Processing of the Human Heparanase Precursor and Evidence That the Active Enzyme Is a Heterodimer*

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Human platelet heparanase has been purified to homogeneity and shown to consist of two, non-covalently associated polypeptide chains of molecular masses 50 and 8 kDa. Protein sequencing provided the basis for determination of the full-length cDNA for this novel protein. Based upon this information and results from protein analysis and mass spectrometry, we propose a scheme to define the structural organization of heparanase in relation to its precursor forms, proheparanase and pre-proheparanase. The 8- and 50-kDa chains which make up the active enzyme reside, respectively, at the NH₂- and COOH-terminal regions of the inactive precursor, proheparanase. The heparanase heterodimer is produced by excision and loss of an internal linking segment. This paper is the first to suggest that human heparanase is a two-chain enzyme.

A vast literature attests to the importance of heparan sulfate proteoglycans (HSPG)¹ in a variety of physiological processes (cf. Refs. 1–8 for reviews and additional references). Not only do these complex molecules provide a physical barrier to movement of cells into tissues, but the carbohydrate, or glycosylamino-glycan moieties of the HSPG bind and sequester a variety of bioactive proteins, including growth factors, chemokines, cytokines, and enzymes. These several proteins may be retained in complexation with the proteoglycans, or released when the HSPG are broken down, thus providing mechanisms for induction of growth, chemotaxis, and extravasation of a diverse set of cells in normal or disease processes (3, 8). This regulatory aspect of HSPG function impacts a wide spectrum of biological phenomena underlying inflammatory and cardiovascular diseases and cancer.

Heparanases, enzymes secreted by activated platelets (2) and neutrophils (9) and by metastatic cells (3), hydrolyze the glycosaminoglycan units of HSPG, thus facilitating release of the many protein modulators of cell function that are bound at these sites. This helps pave the way for migration of neutrophils and cancer cells from the vasculature into tissues and promotes supply of blood vessels to a growing tumor (3). Although the importance of heparanase function has long been recognized (3), and bacterial heparanases have been thoroughly characterized (10), the mammalian enzyme has remained, until very recently, an elusive target. Now, for the first time, Vlodavsky et al. (11), Hulett et al. (12), and Russie et al. (13) have reported the cDNA and derived amino acid sequences of a novel, human heparanase. The molecule is unique among known protein structures and shows no homology to the bacterial enzymes. All three groups agree that the heparanase is a single-chain glycoprotein, roughly 50,000 in molecular weight, that is derived from a precursor of 543 amino acids as defined by the full-length cDNA (11–13). Clearly, this important discovery could have major therapeutic implications for the development of new classes of drugs for cancer, heart disease, and inflammation (3, 14, 15).

The present paper describes the purification and characterization of heparanase from human platelets. During the course of this work, we also determined the cDNA and derived protein sequences for heparanase and its precursor molecules, and our results are in complete accord with those from the aforementioned studies. However, in contrast to the conclusion that heparanase is a single 50-kDa polypeptide (11–13), our findings suggest that the active enzyme is a heterodimer in which this 50-kDa protein works in concert with a tightly associated, but non-covalently linked, 8-kDa peptide. Both chains arise by processing of a single precursor protein, designated herein as proheparanase. A scheme is proposed to define the precursor forms of heparanase and the processing events leading to enzyme activation.

EXPERIMENTAL PROCEDURES

Materials

Fresh human platelet concentrates were obtained by apheresis. Complete protease inhibitor tablets and diisopropyl fluorophosphate were purchased from Roche Molecular Biochemicals. HSPG substrate was prepared as described by Ledbetter et al. (16). Heparin-Sepharose CL6B and Protein A-Sepharose Fast Flow bulk media, and Superdex-75 Hi-Load, and heparin Hi-Trap columns were purchased from Amersham Pharmacia Biotech. Precast 10–20% gradient and 10% homogeneous polyacrylamide gels, Tricine running buffer, and molecular weight marker sets were obtained from Millipore Corp. Polyvinylidene difluoride membranes were from Schleicher and Schuell. Jupiter C4 and C18 microbore columns were obtained from Phenomenex, and HPLC-grade water and acetonitrile were from OmniSolv. Pyroglycaminieaminopidae was from Takara Biomedicals.

