Partial purification of heparanase activities in Chinese hamster ovary cells: evidence for multiple intracellular heparanases

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Heparanases are mammalian endoglycosidases that cleave heparan sulphate glycosaminoglycans from proteoglycan core proteins and degrade them into shorter chains. The enzymes have been proposed to act in a variety of cellular processes, including proteoglycan catabolism, remodelling of basement membranes and release of heparan sulphate-binding ligands from their extracellular storage sites. Additional functions for heparanases may be to generate short heparan sulphate chains that stabilize or activate other proteins. While heparanase activities have been described in a number of tissues and cell lines, it is not known how many different enzymes are responsible for these activities. Our recent studies characterizing the short glycosaminoglycans produced in Chinese hamster ovary (CHO) cells suggested that multiple heparanases are necessary for the formation of the short heparan sulphate chains [Bame and Robson (1997) *J. Biol. Chem.* **272**, 2245–2251]. We examined whether this is the case by purifying heparanase activity from CHO cell homogenates. Based on their ability to bind ion-exchange resins and their elution from gel-filtration columns,

four separate heparanase activities were partially purified. All four activities cleave free glycosaminoglycans over a broad pH range of 3.5–6.0 or 6.5, suggesting that they act in the endosomal/lysosomal pathway. The sizes of the short heparan sulphate chains generated by the partially purified heparanases ranged from 6 to 9 kDa, and for two of the activities the product size is pH-dependent. Three of the four activities degrade proteoglycans as well as the free glycosaminoglycan chain. Interestingly, all four enzymes generate short glycosaminoglycans with a sulphate-rich, modified domain at the non-reducing end of the newly formed chain. Since our previous studies showed that in CHO cells there is also a population of short heparan sulphates with a modified domain at the reducing end of the chain, this suggests that there may be another heparanase in CHO cells that was not purified. Alternatively, our findings suggest that the formation of short heparan sulphate glycosaminoglycans inside CHO cells may be a result of the concerted action of multiple heparanases, and may depend on the proportions of the different enzymes and the environment in which the chains are degraded.

INTRODUCTION

Heparanases are a family of mammalian endoglycosidases that cleave heparan sulphate glycosaminoglycans from proteoglycan core proteins and degrade them into shorter chains [1–3]. In addition to their role in heparan sulphate proteoglycan catabolism, heparanases may act to remodel basement membranes after injury or at inflammation sites [4–7], to release heparan sulphate-binding ligands from their proteoglycan receptors [8], or to prevent the formation of deleterious associations of proteoglycans with other proteins by removing the glycosaminoglycan chains from the core protein [9]. The short heparan sulphate chains produced by heparanases may regulate the interaction of ligands with proteoglycan receptors [10], modulate enzyme activity [8] or associate with internalized ligands and thus protect them from proteases in the endosomal pathway ([11–15]; S. Tumova, B. A. Hatch, D. J. Law and K. J. Bame, unpublished work).

The complete degradation of heparan sulphate proteoglycans and the formation of short, functional heparan sulphate chains may require multiple heparanase enzymes. When bound to a core protein, the glycosaminoglycan chain may not be very accessible to a heparanase; consequently there may be an enzyme that acts on proteoglycans and another that is only able to degrade free chains. If a ligand is bound to the heparan sulphate glycosaminoglycan at a sequence that is recognized or cleaved by a particular heparanase, then another enzyme, which recognizes or cleaves a different sequence, is needed to degrade the chain.

Different heparanases with distinct specificities may also be essential to produce classes of short heparan sulphate chains that, due to the particular arrangement of sulphate groups and uronic acid epimers, have specific functions. Furthermore, since the fine structure of heparan sulphate glycosaminoglycans can vary between different tissues or different developmental states [16], it may be that a complement of heparanases is required to degrade the glycosaminoglycan chain.

Two types of heparanase have been identified, based on the glycosidic bond cleaved and the size of the heparan sulphate product. One type comprises a small, 9 kDa platelet endoglucosaminidase, which cleaves heparan sulphate chains into disaccharides [7]. It was proposed that this platelet enzyme is derived from the chemokine connective tissue activating peptide (CTAP)-III [7], although recent studies suggest that this is not the case [17]. The other type of heparanases are endoglucuronidases that cleave heparan sulphate glycosaminoglycans into short 5–6 kDa oligosaccharides [1–3,18,19]. Endoglucuronidase activities have been purified from platelets [19,20], placenta [21] and B16 melanoma cells [22], and all have different molecular masses, ranging from 45 kDa for the placenta enzyme up to 134 kDa for a platelet protein. It is unclear whether the 97 kDa melanoma protein is actually a heparanase, since it shares N-terminal sequence identity with the heat-shock protein/tumour rejection antigen gp96 [23,24]. No other information about the protein properties of these purified enzymes has been reported, so it is not known whether they are the products of the same or different genes.

Abbreviations used: CHO, Chinese hamster ovary; CP buffer, citrate/phosphate buffer; CPC, cetylpyridinium chloride.

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Recent studies characterizing heparanase products in Chinese hamster ovary (CHO) cells suggested that there are multiple endoglucuronidases responsible for the formation of the short heparan sulphate chains [18]. Heparan sulphate glycosaminoglycans are composed of modified domains, rich in iduronic acid residues and N- and O-sulphate groups, separated by regions of *N*-acetylglucosamine and glucuronic acid disaccharide repeats that contain very little O-sulphate [25]. CHO heparanase activities cleave the glycosaminoglycan at junctions between these modified and unmodified regions [18,26,27], generating two classes of short products that differ in the location of the modified domain on the molecule. Class I heparanase products have a modified domain at the non-reducing end of the chain, while class II products have a modified domain at the newly formed reducing end [18]. The simplest explanation for the formation of these two product classes is that they are generated by separate heparanase activities which recognize and bind the heparan sulphate substrate so that modified domains are oriented differently at the catalytic site.

