Processing and activation of latent heparanase occurs in lysosomes

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Summary

Heparanase is a heparan sulfate degrading endoglycosidase participating in extracellular matrix degradation and remodeling. Heparanase is synthesized as a 65 kDa non-active precursor that subsequently undergoes proteolytic cleavage, yielding 8 kDa and 50 kDa protein subunits that heterodimerize to form an active enzyme. The protease responsible for heparanase processing is currently unknown, as is the sub-cellular processing site. In this study, we characterize an antibody (733) that preferentially recognizes the active 50 kDa heparanase form as compared to the non-active 65 kDa heparanase precursor. We have utilized this and other anti-heparanase antibodies to study the cellular localization of the latent 65 kDa and active 50 kDa heparanase forms during uptake and processing of exogenously added heparanase. Interestingly, not only the processed 50 kDa, but also the 65 kDa heparanase precursor was localized to perinuclear vesicles, suggesting that heparanase processing occurs in lysosomes. Indeed, heparanase processing was completely inhibited by chloroquine and bafilomycin A1, inhibitors of lysosome proteases. Similarly, processing of membrane-targeted heparanase was also chloroquine-sensitive, further ruling out the plasma membrane as the heparanase processing site. Finally, we provide evidence that antibody 733 partially neutralizes the enzymatic activity of heparanase, suggesting that the N-terminal region of the molecule is involved in assuming an active conformation. Monoclonal antibodies directed to this region are likely to provide specific heparanase inhibitors and hence assist in resolving heparanase functions under normal and pathological conditions.

Key words: Heparanase, Localization, Processing, Uptake, Lysosome, Antibody

Introduction

Heparanase is an endo-β-D-glucuronidase involved in cleavage of heparan sulfate (HS) chains, and hence it participates in extracellular matrix (ECM) degradation and remodeling. Heparanase activity has been traditionally correlated with the metastatic potential of tumor-derived cell types (Nakajima et al., 1998; Vlodavsky et al., 1999; Parish et al., 2001; Vlodavsky and Friedmann, 2001). Similarly, heparanase has been shown to facilitate cell invasion associated with autoimmunity, inflammation and angiogenesis (Vlodavsky et al., 1992; Dempsey et al., 2000a; Parish et al., 2001). More recently, heparanase upregulation was detected in a variety of human primary tumors correlating, in some cases, with increased tumor vascularity and poor postoperative survival (El-Assal et al., 2001; Gohji et al., 2001; Koliopanos et al., 2001; Rohloff et al., 2002). In addition, increased heparanase expression has been noted in kidney (Levidiotis et al., 2001), liver (Xiao et al., 2003) and diabetic (Katz et al., 2002) disorders. In the latter case, increased heparanase activity has been detected in patient's urine, suggesting that heparanase may serve as an early marker in diabetes and potentially other pathologies such as tumor metastasis (Goldshmidt et al., 2002b). Increased heparanase in urine and possibly other body fluids strongly implies that heparanase is a secreted enzyme. In addition, exogenously added and endogenous heparanase were localized to endosomes and lysosomes (Nadav et al., 2001; Goldshmidt et al., 2002a).

The heparanase cDNA encodes for a polypeptide of 543 amino acids that appears as a ~65 kDa protein in SDS-PAGE analysis. The protein undergoes proteolytic processing, which is likely to occur at two potential cleavage sites, Glu^{109}-Ser^{110} and Gln^{157}-Lys^{158}, yielding an 8 kDa polypeptide at the N terminus, a 50 kDa polypeptide at the C terminus and a 6 kDa linker polypeptide that resides in between (Fairbanks et al., 1999; Parish et al., 2001). Recently published observations clearly demonstrated that the active heparanase enzyme exists as a heterodimer composed of the 8 kDa polypeptide non-covalently associated with the 50 kDa heparanase subunit, and that heterodimer formation is necessary and sufficient for heparanase enzymatic activity (Levy-Adam et al., 2003; McKenzie et al., 2003). Nevertheless, currently available anti-heparanase antibodies do not distinguish between the latent 65 kDa heparanase precursor and the 50 kDa active enzyme. Thus, specific localization of the latent and active heparanase forms within the cell could not be determined.

We rationalized that heparanase processing may involve conformational changes that are likely to alter antibodies reactivity. More specifically, we hypothesized that cleavage

at the Gln^{157}-Lys^{158} site that ultimately results in formation of the 50 kDa heparanase subunit will generate an epitope specific for the 50 kDa heparanase form. We describe here the characterization of an antibody (733) that was raised against a 14 amino acid peptide mapped at the N terminus region of the 50 kDa heparanase (Lys^{158}-Asn^{171}). This antibody preferentially recognizes the active 50 kDa heparanase form by means of immunoblotting and immunoprecipitation, and labels heparanase in archive paraffin sections subjected to immunohistochemistry. Moreover, this antibody partially neutralizes the enzymatic activity of heparanase, suggesting that the N terminus region of the molecule participates in a three dimensional organization required for proper folding and enzymatic activity. In addition, we provide evidence that heparanase processing is chloroquine-, and bafilomycin A1-sensitive and is therefore likely to take place in lysosomes rather than at the plasma membrane.

Materials and Methods

Antibodies and reagents

Antibody 733 was raised against the peptide ^{158}KKFKNSTYRSSSVD^{171}. A cysteine residue was added following Asp^{171} to enable an efficient coupling of the peptide to kehole limpet hemocyanin (KLH) and to preserve the correct orientation of the peptide. The KLH-conjugated peptide was injected into rabbits and antibody specificity was evaluated by immunoblotting. For affinity purification, the peptide was coupled to agarose beads using the Sulfolink kit, according to the manufacturer's instructions (Pierce, Rockford, IL). Antibody 1453 was raised against the entire 65 kDa heparanase precursor isolated from the conditioned medium of heparanase-transfected 293 cells (Zetser et al., 2003). This antibody was affinity purified on immobilized bacterially expressed 50 kDa heparanase-GST fusion protein (Levy-Adam et al., 2003). Monoclonal anti-heparanase antibody was purchased from Becton-Dickinson (San Diego, CA). This antibody specifically recognizes the latent 65 kDa heparanase precursor in immunoblotting analysis. This antibody also works well in fluorescent immunostaining, but fails to react with archival paraffin sections. Monoclonal anti-heparanase antibody 130, recognizing both the 50 kDa and 65 kDa heparanase forms (Vlodavsky et al., 1999), was kindly provided by InSight Biopharmaceuticals (Rehovot, Israel). Anti-actin and anti-cathepsin D monoclonal antibodies were purchased from Sigma (St Louis, MO). Bafilomycin A1 was purchased from Sigma and dissolved in DMSO. An equivalent volume of the vehicle control was always run in parallel.