Methods

Assay for Heparanase Activity

Samples containing heparanase activity were identified as described earlier (7) by their ability to degrade metabolically radiolabeled and purified high molecular weight HSPG derived from mice bearing the Engelbreth-Holm-Swarm tumor (16) to filterable fragments. We define a unit of activity as that amount of enzyme that produces breakdown of 1% of the substrate per hour, as measured by liberation of counts that pass through a 30,000 nominal molecular weight limit membrane filter.
Purification of the Platelet Heparanase

Heparin-Sepharose Chromatography—The supernatant from activated, centrifuged platelet lysates (7) was made 1 mM in glutathione (GSH) and diithiothreitol (DTT) and loaded (1.0 ml/min) onto a column of heparin-Sepharose (1.6 × 20 cm; 40 ml) equilibrated with phosphate-buffered saline, 1 mM in both GSH and DTT. The column was then washed with 0.01 M sodium acetate, pH 5.6, containing 1 mM GSH, 1 mM DTT, and 0.5% NaCl (buffer A) until the initial base line was reestablished. At this time, a 750-ml linear gradient of increasing NaCl concentration (0.35–1.5 M) in buffer A was applied to elute the active heparanase. The column effluent was monitored by UV detection at 280 nm, and aliquots were taken for heparanase assay. Fractions (9 ml) containing active heparanase were pooled and concentrated using a stirred cell ultrafiltration module.

Superdex-75 Size Exclusion Chromatography—The ultrafiltration retentate (typically 8–10 ml) was loaded in 1-ml batches directly onto a Superdex-75 Hi-Load size exclusion chromatography (SEC) column (1.6 × 60 cm) equilibrated with 0.01 M sodium acetate, pH 5.0, containing 0.5 mM NaCl, 1 mM DTT, and 10 mM β-octyl glucoside (buffer B). The column was run at 1.0 ml/min, and the effluent was monitored at 280 nm. Fractions of 4–6 ml were collected, and aliquots were assayed by SDS-PAGE and for heparanase activity.

Heparin Hi-Trap Chromatography—Pooled fractions from the SEC step were diluted 2-fold with deionized water to lower the ionic strength and loaded directly onto a 1-ml column of heparin Hi-Trap-Sepharose equilibrated with 0.01 mM sodium phosphate, pH 7.0, containing 0.25 mM NaCl, 1 mM DTT, and 10 mM β-octyl glucoside (buffer C). The column was run at 0.5 ml/min and the effluent monitored at 280 nm. Fractions of 4–6 ml were collected, and aliquots were assayed by SDS-PAGE and Western blotting and for heparanase activity.

Protein Characterization

SDS-PAGE—SDS-PAGE (17) was performed under both reducing and non-reducing conditions employing the Tricine buffer system (18) and 10–20% gradient polyacrylamide gels (10 × 10 cm). After electrophoresis, gels were either stained with silver (19) or transferred to polyvinylidene difluoride membranes for Western blot analysis.

Protein Sequence Analysis and Mass Spectrometry—Peptide and protein sequences were obtained by automated Edman degradation in a Perkin-Elmer Applied Biosystems model 494 protein sequencer. Model 610A Version 2.1 software was employed for data acquisition and processing. Matrix-assisted laser desorption time of flight mass spectrometry (MALDI-TOF MS) was performed on a Perseptive Biosystems Voyager Elite mass spectrometer.

Antibody Preparation and Immunological Methods

After the cDNA and derived protein sequences for heparanase were in hand, antibodies were raised against peptide antigens taken from various regions of the enzyme sequence. The following synthetic peptides were prepared by conventional solid phase methods: NH₂- R273TKAAMLKSLKLAAGGVEIGGC-COOH and NH₂- V339WLGET-D105PKKEST-NH₂ from the 50-kDa subunit, and NH₂- D339PKKEST-FEERSGGGC-COOH, overlapping the COOH terminus of the 8-kDa peptide and the segment linking the 8- and 50-kDa peptides. These peptides were conjugated to a maleimide-activated form of keyhole limpet hemocyanin, and the keyhole limpet hemocyanin-conjugated peptides were injected into rabbits. Verification that the antisera specifically recognized the peptides, proheparanase, and the 8- and 50-kDa polyepitides of active platelet heparanase was accomplished by Western blotting.