We have partially purified four heparanase activities from CHO cell homogenates, which differ from each other by apparent molecular mass or their ability to bind to ion-exchange resins. Characterization of the enzymic properties shows that, while there are some differences in substrate specificity and the size of the short heparan sulphate chains produced, all four enzymes generate class I products. This suggests either that a heparanase that creates class II products has not been purified or that the formation of short heparan sulphate chains inside cells is due to the concerted action of all the enzymes.

EXPERIMENTAL

Cell culture

CHO cells (CHO-K1) were obtained from the American Type Culture Collection (CCL-61; Rockville, MD, U.S.A.). Cells were maintained in Ham's F-12 medium (Gibco-BRL, Grand Island, NY, U.S.A.) supplemented with 5.0% (v/v) bovine serum (Gibco-BRL), 100 µg/ml streptomycin sulphate and 100 units/ml penicillin G (Sigma, St. Louis, MO, U.S.A.). Sulphate-free medium (defined F-12 medium) was used for all radioactive labelling experiments and was prepared as described previously [28]. Cells were grown at 37 ± 0.2 °C in an atmosphere of 5% CO₂ in air and 100% relative humidity. They were subcultured every 3–4 days with 0.125% trypsin; after 15–20 passages, fresh cells were revived from frozen stocks stored in liquid nitrogen.

Heparan sulphate glycosaminoglycan and proteoglycan isolation

Heparan [³⁵S]sulphate glycosaminoglycans were isolated from confluent cell monolayers that had been incubated for 8–24 h in defined F-12 medium containing 50 µCi/ml H₂³⁵SO₄ (DuPont-New England Nuclear, Boston, MA, U.S.A.). The radioactive medium was removed, and the cells were washed with PBS and then incubated with trypsin to release cells from the culture dish. The long, nascent [³⁵S]glycosaminoglycans present in the trypsinate were isolated on DEAE-Sephacel columns as previously described [3]. Residual peptides bound to the chains were released by incubating the glycosaminoglycans with 1 M NaBH/0.5 M NaOH for 16 h at 4 °C. The mixture was neutralized with 10 M acetic acid to stop the reduction reaction, precipitated with ethanol, and then treated with 10 m-units/ml chondroitin ABC lyase (ICN Biochemicals, Irvine, CA, U.S.A.) for 16 h at 37 °C to degrade chondroitin sulphate. The long heparan [³⁵S]sulphate chains were separated from the chondroitin [³⁵S]sulphate disaccharides on a Sepharose CL-6B column (104 cm × 1 cm) equili-

brated in 0.2 M NH₄HCO₃. Fractions containing heparan [³⁵S]sulphate were pooled, concentrated on DEAE-Sephacel columns, desalted over a PD10 column (Pharmacia LKB Biotechnology, Inc.) and lyophilized. The purified ³⁵S-labelled chains were resuspended in water to 5000 c.p.m./µl for use in experiments.

Unlabelled long heparan sulphate glycosaminoglycans were prepared from CHO cells in a similar manner, except that larger amounts of starting material were used. CHO cells, grown to confluence in 150 mm-diameter plates, were trypsinized to release the cells from the dish, and the trypsinase was used to purify the long heparan sulphate chains. On average, long chains were isolated from trypsinates combined from about 500 150-mm plates [26]. The Sepharose CL-6B fractions that contained heparan sulphate chains were identified by applying 5–50 µl of each 1 ml fraction to Immobilon membranes and staining the bound glycosaminoglycans with Alcian Blue [29]. Fractions containing long heparan sulphate species were pooled, dialysed against water and lyophilized to dryness. The chains were resuspended in water, and the concentration of the purified heparan sulphate was estimated as follows: the amount of uronic acid (µg/µl) was determined using a carbazole assay [30], and then this value was multiplied by 2 to account for the glucosamine residues.

Heparan sulphate proteoglycans were extracted from confluent CHO cells that had been incubated for 1 h in defined F-12 medium containing 50 µCi/ml H₂³⁵SO₄ and purified by anion-exchange and gel-filtration chromatography as described previously [3,9]. Briefly, proteoglycans in the cell extract were isolated by DEAE-Sephacel chromatography, and applied to anion-exchange HPLC to separate heparan sulphate proteoglycans from chondroitin sulphate proteoglycans [3]. Heparan sulphate proteoglycans were pooled on the basis of their elution position from the anion-exchange column, and applied to a gel-filtration TSK 4000 column (TosoHaas, Montgomeryville, PA, U.S.A.) to separate heparan sulphate chains from intact proteoglycans [3]. Fractions corresponding to the proteoglycans were pooled, and the molecules were concentrated on a DEAE-Sephacel column [3].

Chromatographic materials and methods

High-S cation-exchange and heparin-Affigel resins were purchased from Bio-Rad. DNA-cellulose resins and a prepared Sephacryl 100HR column (1.6 cm × 60 cm) were obtained from Pharmacia. The concentration of salt in the column fractions was determined by measuring the conductivity of the fraction and comparing the value with a standard salt curve. Protein concentrations were determined with the BCA assay (Pierce). Partially purified heparanase activities were concentrated using a stirred-cell system from Amicon Inc. (Beverly, MA, U.S.A.) or Ultrafree centrifugal filtration devices from Millipore. In either case the membranes used for the concentration step had a molecular mass cut-off of 30 kDa. SDS/polyacrylamide gels were run according to the method of Laemmli [30a], fixed with methanol and acetic acid and stained with Coomassie Blue R-250 [31].

