Vol. 211, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 6, 1995 Pages 262-267

FORMATION OF HEPARAN SULFATE OR CHONDROITIN/DERMATAN SULFATE ON RECOMBINANT DOMAIN I OF MOUSE PERLECAN EXPRESSED IN CHINESE HAMSTER OVARY CELLS

Robert Kokenyesi* and Jeremiah E. Silbert

Connective Tissue Research Laboratory, Edith Nourse Rogers Memorial Veterans Hospital, Bedford, MA

Department of Rheumatology and Immunology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA

Received April 26, 1995

Recombinant domain I of mouse perlecan was expressed in Chinese hamster ovary (CHO K1) cells and affinity purified on Ni-agarose. Gel chromatography followed by characterization of glycosaminoglycans by the use of glycosaminoglycan lyases showed that the recombinant proteoglycans contained, on average, three glycosaminoglycan chains of heparan sulfate or chondroitin/dermatan sulfate of approximately 12 kDa median size. These data demonstrate that domain I has functional sites for attachment of glycosaminoglycans and indicate that the glycosaminoglycan chains of native perlecan are grouped at its N-terminal end. This, in turn, suggests that the likely function of domain I in perlecan would be to provide for the addition of glycosaminoglycan chains to the core protein.

The major proteoheparan sulfate of murine basement membranes has been shown to consist of a core protein of 400 kDa reported to carry 3-5 heparan sulfate chains of approximately 65 kDa size (1). This proteoheparan sulfate was named perlecan because of the characteristic "bead on the string"-like appearance of the core protein seen on rotary shadowing electron micrographs (2, 3). The complete amino acid sequence has been established for both mouse and human perlecan and the core protein was shown to consist of five domains (4-6). Domain I (N-terminal end of perlecan) was found to have no homology to any known protein, while domains II-V were found to have various homologies to either LDL receptor, NCAM or laminin. Functions for domains II-V were postulated based on sequence homologies, but no function has been assigned to domain I.

Domain I was found to contain three Ser-Gly-Asp repeats (4-6) where the serine residues were postulated as possible sites for glycosaminoglycan attachment. On the other hand, domain V at the C-terminal end of perlecan contains one (in mouse) (4) or

* To whom correspondence should be addressed at Edith Nourse Rogers Memorial Veterans Hospital, Building 70, 200 Springs Road, Bedford, MA 01730.

0006-291X/95 $5.00

several (in human) (5, 6) Ser-Gly-Xaa-Gly consensus sequences for glycosaminoglycan attachment (7) as well as several other Ser-Gly sequences. Thus, attachment of glycosaminoglycans to domain V as well as to domain I would be consistent with the appearance of glycosaminoglycans grouped at one end of the core protein as described by limited proteolysis (2) and rotary shadowing (3).

We have now expressed domain I of mouse perlecan in CHO cells and shown that the recombinant proteoglycan contains chondroitin/dermatan sulfate and heparan sulfate. Although there appeared to be some heterogeneity in the number of glycosaminoglycan chains per domain I core protein, most of the product appeared to have three chains. Our results indicate that domain I of mouse perlecan can function as an independent unit to accept glycosaminoglycan chains in the absence of the other domains.

Materials and Methods

Materials - CHO K1 cells were from American Type Culture Collection (Rockville, MD). Carrier free $H_2[^{35}S]O_4$ and [^{3}H]glucosamine were from New England Nuclear (Boston, MA). DOTAP was from Boehringer Mannheim (Indianapolis, IN). Opti-MEM and T4 DNA ligase were from GIBCO-BRL (Gaithersburg, MD). Sepharose CL-6B and DEAE-Sephacel were from Pharmacia LKB (Piscataway, NJ). Heparitinase (EC 4.2.2.8.), chondroitin ABC lyase (EC 4.2.2.4.), and chondroitin AC lyase (EC 4.2.2.5. from Flavobacterium) were from Seikagaku America (Rockville, MD). Vent (exo$^-$) DNA polymerase was from New England Biolabs (Beverly, MA). Qiagen Maxi columns and Ni-NTA-agarose were from Qiagen (Chatsworth, CA). All restriction endonucleases were from Promega (Madison, WI). All chemicals were from Sigma (St. Louis, MO).