RESULTS AND DISCUSSION

Purification of Platelet Heparanase—Chromatography of the crude platelet lysate on a column of heparin-Sepharose yielded a profile similar to that published earlier (7). Elution of the column with a gradient of increasing NaCl led to recovery of all of the heparanase activity in a region from about 0.8–1.2 M NaCl. SDS-PAGE analysis of the resulting fractions revealed no hint of the heparanase on stained gels, but Western blot analysis using antibodies against heparanase peptide segments correlated the presence of a 50-kDa band with fractions having heparan sulfate-degrading activity. A 65-kDa band was also observed by Western blotting of this fraction. The second purification step by size exclusion chromatography gave high resolution of heparanase activity in a peak comprising components of about 20–50 kDa as seen on SDS-PAGE. This peak was clearly resolved from the elution position of chemokines (7).

The pool of heparanase activity from the SEC step was loaded onto the final heparin Hi-Trap affinity column, and the elution profile is shown in Fig. 1A. A small peak, shaded in Fig. 1A, was the last to elute from the column, and this peak 5 fraction contained most of the heparanase activity. SDS-PAGE of this peak under non-reducing conditions revealed a high state of purity, with only two species of approximately 8 and 50 kDa, indicated by arrows in Fig. 1B. Both silver staining and immunostaining with anti-heparanase antibodies showed the presence of the 65-kDa protein in fraction 4, where the low heparanase activity would be consistent with the faint 50-kDa band in this fraction (Fig. 1, lane 4). The overall yield of the
purified enzyme (22 µg) was about 6%, with a purification of 160,000-fold over the activity in crude platelet extracts. The specific activity of our purified heparanase was 450,000 units/mg of protein.

Separation and Analysis of the 50- and 8-kDa Peptides—Microbore RP-HPLC (C4) of the purified heparanase resolved two main peaks (Fig. 2A, 1 and 2), corresponding to the 8- and 50-kDa species, respectively (Fig. 2B). Integration of the areas under peaks 1 and 2 revealed that the ratio of 2/1 was about 6.5/1. This is in close agreement with what would be expected from an equimolar amount of the 50- and 8-kDa species in the purified enzyme (Fig. 1, peak 5). NH2-terminal sequence analysis of the individual peaks gave the sequence: KRFKNS... for the 50-kDa protein; the 8-kDa peptide appeared to be blocked.

The 8- and 50-kDa proteins were subjected to cleavage by cyanogen bromide, trypsin, and lysyl-endopeptidase to generate fragments for sequencing. Peptides were isolated by RP-HPLC on a C18 column developed in a standard TFA/acetoni-trile system, and individual peaks were lyophilized and submitted for Edman degradation and/or mass determination by MALDI-TOF MS. These peptide sequences served as the basis for data base searches which led, ultimately, to our elucidation of the cDNA and derived protein sequences for human platelet heparanase. Details with regard to the peptide purification and analysis are not included in this paper, nor are data presented for the cDNA determination. This is because three identical cDNA and derived protein sequences for human heparanase precursor were published while our manuscript was in preparation (11–13), and our sequences are exactly the same.

For purposes of the present discussion, the protein sequence corresponding to the open reading frame for the heparanase precursor, defined herein as pre-proheparanase, is given in Fig. 3. This cDNA codes for a protein of 543 amino acids. There are two Met residues in the signal peptide sequence, Met1 and Met14, and our choice of Met1 as the start site conforms to the previously published sequences (11–13) and is supported by a prediction algorithm for signal peptides (20). Most of the primary structure (86%) deduced from the cDNA sequence was confirmed in our laboratory by peptide sequence analysis; these sequences are designated by arrows and underlining in Fig. 3.

Processing of the Pre-proheparanase—The sequence information for the 50-kDa protein placed it at the COOH-terminal region of the pre-proheparanase, extending from Lys158 to Ile543. This is in accord with results of Huehle et al. (12). The blocked 8-kDa peptide (−0.5 µg) was hydrolyzed overnight with 1 milliunit of Pfu pyroglutamate aminopeptidase, and the resulting protein gave the sequence: Asp37-Val-Val-Asp-Leu... . This placed the 8-kDa peptide in the NH2-terminal region of preheparanase and provided evidence that the signal peptide cleaved the Ala35-Gln36 bond. The NH2-terminal Gln36 of preheparanase then cyclized to PCA 36, thus accounting for the blocked NH2-terminals of the 8-kDa peptide and the 65-kDa proheparanase. To answer the question as to the COOH-terminal processing of the 8-kDa component, purified peptide (Fig. 2A, peak 1) was subjected to mass spectrometry. MALDI-TOF MS yielded two masses of high accuracy and similar molecular weights that were found in a ratio of about 4 to 1 (Fig. 4). The experimentally determined masses are in excellent agreement with the predicted masses corresponding to a major peptide (80%) extending from PCA36 to Glu109 (8247.5 Da) and a minor peptide from PCA36 to Lys109 (8118.4 Da). With reference to Fig. 3, the structural organization of preproheparanase may be represented as shown in Scheme 1.