Heparan sulphate glycosaminoglycan degradation assay [cetylpyridinium chloride (CPC) assay]

Purified nascent ³⁵S-labelled heparan sulphate glycosaminoglycans (5000 c.p.m.) were mixed with unlabelled CHO heparan sulphate or bovine kidney heparan sulphate purchased from Sigma (0.5 ng/µl, final), and incubated for 1–16 h at 37 °C with column fractions or partially purified CHO heparanase. The

Scheme 1 Purification of heparanase activities from CHO cells

Heparanase activity was purified from CHO cells as described in the Experimental section. The activity of CHO heparanases at each step was determined by incubating aliquots of the pooled enzyme(s) with nascent ^{35}S -labelled heparan sulphate chains for 1 h. The short, cleaved chain products were separated from the substrate by their solubility in the detergent CPC. The ^{35}S radioactivity in the CPC supernatant was divided by the time of the assay (1 h) and the amount of protein present in the assayed aliquot to determine the specific activity (c.p.m./h per mg).

volume of the assay was 75 μl , and the pH was maintained at 5.5 with 21 mM citrate/57 mM sodium phosphate. Chain cleavage was determined by precipitating the reaction mixture with 1% (w/v) CPC in 0.32 M NaCl/40 mM sodium acetate (pH 5.5). Under these conditions, uncleaved glycosaminoglycans are precipitated, while cleaved chains remain in the supernatant [3]. The ^{35}S counts in the CPC supernatant were assayed by liquid scintillation counting, and divided by the total amount of ^{35}S radioactivity added to the assay to determine CPC-soluble heparan [^{35}S]sulphate as a percentage. Specific radioactivity was calculated by dividing the % soluble c.p.m. formed in 1 h by the amount of protein assayed (mg).

To examine the size of the heparanase-cleaved glycosaminoglycans by gel-filtration chromatography, the reaction was instead stopped with 75 μl of 0.1 M Tris (pH 8.0)/0.5 M NaCl, and the entire mixture was applied to a TSK 4000 gel-filtration column (7.5 mm $\times$ 30 mm; TosoHaas) equilibrated in 0.1 M KH_2PO_4 (pH 6.0)/0.5 M NaCl/0.2% Zwittergent 3-12 [3]. Heparin [^{35}S]sulphate chains were eluted from the column at a flow rate of 0.5 ml/min [3]. The column was calibrated with

heparin, heparan sulphate and chondroitin sulphate polysaccharides of known molecular mass [26]. By our estimation, the molecular mass (mean $\pm$ S.D.) of nascent CHO heparan sulphate chains is 81.0 ± 5.4 kDa and that of the short, degraded glycosaminoglycans, purified from cells, is 6.0 ± 1.1 kDa [26].

Heparan sulphate proteoglycan degradation assay

Purified CHO heparan [^{35}S]sulphate proteoglycans (5000 c.p.m.) were mixed with unlabelled CHO heparan sulphate (0.5 ng/ μl , final), and incubated for 16–20 h at 37 $^{\circ}\text{C}$ with each partially purified CHO heparanase. The reaction conditions were the same as for the [^{35}S]glycosaminoglycan substrate, except that 0.17% CHAPS was included in the assay. The reaction was stopped with 0.1 M Tris/0.8 M NaCl, pH 8.0, and the reaction mixture was applied to the TSK 4000 gel-filtration column to determine the extent of degradation. To confirm that heparan sulphate chains were still bound to core proteins, the reaction mixture was incubated with 1 M NaBH₄/0.5 M NaOH for 16 h at 4 $^{\circ}\text{C}$, neutralized with 10 M acetic acid and then applied to the TSK 4000 column.

Purification of heparanase activity from CHO cells

Approx. 100 ml of frozen CHO cell pellets (obtained from Genentech) were rapidly thawed at 37 $^{\circ}\text{C}$. Protease inhibitors (1 $\mu\text{g}/\text{ml}$ leupeptin, 0.5 $\mu\text{g}/\text{ml}$ pepstatin, 0.2 mM PMSF and 10 mM *N*-ethylmaleimide) and glutathione were added, and the cells were homogenized using a Dounce homogenizer (Scheme 1). This first homogenization was done without adding any buffer to the thawed cell paste. The homogenized cells were centrifuged (15000 *g*, SM-34 Sorvall, 4 $^{\circ}\text{C}$, 15 min) to remove unbroken cells and large organelles. The 15000 *g* pellets were resuspended in 15 mM citrate/30 mM sodium phosphate (pH 6.0)/1 mM glutathione (CP buffer), re-homogenized and re-centrifuged. The supernatants were combined, ammonium sulphate was added to a final concentration of 30% satn., and precipitated proteins and lipids were collected by centrifugation at 15000 *g* at 4 $^{\circ}\text{C}$ for 15 min. To ensure that all the heparanase activity was released into the 30% satd. ammonium sulphate supernatant, the pellets were resuspended in CP buffer, and proteins and lipids were re-precipitated with 30% ammonium sulphate. Over 90% of the heparanase activity was present in the two ammonium sulphate supernatants. This material was then dialysed against CP buffer to bring the salt concentration to below 6%. Together, the 15000 *g* centrifugation and the ammonium sulphate precipitation removed 70–75% of the protein present in the homogenate. Specific activity, as measured by the CPC assay, increased approx. 10-fold.

The dialysed ammonium sulphate supernatant was applied to a 150 ml High-S cation-exchange column equilibrated in CP buffer, pH 6.0. After the entire sample had been applied, the column was washed with CP buffer containing 0.3 M NaCl until the absorbance at 280 nm stabilized. In addition to removing over 90% of the applied protein, this washing step removed nucleic acids and glycosaminoglycans present in the ammonium sulphate supernatant. Heparanase activity, measured with the CPC assay, was eluted from the column as a single peak as soon as the salt concentration was raised to 1.5 M NaCl. Fractions containing heparanase activity were pooled and dialysed against CP buffer to bring the salt concentration to 0.1 M NaCl. Only 2–3% of the homogenate protein remained in the cation I-purified heparanase fraction, and there was an approx. 60-fold increase in specific activity.