Plasmid DNA constructs

The pSecTag 2 vector containing the full-length heparanase cDNA was kindly provided by Dr Hua-Quan Miao (ImClone Systems Inc, New York, NY) and has been described previously (Levy-Adam et al., 2003). For targeting heparanase to the plasma membrane, heparanase cDNA was sub-cloned into the pDisplay vector (Invitrogen, Carlsbad, CA) which provides the PDGF-R transmembrane domain as a membrane-anchoring domain. The pcDNA3 plasmid containing the full-length heparanase cDNA was applied as template for heparanase amplification, using the forward 5′-GA-AGA-TCT-CAG-GAC-GTC-GTG-GAC-CTG- 3′ and reversed 5′-CCA-ATG-CAT-TTG-TTC-TGC-AGG-ATG-CAA-GCA-GCA-ACT-TTG-GC- 3′ set of primers. The forward primer contained an inserted *Bgl*II restriction site and the reverse primer contained a *Pst*I restriction site, enabling in frame cloning into the pDisplay multiple cloning site. Following PCR reaction with a proofreading enzyme (pfu, Promega, Madison WI), the vector and constructs were digested with *Bgl*II and *Pst*I, and ligated with T4 ligase. *E. coli* strain DH5α was used for transformation, and clones resistant to ampicillin were propagated in bacteria and evaluated for the presence of the insert by digestion with *Bgl*II/*Pst*I.

Cell culture and transfection

Human U87 glioma, MDA-MB-435 breast carcinoma, NMU rat mammary adenocarcinoma and Chinese hamster ovary (CHO) cells were purchased from the American Type Culture Collection (ATCC) and were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics. HEK-293 cells stably transfected with the human heparanase cDNA, were provided by ImClone Systems (New York, NY) and rat C6 glioma cells were kindly provided by Dr Eli Keshet (The Hebrew University School of Medicine, Jerusalem) (Benjamin and Keshet, 1997). For stable transfection, sub-confluent MDA-435, C6, NMU and CHO cells were transfected with the pSecTag2 or pDisplay vectors containing the full-length heparanase cDNA, using Fugene reagent according to the manufacturer's (Roche, Mannheim, Germany) instructions. Transfection proceeded for 48 hours followed by selection with 400 μg/ml Zeocin (pSecTag2 vector) or 800 μg/ml G418 (pDisplay vector) for 2 weeks. Stable transfectant pools were further expanded and analyzed.

Immunoblotting, metabolic labeling and immunoprecipitation

Cell extracts were prepared using a lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, supplemented with a cocktail of protease inhibitors (Roche, Indianapolis, IN). Protein concentration was determined (Bradford reagent, BioRad, Hercules, CA) and 30 μg protein was resolved by SDS-PAGE under reducing conditions using 10% gels. After electrophoresis, proteins were transferred to PVDF membrane (BioRad) and probed with the appropriate antibody followed by HRP-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) and an enhanced chemiluminescent substrate (Pierce).

Metabolic labeling was performed essentially as described previously (Ilan et al., 1996). Briefly, confluent cell cultures were methionine-starved for 30 minutes prior to the addition of 150 μCi/ml [^{35}S]methionine (Amersham, UK). Cells were pulsed for 20 minutes and chased for the indicated times in 1 ml of complete growth medium containing excess of nonradioactive methionine. For immunoprecipitation, equal volumes (0.1 ml) or equal number of TCA-precipitable cpm of lysate samples were brought to a volume of 1 ml with 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl and 0.5% NP-40 (buffer A) and incubated with the indicated antibody for 2 hours at 4°C. Protein A/G Sepharose beads (Santa Cruz) were then added for an additional 30 minutes. Beads were collected by centrifugation and washed three times with buffer A supplemented with 300 mM NaCl and 5% sucrose and finally with buffer A. Sample buffer was then added and after boiling at 100°C for 5 minutes, samples were subjected to electrophoresis as described above. Gels were fixed (30 minutes, 25% isopropanol + 10% acetic acid) and fluorographed (30 minutes, Amplify, Amersham) before drying and autoradiography.

Heparanase purification and uptake studies

The latent 65 kDa heparanase precursor was purified from the culture medium of heparanase-transfected HEK-293 cells, essentially as described previously (Zetser et al., 2003). For uptake studies, the 65 kDa heparanase precursor was added to confluent cell cultures at a concentration of 1 μg/ml under serum-free conditions. At the indicated time points, the medium was aspirated, cells were washed twice with ice-cold PBS and total cell lysates were prepared as

described. Heparanase uptake and processing were analyzed by immunoblotting with antibody 1453.

Immunohistochemistry

All studies were performed with archival paraffin sections. PC3 and MCF7 cell xenografts were generated essentially as described previously (Zetser et al., 2003). At the end of the experiment, xenografts were resected and fixed in 4% paraformaldehyde for 24 hours. Paraffin-embedded 5 μm sections were subjected to antigen retrieval by boiling for 5 minutes in a pressure cooker in 20 mM citrate buffer, pH 6. Sections were blocked with 10% normal goat serum in PBS for 60 minutes followed by overnight incubation with anti-heparanase 733 antibody at 4°C. Slides were then extensively washed with PBS containing 0.01% Triton X-100 and incubated with a secondary reagent (Envision kit) according to the manufacturer's instructions (Dako, Glostrup, Denmark). Following additional washes, color was developed with the AEC reagent (Sigma), sections were counterstained with Hematoxylin and mounted, as described previously (Zetser et al., 2003).

Immunocytochemistry

Breast MDA-MB-435 or glioma U87 cells were left un-treated, or incubated with heparanase (5 μg/ml) for the indicated times and then indirect immunofluorescence staining was performed essentially as described previously (Zetser et al., 2003). Briefly, cells were fixed with cold methanol for 10 minutes. Cells were then washed with PBS and subsequently incubated in PBS containing 10% normal goat serum for 1 hour at room temperature, followed by 2 hours incubation with the indicated primary antibodies. Cells were then extensively washed with PBS and incubated with the relevant (Cy2/Cy3-conjugated) secondary antibody (Jackson ImmunoResearch) for 1 hour, washed and mounted in Vectashield (Vector, Burlingame, CA).

Flow cytometry

Cells were detached with trypsin, centrifuged at 1000 rpm for 4 minutes, washed with PBS and counted. Cells (2×10^5) were centrifuged and the pellet was then resuspended in PBS with 1% FCS and incubated with anti-c-Myc or anti-heparanase monoclonal antibodies for 45 minutes on ice. Cells were then washed twice with PBS and incubated with FITC-conjugated anti-mouse IgG for 30 minutes on ice, washed and analyzed using a FACSCalibur fluorescent activated cell sorter and CellQuest software (Becton Dickinson, Mountain View, CA).

Heparanase activity assay

Preparation of ECM-coated dishes and determination of heparanase activity were performed as described in detail elsewhere (Vlodavsky, 1999; Goldshmidt et al., 2001; Goldshmidt et al., 2002b). Purified active heparanase was kindly provided by Dr H.-Q. Miao (ImClone Systems Inc., New York, NY). For inhibition studies, 20 ng protein were added to 1 ml serum-free RPMI medium and incubated (1 hour, 4°C) with 10 μg of rabbit IgG or 10 μg of affinity-purified antibody 733, followed by 1 hour incubation with ^{35}S-labeled ECM. For heparanase inhibition studies with live cells, heparanase-transfected 293 cells (2×10^5) were resuspended in RPMI medium and incubated (1 hour, 37°C) with ^{35}S-labeled ECM in the presence of 30 μg/ml affinity-purified antibody 733 or control rabbit IgG. The incubation medium containing sulfate-labeled degradation fragments was subjected to gel filtration on a Sepharose CL-6B column. Fractions (0.2 ml) were eluted with PBS and their radioactivity counted in a β-scintillation counter. Degradation fragments of HS side chains were eluted at $0.5< K_{av}<0.8$ (peak II, fractions 15-30). Nearly intact HSPGs were eluted just after the Vo ($K_{av}<0.2$, peak I, fractions 3-15).