Pre-proheparanase: Met1—..........................Ile543
Proheparanase (65 kDa): PCA36—Gln36 + Ile543—
Heparanase (50 kDa): PCA36—Gln36 + Lys109—

Scheme 1.

Edman degradation was performed on all six of the peptides having the consensus sequence for N-linked glycosylation (Fig. 4), and all showed a gap at the positions expected for Asn. This would suggest that all six sites are, indeed, N-glycosylated.

The work described herein presents for the first time an understanding of the processing events leading to activation of heparanase and evidence that the active enzyme is a heterodimer. Others who have studied this enzyme have concluded that the 50-kDa protein alone is responsible for activity (11–13, 21, 22); no one has reported the presence of the 8-kDa peptide. A number of explanations might be considered here. The 8-kDa fragment stains less intensely on acrylamide gels than does the 50-kDa protein (Fig. 1B) and, unless one applies a gel system capable of resolving low molecular weight components, it is likely to go undetected, especially at the low levels of protein encountered with heparanase. Moreover, the 8-kDa peptide is NH2 terminally blocked and refractive to Edman
and this led to speculation that the region NH2-terminal to peak 1 Lys158 may play some functional role in catalysis. Indeed, our results would serve to define this region as the 8-kDa peptide. Furthermore, it is usually the case that processing of proteins from progenitor molecules does not produce the active entity as a stable complex with its activation peptide, unless that activated form is dependent upon the complex itself. An apt example here is procaspase activation, where excision of a peptide bridging the active 2-chain "heterodimeric" caspase (23). These chains pack, and, at present, we lack definitive evidence for an obligate heterodimer.

Finally, an earlier report from this laboratory suggested that heparanase was a post-translationally modified form of a CXC chemokine, namely CTAPIII (7). We have not been able to confirm this observation, nor have others who have purified and characterized human heparanase (11–13, 21, 22). Yet, a recent paper by Rechter et al. (24) reported heparanase activity in a CTAPIII fusion protein with cellulose binding domain. Given this observation, the notion of a chemokine with heparanase activity remains a controversial issue.


degradation, further complicating its identification in a mixture with the 50-kDa protein (12). Of course, it may be that the more extensive purification protocols reported earlier (21, 22) led to dissociation of the 50/8-kDa complex and that the 8-kDa chain is not required for activity. In our hands, however, the complex survived gel filtration at high ionic strength and in the presence of detergent, as well as two affinity purification steps. Furthermore, it is usually the case that processing of proteins from progenitor molecules does not produce the active entity as a stable complex with its activation peptide, unless that activity is dependent upon the complex itself. An apt example here is procaspase activation, where excision of a peptide bridging NH2-terminal and COOH-terminal polypeptides gives rise to the active 2-chain "heterodimeric" caspase (23). These chains remain in tight, non-covalent association, and both are obligatory for function, while the bridge peptide is lost.

A final, and compelling argument for the importance of the 8-kDa chain derives from cloning and expression of heparanase done in other laboratories. Heparanase activity is easily demonstrated in mammalian cell hosts transfected with full-length cDNA corresponding to the 543-residue pre-proheparanase (11–13). However, expression of the truncated 50-kDa protein defined by Lys158 to Ile343 (12) failed to yield active enzyme, and this led to speculation that the region NH2-terminal to Lys158 may play some functional role in catalysis. Indeed, our results would serve to define this region as the 8-kDa peptide.

Considering evidence presently in hand, we conclude that both the 8- and 50-kDa chains are required for enzyme activity, but we lack proof for this assertion. These protein components are easily separated under denaturing conditions on SDS-PAGE (Fig. 1) or by RP-HPLC (Fig. 2). Unfortunately, solvent systems that promote protein unfolding are required for dissociation of the chains, and these conditions lead to irreversible loss of enzyme activity. Therefore, a reconstitution experiment with chains separated as shown in Fig. 2A has not been feasible and, at present, we lack definitive evidence for an obligate heterodimer.

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