Human Heparanase

PURIFICATION, CHARACTERIZATION, CLONING, AND EXPRESSION*

Minako Toyoshima and Motowo Nakajima‡
From Discovery Research, Takarazuka Research Institute, Novartis Pharma K.K., 10-66 Miyuki-cho, Takarazuka 665-8666, Japan

Heparan sulfate and heparan sulfate proteoglycans are present in the extracellular matrix as well as on the external cell surface. They bind various molecules such as growth factors and cytokines and modulate the biological functions of binding proteins. Heparan sulfate proteoglycans are also important structural components of the basement membrane. Heparanase is an endo-β-D-glucuronidase capable of cleaving heparan sulfate and has been implicated in inflammation and tumor angiogenesis and metastasis. In this study, we report the purification of a human heparanase from an SV40-transformed embryonic fibroblast cell line WI38/VA13 by four sequential column chromatographies. The activity was measured by high speed gel permeation chromatography of the degradation products of fluorescein isothiocyanate-labeled heparan sulfate. The enzyme was purified to homogeneity, yielding a peptide with an apparent molecular mass of 50 kDa when analyzed by SDS-polyacrylamide gel electrophoresis. Using the amino acid sequences of the N-terminal and internal heparanase peptides, a cDNA coding for human heparanase was cloned. NIH3T3 and COS-7 cells stably transfected with pBK-CMV expression vectors containing the heparanase cDNA showed high heparanase activities. The homology search revealed that no homologous protein had been reported.

Heparan sulfate (HS)1 and heparan sulfate proteoglycans (HSPG), localized in the extracellular matrix and on the external surface of cell membranes, play a major role in cell-cell and cell-extracellular matrix interactions (1, 2). HSPG are implicated in a number of cellular processes, including cell adhesion, migration, differentiation, and proliferation (3, 4). Various molecules have been reported to interact with HS/HSPG; they are involved in blood coagulation (5–12). HS/HSPG carbohydrate chains are depolymerized enzymatically either by eliminative cleavage with lyases or by hydrolytic cleavage with hydrolases, and these enzymes therefore, in part, modulate the biological functions of HS/HSPG-binding proteins (13).

Heparanase, which cleaves HS into characteristic large molecular weight fragments, was identified in murine metastatic melanoma cells by Nakajima et al. (14). Heparanase activity was correlated with the lung colonization potential of murine B16 melanoma sublines (14). Nakajima et al. (15) concluded that the enzyme responsible for HS degrading is an endogluconidase, cleaving the linkage between GlcUA and GlcNAc, and named it heparanase. Heparanase is a hydrolase distinct from flavobacterium heparitinase and heparinase, which are eliminases capable of specifically degrading heparan sulfate and heparin, respectively, into di- and tetrasaccharides (15–17).

Various methods for detecting heparanase activity have been reported including (i) polyacrylamide gel electrophoresis (14), (ii) gel filtration chromatography (15), (iii) high speed gel permeation chromatography (18), (iv) use of solid-phase substrates of heparanase (19), (v) use of radiolabeled and fluorescein-labeled heparan sulfate (19), and (vi) use of chicken histidine-rich glycoprotein, taking advantage of the fact that heparanase-treated heparan sulfate fragment has low affinity for chicken histidine-rich glycoprotein (20).

Several groups have reported the purification of heparanase from different sources. Jin et al. (21) reported the purification of heparanase from murine melanoma cells. The molecular size of the purified heparanase was 97 kDa, with minor proteins of 205, 125, 76, and 52 kDa also detected (21). Freeman and Parish (22) have recently purified a human platelet heparanase by sequential chromatographies using ConA-Sepharose, Zn2⁺-chelating Sepharose/blue A-agarose, octylagarose, and Superose 12 columns, resulting in a single protein of 50 kDa. Ihrcke et al. (23) also purified a platelet heparanase with a molecular mass of 34 kDa. Gilat et al. (24) purified a 45-kDa heparanase from human placenta. The partial purification of heparanase from Chinese hamster ovary cells was also reported (25). The Chinese hamster ovary heparanase was reported as having a molecular mass greater than 30 kDa.