Plasmid construction - Oligonucleotide primers 5'-ATGTCGACAACGTCAAG-CTGGTGGT-3' (forward) and 5'-TCGTGGATCCTAATGATGATGATGATGATGGA-CTCTTGGGAACTG-3' (reverse) were synthesized by the Oligonucleotide Core Facility in the Department of Rheumatology and Immunology of the Brigham and Women's Hospital. The forward primer matches the nucleotide sequence of mouse perlecan cDNA between 490-506 bp and contains an added restriction endonuclease site for SalI at the 5' end; the reverse primer matches the nucleotide sequence of mouse perlecan cDNA between 1158-1172 bp and contains an added 5' sequence encoding for six histidine residues, a termination codon and a restriction endonuclease site for BamHI. To start PCR, 10 ng of clone 16 of mouse perlecan cDNA (a generous gift of Dr. Douglas M. Noonan, Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy) (4), was added to polymerase buffer (supplied by New England Biolabs) which was adjusted to contain 1 mM of each primer, 0.2 mM of deoxynucleotide triphosphates and 6 mM $MgSO_4$. The mixture was heated to 95°C for 5 min, cooled to the annealing temperature (50°C), and 2 units of Vent (exo$^-$) DNA polymerase and a drop of mineral oil were added, followed by a 2 min extension at 73°C. This was followed by 30 cycles of 1 min at 95°C, 1 min at 50°C and 2 min at 73°C. The PCR-generated DNA was ethanol precipitated, digested with SalI and BamHI restriction enzymes, purified on agarose gel and ligated into pHßAPr-1-neo expression vector (generous gift of Dr. Lawrence Kedes, University of Southern California) (8) to give a transfection vector which we called pRK1. For transfections or for sequencing by the dideoxy termination method (9), pRK1 was purified on a Qiagen Maxi column. The insert in pRK1 was found to have three base changes resulting in the appearance of an asparagine residue instead of aspartic acid 30, a methionine residue instead of valine 114 and a serine residue instead of threonine 141 of the reported sequence.

Cell culture and transient transfection - CHO K1 cells were cultured in Ham's F-12 medium containing 10% heat-inactivated fetal bovine serum and glutamine. Medium was replaced every 3 days. For transfections the cells were plated into 60 mm dishes to reach 50% confluency by next morning. After rinsing the cell layer twice with Opti-

MEM, 3 ml of Opti-MEM containing 50 µg of DOTAP lipofection reagent was added to each dish, and incubated for 1 h at 37°C. Then 10 µg of pRK1 DNA was mixed with 0.5 ml of Opti-MEM and added to the cells. For controls the same amount of pHßAPr-1-neo DNA was added instead of pRK1. The medium was mixed by gentle swirling, and the cells were incubated for another 6 h. The transfection mixture was removed and replaced with fresh medium containing radiolabel. Conditioned media of the transfectants were collected and replaced with fresh media every 48 h up to 6 days.

Metabolic labeling - 100 µCi/ml carrier free $H_2[^{35}S]O_4$ was added immediately after the transfection and was replenished when the medium was changed. At the end of the experiment the cell layer was extracted in a Tris/urea/Triton buffer (10 mM Tris/HCl, pH 7.4; 6 M urea; 1% Triton X-100; 0.15 M NaCl) containing 10 mM EDTA, 10 mM N-ethylmaleimide and 1 mM phenylmethylsulfonyl fluoride, as previously described (10). The conditioned medium and the cell extracts were stored at -20°C until further analysis.

Isolation of recombinant protein - The conditioned medium was added to Ni-NTA-agarose previously equilibrated with Tris-buffered saline (10 mM Tris/HCl, pH 7.4; 0.15 M NaCl) Mixtures were agitated overnight on a rocker at 4°C, washed with 40 bed volumes of the buffer used for equilibration, washed with 10 bed volumes of 5 mM imidazole in Tris buffer (50 mM Tris/HCl, pH 7.4, containing 100 mM NaCl and 0.1% Triton X-100) and eluted either with a 5-50 mM imidazole linear gradient or stepwise with 10, 20, 30, 40, and 50 mM imidazole in the same buffer.