Results

Antibody 733 preferentially recognizes the 50 kDa active heparanase form

Heparanase is synthesized as a ~65 kDa non-active precursor that is subsequently processed into 8 kDa and 50 kDa subunits that heterodimerize to form an active enzyme (Fairbanks et al., 1999; Levy-Adam et al., 2003; McKenzie et al., 2003). We have previously characterized an antibody (810) directed against a peptide located at the C terminus of the 8 kDa heparanase subunit, that preferentially recognizes the 8 kDa fragment as compared to the 65 kDa heparanase precursor (Levy-Adam et al., 2003). We hypothesized that such preferential recognition is due to an epitope that was exposed upon processing of the latent heparanase precursor and adopted a similar approach by raising an antibody against a peptide located at the N terminus of the 50 kDa heparanase subunit. Indeed, the antibody (733) preferentially recognized the 50 kDa heparanase subunit in immunoblot analysis, but failed to react with the 65 kDa heparanase precursor (Fig. 1A, 733). In contrast, an antibody that is commercially available (Becton-Dickinson) reacted only with the 65 kDa heparanase precursor (Fig. 1A, BD), while antibody 1453, which was raised against the entire 65 kDa protein, reacted with both the 65 kDa and 50 kDa heparanase forms (Fig. 1A, 1453). In order to further evaluate the specificity of antibody 733, we employed metabolic labeling and immunoprecipitation analysis. Heparanase-transfected CHO cells were pulsed for 20 minutes with [^{35}S]methionine and then chased for the indicated times in complete growth medium supplemented with an excess of cold methionine (Fig. 1B). Immunoprecipitation of cell lysate samples with mAb 130, which recognizes both the 65 kDa and 50 kDa forms of heparanase (Vlodavsky et al., 1999; Nadav et al., 2001), revealed the synthesis of a single ~65 kDa protein at the end of the pulse period (Fig. 1B, 0). Subsequently, the amount of the 65 kDa heparanase form rapidly declined while a 50 kDa band started to appear after 2 hours and accumulated further by 4 hours of chase (Fig. 2B, upper panel). A similar biosynthesis pattern and kinetics were observed in metabolically labeled heparanase-transfected HEK-293 and U87 glioma cells (not shown). In contrast, immunoprecipitation of the same lysate samples with antibody 733 failed to detect the 65 kDa heparanase precursor found at time 0, but precipitated the 50 kDa heparanase form found after 2 hours and 4 hours of chase (Fig. 1B, lower panel). Thus, under denatured (immunoblotting) and native (immunoprecipitation) conditions, antibody 733 preferentially recognized the 50 kDa active form of heparanase, suggesting that the peptide antigen is less susceptible to denaturation, or that some re-naturation may occur following electrophoresis. Next, we evaluated the ability of this antibody to recognize heparanase in paraffin sections subjected to immunohistochemistry. To this end, we chose placenta, which is known to posses high levels of heparanase activity and, moreover, was used as a source for heparanase purification (Goshen et al., 1996; Vlodavsky et al., 1999; Dempsey et al., 2000b; Haimov-Kochman et al., 2002). As expected, antibody 733 specifically stained the cytotrophoblast cell layer lining the

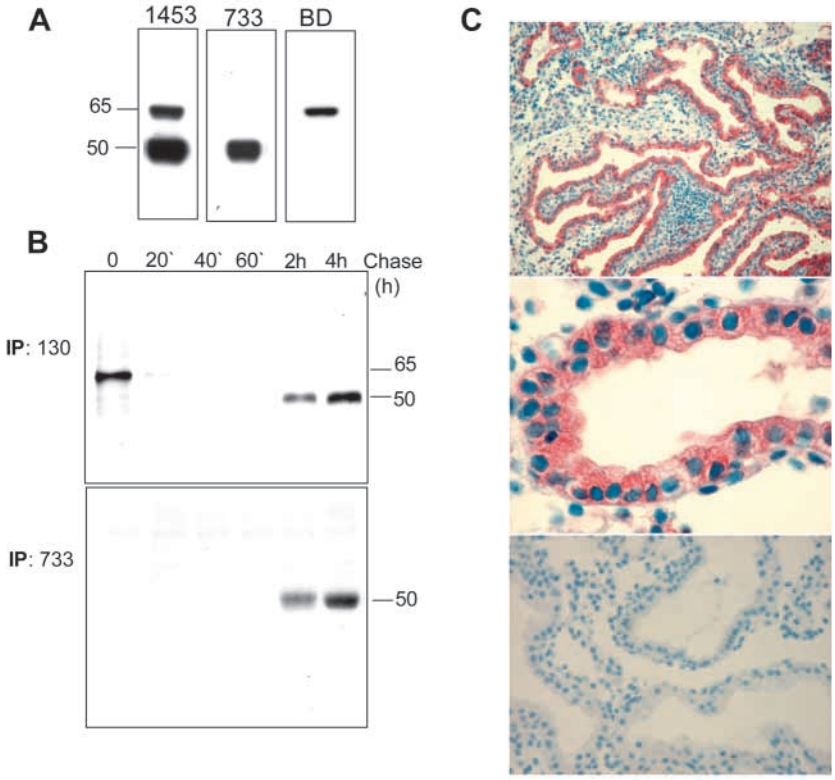


Fig. 1. Characterization of antibody 733 specificity. (A) Immunoblot analysis. Lysates of heparanase-transfected HEK-293 cells were resolved by SDS-PAGE and blots were probed with antibody 1453 (left panel), 733 (middle panel) and anti-heparanase monoclonal antibody purchased from Becton-Dickinson (BD, right panel). (B) Metabolic-labeling followed by immunoprecipitation. Heparanase-transfected CHO cells were pulsed for 20 minutes with [^{35}S]methionine (0) and then chased for the indicated time intervals in complete growth medium containing an excess of non-radioactive methionine. Equal volumes of cell lysate samples were subjected to immunoprecipitation (IP) with mAb 130 (top panel) or antibody 733 (bottom panel), as described in Materials and Methods. (C) Immunohistochemistry. Placenta sections (5 μm) were subjected to immunostaining with antibody 733, as described in Materials and Methods. Negative control, in which the primary antibody was omitted, is shown in the bottom panel. Original magnifications: top and bottom panels ×20, middle panel ×100.

intervillous space (Fig. 1C), in agreement with previously reported placenta staining (Haimov-Kochman et al., 2002).