Hoogewerf et al. (26) purified a heparanase-like enzyme from human platelets. Their purified protein was identified as connective tissue activating peptide III, having a glucosaminidase activity but not a glucuronidase activity and cleaving HS into disaccharides. However, the relationships among HS-degrading endoglycosidases have not yet been elucidated due to a lack of sufficient information regarding their structures and enzymatic characteristics.

In the present study, we purified human heparanase to homogeneity by sequential column chromatographies and cloned a cDNA encoding human heparanase. We report here the enzymatic characteristics of the purified native heparanase, the
cloning of the cDNA coding for the enzyme, and the expression of the enzyme in NIH3T3 and COS-7 cells.

**EXPERIMENTAL PROCEDURES**

**Reagents**

Heparan sulfate (sodium salt) from bovine kidney and heparitinase from *Flavobacterium heparinum* were purchased from Seikagaku Corp. (Tokyo, Japan). TSKgel G3000SWXL was purchased from Tosoh Corp. (Tokyo, Japan). PD-10 columns, Sephacryl S-300 HR, heparin-Sepharose CL-6B, carboxymethyl-Sepharose CL-6B, and phenyl-Sepharose 6FF were from Amersham Pharmacia Biotech. ConA-agarose was purchased from Seikagaku Corp. FBS and reagents for cell cultures were all purchased from Life Technologies, Inc.

**Cell Culture**

SV40 virus-transformed human embryonic fibroblast cells, WI38/VA13, were cultured as monolayers in RPMI supplemented with 10% FBS, 2 mM l-glutamine, penicillin (100 units/ml) and streptomycin (100 μg/ml) in humidified 95% air, 5% CO2 at 37 °C. NIH3T3 and COS-7 cells were also cultured in the same conditions.

**Labeling of Heparan Sulfate**

Heparan sulfate (sodium salt) from bovine kidney was labeled with fluorescein isothiocyanate (FITC). Five milligrams of heparan sulfate and 5 μg of FITC were dissolved in 1 ml of 0.1 M sodium carbonate buffer, pH 9.5, and stirred at 4 °C for overnight in darkness. The solution was then loaded on a PD-10 desalting column to isolate FITC-labeled heparan sulfate (FITC-HS) from unreacted FITC. The FITC-HS solution was then loaded on a PD-10 desalting column to isolate FITC-HS from unreacted FITC. The FITC-HS was further subjected to chromatography through Sephacryl S-300 HR equilibrated with column buffer 1 (25 mM Tris-HCl, 150 mM NaCl, pH 7.5) to fractionate high molecular weight products (Mr >30,000). The fractionated materials were concentrated with a Microcon® 10 concentrator (Millipore, USA) and dialyzed against dilution buffer 2 (0.1 M sodium acetate, pH 6.0, 0.2% CHAPS).

**Heparanase Assay**

Human heparanase activity was determined toward FITC-HS. The reaction was carried out in 100 μl of 50 mM sodium acetate, pH 4.2, containing 0.5–1 μg of FITC-HS. The protein material containing heparanase was added to the reaction mixture without exceeding the salt concentration of 0.25 M and incubated at 37 °C for an appropriate period. The reaction was then stopped by the addition of 100 μg of heparin and subsequent heating at 100 °C for 5 min. The reaction mixture was centrifuged at 15,000 rpm for 5 min to precipitate the insoluble material. The products of FITC-HS yielded by this reaction were analyzed by high speed gel permeation chromatography. Briefly, a 20-μl aliquot of the supernatant was injected into a TSKgel G3000SWXL column (7.8-mm inner diameter × 30 cm) equilibrated with column buffer 1 and run at 0.5 ml/min. The fluorescence in the eluent was measured by a F-1050 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The activity was determined from HPLC chromatograms by measuring a forward half area of the peak of the intact FITC-HS. The decrease of this area following heparanase treatment was measured by using an integrator, and the amount of the degraded FITC-HS was calculated from the decrease of fluorescence intensity (FI). The degradation of FITC-HS was 0–30% of the substrate added to obtain kinetic parameters using this calculation method.

