# Characterization of a novel intracellular heparanase that has a FERM domain

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The catabolism of cell-surface heparan sulphate proteoglycans is initiated by endosomal heparanases, which are endoglycosidases that cleave the glycosaminoglycans off core proteins and degrade them to shorter oligosaccharides. We have purified previously four intracellular heparanase activities from Chinese hamster ovary (CHO) cells [Bame, Hassall, Sanderson, Venkatesan and Sun (1998) Biochem. J. **336**, 191–200], and in the present study we characterize further the most abundant activity (C1A heparanase). This enzyme purifies as a family of 37–48 kDa proteins from both CHO cells and the rat liver, with the major species being 37 and 40 kDa. Amino acid sequence analysis shows the purified C1A heparanase protein is highly homologous with the N-terminal domain, or FERM domain, of the ≈80 kDa proteins ezrin, radixin and moesin (ERM proteins, after ezrin-radixin-moesin). This domain, which is also found in erythrocyte protein 4.1, links cytoplasmic proteins to membranes. Antibodies against the FERM domain recognize all the C1A heparanase proteins on Western blots, suggesting that the smaller species are derived from a larger protein. Activity binds to, and is affected by, molecules known to interact with FERM domains, supporting the hypothesis that the intracellular C1A heparanase is the purified FERM domain protein. Since bacterially expressed FERM domains of radixin and moesin lack heparanase activity, and some tryptic peptides generated from the enzyme do not have a match in any ERM protein, it appears that, rather than being derived from ezrin, radixin or moesin, C1A heparanase may be a new member of the FERM domain family.

Key words: endosomes, ERM (ezrin-radixin-moesin) proteins, heparan sulphate proteoglycan.

## INTRODUCTION

Because of their location in plasma and basement membranes, heparan sulphate proteoglycans (HSPGs) participate in interactions between cells and their environment that influence cell proliferation, differentiation, migration and shape [1,2]. One way to regulate these proteoglycan functions is to degrade the molecule. This process begins via the action of heparanases, which are endoglycosidases that cleave the heparan sulphate glycosaminoglycans (HSs) off the core protein and degrade them to shorter oligosaccharides. Two heparanases that are secreted from cells and degrade basement membrane HSPGs have been described. One enzyme, connective tissue-activating peptide III ('CTAP-III'), is a 9 kDa chemokine derived from platelet basic protein [3,4]. In addition to platelets, the heparanase is expressed in T cells, polymorphonuclear leucocytes and placental extracts [4], suggesting that it may facilitate the movement and migration of immune cells through extravascular tissue. The other enzyme, Hpa1 heparanase, is a novel 50 kDa glycoprotein expressed primarily in placenta and lymphoid organs [5–8]. The enzyme is synthesized as a 65 kDa precursor, which is subsequently processed to the 50 kDa active form. Antibodies against the Hpa1 heparanase show that it is localized to the sub-endothelium [9], suggesting its primary functions are to remodel basement membranes at sites of inflammation or injury, to assist in leucocyte migration and to regulate processes involved in embryo implantation and pregnancy. In addition, evidence indicates that Hpa1 heparanase might have an important role in cancer metastasis [5].

Heparanase activities are also found in the endocytic pathway [10–12], where it degrades HSPGs that have been internalized through normal membrane turnover or specific endocytic events. Some have speculated that Hpa1 heparanase is also responsible for the intracellular catabolism of HSPGs; however, no one has yet shown that the enzyme is active within cells. Several lines of evidence suggest the intracellular activities are different from the secreted enzymes. First, four heparanases' activities, with different physical characteristics, have been purified from Chinese hamster ovary (CHO) cells [13], and although the proteins are comparable in size with Hpa1 heparanase, the activities are not secreted [12]. Secondly, the hpa1 heparanase mRNA is expressed in low levels in heart, liver, lung and kidney [8], tissues known to synthesize cell surface HSPGs, and we cannot detect the hpa1 heparanase transcript in purified CHO cell mRNA (I. Venkatesan and K. J. Bame, unpublished work). Therefore, although Hpa1 heparanase might contribute to the catabolism of internalized HSPGs in non-lymphoid tissues, it is probably not the primary activity. Thirdly, the substrate specificity of the extracellular and intracellular activities appears to be different. Studies indicate that Hpa1 heparanase recognizes a specific sugar sequence or modified residue in the HS chain [14,15], whereas the intracellular activities in CHO cells recognize the substrate using global differences in sulphate content along the glycosaminoglycan [16–18].

In the present study, we describe the characterization of the most abundant CHO activity, C1A heparanase. Amino acid sequence analysis shows it is not related to either of the secreted enzymes, indicating it is a new, unique heparanase. Surprisingly,

Abbreviations used: Caps, 3-(cyclohexylamino)propane-1-sulphonic acid; CHO, Chinese hamster ovary; ERM, ezrin-radixin-moesin; C-ERMAD, C-terminal ERM-associated domain; CTD, C-terminal domain; HSPG, heparan sulphate proteoglycan; HS, heparan sulphate glycosaminoglycan; MALDI, matrix-assisted laser-desorption ionization; ML, mitochondria–lysosome; Ni-NTA, $Ni^{2+}$-nitrilotriacetate; $PIP_2$, phosphatidylinositol 4,5-bisphosphate; Rho GDI, Rho GDP-dissociation inhibitor; TCA, trichloroacetic acid.

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the sequence shows the purified ≈40 kDa C1A heparanase is highly homologous with the N-terminus of the ≈ 80 kDa proteins ezrin, radixin and moesin (ERM proteins; an acronym derived from ezrin-radixin-moesin), which have roles in determining cell shape, adhesion and motility [19–22]. The N-terminus of the ERM proteins is an approx. 300-amino-acid domain that links proteins to the plasma membrane via protein–protein interactions [19–22]. It is called a FERM domain, since it was originally identified in the erythrocyte protein four-point-one [23]. We show that heparanase activity has characteristics of FERM domains, and is also affected by molecules known to interact with FERM domains, strongly suggesting that the enzyme has this protein module. Although the amino acid sequences of C1A heparanase we obtained are identical with sequences in radixin, recombinant radixin and moesin FERM domains lack enzyme activity, suggesting that, instead of being derived from the ERM proteins, C1A heparanase might be a new member of the FERM domain family.

## EXPERIMENTAL

### Chromatographic materials and methods

CHO cell paste was obtained from Genentech Inc. (San Franscisco, CA, U.S.A.), and frozen rat livers were purchased from Pel-Freez Biologicals (Rogers, AR, U.S.A.). C1A heparanase was purified from either 300 ml of CHO cell paste or 100 rat livers, following our published protocol [13]. High-S cation-exchange, heparin–Affigel and hydroxyapatite resins were purchased from Bio-Rad Laboratories (Hercules, CA, U.S.A). DNA-cellulose resins and a prepared Sephacryl S-100HR column (1.6 cm × 60 cm) were obtained from Amersham Biosciences (Piscataway, NJ, U.S.A.). The concentration of salt in the column fractions was determined by measuring the conductivity of the fraction and comparing it with a standard salt curve. Protein concentrations were determined with the BCA assay (Pierce Endogen, Rockford, IL, U.S.A.). Heparanase activities were concentrated with Ultrafree centrifugal filtration devices (Millipore, Bedford, MA, U.S.A.) or by applying the sample to a 1 ml High-S cation-exchange column. Chromatography using SDS/polyacrylamide gels was performed as described by Laemmli, and these were stained with Coomassie Blue R-250 or silver.