Cation-exchange-purified activity was next applied to a 15 ml heparin-Affigel column equilibrated in CP buffer, pH 6.0, containing 0.1 M NaCl. After the sample had been applied, the column was washed with 20 ml of CP buffer/0.1 M NaCl, and then heparanase activity was eluted with a linear NaCl gradient (see Figure 1). Fractions containing heparanase activity were pooled and dialysed against CP buffer to bring the salt concentration to 0.1 M NaCl. Approx. 1% of the homogenate protein remained in the heparan-affinity-purified heparanase fraction, and the specific activity was increased 160-fold.

Heparin-affinity-purified activity was applied to a 30 ml High-S cation-exchange column equilibrated in CP buffer, pH 6.0, containing 0.1 M NaCl. The column was washed first with 100 ml of CP buffer/0.1 M NaCl, followed by 150 ml of CP buffer/0.3 M NaCl, and then heparanase activity was eluted with a linear gradient up to 1.5 M NaCl. Two peaks of heparanase activity were observed when fractions were assayed by the CPC assay (designated C1 and C2; see Figure 1 and Scheme 1). The two heparanase activities were pooled separately, dialysed against CP buffer to bring the salt concentration to 0.1 M NaCl and concentrated with an Amicon system against 30 kDa molecular mass cut-off membranes. C1 activity contained 0.2–0.3% of the homogenate protein, and specific activity had increased approx. 300-fold over that present in the homogenate. C2 activity contained less than 0.1% of the starting homogenate protein, and specific activity was increased approx. 900-fold.

Concentrated C1 and C2 activities were applied separately to a 10 ml DNA-cellulose column equilibrated in CP buffer containing 0.1 M NaCl. The column was washed consecutively with 100 ml portions of CP buffer containing 0.1 M NaCl, 0.3 M NaCl and 0.5 M NaCl. Fractions were collected and the heparanase activity in each was determined by the CPC assay. C1 heparanase activity was eluted as a single peak from the DNA-cellulose column, while C2 activity was eluted from the column in two peaks, which were termed C2A and C2B (see Figure 2). The three heparanase activities were each pooled, dialysed to bring the salt concentration to 0.1 M NaCl, and concentrated using either the Amicon stirred cell system or Millipore Ultrafree centrifugal devices.

The concentrated C1, C2A and C2B activities were applied separately to a Sephacryl S100 gel-filtration column run at a flow rate of 0.5 ml/min. Fractions of 1 ml were collected and the heparanase activity in each was determined by the CPC assay (see Figure 3). C1 activity was associated with both a 45 kDa protein and a 30 kDa protein. Activity associated with the 45 kDa species was pooled as C1A, while the activity associated with the smaller protein was pooled as C1B. The amount of protein in the Sephacryl C1A and C1B pools indicated that the two heparanases represent approx. 0.001% of the CHO homogenate protein. Concentrated C2A and C2B activities were eluted from the Sephacryl column as 40–46 kDa species (see Figure 3). Even when the fractions containing activity were pooled and concentrated, there was not enough C2A or C2B protein to reveal a band on an SDS/PAGE gel, indicating that the enzymes represent less than 0.001% of the starting homogenate protein.

Reduction of heparanase products with NaB³H₄

Unlabelled, nascent CHO heparan sulphate chains were incubated with each purified heparanase for 24 h to generate short glycosaminoglycan products. The reaction conditions were comparable with those used to assay the activity in the column fractions, except that each assay contained 7 ng/ μ l substrate. Identical assays containing heparan [³⁵S]sulphate substrate were run to ensure that cleavage had occurred. Ten assays were done

for each heparanase activity, and then pooled to generate enough product for the subsequent reducing-end labelling. The cleaved, unlabelled product was purified by DEAE-Sephacel chromatography, desalted over a PD10 column and lyophilized to dryness. Then 5 mCi of NaB³H₄ (American Radiolabeled Chemicals, St. Louis, MO, U.S.A.) was resuspended in 50 mM NaOH, and aliquots were added to each lyophilized product. The ratio of NaB³H₄ to heparan sulphate chains was approx. 1.0 mCi for 10 μ g of glycosaminoglycan (dry weight), and the reaction volume was at least 40 μ l. The mixtures were incubated at 45 °C for 24 h, and then the reduction was stopped by the addition of 10 M acetic acid, and neutralized with 1 M NaOH. Sodium acetate buffer (50 mM) was added, and the mixture was applied to a 0.5 ml DEAE-Sephacel column to purify the heparan [³H]sulphate away from the majority of unincorporated radioactivity. The purified [³H]glycosaminoglycans were then precipitated twice with ethanol to remove residual ³H radioactivity that had not been incorporated into the polysaccharide. Carrier heparin was added to the heparan [³H]sulphate to facilitate the ethanol precipitation. Precipitated chains were lyophilized, resuspended in water and stored at 4 °C.

Analysis of ³H-labelled reduced heparan sulphate

To determine the distance of the first modified domain from the newly formed reducing end, a portion of the ³H-labelled reduced heparan sulphate (15000 c.p.m.) was incubated with the bacterial polysaccharide lyase, heparinase (EC 4.2.2.7; Siekagaku America, Inc., Ijamsville, MD, U.S.A.), which cleaves the chain between *N*-sulphoglucosamine and 2-*O*-sulphated iduronic acid residues [32,33]. Heparinase (1.5 m-units) was added to ³H-labelled chains resuspended in 20 mM sodium acetate (pH 7.0)/1 mM calcium acetate (total volume 30 μ l) and incubated at 37 °C. Another 1.5 m-units of enzyme was added after 16 h, and the reaction was incubated for another 24 h to ensure that all the susceptible glycosidic linkages were cleaved. The size and proportions of [³H]oligosaccharides produced in the reaction were examined by chromatography on the Superdex peptide column (Pharmacia) equilibrated in 0.5 M pyridinium acetate, pH 5.0, and run at a flow rate of 500 μ l/min. Fractions of 200 μ l were collected. The column was standardized using BioGel-P2-column purified [³H]oligosaccharides generated by low-pH nitrous acid treatment of [³H]glucosamine-labelled CHO-K1 heparan sulphate or ³H-labelled reduced CHO-K1 heparan sulphate [18].

