Pseudoproteoglycan (pseudoPG) probes that simulate PG macromolecular structure for screening and isolation of PG-binding proteins

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A proteoglycan (PG) monomer is a macromolecule consisting of one or more glycosaminoglycan (GAG) chains attached to a core protein. PGs have signaling roles and modulatory functions in the extracellular matrix and at the cell surface. To elucidate the functions of higher-order PG structures, pseudoPGs that imitate the PG structure were prepared to develop probes and affinity adsorbents. Poly-L-lysine (PLL) or polyacrylamide (PAA) was coupled with various GAGs, then biotinylated, and the remaining amino groups were blocked to obtain the pseudoPG probes, biotinyl PLL (BPL)- or PAA (BPA)-GAGs. Lactoferrin exhibited 30-times higher affinity toward BPL-heparin than the conventional single-strand probe, biotin-hydrazide-heparin.

Heparin-PLL was immobilized on a formyl-Sepharose and compared with the Hep-Sepharose in which heparin was directly immobilized to amino-Sepharose. Screening for ligands in normal rat brain revealed several proteins that specifically bound to either of the two adsorbents, indicating that the heparin-binding proteins exhibit specific recognition depending on the higher-order structure of the PG.

Introduction

Proteoglycan (PG)s play signaling roles and modulate cell functions in important cellular events such as cellular proliferation, differentiation, adhesion and motility, morphological formation, coagulation, and infection (1,2). The structures and number of the glycosoaminoglycan (GAG) chains, in addition to the nature of the core protein, are considered to determine the type of PG function (3). However, information about the significance of the proteoglycan macromolecular structures still remains inadequate. We prepared synthetic probes that simulate the structure of PGs by attaching GAG side chains to a linear polymer strand as the backbone to develop probes and affinity adsorbents to search for and identify PG-binding substances. The new probes were named ‘pseudoproteoglycans (pseudoPGs)’ (see Scheme 1). In this study, pseudoPGs using five kinds of GAGs centering on heparin were prepared, and they were applied to screen for and analyze interactions with ligand proteins in extracts of rat brain.

Recently, significant roles for various proteoglycans in the development, differentiation and morphogenesis of neural tissues have been demonstrated using genetically modified animals (4,5). The protein ligands that specifically recognize

proteoglycans would be of importance to understand the mechanisms of proteoglycan function, and therefore, we tried to identify specific protein ligands in brain extracts using pseudoPGs. The pseudoPGs exhibited remarkable affinity and binding specificity toward several heparin-binding proteins compared to the conventional single-strand heparin probe. The utility of the pseudoPG probes and the significance of the higher-order structure of GAGs in the recognition of PG-binding proteins were shown.

Materials and Methods

Materials – Poly-L-lysine (PLL) with a Mw 15,000-30,000, bromide salt, and streptavidin-biotinylated horseradish peroxidase (HRP-ABC) complex were purchased from Sigma (St. Louis, MO, USA). Polyacrylamide (PAA) with an average Mw 10,000 was purchased from Aldrich (Sigma-Aldrich Japan, K.K., Tokyo, Japan), and PAAs of Mw 600,000-1,000,000 were from Polysciences Inc. (Warrington, PA, USA). Porcine intestinal mucosa heparin (Hep), *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), and unlabelled biotin were purchased from Wako Pure Chemicals (Osaka, Japan). *N*-Hydroxysuccinimide biotin and biotin-LC-hydrazide from Pierce (Rockford, IL, USA) were used for biotin labeling of the probes. Chondroitin sulfate A (CSA) and chondroitin sulfate B (CSB) were purchased from Seikagaku Kogyo (Tokyo, Japan). Colominic acid sodium salt was purchased from Nacalai Tesque Co. (Kyoto, Japan). D-Glucuro-γ-lactone was purchased from Merck Japan (Tokyo, Japan). A pullulan mixture (average Mr= 5,900-788,000 Da) as a standard for size-exclusion HPLC was purchased from Showa Denko Co. (Tokyo, Japan). Rabbit anti-human cyclophilin A

(CypA) antibodies were purchased from Upstate Group Inc. (Charlottesville, VA, USA), and HRP-labeled goat anti-rabbit IgG was from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD, USA).

Preparation of pseudoPG probes – PLL (8 mg) was dissolved in 1 ml of distilled water. To 250 μl of the PLL solution, 80 μl of 0.5 M phosphate-buffer (PB) and 6 mg of Hep or another GAG were added and mixed, then 210 μl of 4 M NaCl was added to dissolve the precipitate. $NaBH_3CN$ solution (10 mg per ml of distilled water) was prepared, and 20 μl was added to the mixture and incubated at 40°C for 9 days with shaking. After the reaction, the mixture was dialyzed against 0.1 M CH_3COONa to remove the reducing agent. If precipitation occurred, 2 M NaCl was added to the mixture to dissolve the precipitate. Then 200 μl of 0.1% sulfo-*N*-hydroxysuccinimide (NHS-)biotin was added, and the mixture was incubated at room temperature for 1 h with shaking (PLL-GAG). For acetylation of the remaining amino groups, 100 μl of acetic anhydride was added to the reaction mixture and incubated for 30 min on ice, then an additional 100 μl was added, and incubated at room temperature for 30 min with shaking to obtain the pseudoPG biotinyl poly-L-lysine (BPL)-GAG probe (Scheme 1 (A)). Then the mixture was dialyzed using a Spectra/Por CE dialysis tube (MWCO: 1,000, Spectrum, Gardena, CA, USA) against 1 M sodium phosphate buffer (pH 7.7). The coupling was monitored by the change in the migration position on SDS-PAGE in which GAG and PLL were detected by silver staining, or by cellulose acetate membrane electrophoresis in which GAG was detected by toluidine blue and PLL was detected by

Coomassie brilliant blue (CBB) staining (6). A BPL-glucuronic acid probe was prepared according to the same procedure by using D-glucurono-γ-lactone that had been dissolved in 0.01 M NaOH, then neutralized with dilute HCl to open the lactone ring. As a control, a BPL probe was prepared without the GAG coupling step.