### Heparanase assay

Nascent [$^{35}$S]HSs (5000 c.p.m.) were purified from CHO cells [17] and incubated for 30–60 min at 37 °C with column fractions or purified C1A heparanase. For most assays, the pH was maintained at 5.5 with 50 mM citrate/100 mM sodium phosphate, and purified heparan sulphate (Sigma, St Louis, MO, U.S.A.) was added to a final concentration of 0.013–0.667 $\mu$g/ml. Chain cleavage was determined by precipitating the reaction mixture with 1 % (w/v) cetylpyridinium chloride 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 [17]. The $^{35}$S counts in the cetylpyridinium chloride supernatant were assayed by liquid-scintillation counting, and amount of soluble HS (in ng) was determined by dividing the soluble $^{35}$S counts by the specific radioactivity of the HS substrate.

### Amino acid sequencing and CNBr treatment

Purified C1A heparanase was precipitated with ethanol or trichloroacetic acid (TCA) and electrophoresed on a 10 % (w/v) Tris/Tricine gel [24]. In order to remove polymerization by-products and radicals that might block the N-terminus of the protein, the gel was pre-electrophoresed for 45 min at 10 mA with upper buffer that contained 0.1 mM thioglycolic acid. The gel then was incubated overnight at room temperature (25 °C) before use the next day. Electrophoresed proteins were blotted on to ProBlott®PVDF membranes (Applied Biosystems, Foster City, CA, U.S.A.) using 3-(cyclohexylamino)propane-1-sulphonic acid (Caps) electroblotting buffer [25]. The bands on the membrane were stained for 1 min with 0.1 % (w/v) Coomassie Blue R-250 in 10 % (v/v) acetic acid/40 % (v/v) methanol, and then destained in 50 % methanol. The membrane was rinsed with deionized water, and the bands were excised with a clean razor blade and placed in Eppendorf tubes for storage and shipment. Amino acid sequencing was performed at the Core Laboratories at Louisiana State University Medical School in New Orleans, LA, U.S.A.

Purified C1A heparanase was ethanol-precipitated, and the resulting pellet was freeze-dried. Of freshly prepared 20 mg/ml CNBr, 20 $\mu$l in 70 % (v/v) formic acid was added to the freeze-dried protein, and the sample was incubated in the dark at room temperature for 48 h. The reaction was stopped by adding 200 $\mu$l of deionized water, and then freeze-drying the sample. The dried CNBr-modified peptides were electrophoresed on 10 % Tris/Tricine gels, blotted on to ProBlott®PVDF membranes, and sent to the LSU Core Laboratories for sequencing.

### Antibodies and Western blotting

Dr Frank Solomon (Massachusetts Institute of Technology, Cambridge, MA, U.S.A.) provided polyclonal antibody 220, which recognizes the radixin FERM domain [26], and Dr S. Tsukita (Kyoto University, Kyoto, Japan) provided monoclonal antibody 2287HT, which recognizes the FERM domain of all the ERM proteins. Proteins were precipitated with ethanol or TCA, and electrophoresed on 10 % Tris/glycine gels. The electrophoresed proteins were blotted on to ProBlott®PVDF membranes using Caps electroblotting buffer [25], and incubated with primary antibody in TTBS buffer [100 mM Tris/27 mM NaCl/0.1 % (v/v) Tween 20 (pH 7.5)]. Bound antibodies were visualized using the Vectastain Elite Kit from Vector Laboratories (Burlingame, CA, U.S.A.).

### Purification of radixin FERM and C-terminal (CTD) domains

*Escherichia coli* DH5aF′IQ cells, which contain the mouse radixin N-terminus (residues 1–317) or the C-terminus (residues 319–585), were generously provided by Dr Frank Solomon. Six histidine residues were added to the C-terminus of each construct to aid in purifying the protein. Expression of the $His_6$-tagged radixin proteins was induced by growth in 1 mM isopropyl $\beta$-D-thiogalactoside ('IPTG') for 3 h. Cells were lysed in 50 mM sodium phosphate, pH 8.0/0.3 M NaCl/20 mM immidazole/1 mg/ml lysozyme containing protease inhibitors, sonicated, and then the solution was clarified by centrifugation. The radixin CTD was purified by incubating the protein with $Ni^{2+}$-nitrilotriacetate (Ni-NTA) Sepharose CL-6B, as described previously [27], dialysed against PBS, and then aliquoted and stored at −80 °C. Because the 250 mM imidazole required to elute $His_6$-tagged proteins from the Ni-NTA resin inactivated C1A heparanase (J. McFarlane and K. J. Bame, unpublished work), the radixin FERM domain was partially purified by applying the clarified lysate to a High-S cation-exchange column. The column was washed with CP buffer [15 mM citrate/30 mM sodium phosphate/1 mM glutathione (pH 6.0)] containing 0.3 M NaCl, until the absorbance at 280 nm had stabilized, and then the radixin FERM domain was eluted from the column with CP buffer containing 1.5 M NaCl. SDS/PAGE was performed to

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determine which fractions contained the radixin FERM domain. Those fractions were pooled, dialysed against CP buffer to bring the NaCl concentration to 0.15 M, and then assayed for heparanase activity.

### Liposome centrifugation assay

Phosphatidylserine (Sigma) was reconstituted in 50 mM Tris, pH 7.4, at a final concentration of 5 mg/ml. C1A heparanase, purified from the Sephacryl S100 column, was diluted 1:20 in 50 mM Tris, pH 7.4, and incubated for 15 min at room temperature in the absence or presence of 0.5 mg/ml phosphatidylserine. The mixtures were centrifuged at 4 °C for 20 min at 20000 ***g***. After the supernatants were removed, the pellets were re-suspended in an equal volume of buffer, and then both the supernatant and pellet were assayed for heparanase activity. Alternatively, the protein in the supernatants and pellets was examined by SDS/PAGE. In this case, the pellet was directly re-suspended in sample buffer while the protein in the supernatant was precipitated with TCA before being suspended in sample buffer.

### Coupling radixin CTD to Sepharose

CNBr-activated Sepharose 4B was reconstituted and washed with 1 mM HCl, then washed three times with cold coupling buffer [0.1 M $NaHCO_3$/0.5 M NaCl (pH 8.3)]. The washed gel was mixed with 1.3 mg of purified radixin CTD (dialysed into coupling buffer) and incubated overnight at 4 °C with gentle inversion. The resin was allowed to settle, unbound protein was removed (post-couple supernatant), and the remaining active groups were blocked by incubating the resin for another 16–24 hours at 4 °C with 0.25 M glycine, pH 8.0. The blocking buffer was removed, and the resin washed five times with coupling buffer. After the last wash, the CTD–Sepharose beads were re-suspended in 50 mM Tris/27 mM NaCl, pH 7.4 (TBS buffer), as a 50 % slurry and stored at 4 °C. To prevent bacterial growth, sodium azide was added to a final concentration of 0.2 %. The efficiency of radixin–CTD coupling was determined by measuring the $A_{280}$ of the starting material and the post-couple supernatant. On the basis of these values, $> 90$ % of the radixin CTD was bound to the Sepharose resin. The same protocol was followed to generate the control Sepharose used in the experiments, except that the initial incubation was carried out in the absence of any protein.