Molecular sieve columns - A Sepharose CL-6B column (47 cm x 0.7 cm) was used to determine the size of recombinant proteoglycans and glycosaminoglycans. It was run at room temperature using 0.5 M ammonium bicarbonate as eluant. Void volume was determined by the elution position of Blue Dextran; total volume was determined by the elution position of [^{3}H]glucosamine.

Degradation of recombinant material - Glycosaminoglycans were obtained from the proteoglycans by alkali elimination overnight at room temperature in 0.5 M NaOH. Digestions with heparitinase (0.003 unit) were for 1 h at 37°C in 0.1 ml Tris/HCl buffer (50 mM, pH 7.4) containing 10 mM sodium acetate, 10 mM EDTA, 10 mM N-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride and 0.1% Triton X-100. Incubations with chondroitin ABC lyase (0.005 unit) and chondroitin AC lyase (0.01 unit) were for 1 h at 37°C in 0.1 ml as described (11).

Ion exchange chromatography - In order to quantitate non-recombinant proteoglycans, the non-binding material from Ni-NTA-agarose columns was adjusted to contain 2 M urea, 0.1% Triton X-100 and 50 mM sodium acetate pH 4.5. Then 1 ml of DEAE-Sephacel (previously equilibrated with the above urea, Triton X-100, sodium acetate buffer plus 0.15 M NaCl) was added and agitated overnight on a rocker at 4°C. The beads were poured onto a column and washed with the same buffer containing 0.2 M NaCl, and then eluted with 1 M NaCl in the same buffer.

Results and Discussion

Domain I of mouse perlecan containing the added polyhistidine tag was transiently expressed in CHO K1 cells that were cultured in the presence of [^{35}S]sulfate with fresh medium and label added every 48 h for a total of 6 days, as described in Materials and Methods. Cells were approximately 80% confluent by the end of the first 48 h incubation and reached confluence during the second 48 h period. The greatest amount of novel ^{35}S-labeled macromolecule was produced during the second 48 h incubation and was used for all subsequent studies. The conditioned medium was found to contain approximately 2/3 of the total proteo[^{35}S]glycan while the cell layer contained approximately 1/3. The material in the cell layer was not examined futher. Conditioned medium was added to the Ni-NTA-agarose column in order to separate the polyhistidine-containing recombinant domain I proteo[^{35}S]glycan from endogenous proteo[^{35}S]glycan

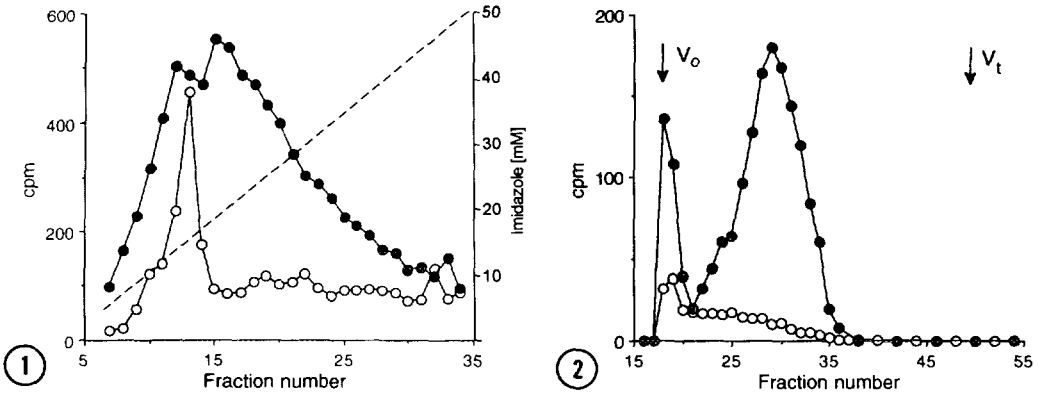

Fig. 1. Gradient elution of proteo[^{35}S]glycans from Ni-NTA-agarose column. Conditioned medium from pRK1 transfectants (●) or from control (pHßAPr-1-neo) transfectants (o) was added to a 0.2 ml Ni-NTA-agarose column, washed and eluted with a 5-50 mM imidazole gradient (- - -) as described in Materials and Methods.