RESULTS

Partial purification of heparanase activities from CHO cells

In order to obtain the amounts of starting material necessary for the partial purification of heparanase activities, we obtained frozen CHO cell pellets from Genentech. Heparanase activities were isolated (Scheme 1) from CHO cell homogenates by centrifugation at 15000 *g*, ammonium sulphate fractionation, cation-exchange chromatography (step elution), heparin-Affigel chromatography, cation-exchange chromatography (gradient elution), DNA-cellulose chromatography and Sephacryl S100 gel-filtration chromatography. All purification steps were done at 4 °C, and all buffers contained 1 mM glutathione, since the reducing agent improved the retention of heparanase activity through the purification steps. The individual steps have been performed between four and ten times, with no marked deviations from the recovery estimates indicated in the Experimental section. Purification of enzyme activity is probably underestimated for

Figure 1 Heparin-Affigel affinity and cation-exchange chromatography of heparanase activities from CHO cells

Heparin affinity chromatography (upper panel): heparanase purified from the first cation-exchange column was applied to a 15 ml heparin-Affigel column, and activity was eluted with a linear NaCl gradient (---) at pH 6.0. Fractions of 8 ml were collected and 5 μ l aliquots were assayed for heparanase activity (●) with the CPC assay (units of % CPC-soluble c.p.m. formed/h). The negatively numbered fractions represent the activity and protein in one flow-through fraction (115 ml) and four washes with 0.1 M NaCl (50 ml each). Activity that eluted between 0.30 and 0.85 M NaCl was pooled as indicated by the bar. Cation-exchange chromatography II (lower panel): heparanase-purified heparanase was applied to a 30 ml High-S cation-exchange column, and activity was eluted with a linear NaCl gradient (---) at pH 6.0. Fractions of 15 ml were collected and 5 μ l aliquots were assayed for heparanase activity (●) with the CPC assay. The negatively numbered fractions represent the flow-through (100 ml) and two washes at 0.1 M NaCl (50 ml each) before the column was washed with 0.3 M NaCl. As indicated by the bars, heparanase activity that eluted between 0.4 and 0.8 M NaCl was pooled as cation 1 (C1) activity, while activity that eluted between 0.9 and 1.2 M NaCl was pooled as cation 2 (C2) activity.

two reasons. First, our heparanase activity assay (CPC assay) only indicates when the 81 kDa substrate is converted into 40 kDa, CPC-soluble chains [26], so that the subsequent cleavages that convert these intermediate chains into the final short 6–10 kDa products are not recorded. In addition, we have found that the partially purified heparanase activities stick avidly to different membranes; therefore, although we wash the membranes as thoroughly as possible, we lose activity when the preparations are concentrated.

CHO heparanase activity is eluted from a heparin-Affigel column as a broad peak between 0.30 and 0.85 M NaCl (Figure 1). When this material was pooled and applied to a High-S cation-exchange column, two peaks of activity were observed. The first peak, designated C1, was eluted from the column between 0.4 and 0.8 M NaCl, while the second peak, designated C2, was eluted from the column between 0.9 and 1.2 M NaCl (Figure 1). The two heparanase activities were pooled separately, dialysed against CP buffer to bring the salt concentration to 0.1 M NaCl and concentrated with an Amicon system against 30000 kDa cut-off membranes. As previously mentioned, a portion of both C1 and C2 activities remained bound to the membrane used for concentration. This activity was recovered by washing the membranes with CP buffer containing 0.15 M NaCl and 0.5% CHAPS, and added to the remaining concentrate.

Figure 2 DNA-cellulose chromatography of heparanase activities from CHO cells

C1 and C2 heparanases were applied to a 10 ml DNA-cellulose column and activity was eluted from the resin with increasing NaCl concentrations (---) at pH 6.0. Fractions of 10 ml were collected and 5 μ l aliquots were assayed for heparanase activity with the CPC assay (units of % CPC-soluble c.p.m./h). The negatively numbered fractions represent flow-through fractions of 5 ml each. C1 heparanase activity eluted at 0.3 M NaCl, while C2 heparanase activity eluted at both 0.3 M NaCl and 0.5 M NaCl. The two C2 heparanase peaks were pooled separately, as indicated by the bars (A and B).

Many transcription factors are purified by binding to heparin affinity resins; therefore it seemed likely that the heparanase activities could be purified further over a DNA column. Concentrated C1 and C2 activities were applied separately to a 10 ml DNA-cellulose column, and activity was eluted by increasing the NaCl concentration. C1 heparanase activity eluted from the resin when the salt concentration was changed to 0.3 M NaCl (Figure 2). Due to the capacity of the column, some C1 activity did not bind to the DNA-cellulose; however, when this flow-through activity was re-applied, it bound to the resin and required 0.3 M NaCl to be eluted (results not shown). C2 heparanase activity eluted from the DNA-cellulose column in two peaks. The majority of the activity eluted from the resin with 0.3 M NaCl (C2A); however, some activity did not elute until the salt concentration was changed to 0.5 M NaCl (C2B) (Figure 2). C1 activity which eluted from the DNA-cellulose resin with 0.3 M NaCl and the two C2 activities were each pooled and concentrated using either the Amicon stirred cell system or Millipore Ultrafree centrifugal devices.

The concentrated enzymes were next applied to a Sephacryl S100 gel-filtration column. C1 heparanase activity eluted from the Sephacryl column as a broad peak, encompassing a wide range of molecular mass values (Figure 3). The protein composition in each fraction was analysed by SDS/PAGE (Figure 4), and two prominent proteins were observed: a 45 kDa species that eluted in fractions 39–44 and a 30 kDa species that eluted in fractions 46–50. Fractions 39–43 were pooled as C1A activity, and fractions 47–50 were pooled as C1B activity. C1A and C1B activities were characterized using these pooled Sephacryl fractions. When concentrated C2A or C2B enzyme was applied to the Sephacryl column, very little activity could be detected,

Figure 3 Sephacryl S100 chromatography of heparanase activities from CHO cells

DNA-cellulose-purified C1, C2A and C2B heparanases were applied to a Sephacryl S100 column. Fractions of 1 ml were collected, and 5–10 μ l aliquots were assayed for activity with the CPC assay (units of % CPC-soluble c.p.m./h).