Differential cellular localization of heparanase forms

Having demonstrated the ability of antibody 733 to specifically react with heparanase in immunohistochemical analysis, we stained xenograft tumor sections derived from prostate PC3 and breast MCF7 cells, cell lines that exhibit low heparanase activity profiles in vitro (Zcharia et al., 2001). These xenograft sections were compared with heparanase expression in human prostate and breast tumor biopsies (Fig. 2A,B). Antibody 733 was able to detect heparanase expression in PC3 (Fig. 2Aa,b) and MCF 7 (Fig. 2Ba,b) xenografts, as well as in prostate (Fig. 2Ac,d) and breast (Fig. 2Bd,e) biopsy specimens. Interestingly, heparanase was localized mainly at perinuclear regions rather than diffusely distributed in the cell cytoplasm. This was best demonstrated in PC3 and MCF7 xenografts and breast biopsy at a high magnification (Fig. 2Ab, Bb,e), and closely resembled the localization of endogenous (Goldshmidt et al., 2002a) or exogenously added (Nadav et al., 2001) heparanase.

In order to further study the sub-cellular localization of the two heparanase forms, we examined the processing of exogenous heparanase added to MDA-MB-435 breast cancer and U87 glioma cells. For this purpose, the latent 65 kDa heparanase precursor was added at a concentration of 1 μg/ml to confluent MDA-435 and U87 cells and heparanase uptake and processing were evaluated at various time intervals by means of immunoblotting. As previously reported for primary fibroblasts (Nadav et al., 2001), heparanase rapidly reacted with MDA-435 and U87 cells and uptake of the 65 kDa heparanase was detected already 15 minutes following its addition (Fig. 3 inset, 15′). The amount of the 65 kDa heparanase continued to accumulate by 30 and 60 minutes without detectable processing, which was noted 2 hours after its addition and apparently completed by 4 hours (Fig. 3, inset). Similar uptake and processing kinetics were observed with 293, C6, PC3 and HeLa cell lines (not shown). This uptake and processing pattern enabled us to study the localization of the two heparanase forms at different time points following application. To this end, MDA-435 cells were left untreated (Fig. 3, Con, upper panel) or incubated with heparanase (5 μg/ml) for 5 minutes (second panel), 1 hour (third panel) or 3 hours (fourth panel) and double stained with antibodies that distinguish between the latent 65 kDa (BD) and active 50 kDa (733) forms of heparanase (Fig. 1A). The 65 kDa heparanase precursor was readily detected by 5 minutes after its addition and seemed to be exclusively localized to the plasma membrane (Fig. 3, second panel, left). At 1 hour, the 65 kDa heparanase appeared more diffusely distributed in the cell cytoplasm, with minimal processing detected with antibody 733 staining (Fig. 3, third panel). Abundant heparanase processing was detected by antibody 733 at 3 hours following the addition of heparanase (Fig. 3, fourth panel, middle), in agreement with the immunoblot analysis (Fig. 3, inset), accumulating at perinuclear areas (Nadav et al., 2001). Double immunostaining with antibody 733 and anti-cathepsin D, a lysosomal marker, clearly revealed these perinuclear vesicles as lysosomes (Fig. 3, sixth panel), suggesting that the 50 kDa active heparanase enzyme resides within the lysosomal compartment. Interestingly, at this time point the non-processed 65 kDa heparanase precursor also appeared perinuclear, partially co-localizing with the processed form (Fig. 3, fourth panel). Such co-localization of the processed and unprocessed heparanase forms was confirmed by additional experiments with human

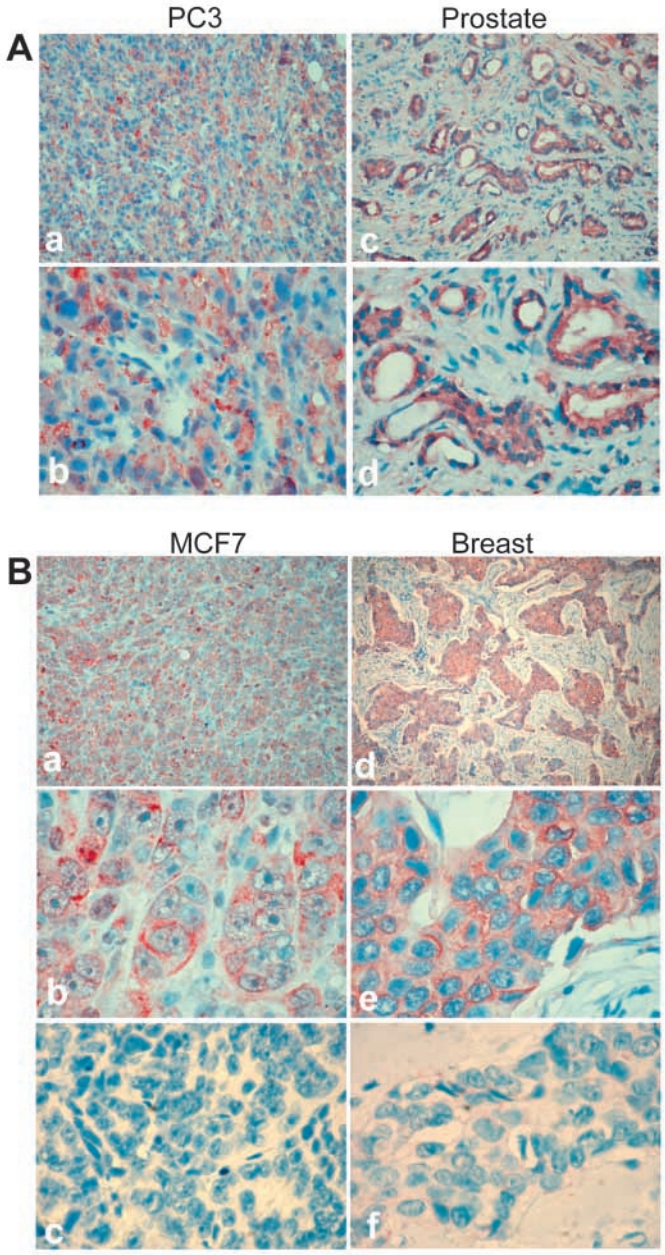


Fig. 2. Heparanase localization in cell xenografts and tumor biopsies. (A) PC3 xenograft (a,b), prostate biopsy (c,d) and (B) MCF7 xenograft (a-c) and breast cancer biopsy (d-f) sections were subjected to antigen retrieval and immunostaining with antibody 733. Negative control, in which the primary antibody was omitted, is shown for MCF7 xenograft (Bc) and breast cancer biopsy (Bf). Original magnifications: Aa,c and Ba,d ×20; Ab,d and Bb,c,e,f ×100.

U87 glioma cells, yielding a similar staining pattern (Fig. 3, fifth panel).