Size-exclusion HPLC – To measure molecular mass (Mr), BPL-Hep was analyzed using an LC-10A HPLC system (Shimadzu, Kyoto, Japan) equipped with a refractive index (RI) detector, UV detector (abs. 220 nm), and multiangle laserlight scattering (MALLS) by Dawn DSP (Wyatt Technology Co., Ltd., Santa Barbara, CA, USA). TSKgel α-6000 (7.8 x 300 mm, Tosoh, Japan), Ultrahydrogel 500 and 250 (7.8 x 300 mm, Waters, Milford, MA, USA), and Protein KW802.5 (8 x 300 mm, Shodex, Tokyo, Japan) were selected as the gel filtration chromatography column, and some of them were tandemly connected to improve peak separation. The temperature of the column oven was set at 40°C, and 0.067 M PB (pH 7.4) was used as the mobile phase at a flow rate of 1 ml/min. The Mr was also calculated from comparison with the retention time of the pullulan standard mixture in the HPLC system.

Preparation of biotin hydrazide-Hep – A single-chain heparin probe, biotin-hydrazide-Hep (Bio-Hep) was prepared by using biotin-LC-hydrazide as described for $GlcNAc_{5\text{-}6}$ (7). Heparin (4 mg) was dissolved in 2 ml of 0.1 M sodium acetate (pH 5.5) and mixed with 1/9 vol. of biotin-LC-hydrazide solution (4 mg in 216 μl DMSO). The mixture was incubated at room temperature for 4 days with shaking

(Scheme 1 (B)). The Bio-Hep probe was dialyzed against distilled water.

Immobilization of pseudoPG probe onto affinity adsorbent – A pseudoPG adsorbent, Hep-PLL-Sepharose, was prepared by immobilizing Hep-PLL to formyl-Sepharose. Hep-PLL was reductively aminated by reacting 32.4 mg of Hep and 10.8 mg of PLL as described above. The Hep-PLL was dialyzed against 1 M sodium phosphate buffer and immobilized on 10 g of formyl-Sepharose (8) by adding 60 mg $NaBH_3CN$ at 4°C for 6 days. The water-washed gel was incubated in 20 ml of 1 M Tris-HCl (pH 7.4), and 60 mg $NaBH_3CN$ was added to block the remaining formyl groups at room temperature for 1 hour with shaking. After washing, the gel was suspended in 5 ml of 0.2 M sodium acetate, acetylated by adding 2.5 ml of acetic anhydride, and incubated for 30 min on ice; then, an additional 2.5 ml was added and incubated at room temperature for 30 min with shaking to block the remaining amino groups (Scheme 1 (C)). The amount of heparin immobilized was 1.02 mg/g wet gel as calculated from the hexosamine concentration after hydrolysis, which was measured according to the method previously described (9). For a precolumn for Hep-PLL-Sepharose, PLL-Sepharose was prepared by coupling PLL with formyl-Sepharose by reductive amination at 4°C for 7 days by the same procedure as described above for Hep-PLL.

Preparation of Hep-Sepharose – Hep was immobilized via its carboxyl groups to amino-Sepharose by a condensation reaction with EEDQ. Hep (200 mg) was dissolved

in 6 ml of distilled water, and EEDQ (200 mg) was dissolved in 4 ml EtOH. The heparin and EEDQ solutions were added to 10 g amino-Sepharose (10), and the mixture was incubated at 40°C for 3 days with shaking. Then the gel was washed with water and the remaining amino groups were blocked by *N*-acetylation using the same procedure as that for Hep-PLL-Sepharose (Scheme 1(D)). The amount of heparin immobilized was calculated to be 7.2 mg per g wet gel according to the method described above (9). Acetoamido-Sepharose was prepared by N-acetylation of amino-Sepharose without coupling to heparin.

Preparation of rat brain extract – Whole rat brain tissues were homogenized with a homogenizer in 4 volumes of 10 mM Na-phosphate buffer (pH 7.7) containing 0.13 M NaCl, 0.02% NaN_3, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 20 mM ε-aminocapronic acid, and extracted at 4°C overnight with shaking. The homogenate was centrifuged at 20,500 g for 50 min at 4°C. The supernatant thus obtained was used as the rat brain extract.

Affinity chromatography – Rat brain extract (8 ml) was applied to a Hep-PLL-Sepharose column equipped with a precolumn of PLL-Sepharose or a Hep-Sepharose column equipped with a precolumn of Sepharose that had been equilibrated with 10 mM Na-phosphate buffer (pH 7.7)-0.13 M NaCl. The bound proteins were eluted stepwise with 10 mM Na-phosphate buffer (pH 7.7) containing 0.5 M NaCl, and then 2 M NaCl. The effluent was monitored by the absorbance at 280 nm.

SDS-PAGE and identification of proteins – SDS-PAGE was carried out as described by Laemmli (11) under reducing or nonreducing conditions. The electrophoresed proteins (10 μg/lane) were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA) in 100 mM Tris-HCl (pH 7.5) containing 192 mM glycine and 20% MeOH at 100 mA for 100 min at room temperature. The blotted membrane was stained with CBB. Direct N-terminal sequencing of each protein on the excised PVDF membrane was performed using a protein sequencer, Procise cLC 492cLC (Applied Biosystems). Proteins were identified by searching the N-terminal 4-10 amino acid sequences in the SWISSPROT database. For immunodetection with anti-CypA antibodies, the electroblotted PVDF membrane was blocked with 5% bovine serum albumin-Tris buffered saline (BSA-TBS) at 4°C overnight, and reacted with rabbit anti-human CypA antibody (1:5000 dilution with TBS) for 1 h. After washing three times with TBS containing 0.1% Tween 20, the membrane was blocked with 5% BSA-TBS for 30 min and reacted with HRP-labeled anti-rabbit IgG antibodies for 2 h. After washing with TBS-Tween 20, the membrane was reacted with HRP-ABC (1:1000 dilution with TBS) for 2 hrs. After washing three times with TBS-Tween 20, the color was developed with 0.02% 3,3'-diaminobenzidine (DAB)-0.02 % H_2O_2-TBS.

Probe-binding assay on membrane – The blotted membrane with proteins electrotransferred from polyacrylamide gels was blocked with 5% BSA-TBS overnight

and incubated with each probe diluted to 24 μg/ml of GAG with 130 mM sodium phosphate buffer for 1.5 h at 4°C. The membrane was washed three times with TBS containing 0.1% Tween 20, then blocked with 5% BSA-TBS for 30 min, and reacted with HRP-ABC (1:1000 dil. with TBS) for 2 h. Further washing and color development were performed as described above for immunoblotting.