**Purification of Human Heparanase**

All of the procedures were performed in a total of 5 days at 4 °C

**Cell Extraction**—Cell lysate was prepared by homogenizing cells in lysis buffer (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% AEBSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 1 μg/ml aprotinin). The lysate was collected from 0 to 30 min and centrifuged at 9000 rpm. The supernatant was then subjected to heparin-Sepharose affinity chromatography. The concentration of protein was measured by BCA protein assay (Pierce).

**Heparin-Sepharose Affinity Chromatography**—The cell lysate obtained as above was loaded on to a heparin-Sepharose column (5.0 × 10 cm) at a flow rate of 2.5 ml/min. The column was washed with 100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% AEBSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 1 μg/ml aprotinin. At the end of the wash, the column was eluted with a step gradient of NaCl from 0.1 M NaCl to 0.5 M. The concentration of protein was measured by BCA protein assay (Pierce).

**Pheny1-Sepharose Hydrophobic Exchange Chromatography**—The purified fractions from heparin-Sepharose were applied to a phenyl-Sepharose column (2.5 × 10 cm) equilibrated with phenyl buffer A (50 mM Tris-HCl, pH 7.5) and washed at a flow rate of 0.5 ml/min from 0 to 40 min. The column was washed with 50 mM Tris-HCl, 0.1% CHAPS (pH 7.5) from 0 to 100% 0-20 min. The heparanase-containing fractions were collected and concentrated with a Centriprep® 10 concentrator (Amicon). The protein quantity was estimated by comparing the staining of various known amounts of bovine serum albumin in a silver-stained SDS-polyacrylamide gel after running according to the method of Laemmli (27).

**Optimum pH Analysis**

A 1-μl aliquot of the purified heparanase solution (20 μg of protein) and 1 μg of FITC-HS was added to GTA solution (50 mM dimethyl
glutaric acid, 50 mM Tris, 50 mM 2-aminomethyl-1,3-propanediol, pH adjusted with HCl and NaOH at various pH values and incubated for 2 h at 37 °C. The heparanase activity was calculated as described for the heparanase assay method.

Amino Acid Sequence of Heparanase Protein and Derived Peptides

The purified heparanase from phenyl-Sepharose chromatography was subjected to SDS-polyacrylamide gel electrophoresis (27) and then electrotransferred onto a ProBlott membrane (Applied Biosystems, Inc.). The transferred protein was visualized with Coomassie Brilliant Blue. The 50-kDa band, which had heparanase activity, was excised and used for N-terminal amino acid sequencing with the HP G105A protein sequencing system (Hewlett Packard). The transferred protein band prepared as above was digested in situ with Lys-C endoproteinase. The digests were subjected to reverse-phase chromatography and eluted with a gradient of 0–100% acetonitrile. The amino acid sequences of the separated peptides were analyzed using the HP G105A protein sequencing system.

Identification of the Human Heparanase cDNA Using Expressed Sequence Tag (EST) Sequences