Heparanase processing requires active lysosomes

The heparanase processing site within the cell has not been identified to date. Nevertheless, several publications raised the possibility of the plasma membrane as the processing organelle (Nadav et al., 2001; Vlodavsky and Friedmann, 2001). The appearance of the non-processed 65 kDa heparanase at perinuclear vesicles (Fig. 3 fourth and fifth panels) argues against this hypothesis and suggests that heparanase processing occurs in acidic vesicles, presumably lysosomes. In order to test this hypothesis, HEK-293 (Fig. 4A, upper panel), MDA-435 (Fig. 4A, second panel), C6 (Fig. 4A, third panel) and NMU (Fig. 4A, fourth panel) cells stably transfected with the human heparanase cDNA were treated with increasing concentrations of chloroquine, an inhibitor of lysosome proteases. Chloroquine, which inhibits lysosomal protease activity by raising the lysosome pH, completely inhibited heparanase processing in a dose-dependent manner, in all the heparanase-transfected cell lines examined (Fig. 4A). This effect of chloroquine was reversible and heparanase processing re-appeared upon chloroquine removal (Fig. 4B). Interestingly, treatment of heparanase-transfected NMU cells with chloroquine resulted in the appearance of at least four different heparanase species (Fig. 4A, fourth panel), suggesting that heparanase processing is more complex then originally thought, involving several steps and possibly different enzymes. Similarly, chloroquine treatment completely inhibited the processing of exogenous heparanase (Fig. 4C), resulting in the accumulation of the unprocessed heparanase in large vesicles (Fig. 4D, right panel). The necessity of acidified lysosomes for heparanase processing was confirmed by treating cells with bafilomycin A1, a specific inhibitor of the vacuolar proton pump (Drose and Altendorf, 1997). As little as 250 nM bafilomycin A1 completely inhibited heparanase processing in transfected C6 glioma and NMU cells (Fig. 4E), supporting the lysosome as the primary heparanase processing organelle.

In order to further rule out the plasma membrane as the heparanase processing site, heparanase was targeted to the cell membrane by introducing the PDGF-R transmembrane domain at the heparanase C terminus. Stably transfected HEK-293 and C6 glioma cells revealed a high expression of this gene construct (Fig. 5A) and membrane localization was verified by FACS analysis (Fig. 5B) and immunofluorescence staining (Fig. 5C). The protein product of this hybrid gene construct was processed into the expected 50 kDa heparanase form (Fig. 5A). This is in contrast to the heparanase-GFP hybrid, which failed to be processed (Goldshmidt et al., 2002a), suggesting that the introduced transmembrane domain does not interfere with heparanase processing. Processing of the membrane-bound heparanase may be brought about by a membranous protease, or can take place in lysosomes as part of membrane recycling. If the latter possibility is correct, processing of the membrane-bound heparanase should also be inhibited by chloroquine. Indeed, incubation of HEK-293 and rat C6 glioma cells expressing the membrane-targeted heparanase with chloroquine completely abolished heparanase processing (Fig. 5D), suggesting that the protease(s) responsible for heparanase processing resides within the lysosome and requires acidic pH.

Antibody 733 inhibits heparanase enzymatic activity

The preferential recognition by antibody 733 of the processed 50 kDa heparanase as compared to the non-processed 65 kDa form (Fig. 1A) suggests that the N terminus of the 50 kDa protein undergoes conformational changes upon heparanase processing, exposing an epitope that is not present in the 65

kDa heparanase precursor. Although this region is not considered to be part of the heparanase active site (Hulett et al., 2000), it may well be involved in a three-dimensional organization assumed by the 50 kDa heparanase upon processing, and that is necessary for enzymatic activity. To test this hypothesis, recombinant purified heparanase was incubated with affinity-purified antibody 733 or control rabbit IgG, and enzymatic activity was determined. As shown in Fig. 6A, antibody 733 significantly inhibited heparanase enzymatic activity at concentration of 10 μg/ml. Higher antibody concentrations (30 μg/ml) produced no further inhibition while lower concentrations (2 μg/ml) were significantly less effective (data not shown). Moreover, antibody 733 also inhibited heparanase activity in live 293 cells (Fig. 6B). Raising monoclonal antibodies directed against the peptide recognized by antibody 733 may result in a better inhibitory antibody and provide a specific molecular tool to study heparanase function under normal and pathological conditions.

Discussion

Traditionally correlated with the metastatic potential of tumor-derived cells, heparanase up-regulation has been documented in an increasing number of primary human tumors (Zcharia et al., 2001; Vlodavsky et al., 2002). More recently, increased heparanase expression was observed in several other disorders such as nephrosis (Levidiotis et al., 2001) and cirrhosis (Xiao et al., 2003). The role that heparanase may play in these and other pathologies (Dempsey et al., 2000b) is only poorly understood. Moreover, heparanase expression is not restricted to pathological conditions and high levels of activity have long been found in placenta and more recently in the skin (Bernard et al., 2001) and other tissues (Dempsey et al., 2000a). Specific heparanase inhibitors are expected to provide pivotal tools for studying heparanase functions under normal and pathological conditions. Currently available heparanase inhibitors are various sulfated poly- and oligosaccharides such as heparin fragments, laminaran sulfate and PI-88 (Vlodavsky et al., 1994; Miao et al., 1999; Parish et al., 1999). These compounds were shown to inhibit heparanase activity and produce anti-metastatic and anti-angiogenic effects (Miao et al., 1999; Parish et al., 1999; Vlodavsky et al., 1994). Nevertheless, laminaran sulfate and species of heparin also inhibit bFGF binding to its receptor, resulting in inhibition of endothelial cell proliferation (Hoffman et al., 1995) and angiogenesis (Hoffman et al., 1996). These compounds also inhibit selectin-mediated cell adhesion (Koenig et al., 1998; Varki and Varki, 2002). The lack of specificity makes interpretation questionable when using these and other polysulfated reagents (Nakajima et al., 1991; Marchetti et al., 2003).

In this report, we have characterized an antibody (733) that was raised against a 14 amino acid sequence located at the N terminus of the 50 kDa heparanase enzyme. This antibody