Quantification of interactions between probes and various heparin-binding proteins by SPR – Quantitative interaction analyses were performed with a BIAcore2000 surface plasmon resonance (SPR) biosensor. The SA sensorchip, which is streptavidin covalently immobilized on a carboxymethylated dextran matrix (GE Healthcare Bio-Science Corp., Piscataway, USA), was equilibrated with 10 mM HEPES-buffered saline (pH 7.4), 3 mM EDTA, and 0.005% Surfactant P20 (HBS-EP), and then activated with three consecutive 2-min injections of 40 μl activating buffer (1 M NaCl and 50 mM NaOH) prior to the immobilization. The flow cells were saturated with a 2-min injection of a biotinylated probe, BPL-Hep or biotin-hydrazide-Hep (each 10 μg/ml in HBS-EP as heparin concentration), at a constant flow rate of 10 μl/min at 25°C and were blocked with sulfo-NHS-biotin (10 μg/ml). The reference flow cell was prepared with BPL for BPL-Hep or biotin for biotin-hydrazide-Hep as a ligand and used for correction of the non-specific binding of analytes to the sample flow cell.

To measure the binding curves, three known heparin-binding proteins, lactoferrin, thrombin, and anti-thrombin III (AT-III), were separately injected onto the sensor chip at various two-fold dilutions in HBS-EP for 120 s at a flow rate of 20 μl/min at 25°C.

After the injection of each HBP concentration, binding surfaces were regenerated with two consecutive 30-s injections of 10 mM HCl. Kinetic parameters were calculated by global analysis using the BIAevaluation software version 3.1.

Results

Preparation of probes – The electrophoretic mobility of PLL to the anode, which was detected with CBB, on the cellulose acetate membrane was reduced by the coupling of PLL with D-glucuronic acid by reductive amination. Heparin migrated to a higher molecular weight position on SDS-PAGE due to the coupling of PLL with heparin, as detected with toluidine blue (data not shown). After N-acetylation, the BPL-Hep was subjected to MALLS on size-exclusion HPLC. As shown in Fig. 1(A)-(D), as the molar ratio of PLL to heparin increased, the area ratios of peak 1 to that of the heparin peak (peak 2) increased, indicating that peak 1 corresponds to BPL-Hep and peak 2 to unreacted heparin. Similar results were obtained for BPL-CSA (data not shown). PLL before N-acetylation was completely adsorbed onto the HPLC columns of Shodex KW and TSK gel, while unconjugated but N-acetylated PLL was detected at peak 2 with a smaller refractive index (RI) signal than that of heparin. Using the four columns in tandem, the void volume (V_o) and total volume (V_t) were calculated according to the manufacturer's instructions to be 24 ml and 46 ml, respectively.

Although an elevation of the molecular weight of BPL-Hep compared to that of heparin was suggested by SDS-PAGE, the yield of BPL-Hep was considered to be small because the refractive index (RI) of peak 1 was scarcely detected. Therefore, the

absolute molecular weight of BPL-Hep could not be determined by SEC-MALLS, but the relative molecular mass was calculated from the elution position of peak 1 using pullulan as a standard. As shown in Fig. 1(E), it was 160-230 kDa. The molecular mass of free heparin was calculated by the elution position to be 24.5±1.3 kDa, and 17.8 kDa from MALLS and RI signals, respectively, by using ASTRA software (Wyatt Technologies Inc.). Because the molecular mass of PLL was 15-30 kDa according to the software results, 5-12 heparin chains were immobilized on a PLL chain in BPL-Hep. The reaction mixtures containing BPL-Hep or BPL were dialyzed against the buffer and used for immobilization onto the sensorchip by biotin-avidin interaction.

Interactions between pseudoPG probes and heparin-binding proteins by SPR – The total amounts of immobilized BPL-Hep, BPL, and biotin-hydrazide-Hep corresponded to 350, 260, and 130 Biacore resonance units (RU, 1000 RU = 1 ng/mm^2), respectively. The bulk effect changes in RU induced by binding of the analytes to the probe-immobilized flow cell were corrected by subtracting the changes of the respective control flow cells. This correction removed the interference by non-specific binding of the analytes to the backbone PLL or biotin moiety of the probes at the same time. AT-III, thrombin, and lactoferrin concentration-dependently bound to BPL-Hep (Fig. 2) and biotin-hydrazide-Hep, but they did not bind to BPL and biotin (data not shown). The association rate constant *(ka*), dissociation rate constant (*kd*), and dissociation constant (Kd) were calculated and are summarized in Table 1. Among the samples, β-lactoferrin bound best to the BPL-Hep-immobilized flow cell with a Kd of 1.2 x 10^{-9} M^{-1}, which is

forty-fold lower than the Kd of biotin-hydrazide-Hep, 4.4 x 10^{-8} M^{-1}. Thrombin bound to the immobilized BPL-Hep at a Kd that was 3-fold lower than the Kd of biotin-hydrazide-Hep. While AT-III showed considerable binding to BPL-Hep, it bound very little to biotin-hydrazide-Hep, and the fitting to calculate the parameters was low. The low *kds* of BPL-Hep to β-lactoferrin and AT-III indicate that the bound β-lactoferrin and AT-III only slowly dissociate from the BPL-Hep probe, which caused the high affinity of pseudoPG. These results indicate that the heparin-binding proteins examined showed remarkably higher affinity to the Hep-pseudoPG probe than that to the single-chain heparin probe.

Membrane detection of GAG-binding proteins – To examine whether the binding specificity depends on the glycan moiety or skeletal polymer, pseudoPG probes containing various GAGs were applied to the detection of binding proteins in brain extract from rats that had been denatured by SDS-PAGE and electroblotted onto a PVDF membrane. As shown in Fig. 3, each probe showed differential binding to many proteins. The BPL-Hep probe bound to several bands distinct from those of the biotin hydrazide-Hep probe, except that some proteins commonly bound to both probes. The BPL-Hep probe showed higher staining intensity at the same heparin concentration, probably because it had higher affinity in addition to a higher biotin concentration than the biotin-hydrazide-Hep probe. BPL-chondroitin sulfates A and B and BPL-colominic acid probes showed similar binding patterns, which were quite different from those of Hep probes and the BPL-glucuronic acid probe. Only endogenous avidin was detected

with a control BPL probe, at a position corresponding to 68 kDa, indicating that BPL did not bind to any of the proteins on the membrane. Essentially the same reactivity was observed between the heparin pseudoPGs of different skeletal polymers, BPL-Hep and BPA-Hep.