The peptide sequences identified from the purified human heparanase were used to search for homologues in the gene data bases stored at the NCBI by using the tBLASTn sequence alignment program. Translation of EST sequences (yw67t02.r1 Homo sapiens cDNA clone 260138 5', GenBank™ accession no. N45867 and yw70a013.r1 Homo sapiens cDNA clone 257548 3', GenBank™ accession no. N30824) in one frame presented a significant sequence similarity with three internal peptide sequences of the human heparanase. These sequences were originally obtained from a human placenta 8–9-week cDNA library primed with oligo(dT) primer. Aligning overlapping regions, the sequences of these EST clones were connected to make one DNA fragment 968 bp in length. A 731-bp DNA fragment corresponding to the connected DNA of these EST clones was then amplified by reverse transcriptase-PCR using WI38/Va13 cDNA. For this purpose, a total RNA was prepared from the cultured WI38/Va13 cells using ISOGEN (Nippon Gene, Corp. Japan), and poly(A) RNAs were purified with an mRNA purification kit (Amersham Pharmacia Biotech). poly(A) RNA was reverse-transcribed with the avian myeloblastosis virus reverse transcriptase (Takara, Japan) according to the manufacturer's protocol and used for PCR with a sense oligonucleotide primer (5'-CCTTCTAA-GAAGTCCACCTTC-3') and an antisense oligonucleotide primer (5'-AAACTATATGAGAAAGCTGGC-3') of 72,000 cm$^{-2}$ 4). PCR was performed using the LA PCR™ kit (Takara). PCR conditions were as follows: 98 °C for 20 s and 45 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. PCR products were then subcloned into pCR™ 2.1 cloning vector using the TA Cloning® kit (Invitrogen). The clone was sequenced to confirm its length and identity with the EST sequence by automated sequencer (Applied Biosystems model 377).

The purified heparanase from phenyl-Sepharose chromatography was subjected to SDS-polyacrylamide gel electrophoresis (27) and then electrotransferred onto a ProBlott membrane (Applied Biosystems, Inc.). The transferred protein was visualized with Coomassie Brilliant Blue. The 50-kDa band, which had heparanase activity, was excised and used for N-terminal amino acid sequencing with the HP G105A protein sequencing system (Hewlett Packard). The transferred protein band prepared as above was digested in situ with Lys-C endoproteinase. The digests were subjected to reverse-phase chromatography and eluted with a gradient of 0–100% acetonitrile. The amino acid sequences of the separated peptides were analyzed using the HP G105A protein sequencing system.

Identification of the Human Heparanase cDNA Using Expressed Sequence Tag (EST) Sequences

The peptide sequences identified from the purified human heparanase were used to search for homologues in the gene data bases stored at the NCBI by using the tBLASTn sequence alignment program. Translation of EST sequences (yw67t02.r1 Homo sapiens cDNA clone 260138 5', GenBank™ accession no. N45867 and yw70a013.r1 Homo sapiens cDNA clone 257548 3', GenBank™ accession no. N30824) in one frame presented a significant sequence similarity with three internal peptide sequences of the human heparanase. These sequences were originally obtained from a human placenta 8–9-week cDNA library primed with oligo(dT) primer. Aligning overlapping regions, the sequences of these EST clones were connected to make one DNA fragment 968 bp in length. A 731-bp DNA fragment corresponding to the connected DNA of these EST clones was then amplified by reverse transcriptase-PCR using WI38/Va13 cDNA. For this purpose, a total RNA was prepared from the cultured WI38/Va13 cells using ISOGEN (Nippon Gene, Corp. Japan), and poly(A) RNAs were purified with an mRNA purification kit (Amersham Pharmacia Biotech). poly(A) RNA was reverse-transcribed with the avian myeloblastosis virus reverse transcriptase (Takara, Japan) according to the manufacturer's protocol and used for PCR with a sense oligonucleotide primer (5'-CCTTCTAA-GAAGTCCACCTTC-3') and an antisense oligonucleotide primer (5'-AAACTATATGAGAAAGCTGGC-3') of 72,000 cm$^{-2}$ 4). PCR was performed using the LA PCR™ kit (Takara). PCR conditions were as follows: 98 °C for 20 s and 45 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. PCR products were then subcloned into pCR™ 2.1 cloning vector using the TA Cloning® kit (Invitrogen). The clone was sequenced to confirm its length and identity with the EST sequence by automated sequencer (Applied Biosystems model 377).
stopped by adding an excess of heparin followed by boiling of the reaction mixture. The heparin was added before boiling to prevent FITC-HS from precipitating with the protein. The degraded products were analyzed by HPLC equipped with an automated injector. A 20-μl aliquot of the reaction mixture was injected into a G3000 SWXL column to analyze the products by molecular size. Fig. 1a shows a chromatographic profile of the intact FITC-HS with a sharp peak and retention time of 15.99 min. When the products of FITC-HS incubated with the cell extract prepared from SV40-transformed human embryonic fibroblast WI38/VA13 cells were analyzed, a very broad peak with decreased FI appeared in the lower molecular weight range as compared with the intact FITC-HS (Fig. 1b). From the retention time, the average molecular mass of the degradation products was determined to be 20–30 kDa. The FITC-HS was also tested for degradation following a treatment with 1 milli-unit of heparitinase of bacterial origin (Fig. 1c). The peaks at retention times of 29.98 and 31.94 min represent tetra- and disaccharides produced by heparitinase treatment, respectively.