### CTD–Sepharose chromatography

C1A heparanase, purified from the Sephacryl S100 column, was diluted 1:30 with TBS buffer. Of the diluted enzyme, 100 $\mu$l was added to 50 $\mu$l of CTD–Sepharose in an Eppendorf tube, and the mixture was incubated at 4 °C with rotation for 1 h. The mixture was centrifuged for 20 s to pellet the resin, and the buffer was removed and placed on ice. The resin was then washed seven times with 100 $\mu$l of TBS buffer, and each wash was saved separately on ice. Subsequently, 100 $\mu$l of 50 mM Tris/1.0 M NaCl, pH 7.4, was added to the washed resin, and the mixture was incubated at 4 °C with rotation for 1 h. The resin was washed four more times with 100 $\mu$l of the 1.0 M NaCl buffer, and each wash was saved separately. Two 10 $\mu$l aliquots of each fraction were assayed for heparanase activity over a 30 min period. Since NaCl affects enzyme activity (K. J. Bame, unpublished work), each assay was adjusted so that the salt concentration was identical. To examine whether the C1A heparanase protein was bound to the CTD–Sepharose, the TBS-washed resin was re-suspended with sample buffer and boiled at 100 °C for 5 min, and the released protein was analysed by SDS/PAGE.

### Differential centrifugation of CHO cells

CHO cells, grown to confluence in 150 mm plates, were scraped from the dish with a solution containing 10 mM Hepes, pH 7.4, 100 mM NaCl and 20 mM KCl, and then pelleted in a clinical centrifuge. The cell pellet was re-suspended in TEA buffer [10 mM triethanolamine/10 mM sodium acetate (pH 7.4)/0.25 M sucrose/1 mM EDTA] containing protease inhibitors, and homogenized using a glass/glass Dounce homogenizer. After centrifuging the homogenate in an HS-4 Sorvall rotor (750 ***g***) to remove unbroken cells and nuclei, the post-nuclear supernatant was centrifuged in an SS-34 rotor (15000 ***g***) to pellet the mitochondria and lysosomes [the mitochondria–lysosome (ML) fraction]. The supernatant, representing the cytoplasmic fraction, was removed and saved, and the ML pellet was then re-suspended in TEA buffer.

### Matrix-assisted laser-desorption ionization (MALDI)-MS analysis of tryptic peptides

Sephacryl-purified C1A heparanase was TCA-precipitated, electrophoresed, and visualized by Coomassie staining. The 40 kDa protein was cut out of the gel, diced into small pieces and placed in an Eppendorf tube. A piece of blank gel was also cut out and diced to serve as a control. Enough 25 mM ammonium bicarbonate/50 % acetonitrile was added to cover the gel and the pieces were vortex-mixed for 10 min to extract the Coomassie stain. The buffer was discarded, and the gel pieces were washed with the 25 mM ammonium bicarbonate/50 % acetonitrile solution two more times. Washed gel pieces were freeze-dried, rehydrated with 10 mM dithiothreitol in 25 mM ammonium bicarbonate, and incubated at 56 °C for 1 h to reduce disulphide bonds. The dithiothreitol buffer was removed, 5 mM iodoacetamide was added to the gel pieces and incubated for a further hour at 56 °C to modify thiol groups. After the iodoacetamide buffer was removed, the gel pieces were washed twice with 25 mM ammonium bicarbonate/50 % acetonitrile, and then freeze-dried. The dried gel pieces were re-suspended with 25–60 $\mu$l of 1.25 mg/ml trypsin in 25 mM ammonium bicarbonate, vortex-mixed for 10 min, sonicated for 3 min, and then incubated at 4 °C for 30 min. Any trypsin solution remaining from the rehydration of the gel pieces was removed, and replaced with 25 mM ammonium bicarbonate so that the gel pieces were covered. The samples were then incubated overnight at 37 °C. To extract the tryptic peptides from the gel pieces, 100 $\mu$l of water was added, and the pieces were vortex-mixed for 10 min and sonicated for 5 min. The digest solution was transferred to a clean Eppendorf tube, to which 5 $\mu$l of 50 % acetonitrile/5 % formic acid had been added. Fresh 50 % acetonitrile/5 % formic acid solution was added to the gel pieces, and additional peptides were extracted from the gel by vortex-mixing and sonicating. All the peptide extracts were combined and freeze-dried to 10 $\mu$l. Of the trypsin digest, 2–4 $\mu$l was spotted on to a matrix of $\alpha$-cyano-4-hydroxy-cinnamic acid, and analysed by MALDI MS, using a Voyager DE-Pro mass spectrometer. Comparing the protein and blank sample profiles identified peptides unique to the C1A heparanase. The PAWS computer program was used to compare the masses of the tryptic peptides generated from the C1A heparanase proteins and the FERM domains of mouse radixin, moesin and ezrin.

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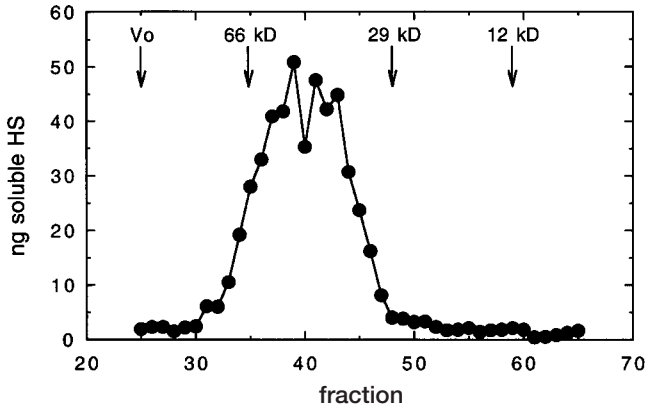


**Figure 1 Sephacryl S100 profile of C1A heparanase**

C1A heparanase was purified from CHO cell homogenates by cation exchange, heparin affinity and DNA-affinity columns [13], concentrated, and applied to a Sephacryl S100 column. Aliquots of each fraction were assayed for heparanase activity at pH 5.5, as described in the Experimental section. Vo, void volume.

## RESULTS

### Purification and initial characterization of C1A heparanase

We reported previously the purification of four heparanase activities from CHO cell homogenates that differed in molecular masses or physical properties [13]. Most of the enzymic activity in CHO cells was associated with an approx. 40 kDa protein that we named 'C1A heparanase'. In order to generate the amounts of enzyme needed to physically characterize the protein, we adapted our purification protocol to frozen rat liver, a more convenient tissue source. Although we were able to purify C1A heparanase from rat liver homogenates, the yields of purified protein were inconsistent, due primarily to problems in solubilizing the activity in ammonium sulphate precipitates. Because of these problems, we have returned to CHO cell paste, obtained from Genentech, Inc., as our starting material. We now start with higher amounts of CHO cell protein (300–400 ml of CHO cell paste) and see similar problems solubilizing activity from ammonium sulphate precipitates, although not to the extent seen with rat liver tissue. The physical characterization of the C1A heparanase has been achieved with proteins purified from both CHO cells and rat liver, whereas the studies examining C1A heparanase activity have been done with the purified CHO enzyme.