Figure 4 SDS/PAGE of C1 activities eluted from the Sephacryl column

Sephacryl fractions containing C1 activity were dialysed to remove salt, and an aliquot was analysed by SDS/PAGE. Proteins were visualized with Coomassie Blue. For enzymic analysis, fractions 38–42 were pooled for C1A activity and fractions 47–51 were pooled for C1B activity.

but that which could be eluted from the Sephacryl column as 40–46-kDa species (Figure 3). There are several possibilities to explain the loss of heparanase activity on the Sephacryl column. It may be that the C2 enzymes form non-covalent associations with some other factor that becomes separated from the enzyme on the Sephacryl column, and without this factor neither C2A nor C2B heparanase is as active. Alternatively, we have found that both C2 activities are not as stable in storage as the C1 heparanases, so the lack of activity from the Sephacryl column could be due to the general instability of the enzymes. Because of the instability of the C2 activities, the characterization studies were done with the DNA-cellulose-purified enzymes.

Figure 5 CHO heparanase activities as a function of pH

Concentrated C1A, C1B, C2A and C2B activities were each incubated with nascent CHO 35 S-labelled heparan sulphate for 1 h at 37 $^{\circ}$ C. The pH of the solution was varied from pH 3.0 to 8.0 using different combinations of 15 mM citric acid and 30 mM sodium phosphate. Formation of short heparan sulphate chains was determined by the CPC assay (units of % CPC-soluble c.p.m./h). Two to four assays were performed at each pH.

Characterization of CHO heparanase activities

Activity and pH

To determine the optimal pH for the heparanase reactions, each of the partially purified enzymes was incubated with nascent heparan [35 S]sulphate chains in CP buffers of different pH values. After 1 h, the formation of short chains was assessed by CPC precipitation. All four heparanases are active over a broad pH range (Figure 5). C1A and C1B heparanases have similar pH profiles, with optimal activity at pH 3.5–4.0 and again at pH 5.0–5.5. Neither enzyme is very active above pH 6.0. C2A and C2B heparanases are also active at the lower pH values, but both enzymes have a reproducible spike of activity at pH 6.5. Although all four heparanases cleave the heparan sulphate substrate to short, 6 kDa products at pH 3.5 or 4.0 (results not shown), this activity may not be physiologically relevant. Others have shown that heparanases degrade the glycosaminoglycan chains in endosomes [1,34], where the pH is thought to be between 5.0 and 6.5 [35]. However, it is possible that there is a population of endosomes where the pH is as low as 3.5, and that this is where heparanases cleave the polysaccharide. Alternatively, it may be that in order to cleave the heparan sulphate substrate the heparanase active site maintains a pH around 3.5–4.0, even though the overall endosomal pH is higher. In any case, these pH experiments suggest that the heparanase proteins have interesting properties with regard to the amino acids involved in the enzymic reaction.

Figure 6 Size analysis of the ^{35}S -labelled heparan sulphate products generated from nascent glycosaminoglycans

Concentrated C1A, C1B, C2A and C2B heparanases were incubated overnight at 37 °C with nascent ^{35}S -labelled heparan sulphate substrate at pH 5.5 or pH 6.5. The long incubation time was chosen to ensure that all the nascent chains were degraded completely. The degradation products were analysed on a TSK 4000 gel-filtration column. The elution position of the nascent heparan sulphate substrate (HS sub) is indicated by the broken line. ○, C1A heparanase; ●, C1B heparanase; △, DNA-cellulose-purified C1 activity; ◇, C2A heparanase incubated at pH 5.5; ◆, C2A heparanase incubated at pH 6.5; □, C2B heparanase incubated at pH 5.5; ■, C2B heparanase incubated at pH 6.5.

Size of products

Nascent, 81 kDa heparan [^{35}S]sulphate chains were incubated exhaustively with all four heparanases at pH 5.5, and with the C2 enzymes at pH 6.5, to allow the glycosaminoglycan substrate to be completely degraded. The reaction mixtures were then analysed by TSK 4000 gel-filtration chromatography to determine the size of the heparan sulphate products (Figure 6). All the enzymes cleave the nascent chains to short glycosaminoglycans that are comparable in size to the short chains produced *in vivo* [3,26]; however, there are some differences between the heparanases. C1A heparanase cleaves the heparan sulphate substrate to short chains of about 6 kDa, while C1B heparanase generates slightly longer products of about 9 kDa (Figure 6). Interestingly, if the DNA-cellulose-purified C1 enzyme that contains both C1A and C1B activities is incubated with the [^{35}S]glycosaminoglycan substrate, the heparan sulphate chains generated are shorter than the products formed by either enzyme alone (Figure 6), suggesting that the two heparanases may recognize and cleave different sequences. C2A and C2B heparanases form similar-sized products, but the heparan sulphate fragments generated are dependent on the pH of the reaction. At pH 6.5, both enzymes generate 9 kDa products, while at pH 5.5 the shorter 6 kDa glycosaminoglycans are produced (Figure 6).

Figure 7 Size analysis of the ^{35}S -labelled heparan sulphate products generated from heparan sulphate proteoglycans

Concentrated C1A, C1B, C2A and C2B heparanases were incubated overnight at 37 °C with ^{35}S -labelled heparan sulphate proteoglycan substrate at pH 5.5 or 6.5, and the degradation products were analysed on a TSK 4000 gel-filtration column. The elution position of the heparan sulphate proteoglycan substrates (HSPG sub) is indicated by the broken line, and the arrow indicates the elution position of the nascent glycosaminoglycans released from protein cores by base treatment. ○, C1A heparanase; ●, C1B heparanase; ◇, C2A heparanase incubated at pH 5.5; ◆, C2A heparanase incubated at pH 6.5; □, C2B heparanase incubated at pH 5.5; ■, C2B heparanase incubated at pH 6.5.