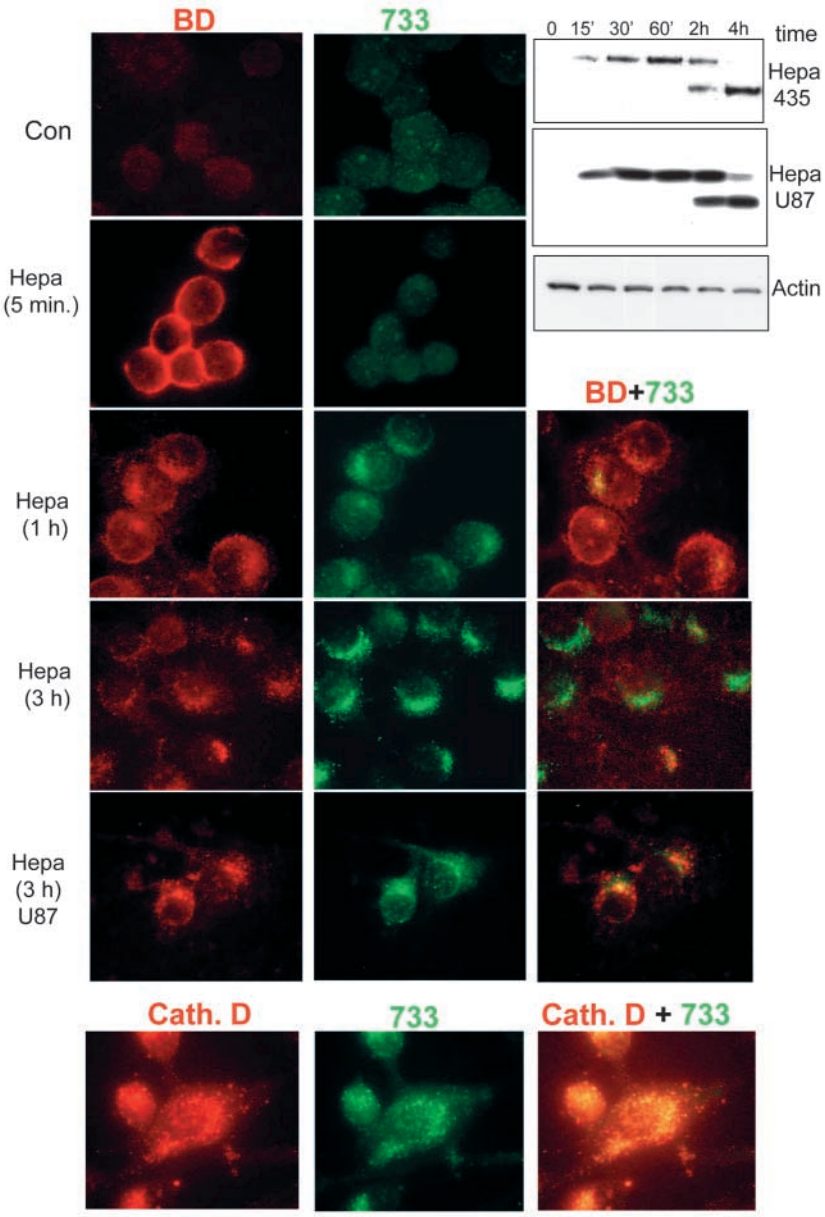


Fig. 3. Heparanase uptake, processing and localization in human MDA-435 breast carcinoma and U87 glioma cells. MDA-435 cells were left untreated (Con, top row) or incubated with the 65 kDa heparanase precursor (5 μg/ml) for 5 minutes (second row), 1 hour (third row) or 3 hours (fourth row) and stained with monoclonal anti-heparanase antibody (BD, left column, red) or with antibody 733 (middle column, green). Merged images are shown in the right column. U87 cells were incubated with heparanase for 3 hours and similarly stained (fifth row). Original magnification: ×100. MDA-435 cells were also stained with monoclonal anti-cathepsin D antibody, or with antibody 733. Merged image is shown in the right panel (bottom row). (Inset: top right) Heparanase uptake and processing. MDA-435 (upper panel) and U87 glioma cells (second panel) were left untreated (0) or incubated with the 65 kDa heparanase precursor. At the indicated time points, cells were washed and total cell lysates were subjected to SDS-PAGE followed by immunoblotting with antibody 1453 (first and second panels), or with anti-actin antibody (third panel).