**Purification of Human Heparanase**—A human embryonic lung fibroblast cell line WI38 did not show any heparanase activity toward FITC-HS when analyzed by HPLC (data not shown), while the SV40-transformed clone WI38/VA13 showed very high heparanase activity. Therefore, WI38/VA13 cells were used as a source of the human heparanase in this purification. The cell extract containing 880 mg of protein was first subjected to heparin-Sepharose affinity column chromatography at pH 7.5. The amount of protein eluted from this column was 10.2 mg, which was approximately 1% of the original cell extract (Table 1). Then ConA-agarose affinity chromatography was employed. This procedure again resulted in significant enrichment of the heparanase in the eluate, but the recovery of the enzyme activity was less than 5%. The third step was carboxymethyl-Sepharose cation exchange chromatography, which did not result in any reduction in the activity recovered. The final step was performed utilizing hydrophobic interaction matrices tested, phenyl-Sepharose showed the highest binding to heparanase. Fig. 2A shows the chromatographic profile of phenyl-Sepharose chromatography and the catalytic activity toward FITC-labeled heparan sulfate. The heparanase was detected in fractions 62–78. Although the recovery of the enzyme activity was less than 5%, the degree of purification was ele-
The peptides derived from Lys-C endoproteinase digestion of the purified human heparanase were separated by reverse phase chromatography. All amino acid sequences were obtained as described under “Experimental Procedures.” X represents an unidentified amino acid residue.

| Protein/peptides          | N-terminal amino acid sequence                  |
|---------------------------|------------------------------------------------|
| Intact heparanase (50-kDa protein) | Lys-Lys-Phe-Lys-X-Ser-Thr-Tyr-Ser-Arg-Ser     |
| Peptide 45                | Val-Phe-Gln-Val-Val-Glu-Ser-Thr-Arg-Pro-Gly-Lys |
| Peptide 58                | Glu-Gly-Asp-Leu-Thr-Leu-Tyr-Ala-Ile-Asn-Leu-His-Asn-Val-Thr-Lys |
| Peptide 61                | Tyr-Leu-Leu-Arg-Pro-Leu-Gly-Pro-Gly-Leu-Leu-Ser-Lys |

**DISCUSSION**

A highly sensitive heparanase assay was developed by labeling heparan sulfate with FITC and using high speed gel permeation chromatography. The FI of FITC-HS was high enough to detect a very low level of heparanase activity. Most of all, the advantage of using fluorescence for detection was that the sensitivity was almost equivalent to that achieved by use of radioisotopes, but experiments could be performed safely and easily. The degraded products were analyzed by gel permeation column chromatography. Continuous routine analyses with a 7-min interval were performed in our assay system. Calculating the reduction of FI in the forward half area of the intact FITC-HS peak enabled us to perform quantitative analysis. We employed this calculation procedure rather than measuring retention time shift or the area of the fully cleaved heparan sulfate because several heparanase cleavage sites have been reported to exist in the heparan sulfate sequence (14, 15). The cleavage of only one susceptible site by heparanase was enough to cause a reduction of the forward area of the intact FITC-HS peak (15, 18, 19).