### C1A heparanase purifies as a family of proteins

When purified from CHO cell paste or frozen rat livers, C1A heparanase activity elutes from the Sephacryl column as a broad peak, with most of the activity eluting with an apparent molecular mass of approx. 40 kDa (Figure 1). An aliquot of Sephacryl fraction 42, analysed by SDS/PAGE, showed that activity is associated with a family of proteins that range from 37–48 kDa (Figure 2A, lane 1). In this particular preparation, the most prominent Coomassie-Blue-stained band migrates as a 37 kDa protein, which is slightly smaller than the 40 kDa protein we purified previously [13]. This might be due to incomplete inhibition of proteolysis during the purification steps, since the experiments were performed with between three and four times more CHO cell paste than in our previous report [13]. With less material, the predominant Coomassie-stained proteins in the preparation migrate as 48- and 40-kDa species, with very little 37 kDa protein present (results not shown). We did observe the higher-molecular-mass species in our reported purification of CHO heparanases ([13]; also see Figure 4); however, the majority of the activity eluting from the gel filtration column in that study

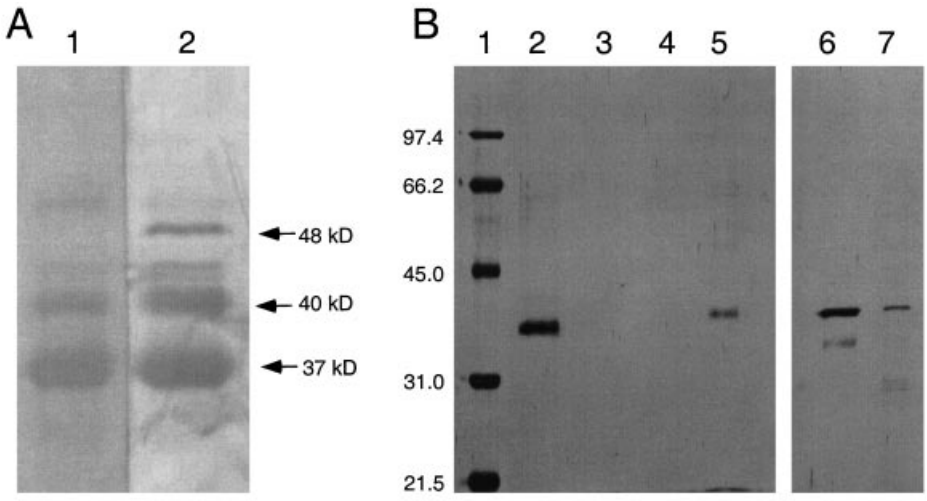


**Figure 2 C1A heparanase purifies as a family of proteins**

(**A**) Equal aliquots of Sephacryl fraction 42 (see Figure 1) were electrophoresed, and the separated proteins were transferred to PVDF membranes and stained for protein with Coomassie Blue (lane 1), or probed with monoclonal antibody 2287HT (lane 2). Similar results were observed when the blotted C1A heparanase preparation was probed with rabbit polyclonal antibody 220 (results not shown). (**B**) Sephacryl-purified C1A heparanase was centrifuged in the absence or presence of 0.5 mg/ml phosphatidylserine, and the proteins remaining in the supernatant or pelleted with the lipid were electrophoresed and detected with silver stain (lanes 2–5). Sephacryl-purified heparanase was incubated with CTD–Sepharose for 1 h at 4 °C, and the proteins remaining bound to the TBS-washed resin were released by boiling in sample buffer, electrophoresed and detected with silver stain (lanes 6 and 7). Lane 1, protein standards; lane 2, supernatant in the absence of phospholipid; lane 3, pellet in the absence of phospholipid; lane 4, supernatant in the presence of phospholipid; lane 5, pellet in the presence of phospholipid; lane 6, Sephacryl-purified C1A heparanase control; lane 7, protein released from CTD-Sepharose.

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**Table 1 Sequences of radixin and the N-terminus and CNBr-modified fragments of C1A heparanase**

Purified C1A heparanase proteins were electrophoresed and blotted to PVDF membranes as described in the Experimental section. The blotted proteins were sent to the LSU Core Facility for amino acid sequencing. The CHO C1A heparanase N-terminal sequence matches the N-terminus of mouse moesin.

| Protein | Sequence |
|---|---|
| Mouse radixin | 1 – MPKPINVRVTTMDAELEFAIQPNTTGKQLFDQVVK – 35 |
| Rat C1A N-terminus | PKPINVRVTTMDAELEFAIQ |
| CHO C1A N-terminus | PKTISVRVTTMDAELEFAIQ |
| Rat C1A CNBr-1 | DAELEFAIQPNTTGKQLFDQ |
| Mouse radixin | 191 – YLKIAQDLEMYGVNYGEIKNKKGTELWLGVDALGL – 225 |
| Rat C1A CNBr-2 | YGVNYGEIKNKKGTELWLGV |

was associated with a 40 kDa protein. Unlike Hpa1 heparanase, C1A activity does not bind to a concanavalin A column, suggesting that the protein is not glycosylated. The absence of sugars on any of the protein species was confirmed using a Bio-Rad Immunoblot glycoprotein detection kit (results not shown).

Since the purified C1A heparanase preparation contained a number of bands, it was important to establish which of the proteins have enzymic activity. The 37 kDa protein could be purified apart from the other higher-molecular-mass species using a heparin column, equilibrated in 0.1 M Tris/HCl, pH 7.85. In the preparation where we purified the 40 and 48 kDa species, we were able to separate the proteins by reapplying pooled and concentrated Sephacryl fractions on the gel filtration column. All three purified proteins have heparanase activity, and have similar enzymic properties on the basis of the pH-dependence of the activity (results not shown).

### Purified C1A heparanase has homology with the ERM proteins

To examine whether the C1A heparanase was related to the newly identified Hpa1 heparanase, the N-terminal sequence of the major 40 kDa protein from rat liver and two CNBr-modified peptides derived from this protein were determined. Unexpectedly, the C1A heparanase sequences matched sequences found in the N-terminal domain of the 80 kDa protein radixin, a member of the ERM family. The N-terminus of rat liver C1A heparanase matched residues 2–21 of radixin, whereas the two CNBr-peptides matched residues 13–27 and 201–220 (Table 1). Interestingly, the first 20 amino acids of the CHO enzyme match the N-terminus of moesin, which differs from radixin with respect to residues 4 and 6 [22]. The sequences of the $\approx$ 300-amino-acid FERM domains of ezrin, radixin and moesin are 85% identical [22]. We also sequenced the first five residues of the minor, higher-molecular-mass rat liver heparanase protein, and found that it too matches the radixin sequence. This result indicates that the higher-molecular-mass proteins are related to the major species, and supports the observation that they also are enzymically active. Presumably, proteolysis is occurring at the C-terminus of the C1A heparanase enzyme, which results in the family of proteins purified in the present study (Figure 2A). We do not know whether this proteolysis occurs as part of the post-translational processing of heparanase, or as a consequence of purification.