Identification of pseudoPG-binding proteins by affinity chromatography – The binding specificities of pseudoPG for native proteins were compared by affinity chromatography using the immobilized Hep-PLL, Hep-PLL-Sepharose, and a heparin adsorbent prepared by the conventional condensation method, Hep-Sepharose. An *N*-acetylated PLL-Sepharose or Sepharose column was used as a precolumn for the Hep-PLL-Sepharose and Hep-Sepharose columns, respectively, to remove nonspecific binding of each adsorbent caused by the PLL moiety and Sepharose matrix. Therefore, the differences in the proteins that bound to Hep-PLL-Sepharose from those that bound to heparin-Sepharose are attributable to the binding to the PG structure. The rat brain extract was applied to the columns, and the bound proteins were eluted with increasing concentrations of NaCl. As shown in Fig. 4, the proteins eluted with 0.5 M NaCl showed different patterns between the columns on SDS-PAGE (Fig. 4(C)), while the proteins were scarcely observed in the 2 M NaCl fractions.

The proteins that specifically bound to either of the two adsorbents were identified by direct N-terminal sequencing of each protein on the excised PVDF membrane, and they are summarized in Fig. 4(D). Among the proteins that specifically bound to the Hep-PLL-Sepharose column, band ‘c’ (18 kDa) was identified as a small immunophilin,

CypA. The N-terminal sequences of band ‘d’ (95 kDa) and band ‘e’ (96 kDa) coincided with those of heat-shock proteins, HSP90α and HSP90β, respectively. Band ‘b’ was N-terminally blocked. The 32 kDa protein N-terminal sequence (band a) that bound to Hep-Sepharose was identified as a neurite-promoting factor, amphoterin (HMG1).

The affinity chromatography was also carried out for a normal porcine brain extract eluted with 0.5 M NaCl (Fig. 5). As shown in Fig. 5(C), the anti-CypA antibody strongly bound to the 18-kDa band in lane 4, and the binding to the band was completely inhibited by recombinant human CypA (data not shown). Therefore, CypA was more enriched in the fraction eluted from Hep-PLL-Sepharose by 0.5 M NaCl than that from Hep-Sepharose, as shown by antibody reactivity, while the specific binding of HMG1 to Hep-Sepharose was confirmed by N-terminal sequencing (data not shown). The results indicate that the CypA of both rat and pig binds to the pseudoPG adsorbents. A heparin-binding site of CypA is highly conserved among human, pig, and rat in a short amino acid sequence, ‘RNGKTSKK’ (4 essential amino acids are underlined), that is present at the C-terminal region (12). If this region is responsible for the binding of CypA to the BPL-Hep probe, multimerization of CypA may be necessary for recognition of the macromolecular structure of pseudoPG. Thus, SDS-PAGE was carried out under a nonreducing condition to deduce how CypA recognized the higher-order structure of pseudoPG. As shown in Fig. 5(D), CypA in the porcine brain extract was found at the upper edge of the separation gel, indicating that CypA was multimerized or complexed with other protein(s) to become a high-molecular weight complex that is stabilized by disulfide bridges in the brain

extract.

Discussion

In this study, we synthesized pseudoPGs for the first time. There have been many conjugation studies by several groups, in which poysaccharides were coupled with synthetic polymers. However, most conjugates in those studies were synthesized using nonpolypeptide backbone materials as backbone molecules; such as polyethylene, (13), (14) and a hydrophobic 1-octanethiol-coated sensorchip surface (15). Even in the studies where poly L-lysine was used as a backbone, neutral oligosaccharides or unsulfated polysaccharide such as fucose, lactose, dextran, or hyaluronic acid were used as glycan moieties (16),(17). The conjugates have a clustered carbohydrate determinant; however, the glycan chains, which are important to characterize the ligand-binding activity of proteoglycans, are quite different from those of natural PG monomers. On this context, the pseudoPG is the first to simulate a natural proteoglycan structure having a linear neutral polypeptide backbone and sulfated GAGs.

Glycosylated PLLs have been conventionally developed to target oligonucleotides and genes as nonviral carriers using oligosaccharides (16), dextran, and hyaluronic acid (17,18). The greatest difference between the pseudoPGs and the gene carriers is that the gene carriers retain free amino groups of PLL to interact with DNA, while the amino groups in the pseudoPGs were blocked after coupling with GAG by biotinylation and subsequent N-acetylation to suppress electrostatic interaction of the core polymer. Heparin rather than heparan sulfate was mostly used to detect the PG-binding proteins

in this study because of its high reactivity with the heparin/heparan sulfate-binding proteins. The heparin-pseudoPG best simulates a natural heparin-clustered proteoglycan such as the serglycin of the secretory vesicles in mast cells (19), and it also simulates heparan sulfate proteoglycan such as syndecan (20).

The binding characteristics of pseudoPG probes were evaluated by SPR, membrane analysis, and affinity chromatography, taking appropriate controls for each assay. A pseudoPG probe, BPL-Hep, exhibited higher affinity toward the known heparin-binding plasma proteins on SPR than the single-strand probe biotin-hydrazide-Hep (Fig. 2 and Table 1). The increased affinity of BPL-Hep over biotin-hydrazide-Hep would be attributable to the multivalent, myriapod-like structure of the proteoglycan. The applicability of pseudoPG probes containing different GAGs to the sensitive detection on a membrane was shown for the binding proteins even after denaturation by SDS and electroblotting. PseudoPG adsorbents showed remarkable binding specificity toward several heparin-binding proteins in the rat brain extract (Fig. 3 and Fig. 4). The proteins that specifically bound to Hep-PLL-Sepharose were CypA and HSP90α and -β. The pseudoPG probes prepared here proved useful in detection and separation of the PG-binding substances, and they will be applied in finding recognition events that involve higher-order PG structures.