In the present study, human heparanase was purified from SV40 virus-transformed WI38/VA13 cells. The parental human embryonic lung fibroblast WI38 cells did not show significant heparanase activity, suggesting that heparanase expression was activated by SV40 transformation in WI38/VA13 cells. Similar results were reported on hyaluronidase expression in SV40-transformed T3 cells by Orkin et al. (30). Thus, the expression of these endoglycosidases may be controlled by the same transcriptional regulation mechanism and closely linked to malignant transformation. Alternatively, the production of
endogenous inhibitors may be lost by SV40 transformation. It is thus of great interest that the heparanase activity of various malignant tumor cells correlates well with their metastatic potential as reviewed by Nakajima et al. (31). High expression of such enzymes is probably necessary for malignant cells to invade basement membranes and cause angiogenesis (31, 32).

In our purification, following the initial use of the heparin-Sepharose column, total enzyme activity rose from 5.1 to 82.5 FI/h. This result suggests that in the cellular extract, there is an endogenous inhibitor(s) of heparanase. The possible

FIG. 4. Nucleotide sequence of the human heparanase cDNA and the predicted amino acid sequence. The nucleotide sequence and the predicted amino acid sequence are shown. Six potential glycosylation sites are indicated by a triangle. The N-terminal amino acid sequence of the purified human heparanase is double underlined. Internal amino acid sequences obtained by Lys-C endoproteinase treatment are underlined.
existence of such an endogenous inhibitor(s) has been implied by Ihrcke et al. (23) working on heparanase purification from platelets. The high recovery of heparanase activity after heparin-Sepharose chromatography cannot be explained solely by the removal of HS/HSPG present in the cell extract. In fact, Keren et al. (33) have partially purified an endogenous heparanase inhibitor(s) of $M_r$ 2,000–10,000 with an isoelectric point of pH 5.6–5.8 from murine melanoma cells. There might also be endogenous small proteins other than HS/HSPG that strongly inhibit heparanase activity in WI38/VA13 cells.

The second purification step we applied was ConA affinity chromatography. Heparanase bound strongly to ConA-agarose, suggesting that it had several high mannose-type oligosaccharides linked to asparagine residues. Incubation of heparanase bound to ConA-agarose in elution buffer even overnight yielded less than 50% recovery of the heparanase activity, suggesting not only that there is a high affinity of heparanase oligosaccharides for ConA but also that strong hydrophobic interactions exist among these proteins. The third step of purification was carboxymethyl-Sepharose cationic exchange chromatography in which heparanase was eluted at a very low concentration of NaCl.

The final and very efficient purification step was phenyl-Sepharose hydrophobic interaction chromatography. This step was chosen after many failed attempts with various column chromatographies. Although the recovery of the enzyme was 3.6% of that recovered in the eluate from the heparin-Sepharose column, we detected only a single band of molecular size 50 kDa in the purified fraction from phenyl-Sepharose chromatography. The molecular mass of the purified heparanase reported so far varies from 10 up to 98 kDa. There might be a post-translation modification and/or an activation of heparanase enzymes in an organ-specific manner, resulting in the different sizes of heparanase observed. Alternatively, they might be truly different enzymes.

The optimal pH for heparanase enzymatic activity was 4.2. This result coincides with previous reports from several groups. From the optimal pH, it is suggested that heparanase is localized in lysosomes. Mollinedo et al. (34) have reported that heparanase is localized in the tertiary granules of human neutrophils. Since there have been extensive reports concerning glycosaminoglycans in the nucleus modulating cell growth, heparanase might also be localized in the nucleus as a regulator of the cell cycle (35). In relation to this, there is an interesting report that the activity of topoisomerase I was inhibited by HS of approximately 15 kDa (11), which is equivalent to the average size of HS degradation products following heparanase treatment.

Heparanase was reported to act as an adhesion molecule above pH 7.0 (24). It would be very interesting to know whether the enzyme we have purified could also act as adhesion molecules on the cell surface. We know that at least at neutral pH, the enzyme still possesses a strong affinity toward HS and heparin. Heparanase may have the ability to adhere to HSPG expressed on the cell surface or HS existing in the extracellular matrix at physiological pH. We are currently attempting to produce a very specific antibody against heparanase. When this has been obtained, further studies regarding the localization as well as the function of heparanase on the cell surface could be carried out.