Since the amino acid sequence data suggest C1A heparanase has high homology with the N-terminus of the ERM proteins, we obtained antibodies against the FERM domain of ERM to examine whether they could recognize the C1A heparanase protein. Antibody 220 is a polyclonal antibody raised against the radixin sequence 194–200 [26], and antibody 2287HT is a monoclonal antibody that recognizes the N-terminal domain of all three ERM proteins. Both antibodies recognize the 37 kDa C1A heparanase on Western blotting (Figure 2A, lane 2), indicating that the purified protein shares epitopes with ezrin, radixin and moesin. The antibodies also recognize the 40 and 48 kDa species present in the heparanase preparation (Figure 2A, lane 2), confirming further that all three proteins are related. An antibody raised against Hpa1 heparanase [9] did not recognize any proteins in our purified C1A heparanase preparation (results not shown), providing additional support that the intracellular heparanase is different from the extracellular enzyme.

### FERM domain protein as a putative contaminant in the preparation

The physical characterization of the purified C1A heparanase suggests the activity resides in a protein with high homology with the N-terminal domain of the ERM proteins. There are at least three possible explanations for our findings: (i) the FERM protein in the preparation is a proteolytic fragment of the ERM proteins, which co-purifies with a different heparanase enzyme protein that we cannot detect; (ii) C1A heparanase has a FERM domain and is derived from the ERM proteins; or (iii) C1A heparanase has a FERM domain, and is a new member of the FERM domain family. It was not surprising that a protein with a FERM domain could be purified by our protocol, since moesin was originally purified as a heparin-binding protein [28] and heparin affinity chromatography has been used to purify recombinant ERM proteins [29]. We tried to immunoprecipitate heparanase activity with the anti-FERM domain antibodies to show that activity was associated with the FERM domain protein. Unfortunately, neither antibody immunoprecipitated the purified 37 kDa protein, so this approach could not be used. The FERM domain protein might co-purify with the C1A heparanase owing to intermolecular interactions [19–22]; however, Sephacryl-purified C1A heparanase activity did not bind to a moesin FERM domain affinity resin (results not shown), suggesting that the presence of the FERM domain protein in the heparanase preparation was probably not because of physical interactions with the enzyme. We tried to remove FERM domain contaminants by applying the partially purified heparanase preparation to a hydroxyapatite column, since this approach has been used to purify recombinant FERM domains [30]. However, heparanase activity bound the hydroxyapatite and was eluted from the resin with similar phosphate concentrations, as predicted for the FERM domains (results not shown).

### Possible association of heparanase activity with a FERM domain

The hydroxyapatite chromatography suggests heparanase activity has characteristics similar to those of the FERM domain, so we decided to examine whether activity shared other properties of ezrin, radixin and moesin. The ERM proteins link the plasma membrane to the cytoskeleton via the action of two domains. The FERM domain binds directly or indirectly to the plasma membrane, whereas the last 50–100 amino acids of the protein, known as the C-terminal ERM-associated domain (C-ERMAD) bind F-actin [20,21]. A number of studies have suggested that the activity of the ERM proteins is regulated by intramolecular interactions between the FERM domain and the C-ERMAD. When associated, the sites that allow the domains to bind to membrane proteins or F-actin are blocked, keeping the ERM protein in an inactive state [20,21]. Activation of the ERM proteins is thought to occur when phosphatidylinositol 4,5-bisphosphate ($PIP_2$) binds the FERM domain, disrupting its association with the C-ERMAD [20,21].

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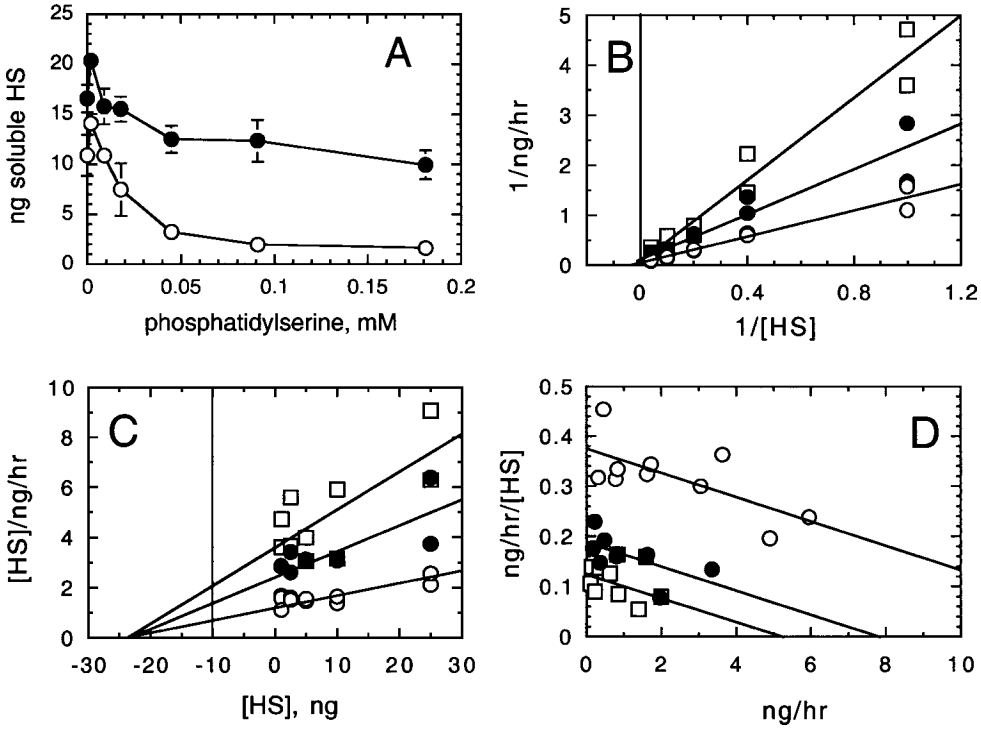


**Figure 3 C1A heparanase activity is inhibited by phosphatidylserine**

(**A**) The activity of purified C1A heparanase at pH 5.5 with increasing concentrations of phosphatidylserine was determined in the absence (○) or presence (●) of Triton X-100. Each point is the average of two to four assays. (**B**–**D**) The activity of purified C1A heparanase at pH 5.5 with different concentrations of HS substrate and phosphatidylserine was determined. To ascertain the type of inhibition, the data were plotted by the method of (**B**) Lineweaver–Burk ($1/v$ versus $1/S$; [33]), (**C**) Hanes–Woolf [$S/v$ versus $S$; [33]) or (**D**) Eadie–Scatchard ($v/S$ versus $v$; [33]), and the lines were determined by best-fit analysis. Phosphatidylserine concentrations of 0 mM (○), 0.01 mM (●) or 0.02 mM (□) were used. The data presented are from a representative of three separate experiments, which all gave comparable results.