HSP90 is a major molecular chaperone involved in the folding and activation of substrate proteins (21); it presents two isoforms, HSP 90α and HSP 90β, the functional differences of which have not been clarified yet (22). HSP90 has two heparin-binding sites (23) that may be responsible for the binding with Hep-PLL. On the one hand,

HSP90 forms a complex with client proteins to stabilize them. On the other hand, CypA was recently found in a heterocomplex of potential sorting proteins containing HSP90 and other chaperonins to traffic asialoglycoprotein receptors between the plasma membrane and the endosomal pool (24). The high-molecular-weight band observed on SDS-PAGE under the nonreducing condition (Fig. 5(D)) might be such a heterocomplex that includes CypA. Furthermore, CypA and Hsp90s are secreted into the extracellular space by the above mentioned vesicular traffic pathways (25,26), which allows them to encounter heparan sulfate proteoglycans.

CypA is a peptidyl-prolyl isomerase that binds the immunosuppressive drug cyclosporin A to modulate T cell differentiation and proliferation. It is widely expressed in multiple tissues, especially abundantly in the brain (27). In this study, CypA was found to bind pseudoPG Sepharose in spite of its small heparin-binding site by forming multimers or complexes with other protein(s) to become a high-molecular-weight complex (Fig. 5(D)). CypA has been reported to be involved in an initial process of HIV-1 attachment to macrophages by interacting with a heparan sulfate PG, syndecan, which serves as an attachment receptor at the cell surface (12,28). In addition, CypA was shown to contribute to the subsequent infection process by modulating the response of HIV-1to host restriction factors (29). Interestingly, it has been reported that Hsp60 and HSP70 family proteins are incorporated into HIV-1 and -2 similar to the incorporation of CypA into HIV-1. In this context, this study proposes that a PG structure of cell surface heparan sulfate is important for recognition of CypA and HSP family proteins at the initial attachment of HIV-1. In that case, pseudoPG may serve as a

potent inhibitor or investigation probe for syndecan-HIV-1 interaction.

A few problems still remain for the efficient synthesis of pseudoPGs; one question is the possibility that an unsubstituted amino group in the glucosamine present in the heparin chain might react during biotinylation of Hep-PLL with NHS-biotin, or immobilization of Hep-PLL to formyl Sepharose. However, the concentration of free amino groups in heparin is small and has been reported by Osmond et al. to be 1/10 (mol/mol heparin) (30). Under the reaction condition in this study, the amino groups in PLL are present in about 10^4-fold excess of that of free amino groups in heparin. Therefore, the possibility of interference by heparin underivatized with PLL would be negligible even if N-unsubstituted glucosamine is present and biotinylated before injection onto the sensor chip, or if it reacts to formyl Sepharose during the preparation of affinity adsorbent.

The low yield of reductive amination for sulfated GAGs is a challenge that remains to be solved. Although the RI signal indicated that the generation of pseudoPGs is minute (Fig. 1), the binding specificities of pseudoPGs and the biotin-hydrazide-heparin probe are distinct from each other (Table 1, Figs. 3, 4, and 5) and the reactivity is different among the pseudoPG probes, depending on the glycan chains (Fig. 3). The introduction of a biotin group into the pseudoPG probe allows concentration of the probe on the SA-sensor chip and detection of the binding proteins on the membrane at high sensitivity. Suda et al. reported that reductive amination of sulfated GAG proceeds almost quantitatively with aromatic amines at pH 3-4, and this may improve the yield of pseudoPGs (15,31). Using polymers of an aromatic amine

instead of PLL as the backbone may also enhance the production of pseudoPGs. To improve the efficiency of the pseudoPG probes, preparations using various skeletal polymers are under examination in our laboratory.

Acknowledgements

This work was supported in part by Grants-in-aid for Scientific Research on Priority Areas 15040209 and 17046004 (HO) from the Ministry of Education, Culture, Sports, Science, and Technology and by a Research Grant on HIV/AIDS from the Ministry of Health Labour Sciences Research. We thank K. Ono for editing the English.

Legends to Figures

Figure 1 **Elution of BPL-Hep probe by SEC-MALLS**

BPL-Hep probes were prepared from the materials in the molar ratios PLL:Hep=1:6 (A), 1:10 (B), 1:50 (C), and were injected on a Dawn DSP HPLC system of SEC and detected by MALLS and refractive index (RI), as described in the text. Underivatized free heparin was injected as a control (D). The elution patterns detected by MALLS are expressed as dots, and those of RI are expressed as solid lines.

Figure 2 **Quantification of interaction between Hep-BPL and Hep-binding proteins by SPR.**

Hep-BPL was immobilized on a CM5 sensor chip as described in the text. Plasma glycoproteins were serially diluted two-fold and injected onto a Hep-BPL-immobilized sensor chip in 10 mM HB-EPS (pH 7.5) for 150 s at a flow rate of 20 μl/min at 25°C. The response was expressed as the change in resonance units (RU) induced by binding of the protein to the Hep-BPL immobilized flow cell, which was corrected for bulk effect by subtracting the change on the BPL-immobilized reference cell. Binding curves of various concentrations of glycoproteins on the sensor chip were 11.9-190 nM lactoferrin (A), 0.42-27.0 μM thrombin (B), and 0.26-17.4 μM AT-III (C).

Figure 3 **Reactivity of biotin hydrazide Hep, BPL-GAGs, and BPA-Hep probes with rat brain extracts**

Extracts (8 μg) were subjected to SDS-PAGE on 9.5% polyacrylamide gels, and

Western blotting was performed on PVDF membranes. Proteins were stained with CBB or allowed to react with biotin hydrazide-Hep, BPL-GAGs, and BPA-Hep probes, as described in the text. Molecular mass markers are shown on the left-hand side.