Proteoglycan substrates

The initial substrate encountered by the intracellular heparanases will be intact proteoglycans. With proteoglycan substrates, the heparan sulphate chains are tethered to a core protein, and this could affect the ability of the enzyme to access the cleavage sites on the glycosaminoglycan chain. Therefore it is important to examine whether the four CHO heparanase activities can degrade proteoglycans. All four heparanases were incubated exhaustively with purified CHO heparan [^{35}S]sulphate proteoglycans at pH 5.5 or 6.5, and the products were analysed by applying the reaction mixtures to a TSK 4000 gel-filtration column. At pH 5.5, both C1A and C1B heparanases cleaved heparan [^{35}S]sulphate proteoglycans to short [^{35}S]glycosaminoglycans that are comparable with the species produced from the free-chain substrate, as well as ^{35}S -labelled molecules that are larger than the nascent 81 kDa chains (Figure 7). These larger molecules (C1A, $K_{av} = 0.38$; C1B, $K_{av} = 0.25$) are partially degraded proteoglycans, since they disappeared when the reaction mixture was treated with base to release glycosaminoglycans from core proteins. After base treatment, the ^{35}S -labelled chains which were bound to the core protein migrated as shorter glycosaminoglycans that eluted in similar positions to the species initially released from the proteoglycan by the heparanase (results not shown). Similar results were observed when C2B heparanase was incubated with [^{35}S]proteoglycans (Figure 7). At pH 5.5, C2B heparanase acts in a comparable manner to C1A heparanase, while at pH 6.5, the

Figure 8 Susceptibility of ^3H -labelled reduced heparan sulphate products to bacterial heparinase

Short heparan sulphate chains generated by the different CHO heparanase activities were labelled at the reducing end with NaB^3H_4 (see the Experimental section) and analysed on a Superdex peptide column before (open symbols) and after (closed symbols) incubation with bacterial heparinase I. The arrows indicate the elution positions of the different sized oligosaccharides on the column (numbers of residues are indicated).

former enzyme cleaves proteoglycan in a manner analogous to C1B heparanase. These results indicate that C1A, C1B and C2B heparanases can degrade glycosaminoglycans covalently bound to core proteins.

Unlike the other activities, C2A heparanase does not significantly cleave the heparan [^3S]sulphate proteoglycan substrate at either pH 5.5 or 6.5 (Figure 7). Two lines of evidence suggest that this lack of degradation is not due to inactivation of the enzyme. First, these assays were done at the same time as the assays in which the nascent glycosaminoglycan substrate was cleaved to the short 6–9 kDa products (Figure 6), indicating that the C2A heparanase is active. Secondly, adding another aliquot of C2A heparanase to the assay did not change the amounts of shorter products generated, suggesting that the reaction had gone to completion during the initial incubation. These results suggest that C2A heparanase is unable to access the glycosaminoglycan substrate when it is covalently bound to a core protein.

Heparanase cleavage sites

To determine the class of products generated by the CHO heparanases, unlabelled CHO heparan sulphate chains were incubated with the individual activities at pH 5.5, and the short glycosaminoglycan products were labelled at the reducing end with NaB^3H_4 . As expected from the TSK 4000 profiles, all the

^3H -labelled reduced heparan sulphate products eluted near the void volume of a Superdex peptide gel-filtration column (Figure 8). C1A and C2B activities generated a homogeneous population of short chains with an average of 22 residues. The sizes of the products created by C1B were similar, although 25% of the molecules eluted as chains greater than 26 residues. Interestingly, C2A generated a heterogeneous mixture of products that ranged from 13 to over 26 residues. All the ^3H -labelled reduced chains were susceptible to low-pH nitrous acid treatment, producing shorter oligosaccharides ranging in size from trisaccharides to 19 sugars (results not shown). The patterns of oligosaccharides generated by low-pH nitrous acid treatment of each ^3H -labelled reduced product were comparable, suggesting that they all have a similar distribution of N-sulphate groups.

When the ^3H -labelled reduced products were treated with the bacterial enzyme heparinase, which cleaves the glycosidic bond between N-sulphoglucosamine and 2-O-sulphated iduronate residues [32,33], there were slight shifts in the distribution of ^3H -labelled species, but most of the radioactivity remained near the void volume of the Superdex column, eluting as oligosaccharides of between 13 and 21 residues (Figure 8). These results suggest that the heparinase-susceptible sequence is at or near the non-reducing end of the CHO heparanase products, and indicates that all four heparinase activities cleave the glycosaminoglycan substrate to class I chains that have a modified domain at the non-reducing end of the short oligosaccharides [18]. Similar results were observed when C2 heparanases were incubated with unlabelled substrate at pH 6.5 (not shown).

DISCUSSION

Four intracellular heparanase activities have been partially purified from CHO cells, based on differences in molecular mass or on the ability of the enzymes to bind to cation-exchange and DNA resins. C1A and C1B heparanases co-elute from cation-exchange and DNA-cellulose columns and, so far, can only be separated by gel-filtration chromatography. The two C1 heparanases have apparent molecular masses of 45 and 30 kDa. The activities have similar pH optima (Figure 5) and degrade nascent heparan sulphate chains and proteoglycans to shorter oligosaccharides (Figures 6 and 7). Although both enzymes cleave the heparan sulphate substrate so that the first modified domain is at least 13 residues from the newly formed reducing end (Figure 8), C1A heparanase produces short, 6 kDa oligosaccharides, while C1B heparanase generates longer, 9 kDa molecules (Figures 6 and 7). Together the enzymes degrade the glycosaminoglycan chain to oligosaccharides that are shorter than 6 kDa (Figure 6), suggesting that each heparanase may cleave a different heparan sulphate sequence.