### Association with acidic phospholipids

The ERM proteins can be pelleted with phosphatidylserine liposomes, since their FERM domains have a binding site for $PIP_2$ [31]. To see whether heparanase activity would also associate with phospholipids, we incubated Sephacryl-purified C1A heparanase in the absence or presence of 0.5 mg/ml phosphatidylserine, centrifuged the mixture to pellet the liposomes, and assayed the supernatants and pellets for heparanase activity. Triton X-100 was included in the reaction mixture to ensure that the enzyme and HS substrate could interact. In the absence of lipid, $93 \pm 4\%$ of the heparanase activity remained soluble, whereas, in the presence of phosphatidylserine, $58 \pm 4\%$ of the measurable heparanase activity is pelleted. The distribution of heparanase activity in the supernatant and liposome pellet is similar to that observed with ezrin [31], indicating that the C1A heparanase, like the ezrin FERM domain, interacts with acidic liposomes. As with ezrin, the association of C1A heparanase with phosphatidylserine liposomes is sensitive to ionic strength [31], since only $22 \pm 7\%$ of the activity is pelleted if 0.13 M KCl is present when the protein is incubated with the lipid. As predicted, the purified 37 kDa C1A heparanase protein remains soluble in the absence of phosphatidylserine, but is pelleted in the presence of the phospholipid (Figure 2B).

Since C1A heparanase interacts with phosphatidylserine liposomes, we then asked whether the phospholipid had any effect on enzymic activity. The purified 37 kDa enzyme was assayed in the presence of increasing concentrations of phosphatidylserine. After an initial activation, the presence of the phospholipid inhibits C1A heparanase (Figure 3A, open symbols). At every point, the concentration of phosphatidylserine is below the critical micellar concentration of the lipid (0.25 mM; [32]), indicating that the observed inhibition is not due to sequestering the enzyme in liposomes. If Triton X-100 is added to the enzyme assay, the inhibition by the lipid is relieved (Figure 3A, closed symbols), which is most likely due to the detergent associating with the phospholipid and pulling it away from the enzyme protein. Triton X-100 molecules might also be interacting with the enzyme, since C1A heparanase activity is stimulated in the presence of the non-ionic detergent. To see whether phosphatidylserine was binding to the HS substrate site, heparanase activity at different substrate and lipid concentrations was measured. Analysis of the data by a Lineweaver–Burk plot (Figure 3B) could not discriminate between competitive and non-competitive inhibition, and so two additional methods were used [33]. When the data were analysed by a Hanes–Woolf plot (Figure 3C), the lines intersected at the $x$-axis, while analysis by an Eadie–Scatchard plot (Figure 3D) yielded parallel lines. The best-fit lines in the latter two cases indicate that phosphatidylserine is a non-competitive inhibitor [33], and is binding at a different site on the enzyme than the HS substrate.

### Associations with the radixin C-terminus

Another property of FERM domains is that they bind to the C-ERMAD of the ERM proteins. To test whether heparanase activity could bind the C-ERMAD, we made an affinity resin using a recombinant radixin C-terminal end (CTD). In addition to the C-ERMAD, this recombinant protein contains approx. 200 amino acids of the $\alpha$-domain, which links the FERM domain to the C-ERMAD [21,22]. C1A heparanase, purified from the

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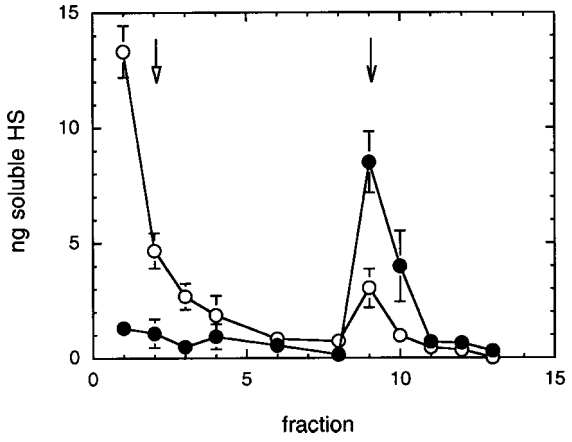


**Figure 4 C1A heparanase activity binds the CTD of radixin**

Sephacryl-purified C1A heparanase was diluted in TBS buffer and incubated with CTD-Sepharose (●) or Sepharose (○) for 1 h at 4 °C. The unbound protein was removed, and the resin was washed seven times with TBS buffer (indicated by the arrow on the left) before being incubated with 50 mM Tris containing 1 M NaCl (indicated by the arrow on the right). Aliquots of each fraction were assayed for heparanase activity at pH 5.5. Each point represents the average of duplicate assays from two (Sepharose) or three (CTD–Sepharose) independent experiments.

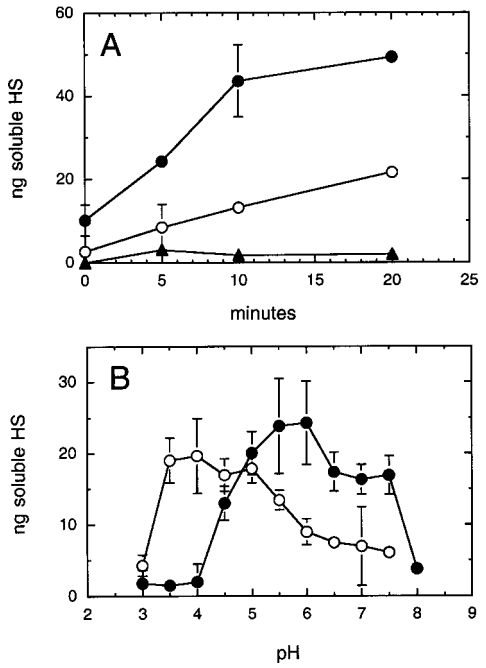


**Figure 5 C1A heparanase activity is altered in the presence of the radixin CTD**

(**A**) The activity of Sephacryl-purified C1A heparanase at pH 5.5 was determined in the absence (○) or presence (●) of 0.1 $\mu$g/$\mu$l radixin CTD. (▲) The activity of radixin CTD alone. Each point is the average of duplicate assays. (**B**) The activity of Sephacryl-purified C1A heparanase was determined in the absence (○) or presence (●) of 0.05 $\mu$g/$\mu$l CTD. The pH of the reaction was maintained with 50 mM citrate, 100 mM sodium phosphate (pH 3.0–7.5) or 100 mM sodium phosphate (pH 8.0). Each point is the average of three to five assays.

Sephacryl column and containing primarily the 37 kDa protein, was diluted with TBS buffer and mixed with the CTD–Sepharose. After allowing the proteins to interact, the solution was removed, the CTD-resin was washed with TBS to remove any unbound activity, and this was then incubated with Tris buffer containing 1 M NaCl to disrupt protein interactions. The volumes of the binding, washing and elution buffers were kept constant so that differences in heparanase activity in each fraction would not be due to dilution of the enzyme. A small amount of heparanase activity remained in the initial binding and wash fractions, but the majority of the activity required 1 M NaCl to be released from the CTD-resin (Figure 4A, closed symbols). Binding is specific for the radixin CTD, since most of the C1A heparanase activity does not bind to Sepharose (Figure 4A, open symbols). The 37 kDa protein in the C1A heparanase preparation remains bound to the CTD–Sepharose after the TBS washes (Figure 2C), strongly suggesting that the heparanase activity resides in this FERM protein.