Fig. 4 **Affinity chromatography of rat brain extract on Hep-Sepharose (A) or Hep-PLL-Sepharose (B) column. Identification of the bound proteins by SDS-PAGE (C) and N-terminal amino acid sequences (D)**

(A) Eight milliliters of rat brain extract was incubated with 8 ml of acetoamido-Sepharose for 3 h at 4℃ with shaking and packed into a column (2×2.6 cm). The flow-through fractions were collected, incubated with 2 ml of Hep-Sepharose overnight at 4℃ with shaking, and packed into a column (0.75×4.5 cm). After washing the column with 0.13 M PBS, the bound materials were eluted with 0.5 M PBS and subsequently with 2 M PBS at the points indicated by arrows. Elution was monitored at 280 nm for protein. (B) Ten milliliters of rat brain extract was incubated with 10 ml of PLL-Sepharose. The subsequent procedure was the same as described in (A) except incubation with 2 ml of Hep-PLL-Sepharose instead of Hep-Sepharose as the second affinity adsorbent. (C) SDS-polyacrylamide gel electrophoresis of 0.5 M NaCl-eluted fractions from a Hep-Sepharose or Hep-PLL-Sepharose column. Each fraction (8 μg protein/lane) was subjected to SDS-PAGE on 13% (A) or 6% (B) polyacrylamide gels under the reducing condition and transferred to a PVDF membrane. Proteins were stained with CBB. Lanes 1 and 3: fractions eluted from Hep-Sepharose, lanes 3 and 4: fractions eluted from Hep-PLL-Sepharose. (D) Direct N-terminal sequencing of each

protein in (C) was performed on the excised PVDF membrane using a protein sequencer, and proteins were identified by searching the N-terminal 4-10 amino acid sequences in the SWISSPROT database.

Fig. 5 Affinity chromatography of pig brain extract on a Hep-Sepharose column (A) or Hep-PLL-Sepharose column (B). SDS-PAGE of 0.5 M NaCl-eluted fractions (C, D).

(A) Extracts from pig brain (90 ml, 2.92 mg/ml protein) were applied to an *N*-acetyl Sepharose precolumn (Vt=6 ml, 1.5×3.5 cm). The flow-through fractions were collected and applied to a Hep-Sepharose column (Vt=4 ml, 1.5×2.5cm). After washing the column with 0.13 M NaCl-PB (first arrow), the bound proteins were eluted with 0.5 M and 2 M NaCl-PB at points indicated by arrows. Elution was monitored at 280 nm for protein. (B) Extracts from pig brain were applied to a PLL-Sepharose precolumn (Vt=6 ml, 1.5×3.5cm). The flow-through fractions were collected and applied to a Hep-PLL-Sepharose column (Vt=3 ml, 1.5×2cm). Elution and protein monitoring were performed in the same way as in A. (C) Fractions eluted from Hep-Sepharose or Hep-PLL-Sepharose (10 μg protein/lane) were subjected to SDS-PAGE on 15% polyacrylamide gels under the reducing condition, and Western blotting on a PVDF membrane was performed. Proteins were stained with CBB (lanes 1, 2) or reacted with anti-CypA (lanes 3, 4). Lanes 1, 3: Fractions eluted 0.5 M NaCl from a Hep-Sepharose column. Lanes 2, 4: Fractions eluted by 0.5 M NaCl from a Hep-PLL-Sepharose column. Molecular weight markers are shown on the left. (D) The

fraction eluted by 0.5 M NaCl from a Hep-PLL-Sepharose column was electrophoresed under reducing (lanes 1, 2) or non-reducing condition (lanes 3, 4) and transferred onto a PVDF membrane. Proteins were stained with CBB (lanes 1, 3) or reacted with anti-CypA and developed with DAB/H_2O_2 (lanes 2, 4). Molecular weight markers are shown on the left.

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Scheme 1　Probes and adsorbents prepared in this study

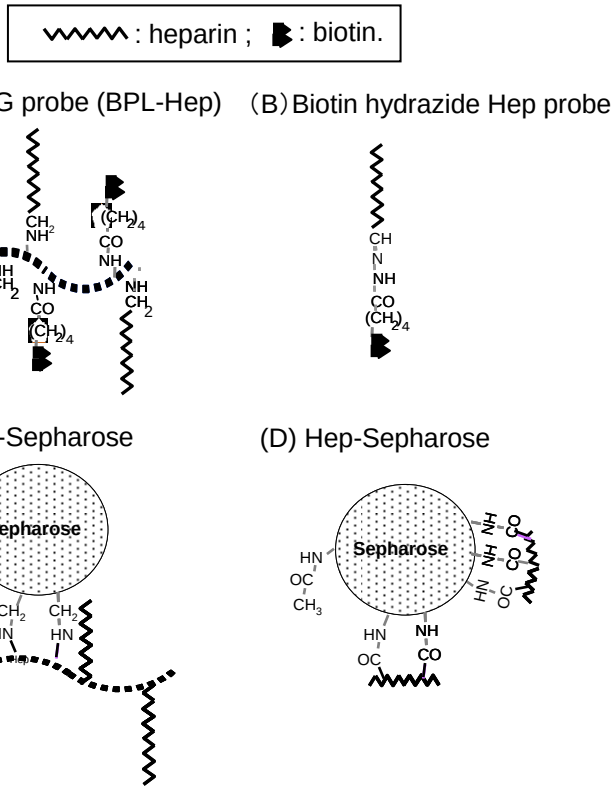
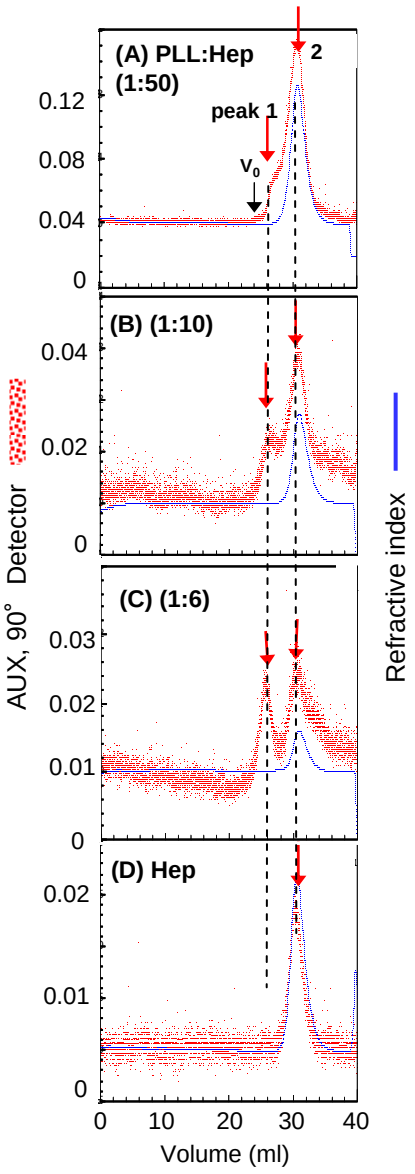