C2A and C2B heparanases can be separated from the C1 enzymes on a cation-exchange column, and then from each other on a DNA-cellulose column. Both enzymes elute from the Sephacryl S100 gel-filtration column as species with molecular masses of around 45 kDa. As with the C1 activities, C2A and C2B heparanases have similar pH profiles, but unlike the C1 enzymes both have a reproducible spike of activity at pH 6.5 (Figure 5). The size of the heparan sulphate products generated by C2A and C2B heparanases is pH-dependent, with shorter 6 kDa species being formed at pH 5.5, and longer 9 kDa molecules created at pH 6.5 (Figure 6). At either pH, both heparanase activities cleave the heparan sulphate chain so that modified domains are at the non-reducing end of the shorter oligosaccharides (Figure 8). C2B heparanase is also able to degrade heparan sulphate proteoglycans in a pH-dependent manner, but C2A heparanase does not (Figure 7), suggesting

that the C2A enzyme is unable to access the glycosaminoglycan substrate when it is tethered to a core protein.

Based on the characterization of the short heparan sulphate oligosaccharides formed in intact cells, we proposed that CHO heparanases cleave the glycosaminoglycan at junctions between modified and unmodified domains, so that the short-chain products have modified residues at either the newly formed reducing or non-reducing end [18,26,27]. Analysis of the chains produced by the four CHO heparanase activities supports this hypothesis. C1A, C2A and C2B heparanases created short glycosaminoglycans that were virtually insensitive to bacterial heparinase (Figure 8), indicating that the susceptible bond between *N*-sulphoglucosamine and 2-*O*-sulphated iduronic acid is at, or very close to, the non-reducing end of the product. The longer glycosaminoglycans produced by C1B heparanase were sensitive to the bacterial enzyme; however, the heparinase-cleaved chains were still large, indicating that the closest modified domain is at least 17 residues from the reducing end (Figure 8).

Interestingly, none of the CHO heparanases generated shorter chains that had a modified domain at the reducing end, even though these products were formed in intact cells [18]. One explanation for the lack of these 'class II' products [18] may be that there is another heparanase activity in CHO cells that generates these chains which was not purified using our protocol. Approx. 10 % of the heparanase activity remains associated with the 30 % ammonium sulphate pellet, and it is possible that this activity is the class II heparanase. A class II heparanase may also be lost during the purification steps if it is more sensitive to inactivation, or if it sticks tightly to the concentration devices. Alternatively, it may be that all the CHO heparanase activities preferentially cleave the glycosaminoglycan chain to produce class I-type chains *in vitro*, but inside cells their concerted action may also generate class II products. This hypothesis is supported by the observation that the DNA-cellulose-purified C1 enzyme, which contains both C1A and C1B activities, generates shorter heparan sulphate products than either heparanase alone (Figure 6). It may be that the proportions of the various enzymes and changes in pH during endocytosis affect the specificities of the enzymes, so that chain products which have modified domains at the reducing end are generated. Class II chains may also be formed inside cells because of other molecules that associate with either the heparanase proteins or the heparan sulphate substrate and alter the specificity of the enzymes. These molecules may have been lost during the purification steps, so that now only the inherent specificity of the enzymes is observed.

The similarities in molecular masses and enzymic properties of the four heparanases suggest that the proteins may be related. Potentially, all four enzymes could be derived from the same primary gene product, with post-translational modifications or processing having altered their abilities to bind to cation-exchange and DNA resins or affected the size of the protein. It is also possible that the four enzymes are products of different genes, but since they all degrade the same substrate their enzymic and physical properties are nearly identical. At the present time we have no protein sequence information for any of the enzymes, nor any evidence of whether post-translational modifications such as glycosylation or phosphorylation have occurred.

It is possible that one or more of the CHO heparanases is related to the activity in human placenta. In addition to having a comparable apparent molecular mass [21], the pH profile of a partially purified placental heparanase indicates that this activity can also degrade heparan sulphate chains at low, non-physiological pH [36]. However, our studies provide additional evidence that the CHO heparanases are different enzymes from platelet and melanoma endoglucuronidases. Unlike the CHO heparan-

ases, the platelet enzymes are not active below pH 4.5 [19,20], and can cleave the heparan sulphate chain within the highly modified anti-thrombin III binding domain [4]. Although the CHO cell heparan sulphate substrate lacks the sequence that binds anti-thrombin III [37], characterization of the cleavage site [18,26,27] suggests that the CHO heparanases would not act at this sequence. Studies with the melanoma heparanase indicate that it recognizes a specific structure or sequence that is present on plasma-membrane proteoglycans, but not on the heparan sulphate chains released from the cell surface [38]. This sequence appears to be different from that required for the CHO heparanases, since the melanoma activity is not affected by basic fibroblast growth factor *in vitro* [38], while the addition of the growth factor to the reaction mixture prevents all the CHO enzymes from degrading the heparan sulphate substrate [26]; S. Tumova and K. J. Bame, unpublished work).

The partial purification of four heparanases from CHO cells raises the question of why multiple enzymes may be required to degrade heparan sulphate chains to short oligosaccharides. Recent studies with a CHO cell mutant defective in 2-*O*-sulphation of uronic acids suggested that heparanase cleavage is not essential for the complete lysosomal degradation of the glycosaminoglycan to monosaccharides and free sulphate [27], although this may not be the case *in vivo*. In an organism, degradation of heparan sulphate chains by heparanases may be crucial for releasing ligands from internalized proteoglycans, or for creating the variety of short heparan sulphate fragments needed to regulate proteoglycan interactions [10] or to protect ligands from inactivation and degradation ([8,11]; S. Tumova, B. A. Hatch, D. J. Law and K. J. Bame, unpublished work). Differential expression of a complement of heparanase enzymes may be a mechanism involved in regulation of the formation of short heparan sulphate chains during development or in various tissues.

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