The affinity between C1A heparanase and the radixin CTD is not as strong as that observed between the ezrin FERM domain and C-ERMAD, which remain associated in 2 M NaCl [34]. A possible explanation for the absence of a similar affinity might be that in the present study the entire C-terminal end of radixin is being used, and the extra 200 amino acids in the $\alpha$-domain affect how the FERM domain and C-ERMAD interact. However, the lower affinity might also indicate differences in the amino acid sequences of the C1A heparanase and radixin FERM domains. Merlin (moesin, ezrin, radixin-like protein) has a FERM domain that is only 65% identical with the FERM domains of ERM [22], and it binds to the ezrin C-ERMAD with less affinity than to the ezrin FERM domain [34].

When the FERM domain and C-ERMAD interact, the ERM proteins are unable to link to the cytoskeleton or plasma membrane [20–22], so we looked at whether this association would also affect heparanase activity at pH 5.5. In the presence of purified recombinant radixin CTD, the activity of Sephacryl-purified C1A heparanase is stimulated compared with the activity of the enzyme in the absence of the added domain (Figure 5A). Maximal stimulation is observed with as little as 0.01 $\mu$g/$\mu$l radixin CTD (results not shown). This finding suggests that the interaction of C1A heparanase with the radixin CTD does not block access of the HS chain to its binding or catalytic sites, but rather enhances substrate binding or catalysis. Since we had previously shown that C1A heparanase was active over a wide pH range [13], we next examined whether the stimulation by the radixin CTD occurred at different pHs. As we observed previously, in the absence of the radixin CTD, C1A heparanase is most active in the pH range of 3.5–5.0, although it still has activity up to pH 7.0 (Figure 5B, open symbols). A much different profile is seen when C1A heparanase is assayed in the presence of the radixin CTD. Now the enzyme is completely inactive at or below pH 4 (Figure 5B, closed symbols). Maximal activity is seen at pH 5.5–6.0, and HS chains are still being cleaved well at pH 7.5. These findings indicate that, without the radixin CTD, C1A heparanase requires an acidic pH for optimal activity. Presumably, the acidic buffer protonates specific amino acid side chains so that substrate binding and/or catalysis can occur. When the C1A heparanase interacts with the radixin CTD, the enzyme reaction can now occur at more neutral pH. Whether this is due to the radixin CTD inducing conformational changes in the heparanase protein or providing the proton donors is unknown.

## Possible derivation of C1A heparanase from ezrin, radixin and/or moesin

Our evidence that heparanase activity binds acidic phospholipids and the radixin CTD, and is affected by interactions with these

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molecules, strongly suggests that the intracellular C1A heparanase has a FERM domain. The next question is whether the 37–40 kDa enzyme is derived from the 80 kDa ERM proteins, or whether it is a new member of the FERM domain family. The size of the purified C1A heparanase suggests that, if the enzyme is derived from the ERM proteins, only the N-terminal FERM domain sequences are required for activity. Therefore we obtained purified human moesin FERM domain (residues 1–296) from Professor Anthony Bretscher at Cornell University (Ithaca, NY, U.S.A.) and an *E. coli* strain that expresses the murine radixin FERM domain (residues 1–317) from Dr Frank Solomon at Massachusetts Institute of Technology, and assayed the recombinant proteins for activity. We were unable to detect heparanase activity with either recombinant protein, even when the assay was conducted at pH 4, the optimal pH for the purified enzyme. It is possible that the lack of activity in the recombinant FERM domains is due to conditions of the prokaryotic expression system that inactivate the enzyme, or that the full-length ERM protein must be synthesized and proteolytically processed to generate an active heparanase. However, the simplest explanation is that the C1A heparanase is not derived from the ERM proteins, but might be a novel protein with significant homology with N-terminus of ezrin, radixin or moesin.

Additional evidence that the ERM proteins might not be heparanases comes from our previous observation that heparanase activity is only associated with endosomes [12]. Others have shown that ezrin, radixin and moesin are equally distributed between the plasma membrane and cytoplasm [35]. Since C1A heparanase is still active in the presence of the radixin CTD (Figure 5), we would predict that, if the enzyme is derived from the ERM proteins, the cytoplasmic forms would also be active when assayed at pH 5.5. We homogenized CHO cells in TEA buffer containing 0.25 M sucrose, and separated the intracellular organelles from the cytoplasm by differential centrifugation. The sucrose in the buffer keeps the organelle membranes intact, and prevents the release of their contents into the cytoplasmic fraction. Under these conditions, 96 % of the heparanase activity is found in the ML fraction, which should include endosomes. Only 4 % of the measured heparanase activity is present in the cytoplasm. The lack of significant heparanase activity in the cytoplasmic fraction suggests C1A heparanase is not derived from the ERM proteins. To examine whether a FERM domain protein was present in the ML fraction, we partially purified heparanase activity in the solubilized ML pellet by cation-exchange chromatography. This step removed 99 % of the measurable protein. An aliquot of the cation-exchange-purified ML fraction was electrophoresed, blotted on to membranes and probed with the polyclonal antibody 220. The antibody recognizes a number of proteins in this preparation, including three that have molecular masses similar to that of the purified C1A heparanase (results not shown). Since the antibody reacts with a number of proteins in the preparation, we cannot conclusively say that these antibody-reactive bands are C1A heparanase proteins; however, their presence supports the hypothesis that a FERM domain protein might be the enzyme in the endosomal–lysosomal pathway of CHO cells.

So far, we have only sequenced 17–18 % of the C1A heparanase proteins, so it is likely there are amino acid sequences unique to the protein that are responsible for the enzymic activity. In order to examine whether the C1A heparanase proteins have sequences other than those in ezrin, radixin and moesin, we measured the monoisotopic masses of tryptic peptides generated from the 37 and 40 kDa proteins by MALDI-MS, and compared them with the expected masses of tryptic peptides from the ERM proteins using the computer program PAWS. Tryptic peptides were generated by incubating SDS/polyacrylamide gel slices of the 37 and 40 kDa proteins with trypsin, and extracting the peptides from the gel with acetonitrile and formic acid. A blank gel slice was treated identically as a control. The peptide mixture was spotted on to a matrix of α-cyano-4-hydroxy-cinnamic acid, and then analysed by MALDI-MS. Only a subset of tryptic peptides should be observed, since some might not be eluted well from the gel; nor will all the extracted peptides be equally desorbed from the matrix. Twenty-four peptides derived from the C1A heparanase proteins were observed by the MALDI-MS analysis (Table 2). Of these, 19 matched masses of tryptic peptides in the first 360 residues of radixin, moesin or ezrin. However, five peptides, which ranged in size from 841–4975 Da, did not have a match in any ERM protein. These results suggest the C1A heparanase protein may have different sequences in its FERM domain that give it enzymic activity.