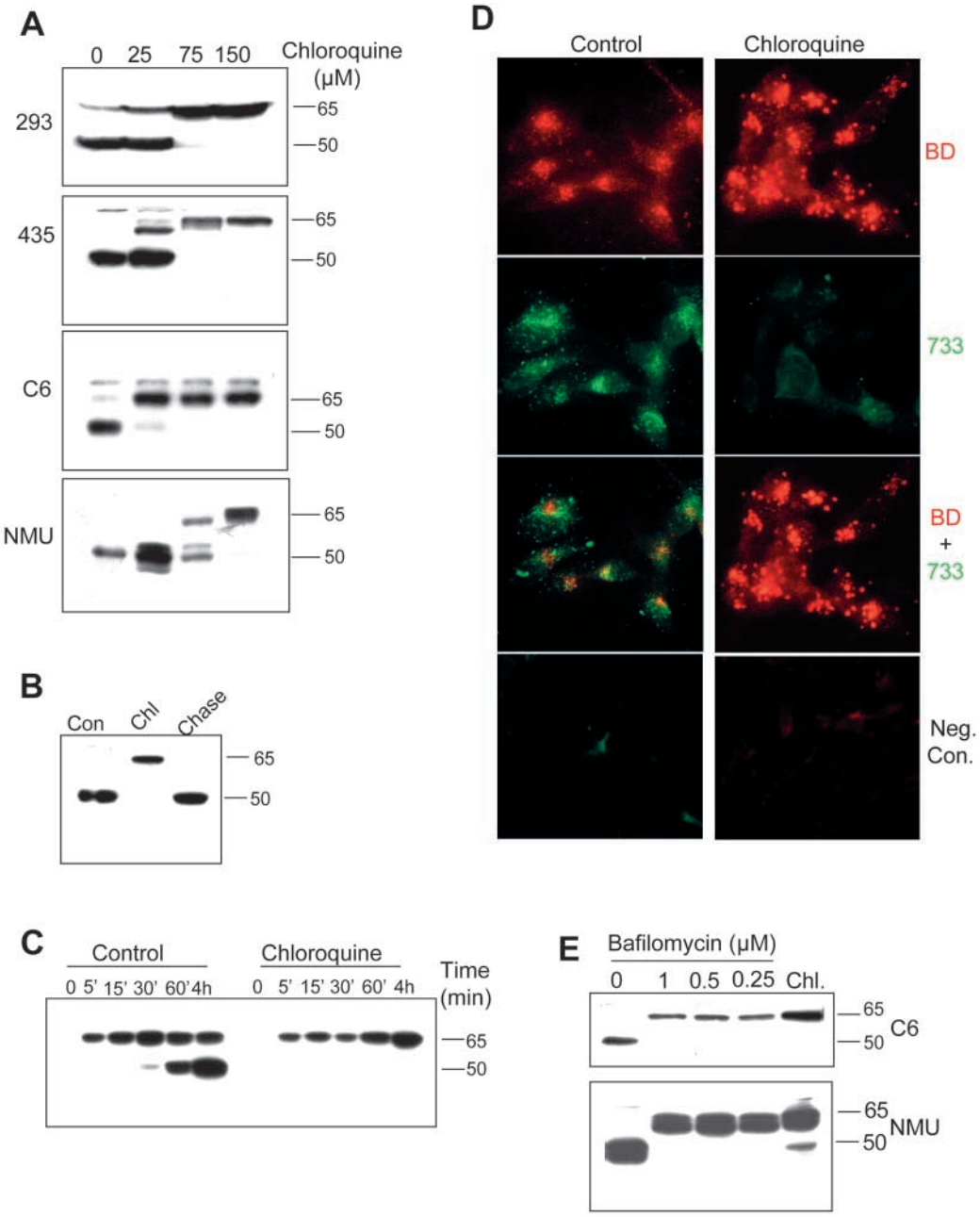


Fig. 4. Heparanase processing is inhibited by lysosomal proteinase inhibitors. (A-D) Chloroquine treatment. (A) Heparanase-transfected 293 (upper panel), MDA-435 breast carcinoma (second panel), C6 rat glioma (third panel) and NMU rat mammary adenocarcinoma (fourth panel) cells were left untreated (0) or incubated for 20 hours with the indicated concentrations (μM) of chloroquine. Total cell lysates were immunoblotted with anti-heparanase antibody 1453. Note a dose-response inhibition of heparanase processing and accumulation of the unprocessed 65 kDa heparanase precursor. (B) Chloroquine treatment is reversible. Heparanase-transfected C6 glioma cells were left untreated (Con) or treated with 50 μM chloroquine for 20 hours. Cells were then lysed (Chl) or washed and chased for an additional 24 hours with chloroquine-free medium (Chase). Total cell lysates were then analyzed for heparanase processing by immunoblotting as above. Note re-appearance of the processed 50 kDa heparanase form upon chloroquine removal. (C,D) Uptake studies. (C) U87 glioma cells were left untreated (Control) or pre-treated with 100 μM chloroquine (Chloroquine) for 2 hours. The latent 65 kDa heparanase protein was then added for the indicated time points and total cell lysates were analyzed for heparanase processing by immunoblotting as above. Note complete inhibition of exogenously added heparanase processing upon chloroquine pre-treatment. (D) U87 glioma cells were left untreated (Control) or incubated with chloroquine (100 μM) for 2 hours, followed by the addition of the 65 kDa heparanase protein for additional 2 hours. Cells were then fixed and immunostained with anti-heparanase monoclonal antibody (BD) and 733 anti-heparanase (733) antibodies. Merged images are shown in the third row. Negative control, in which the primary antibody was omitted, is shown in the bottom row. Note the complete absence of heparanase processing as evident by the lack of reactivity with antibody 733 upon chloroquine treatment (second panel, right), and the accumulation of the latent heparanase form in large vesicles (upper and 3rd panels, right) . Original magnifications ×100. (E) Heparanase-transfected C6 glioma (upper panel) and NMU (lower panel) cells were left untreated (0) or incubated with the indicated concentrations (μM) of bafilomycin A1 or chloroquine (Chl, 50 μM) for 20 hours. Total cell lysates were analyzed by immunoblotting as above. Note complete inhibition of heparanase processing upon bafilomycin treatment.

reacts specifically with the 50 kDa heparanase form, as evaluated by immunoblotting (Fig. 1A) and immunoprecipitation (Fig. 1B) analyses. Importantly, antibody 733 was able to partially neutralize the activity of purified active heparanase (Fig. 6A) and to significantly inhibit heparanase activity in live cells (Fig. 6B). This finding suggests that the N-terminal region of the 50 kDa heparanase enzyme participates in the formation of a three-dimensional structure necessary for enzymatic activity, and that antibody 733 binding to this region prevents proper enzyme folding. Alternatively, the neutralizing ability may be due to antibody 733 interfering with the interaction of heparanase with its substrate. Given the

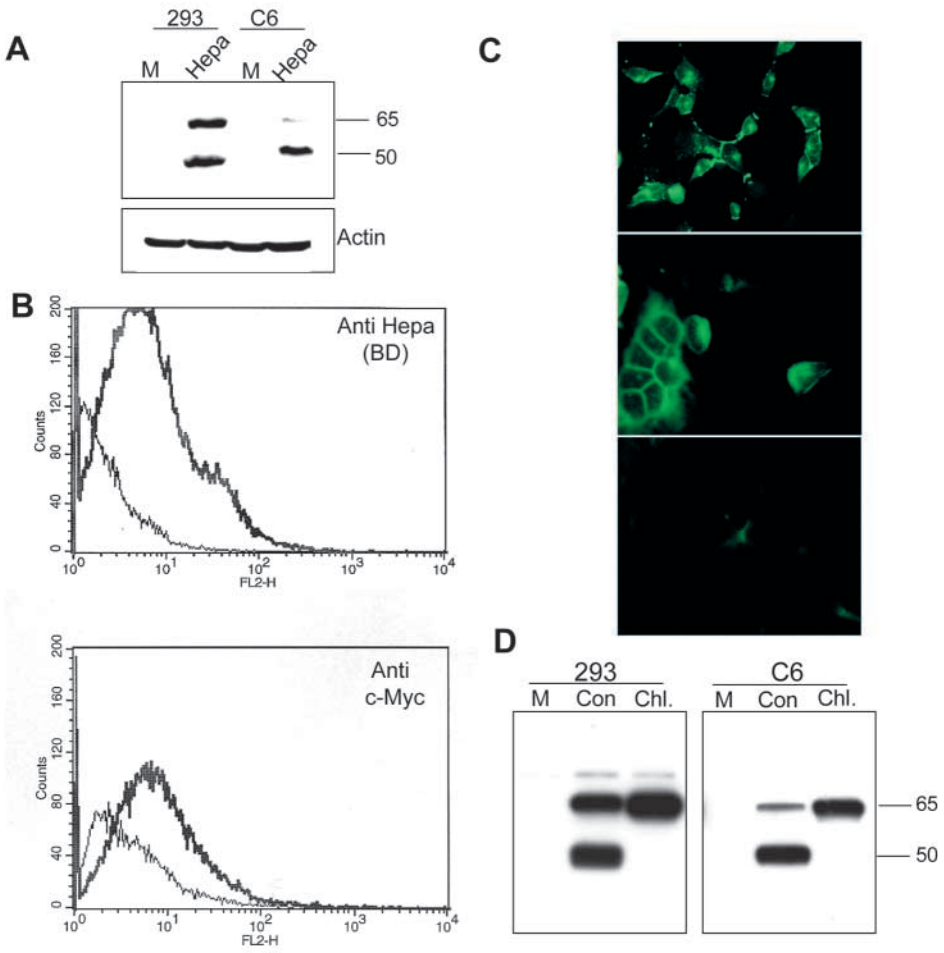
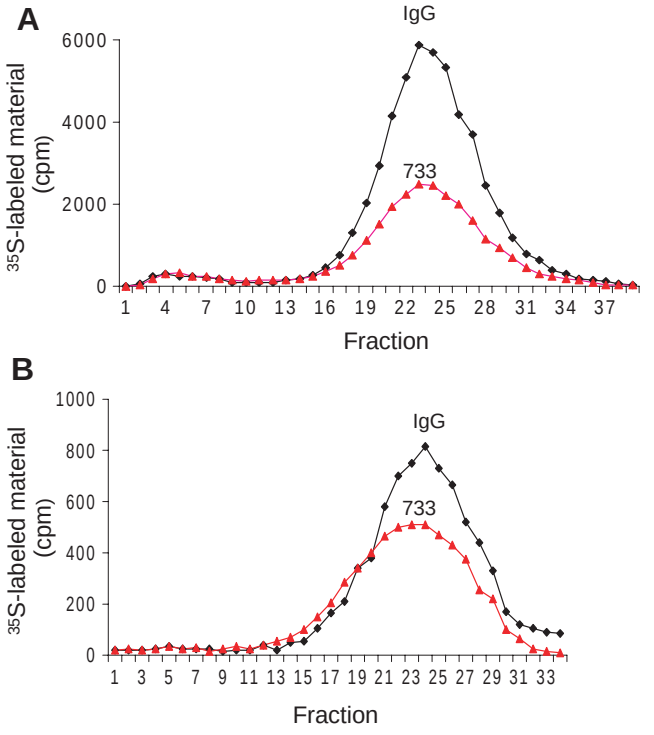


Fig. 5. Processing of membrane-targeted heparanase is chloroquine sensitive. (A) Expression of membrane-targeted heparanase in stably transfected 293 and C6 cells. Control (M) and heparanase (Hepa) transfected cells were lysed and subjected to immunoblot analysis with anti-heparanase antibody 1453 (upper panel) or anti-actin antibodies (lower panel). 293 cells expressing the membrane-targeted heparanase gene construct were analyzed by FACS (B) with anti-heparanase monoclonal antibody (BD, upper panel) and anti-c-Myc epitope-tagged antibodies (lower panel), or stained by immunofluorescence (C) with anti-heparanase monoclonal antibodies (BD). Negative control, in which the primary antibody was omitted, is shown in the lower panel. Note heparanase accumulation at areas of cell-cell junctions in sparse cultures (C, top panel) and exclusive localization at the cell borders in confluent cells (C, middle panel). (D) Processing of membrane-targeted heparanase is chloroquine sensitive. 293 (left panel) and C6 (right panel) cells stably transfected with the membrane-targeted heparanase gene construct were left untreated (Con) or treated for 20 hours with 100 μM chloroquine (Chl). Total cell lysates were immunoblotted with anti-heparanase antibody 1453. Non-transfected cell lysates were included as control (M).



specificity of antibody reaction and the recent therapeutic use of antibodies in the clinic (Ludwig et al., 2003; Nahta et al., 2003), neutralizing anti-heparanase antibodies are extremely important reagents for basic heparanase research and, possibly, clinical applications. Raising monoclonal antibodies against the 14 amino acid sequence recognized by antibody 733 is hoped to yield an even better neutralizing ability and is currently in progress.