Fig. 2. CL-6B chromatography of the proteo[^{35}S]glycans eluted from Ni-NTA-agarose. Affinity-isolated material from pRK1 transfectants (●) or from control (pHßAPr-1-neo) transfectants (o) was chromatographed on a Sepharose CL-6B column (49 X 0.7 cm) in 0.5 M NH_4HCO_3; 1 ml fractions were collected.

which would not have the polyhistidine tag necessary for specific binding to the column. Approximately 6% of the proteo[^{35}S]glycans from the conditioned medium of pRK1 transfectants bound to the column, while only 1% of the total proteo[^{35}S]glycans from the conditioned medium of control transfectants bound. Elution of bound material with a linear imidazole gradient (Fig. 1) showed that most of the proteo[^{35}S]glycans from pRK1 transfectants required higher imidazole concentrations for elution than were required for the elution of the control. Consequently, we considered this novel proteo[^{35}S]glycan (fraction 15-27) to be the newly expressed domain I of perlecan.

To demonstrate the unique overall size of the novel proteo[^{35}S]glycan we took an aliquot of fraction 17 from the gradient elution (Fig. 1) of pRK1 transfectant-derived material and analyzed it on a Sepharose CL-6B column (Fig. 2). This material ran as a main peak at K_{av} = 0.35 (approximately 40 kDa median size compared to glycosaminoglycan markers (12)) with a small amount (15%) of larger material eluting in the void volume. We also analyzed an equal aliquot of fraction 17 from the gradient elution of control transfectant-derived material which was also partially excluded from the column, while the heterogeneous included material indicated a considerably larger size than that of the novel proteo[^{35}S]glycan from pRK1 transfectants (Table 1).

Following treatment of the novel proteo[^{35}S]glycan (fractions 25-34 from the Sepharose CL-6B column) with chondroitin ABC lyase or chondroitin AC lyase, approximately 55% was degraded to disaccharides or to various sized oligosaccharides and disaccharides, respectively, indicating that chondroiotin/dermatan sulfate was present. The remaining 45% maintained the original elution position of K_{av} = 0.35; this

Table 1
Size of proteo[^{35}S]glycans and [^{35}S]glycosaminoglycans obtained from transfectants

	Proteoglycan		Glycosaminoglycan	
Transfection	K_{av}	Median kDa	K_{av}	Median kDa
Control	0.18	>100	0.50	20
pRK1	0.35	40	0.62	12

non-degraded material represented proteoheparan[^{35}S]sulfate. Treatment of the novel proteo[^{35}S]glycan with heparitinase resulted in the degradation of approximately 45% to various sized oligosaccharides, while the remaining 55% maintained the original elution position of K_{av} = 0.35. This non-degraded material represented proteochondroitin/dermatan[^{35}S]sulfate. The elution position of the non-degraded material suggested that both the proteochondroitin/dermatan[^{35}S]sulfate and the proteoheparan[^{35}S]sulfate were distinct populations, since degradation of one type of glycosaminoglycan chains on a hybrid proteoglycan molecule would have resulted in a smaller size proteo[^{35}S]glycan, represented by a shift of the elution position to a higher K_{av}.