**Table 2 Tryptic fragments from the 37- and 40-kDa C1A heparanases**

Purified C1A heparanase proteins were electrophoresed, and the Coomassie-Blue-stained bands were cut out of the gel and incubated with trypsin, as described in the Experimental section. The tryptic peptides were eluted from the gel, and the masses were then determined by MALDI-MS and examined for whether there was a match in the first 360 residues of ezrin, radixin or moesin (indicated in the Table as E, R and M respectively).

| Peptide mass (Da) | ERM sequence |
|---|---|
| 626.7 | ERM 152–156 |
| 745.7 | RM 330–335 |
| 841.3 | No match |
| 959.4 | ERM 255–262 |
| 968.5 | No match |
| 976.4 | ERM 28–35 |
| 989.5 | E 72–79 |
| 1104.5 | ERM 238–246 |
| 1175.8 | E 172–180 |
| 1182.5 | ERM 264–273 |
| 1254.6 | No match |
| 1310.7 | ERM 263–273 |
| 1412.7 | R 140–151 |
| 1504.8 | R 72–83 or E 144–156 |
| 1536.8 | M 181–193 |
| 1660.8 | M 41–53 |
| 1748.8 | R 338–350 |
| 1851.9 | No match |
| 1947.9 | RM 157–171 |
| 2005.8 | R 336–350 or M 345–360 or E 166–180 |
| 2066.8 | ERM 9–27 |
| 2081.9 | RM 84–100 |
| 2280.9 | RM 82–100 |
| 4973.4 | No match |

## DISCUSSION

It has been known for many years that the first steps in the degradation of cell-associated HSPGs are catalysed by heparanases in the endosomal pathway [10–12]. When Hpa1 heparanase was purified from placenta and platelets, an immediate question was whether this secreted enzyme also degrades HSPGs inside cells. Even though several lines of evidence suggested the intracellular and extracellular enzymes were different, there was no physical characterization of an intracellular heparanase to verify this hypothesis. In the present study, we physically characterize the major heparanase activity in CHO cells, C1A heparanase, and identify it as a protein different from the extracellular enzymes. Our evidence indicates that C1A hepara-

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nase has a FERM domain, a unique module that binds cytoplasmic proteins to membranes [19–22], since it has amino acid sequences that match sequences in the FERM domain of the ERM proteins, and because enzyme activity both binds to and is affected by molecules known to interact with the domain. The predominant form of the enzyme purified from CHO cells or rat liver has a molecular mass of 37–40 kDa, but the presence of 48 kDa species in the preparation suggests the enzyme may be synthesized as a larger protein that is processed proteolytically.

Even though C1A heparanase has amino acid sequences that are identical with sequences in radixin, recombinant radixin and moesin, FERM domains lack enzymic activity. The simplest explanation for this observation is that the enzyme is not derived from the ERM proteins. This hypothesis is supported by the absence of heparanase activity in the cytoplasm of CHO cells, and the presence of tryptic peptides generated from the C1A heparanase that do not have a mass match in any of the ERM proteins. Instead, our results suggest C1A heparanase may be a new member of the FERM domain family [23] that has extremely high homology with the ERM proteins. There is evidence for additional ERM family members. Takeuchi et al. [36] isolated over 200 PCR products using primers from the FERM domain of band 4.1 and a mouse cDNA library, and discovered two new cDNAs that had high similarity to ezrin, radixin, moesin and merlin. These cDNAs probably do not encode the C1A heparanase, since their expression was restricted to brain and thymus; however, it indicates that other ERM-family members may exist. When we used the N-terminal sequence of the putative C1A heparanase to search the human genome database, we found that, in addition to the ezrin, radixin and moesin sequences on chromosomes 6 [37], 11 [38] and the X-chromosome [39], a match was made with a sequence on chromosome 5. It is possible that this sequence may be the C1A heparanase gene. Alternatively, it might be that the ERM proteins do encode C1A heparanase, but the bacterially expressed FERM domains are not enzymically active. It might be that the full-length ERM protein must be synthesized and proteolytically processed to generate an active heparanase, or that a post-translational modification is required for the FERM domain to exhibit heparanase activity, and this modification does not occur in a bacterial cell. We are currently performing experiments to identify, purify and sequence the tryptic peptides from C1A heparanase that do not have a match in ezrin, radixin or moesin to determine if the enzyme is a previously unidentified protein. These sequences can then be used to specifically clone the C1A heparanase gene, since ultimately this must be done to verify that enzyme activity is associated with a protein with a FERM domain.

Why would an intracellular heparanase have a FERM domain? Studies show the FERM domain binds directly or indirectly to membrane proteins [19–22], so this region might be essential for bringing C1A heparanase to its HSPG substrates in plasma or endosomal membranes. In fact, the FERM domain of ezrin binds the core protein of the cell-associated HSPG, syndecan-2 [40], so C1A heparanase might actually find its substrate via its FERM domain. One problem with this hypothesis is that the enzyme would be found on the opposite side of the membrane from the HS substrate. The sequence and crystal structure of the FERM domains of radixin and moesin suggest they are not incorporated into the membrane in a 'classical' way [41–43]. However, moesin has been identified as a cell-surface receptor for HS and heparin [28], measles virus [44], lipopolysaccharides [45] and interleukin-2-derived peptides [46], suggesting that some portion of the protein is exposed to the extracellular surface. It might be that the FERM domain of C1A heparanase interacts with the membrane in a similar manner so that the substrate binding and catalytic sites are exposed to the insides of endosomes.

Having a FERM domain might also be a mechanism to coordinate HSPG catabolism with the reorganization of the cytoskeleton that occurs when cells move, proliferate or differentiate. C1A heparanase will directly disrupt interactions between cells and the extracellular matrix by degrading the cell-associated proteoglycans. Because it has a FERM domain, the enzyme might also disrupt the link between the plasma membrane and the cytoskeleton by displacing the ERM proteins from their binding partners. An example of this kind of competition between FERM domains has been observed with ezrin and merlin binding to the cell adhesion protein, CD44. Merlin, which lacks the actin-binding site in its C-terminus, has been implicated in cell proliferation and differentiation [22]. During conditions where cell growth is promoted, both ezrin and merlin bind to CD44, but at a high cellular density when growth is inhibited, only merlin binds the transmembrane receptor [47]. If cells over-express ezrin, they exhibit multilayer growth [48], and it might be that, in this situation, ezrin prevents merlin from binding to CD44, keeping the cells in a proliferative state. Another protein that binds to FERM domains is the Rho GDP-dissociation inhibitor (Rho GDI) [49], which regulates the Rho signalling pathway. The Rho family of small G-proteins regulate cell-shape change, cell motility and cytokinesis by reorganization of actin filaments [49], and are kept in an inactive state via a complex formed with Rho GDI. It has been proposed that FERM domains bind Rho GDI [35,49], which releases Rho protein from the inhibitor. Because C1A heparanase has a FERM domain, it might regulate the Rho signalling pathway in a similar manner.

We previously purified two populations of short, heparanase-derived HS chains from CHO cells that differed in the position of the S-domain to the reducing end of the glycosaminoglycan [16]. This finding led us to propose that there must be at least two heparanases inside cells, since it was difficult to imagine how one enzyme would generate both types of oligosaccharides. The observations that C1A heparanase may be synthesized as a larger protein (Figure 2A) and that intermolecular interactions between the 37 kDa C1A heparanase and the radixin CTD changes the enzymic properties of the enzyme (Figure 5B) suggest another mechanism to generate different types of short HS chains. Once we know the size of the nascent enzyme protein, it will be interesting to test whether proteolytic processing of the enzyme or the pH changes in the endosomal pathway modulate C1A heparanase specificity, resulting in the two populations of short HS products we observed.

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