We have used the 733 antibody to study heparanase localization and its sub-cellular processing site, two important aspects of heparanase biology (Goldshmidt et al., 2002a; Goldshmidt et al., 2002b). Previously, we have shown that exogenously added heparanase rapidly interacts with primary human fibroblasts, followed by processing of the 65 kDa

Fig. 6. Antibody 733 inhibits heparanase enzymatic activity. (A) Purified, active recombinant heparanase (20 ng) was added to 1 ml RPMI medium and incubated with affinity-purified antibody 733 (10 μg, red symbols), or rabbit IgG (black symbols) for 1 hour on ice, followed by 1 hour incubation with ^{35}S-labeled ECM. Heparanase activity was determined as described in Materials and Methods. (B) Heparanase-transfected 293 cells (2×10^5) were plated on ^{35}S-labeled ECM in the presence of affinity-purified antibody 733 (30 μg/ml, red symbols) or control rabbit IgG (black symbols) for 2 hours. The incubation medium containing sulfate-labeled degradation fragments was subjected to gel filtration on a Sepharose CL-6B column.

precursor into an active 50 kDa enzyme (Nadav et al., 2001). This uptake and processing pathway was now demonstrated with tumor-derived MDA-435 breast carcinoma and U87 glioma cell cultures (Fig. 3, inset). In fact, heparanase processing was evident in all primary and tumor-derived cell types examined, suggesting that the involved protease(s) is constitutively active and highly abundant. Moreover, processing of exogenously added heparanase has led to the hypothesis that the protease is a membrane-associated enzyme (Nadav et al., 2001; Vlodavsky and Friedmann, 2001). We utilized this experimental system to determine the localization of the two heparanase forms during uptake and processing of the latent enzyme, using fluorescent immunostaining. As expected, shortly after its application, the 65 kDa heparanase was exclusively localized to the plasma membrane (Fig. 3, second panel, red), presumably interacting with HSPG in the membrane (S.G.-V., A.Z., M.Y.F., I.V. and N.I., unpublished) (Nadav et al., 2001). By 1 hour following application, heparanase staining appeared diffuse with minimal processing evident by a weak staining with antibody 733 (Fig. 3, third panel) that became intense by 3 hours following heparanase application (Fig. 3, fourth and fifth panels, green), a time point at which the 50 kDa heparanase appeared mainly in perinuclear vesicles, identified as lysosomes (Fig. 3, sixth panel). Interestingly, we noted that the 65 kDa heparanase similarly appeared in vesicles that co-localized, at least in part, with the 50 kDa processed form. This suggests that heparanase processing occurs after its internalization and away from the cell membrane. Lysosomal processing was confirmed by the inhibitors chloroquine (Fig. 4A-D) and bafilomycin A1 (Fig. 4E), treatments that completely inhibited heparanase processing in a number of heparanase-transfected cell types (Fig. 4A) and in a reversible manner (Fig. 4B). Moreover, chloroquine also inhibited the processing of membrane-targeted heparanase (Fig. 5D), further arguing for lysosomes, rather than the plasma membrane, as the processing site. The exact lysosomal protease that is responsible for heparanase processing is, however, still to be discovered.

Heparanase biosynthesis has not yet been followed by metabolic labeling. A single 65 kDa band appeared after a short metabolic pulse, corresponding to the 65 kDa latent heparanase form (Fig. 1B). Interestingly, this band rapidly disappeared, while a 50 kDa protein was detected instead, starting at 2 hours, and even more so at 4 hours of chase (Fig. 1B). The lag between the synthesis of the latent 65 kDa heparanase and the appearance of the processed 50 kDa form is not entirely clear, but may result from secretion of the 65 kDa latent form, followed by uptake, internalization and processing. If this is indeed the case, our uptake studies (Fig. 3) may reflect the in vivo course of heparanase trafficking. This scenario is supported by analysis of the secreted heparanase found in the culture medium. When samples of medium are analyzed, the latent 65 kDa heparanase protein is first detected at 1 hour and further accumulates by 2 and 4 hours of chase (data not shown). Thus, the appearance of the processed 50 kDa heparanase form in the cell lysate correlates with heparanase accumulation in the culture medium. This may suggest that upon de-novo synthesis, heparanase is first secreted and subsequently is subjected to uptake and processing. Accumulation of the 50 kDa processed form by 4 hours of chase (Fig. 1B) and even more so by 24 hours (data not shown) may suggest that heparanase half-life is at least 24 hours. This stands in contrast to the relatively short half life of HSPG with transmembrane domain, estimated to be 2-3 hours for cultured rat hepatocytes and 5-6 hours for rat ovarian granulosa cells (Egeberg et al., 2001), or even shorter ($T_{1/2}$ ~25 minutes) for glycosylphosphatidyl-inositol-anchored HSPG (Egeberg et al., 2001). This suggests that heparanase may normally function in the turnover of lysosomal HSPG, while heparanase secretion may be involved in its pathological aspects.

High levels of heparanase activity found in the urine of diabetic patients (Katz et al., 2002) and the more traditional correlation between heparanase activity levels and the metastatic potential of tumor-derived cells (Nakajima et al., 1998; Vlodavsky and Friedmann, 2001), argue for heparanase being a secreted enzyme. In fact, the enzyme is readily released by activated platelets and cells of the immune system (Vlodavsky et al., 1992). Antibody 733 and anti-heparanase monoclonal antibodies yet to be characterized, may provide the basis for a sensitive screening assay to detect heparanase in body fluids. This will enable a comprehensive study to establish heparanase as a diagnostic marker for human pathologies. These studies are currently in progress.

Since antibody 733 preferentially recognizes the active form of heparanase, positive staining of archival paraffin sections indicated not only the presence of heparanase, but also its being active. Importantly, in MCF7 and PC3 cell xenografts, as well as in a breast tumor biopsy, heparanase localization resembled the in vitro localization, accumulating perinuclearly in a vesicle-like pattern (Fig. 2Ab,Bb,e), suggesting that our in vitro studies reflect heparanase localization in vivo. In other cases, heparanase appeared less localized and more diffusely distributed in the cytoplasm (Fig. 1C, Fig. 2Ac,d). This suggests that under different biological situations, heparanase may be localized in different cellular compartments and hence may exert diverse functions.

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