Fig. 1


(A) PLL:Hep
(1:50)
peak 1
2
V_0
(B) (1:10)
(C) (1:6)
(D) Hep
AUX, 90° Detector
Refractive index
Volume (ml)

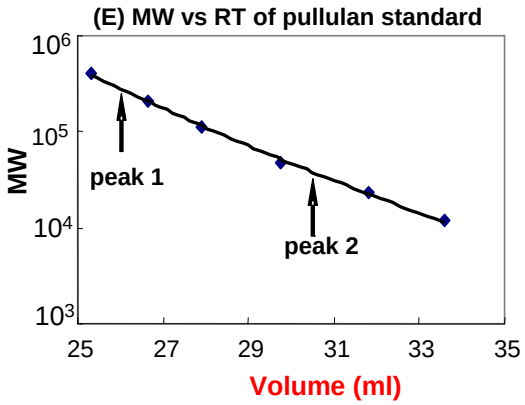

(E) MW vs RT of pullulan standard
MW
peak 1
peak 2
Volume (ml)

Fig. 2

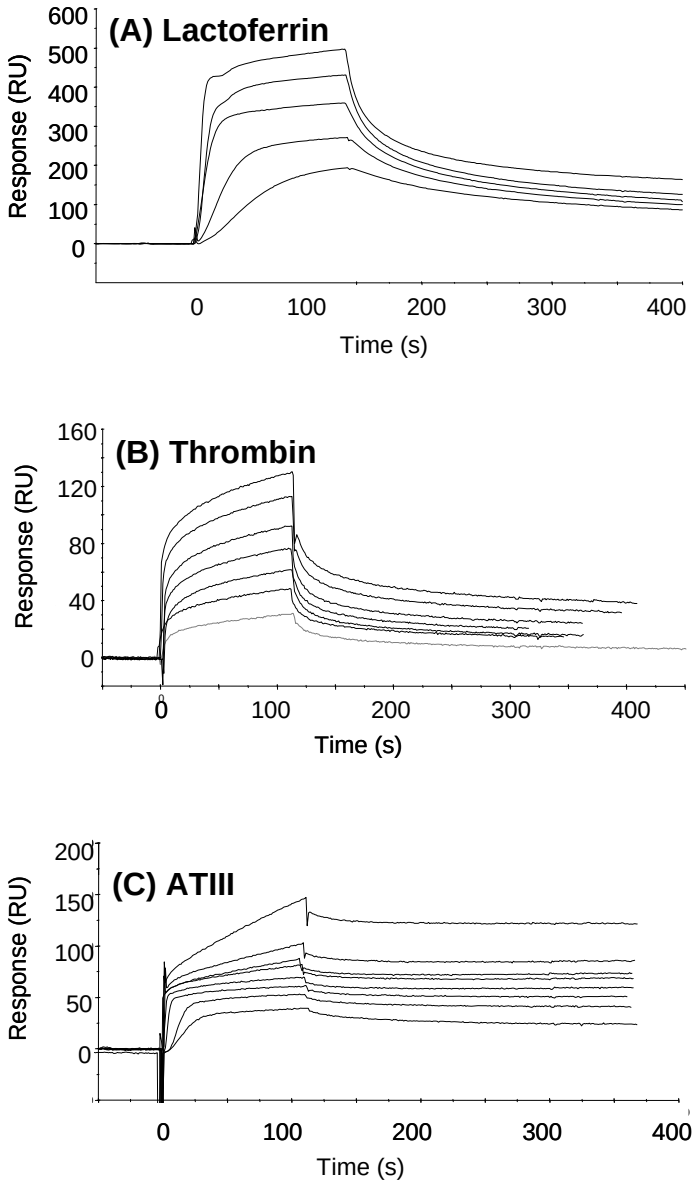

(A) Lactoferrin
Response (RU)
600
500
400
300
200
100
0
0
100
200
300
400
Time (s)
(B) Thrombin
Response (RU)
160
120
80
40
0
0
100
200
300
400
Time (s)
(C) ATIII
Response (RU)
200
150
100
50
0
0
100
200
300
400
Time (s)

Table 1. Kinetic parameters for the interaction of lactoferrin, thrombin and AT III with immobilized BPL-Hep or biotin hydrazide-Hep.

BPL-Hep- and biotin hydrazide-Hep-immobilized sensor chips were prepared as described in the text. Immobilized amounts were 350 RU for BPL-Hep, and 285 RU for biotin hydrazide-Hep. The ka, kd, and Kd values were calculated by global fittingusing BIAevaluation 3.1 software.

	BPL-Hep probe			Biotin hydrazide-Hep probe		
	ka (s)	*kd (s^{-1})*	*Kd (M)*	*ka (s)*	*kd (s^{-1})*	*Kd (M)*
Lactoferrin	1.9×10^{6}	2.3×10^{-3}	1.2×10^{-9}	1.5×10^{6}	6.4×10^{-2}	4.4×10^{-8}
Thrombin	3.1×10^{3}	2.0×10^{-3}	6.6×10^{-7}	9.5×10^{2}	1.7×10^{-3}	1.8×10^{-6}
AT III	54	1.5×10^{-4}	2.8×10^{-6}	–	–	–