Sepharose CL-6B chromatography of the glycosaminoglycan chains obtained by alkali treatment of the novel proteo[^{35}S]glycan indicated a median size of approximately 12 kDa (K_{av} = 0.62; Table 1) for both chondroitin/dermatan sulfate and heparan sulfate. Since this was approximately 1/3 the size of the proteo[^{35}S]glycans, it appeared that both domain I proteochondroitin/dermatan[^{35}S]sulfate and domain I proteoheparan[^{35}S]sulfate contained an average of three glycosaminoglycan chains, matching the number of potential glycosaminoglycan attachment sites on domain I of mouse perlecan (4). Alkali degradation of the material from control transfectants gave a broad peak with median size of approximately 20 kDa (Table 1), indicating an average of 5 or more glycosaminoglycan chains. These data showed that both the overall size of the pRK1 transfectant-derived proteo[^{35}S]glycan and [^{35}S]glycosaminoglycan chains were much smaller than the respective sizes of the material from control transfectants, indicating that this novel proteo[^{35}S]glycan was produced as a consequence of transfection of cells with cDNA corresponding to domain I of mouse perlecan.

These results provide the first direct evidence that domain I (N-terminal end) of mouse perlecan can provide attachment sites for glycosaminoglycans, indicating that this is a main function of domain I in perlecan. Our finding that chondroitin/dermatan sulfate can be attached to perlecan domain I is consistent with previously published information.

Perlecan containing both heparan sulfate and chondroitin/dermatan sulfate on the same core protein has been isolated from human placenta (13) and BAM cells (established from the Engelbreth-Holm-Swarm tumor) (14). A perlecan-related proteoglycan from rabbit renal cells was also shown to have both heparan sulfate and chondroitin sulfate chains (15). These data suggest that the overall structure and conformation of domain I, whether separate or joined to the rest of the perlecan core protein, permits the attachment of either chondroitin/dermatan sulfate or heparan sulfate chains.

Acknowledgments: We are grateful to Dr. Douglas M. Noonan for clone 16 of mouse perlecan. This research was supported by the Medical Research Service of the Department of Veterans Affairs, an Arthritis Foundation Investigator Award to R.K., and by core facility research grant AR-36308 from the National Institutes of Health.

References

1. Hassell, J.R., Noonan, D.M., Ledbetter, S.R., and Laurie, G.W. (1986) in Functions of Proteoglycans (Evered, D., and Whelan, J., eds) pp. 204-222, John Wiley & Sons, Chichester
2. Yurchenko, P.D., Cheng, Y-S., and Ruben, G.C. (1987) J. Biol. Chem. 262, 17668-17676
3. Laurie, G.W., Inoue, S., Bing, T.J., and Hassell, J.R. (1988) Am. J. Anat. 181, 320-326
4. Noonan, D.M., Fuelle, A., Valente, P., Cai, S., Horigan, E., Sasaki, M., Yamada, Y., and Hassell, J.R. (1991) J. Biol. Chem. 266, 22939-22947
5. Kallunki, P., and Tryggvason, K. (1992) J. Cell Biol. 116, 559-571
6. Murdoch, A.D., Dodge, G.R., Cohen, I., Tuan, R.S., and Iozzo, R.V. (1992) J. Biol. Chem. 267, 8544-8557
7. Bourdon, M.A., Krusius, T., Campbell, S., Schwartz, N.B., and Ruoslahti, E. (1987) Proc. Natl. Acad. Sci. USA 84, 3194-3198
8. Gunning, P., Leavitt, J., Muscat, G., Sun-Yu, N., and Kedes, L. (1987) Proc. Natl. Acad. Sci. USA 84, 4831-4835
9. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467
10. Sanderson, R.D., and Bernfield, M. (1988) Proc. Natl. Acad. Sci. USA 85, 9562-9566
11. Saito, T., Yamagata, T., and Suzuki, S. (1968) J. Biol. Chem. 243, 1536-1542
12. Wasteson, A. (1971) J. Chromatogr. 59, 87-97
13. Isemura, M., Sato, N., Yamaguchi, Y., Aikawa, J., Munakata, H., Hayashi, N., Yosizawa, Z., Nakamura, T., Kubota, A., Arakawa, M., and Hsu, C-C. (1987) J. Biol. Chem. 262, 8926-8933
14. Danielson, K.G., Martinez-Hernandez, A., Hassell, J.R., Iozzo, R.V. (1992) Matrix 11, 22-35
15. Piedagnel, R., Prie, D., Cassingena, R., Ronco, P.M., and Lelongt, B., (1994) J. Biol. Chem. 269, 17469-17476