We report here, for the first time, amino acid sequences of human heparanase peptides. In a search against the SwissProt protein data base, the sequences showed no homology to any known protein sequences. Thus, we performed molecular cloning of human heparanase cDNA from the WI38/VA13 cDNA library by using the obtained internal amino acid sequences of human heparanase, and one cDNA coding a full-length heparanase was cloned. This cDNA had no homology with connective tissue activating peptide III, which was reported to have heparan sulfate-degrading endoglucosaminidase activity (26). There were six potential $N$-glycosylation sites in the protein sequence, and the enzyme may be further modified by $N$-glycosylation.
sites in the open reading frame, which coincided with our findings that the purified heparanase had high mannose-type N-linked oligosaccharide chains.

The expression study clearly demonstrated that the clone is the cDNA encoding for human heparanase. When the full-length heparanase was expressed in mammalian cell lines, NIH3T3 and COS-7, both transfectants showed high levels of heparanase activity against FITC-HS.

In summary, our studies reveal for the first time the cDNA encoding human heparanase. Bame and Robson (36) proposed that there might be at least two different kinds of heparan sulfate-degrading enzyme. In their study, the products of heparan sulfate degraded by heparanase were grouped into two distinct classes, one of each recognizing the substrate by differences in sulfate content between modified and unmodified regions. Their work suggests that there might be another homologous heparanase, or alternatively the heparanase coded by the same cDNA may express different substrate specificities when modified by proteinases post-translation. In the latter model, differences in sizes of the heparanase reported so far would provide a supportive observation. It would be very interesting to elucidate the actual site of heparan sulfate that the heparanase catalytically cleaves. Once we identify concrete structures of the cleavage sites, we might be able to carry out the kinetic study using a substrate molecule having a single heparanase cleavage site.

Further studies will be needed to clarify the roles of heparanase in regulating biological activities of heparan sulfate and heparan sulfate proteoglycans. The expression of a recombinant human heparanase as well as the production of antibodies against it has been well advanced. These materials will be useful in studying heparanase functions (cellular localization, substrate specificity, etc.) in relation to diseases including cancer, inflammation, wound healing, and central nervous diseases.

Acknowledgments—We thank N. Uchida and N. Uodome for excellent technical assistance. We thank Drs. Y. Shibanaka, T. Kasaoka, T. Watanabe, H. Nishiyama, M. Okada, N. Uchiyama, T. Matsushita, J. Dong, and A. Kukula for helpful suggestions and discussion. We also thank Dr. A. Kukula for critical reading of the manuscript.