Fig. 3

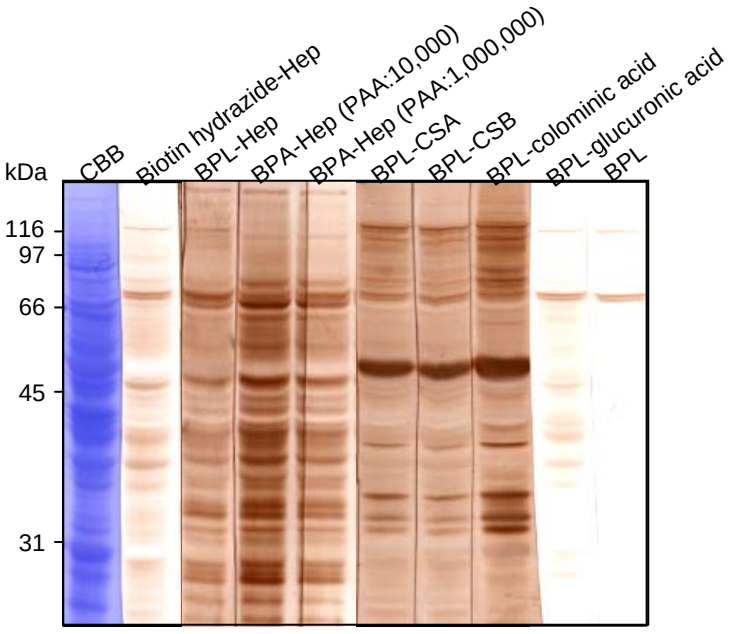
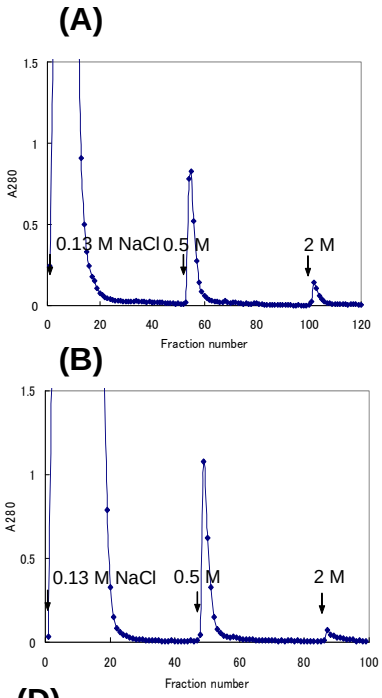
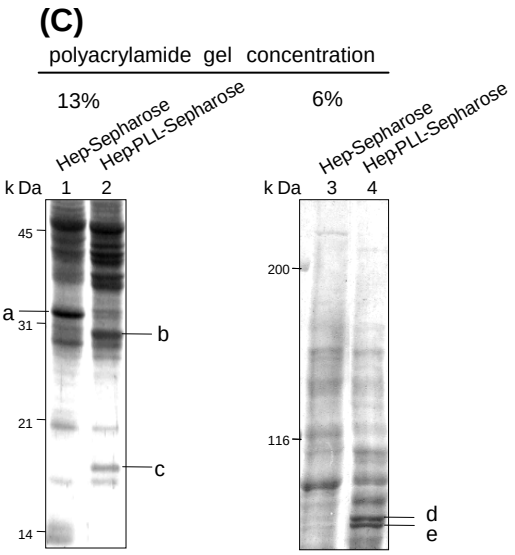

kDa
CBB
Biotin hydrazide-Hep
BPL-Hep
BPA-Hep (PAA:10,000)
BPA-Hep (PAA:1,000,000)
BPL-CSA
BPL-CSB
BPL-colominic acid
BPL-glucuronic acid
BPL
116
97
66
45
31

Fig. 4

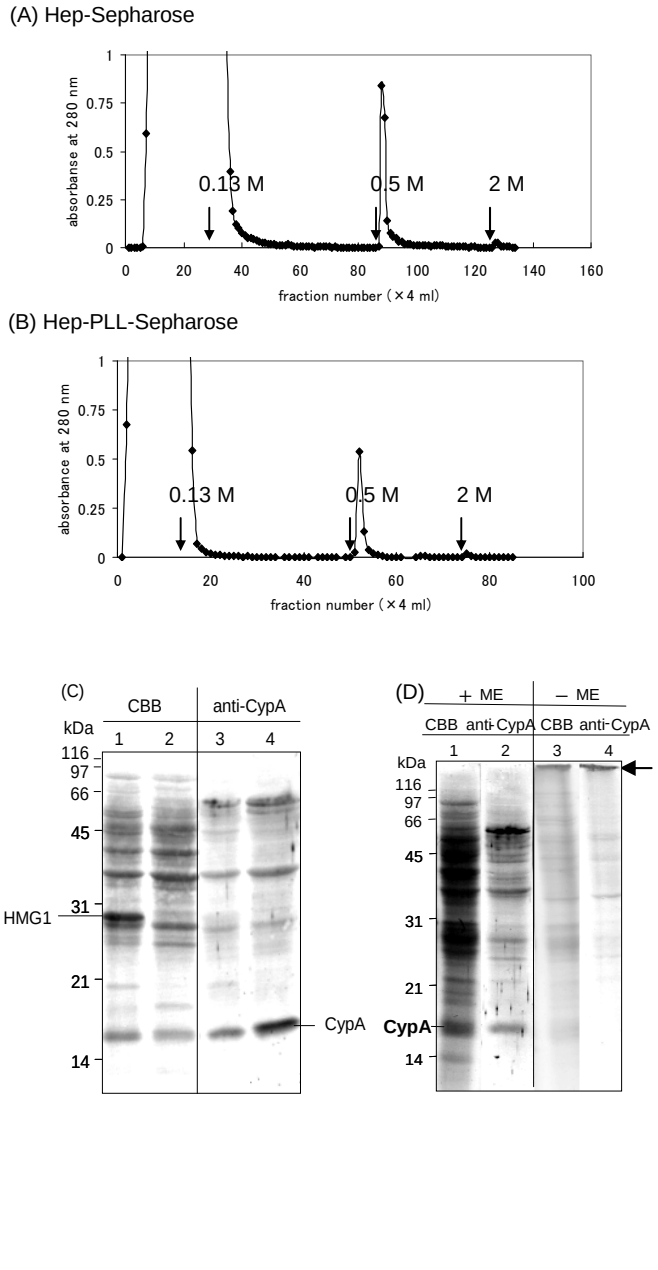




(D)

Band No.	MW (kDa)	N-terminal sequence	Identified protein	Bound & eluted from
a	32	GKGDPKKPRG	amphoterin (HMG1)	Hep-Sepharose
b	30	blocked N-terminal	—	Hep-PLL-Sepharose
c	18	VNPTVFFDIT	cyclophilin A (CypA)	Hep-PLL-Sepharose
d	95	PEETQTQD	HSP90-α	Hep-PLL-Sepharose
e	94	PEEV	HSP90-β	Hep-PLL-Sepharose

Fig. 5


(A) Hep-Sepharose
absorbanse at 280 nm
0.13 M
0.5 M
2 M
fraction number (×4 ml)
(B) Hep-PLL-Sepharose
absorbance at 280 nm
0.13 M
0.5 M
2 M
fraction number (×4 ml)
(C)
CBB
anti-CypA
kDa
HMG1
CypA
(D)
+ ME
− ME
CBB anti-CypA CBB anti-CypA
kDa
CypA