will be needed to discover the structural specificity with which HappY binds to heparin and/or HS.

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