REFERENCES
1. Hileman, R. E., Fromm, J. R., Weiler, J. M., and Linhardt R. J. (1998) BioEssays 20, 156–167
2. Stringer, S. E., and Gallagher, J. T. (1997) Int. J. Biochem. Cell Biol. 29, 709–714
3. Rappaz, A. C. (1993) Curr. Opin. Cell Biol. 5, 844–853
4. Bernfeld, M., Hinkes, M. T., Gallo, R. L. (1993) Development 1993 Suppl., 205–212
5. Kjellen, L., and Lindahl, U. (1991) Annu. Rev. Biochem. 60, 443–475
6. Kovalszky, I., Dudas, J., Olah-Nagy, J., Pogany, G., Tovary, J., Timar, J., Kopper, L., Jeney, A., Iozzo, R. V. (1998) Mol. Cell. Biochem. 183, 11–23
7. Schulz, J. G., Megow, D., Roszka, R., Villerenger, A., Einhaupl, K. M., and Dinagl, U. (1998) Eur. J. Neurosci. 10, 2085–2093
8. Ernst, S., Langer, R., Cooney, C., and Sassekharan, R. (1995) Crit. Rev. Biochem. Mol. Biol. 30, 387–444
9. Nakajima, M., Irimura, T., Di Ferrante, D., Di Ferrante, N., Nicolson, G. L. (1983) Science 260, 661–663
10. Pillarisetti, S., Paku, L., Sasaki, A., Vanni-Reyes, T., Yin, B., Puthasra, N., Wagner, W. D., Goldberg, I. J. (1997) J. Biol. Chem. 272, 15753–15759
11. Nakajima, M., Irimura, T., Di Ferrante, N., and Nicolson, G. L. (1986) Mol. Cell. Biochem. 63, 391–395
12. Nakajima, M., Irimura, T., Di Ferrante, N., Nicolson, G. L. (1986) Anal. Biochem. 157, 162–171
13. Ernst, S., Langer, R., Cooney, C., and Sasisekharan, R. (1995) Anal. Biochem. 230, 2283–2290
14. Ototani, N., Kikuchi, M., and Yoshizawa, Z. (1981) Carbohydrate Res. 88, 291–303
15. Pillarisetti, S., Paku, L., Sasaki, A., Vanni-Reyes, T., Yin, B., Puthasra, N., Wagner, W. D., Goldberg, I. J. (1997) J. Biol. Chem. 272, 15753–15759
16. Nakajima, M., Irimura, T., Di Ferrante, N., and Nicolson, G. L. (1984) J. Biol. Chem. 259, 709–714
17. Groves, R., and Sassekharan, R. (1996) Biochem. Biophys. Res. Commun. 229, 770–777
18. Irimura, T., Nakajima, M., Di Ferrante, N., Nicolson, G. L. (1983) Anal. Biochem. 130, 461–468
19. Nakajima, M., Irimura, T., Nicolson, G. L. (1986) Anal. Biochem. 157, 162–171
20. Freeman, C., and Parish, C. R. (1997) J. Biol. Chem. 272, 229–237
21. Jin, L., Nakajima, M., Nicolson, G. L. (1990) Int. J. Cancer, 45, 1088–1095
22. Itoh, N., Nakajima, M., Nicolson, G. L. (1990) Crit. Rev. Biochem. Mol. Biol. 30, 387–444
23. Ikeda, E., Nakajima, M., and Irimura, T. (1998) Cell. Physiol. 175, 255–267
24. Gilat, D., Hershkoviz, R., Goldkorn, I., Cahalon, L., Kornerr, G., Vladovskiy, I., and Lider, O. (1995) J. Exp. Med. 181, 1929–1934
25. Tumova, S., and Bame, K. J. (1997) J. Biol. Chem. 272, 9078–9085
26. Hoogewerf, A. J., Leone, J. W., Reardon, I. M., Howe, W. J., Asa, D., Heinrikson, R. L., and Ledbetter, S. R. (1995) J. Biol. Chem. 270, 3268–3277
27. Laemmli, U. K. (1970) Nature 227, 680–685
28. Arisaka, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Nucleic Acids Res. 25, 3389–3402
29. Azizkhan, J. C., Jensen, D. E., Pierce, A. J., and Wade, M. (1993) Critical Rev. Eukaryotic Gene Expression 3, 229–254
30. Orkin, R. W., Underhill, C. B., and Toole, B. P. (1982) J. Biol. Chem. 257, 5821–5826
31. Nakajima, M., Irimura, T., and Nicolson, G. L. (1988) J. Cell. Biochem. 36, 157–167
32. Vladovskiy, I., Kornerr, G., Ishi-Michaeli, B., Bashkin, P., Bar-Shavit, R., Fuks, Z. (1990) Cancer Metastasis Rev. 9, 203–226
33. Keren, Z., Leland, F., Nakajima, M., and LeTruc, S. J. (1989) Cancer Res. 49, 295–300
34. Molinendo, F., Nakajima, M., Lichtenstein, E., Callejo, S., Gajate, C., and Fabra, A. (1997) Biochem. J. 327, 917–923
35. Liang, Y., Haring, M., Roughley, P. J., Margolis, R. K., and Margolis, R. U. (1997) J. Biol. Chem. 272, 851–864
36. Bame, K. J., and Robson, K. (1997) J. Biol. Chem. 272, 2245–2251