Activation, processing and trafficking of extracellular heparanase by primary human fibroblasts

Liat Nadav[1], Amiram Eldor[1], Oron Yacoby-Zeevi[2], Eli Zamir[3], Iris Pecker[2], Neta Ilan[2], Benjamin Geiger[3], Israel Vlodavsky[4] and Ben-Zion Katz[1,*]

[1]Tel-Aviv Sourasky Medical Center, Tel-Aviv, Israel
[2]InSight Ltd., Rehovot, Israel
[3]Weizmann Institute of Science, Rehovot, Israel
[4]Hadassah-Hebrew University Hospital, Jerusalem, Israel
*Author for corrrespondence (e-mail: sbkatz@netvision.net.il)

This study is dedicated to the memory of Professor Amiran Eldor, a clinician and a leading scientist in the field of glycosaminoglycans and heparanase, who died on 25 November 2001 in a tragic plane crash

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Summary

Heparanase is a heparan-sulfate-degrading endoglycosidase that has important roles in various biological processes, including angiogenesis, wound healing and metastatsis. Human heparanase is synthesized as a 65 kDa latent precursor, which is proteolytically processed into a highly active 50 kDa form. Extracelluar heparanase is found in various tissues and is utilized by both normal cells and metastatic cancer cells to degrade heparan sulfate moieties in basement membranes and extracellular matrices. This study characterizes the processing and trafficking events associated with cellular activation of extracellular heparanase. We show that primary human fibroblasts are capable of binding and converting the 65 kDa heparanase precursor into its highly active 50 kDa form, concomitantly with its cytoplasmic accumulation. Heparanase uptake depends on the actin cytoskeleton integrity, resulting in a prolonged storage of the enzyme, mainly in endosomal structures. Heparanase endocytosis and its proteolytic activation are independent processes, indicating that heparanase cleavage is a cell surface event. Heparin completely inhibits heparanase endocytosis but only partially inhibits its association with the cells, suggesting that cell surface heparan sulfate moieties play a specific role in its endocytosis. Cellular binding and uptake of extracellular heparanase control its activation, clearance rate and storage within the cells.

Key words: Endocytosis, Heparan sulfate proteoglycans, Heparanase, Processing

Introduction

Endoglycosidic heparan-sulfate-degrading heparanase has important roles in a variety of biological processes, including angiogenesis, inflammation, wound healing and metastatsis (Dempsey et al., 2000a; Irimura et al., 1986; Parish et al., 2001; Vlodavsky et al., 1990; Vlodavsky et al., 1994). A predominant cDNA encoding heparanase was independently cloned by several groups, and its gene localized to chromosome 4q.21.3 (Dempsey et al., 2000b; Hulett et al., 1999; Toyoshima et al., 1999; Vlodavsky et al., 1999a). Expression of the full-length heparanase cDNA yields a 65 kDa latent protein that is proteolytically processed at the N-terminus to yield a highly active 50 kDa form of the enzyme (Fairbanks et al., 1999; Hulett et al., 1999; Kussie et al., 1999; Toyoshima et al., 1999; Vlodavsky et al., 1999a). The major heparanase substrate, heparan sulfate proteoglycans (HSPGs), resides within the extracellular milieu of a variety of tissues. HSPGs are important constituents of the extracellular microenvironment, particularly basement membranes (BM), where they bind and assemble extracellular matrix (ECM) constituents (e.g. laminin, collagen) and hence contribute to the assembly and stability of these supramolecular structures (Bernfield et al., 1999; Iozzo, 1998; Kjellen and Lindahl, 1991). HSPGs are also utilized to sequester and store growth promoting factors, cytokines and enzymes in the microenvironment of cells (Bernfield et al., 1999; Iozzo, 1998; Vlodavsky et al., 1993; Vlodavsky et al., 1996; Wight et al., 1992). Heparanase is utilized by hematopoietic cells and by blood-borne metastatic tumor cells to disintegrate the structure of the subendothelial BM, thereby facilitating their trafficking through blood vessel walls (Dempsey et al., 2000a; Irimura et al., 1986; Parish et al., 2001; Vlodavsky et al., 1990; Vlodavsky et al., 1992; Vlodavsky et al., 1994). Highly metastatic cells and human tumors express high levels of heparanase compared with the respective non-invasive cells and normal tissues (Friedmann et al., 2000; Hulett et al., 1999; McKenzie et al., 2000; Vlodavsky et al., 1999a). Low metastatic murine T-lymphoma and melanoma cells become highly metastatic following transfection of the heparanase cDNA and expression of the protein, demonstrating a cause and effect relationship between heparanase expression and metastasis (Vlodavsky et al., 1999a). The extracellular location of HSPGs requires heparanase to be accessible to the extracellular milieu. Indeed, sequence analysis of the heparanase protein reveals a hydrophobic amino acid stretch at the N-terminus, which might function as a signal peptide for its secretion (Hulett et al., 1999; Vlodavsky et al., 1999a). Secretion of heparanase was found in activated platelets, granulocytes and lymphocytes and in highly metastatic tumor cells (Parish et al., 2001; Vlodavsky et al., 1992; Vlodavsky et al., 1994). Accumulation of

extracellular heparanase was detected in several tissues including human placenta and in organs undergoing graft rejection and tissue repair (Dempsey et al., 2000b) (I.V., unpublished).

Transfection experiments indicated that the major heparanase form produced by insect and mammalian cells is the non-processed, inactive 65 kDa precursor (Vlodavsky et al., 1999a). In fact, attempts to express the truncated 50 kDa (Lys^{158}-Ile^{543}) protein failed to yield an active enzyme, suggesting that the region starting at the N-terminus to Lys^{158} plays a role in mediating expression and/or function of heparanase (Fairbanks et al., 1999; Hulett et al., 1999; Kussie et al., 1999; Toyoshima et al., 1999; Vlodavsky et al., 1999a). In the present study we investigated the interactions of the extracellular inactive 65 kDa heparanase with primary human fibroblasts devoid of endogenous heparanase. We found that human fibroblasts readily convert the precursor form of heparanase into its highly active proteolytically processed form. During this process, heparanase binds to the cells, and following cleavage the processed form of the enzyme is endocytosed. Heparanase endocytosis is inhibited in the presence of excess heparin and requires integrity of the actin cytoskeleton. Endocytosis of the 50 kDa, processed form is significantly enhanced compared with the non-processed proenzyme. Following endocytosis, the truncated active enzyme is stored within the cytoplasm for a prolonged period of time (at least 16 hours). This pattern of events provides a novel regulatory mechanism by which extracellular heparanase, an important source of heparanase activity, is activated and later on may enhance the invasive behavior of malignant cells.

Materials and Methods

Cells, antibodies and recombinant heparanase

Human foreskin fibroblasts (HFF) were kindly provided by Susan S. Yamada (NIDCR, NIH, Bethesda, MD) (Katz et al., 1999). HFF were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 mM glutamine, 50 μg/ml streptomycin, 50 U/ml penicillin and 10% heat-inactivated bovine serum (Biological Industries, Beit-Ha'Emek, Israel) at 37°C in a 5% CO_2 humidified incubator. Monoclonal mouse anti-human heparanase antibody (mAb 130, which was directed against the C-terminal region of the enzyme) has been previously described (Vlodavsky et al., 1999a). Polyclonal goat anti-EEA1, Lamp-1 and caveolin-1 were from Santa Cruz (Santa Cruz, CA). Cy3 or Alexa-conjugated goat anti-mouse antibodies were from Jackson Laboratories (Bar-Harbor, MA). Rhodamine-conjugated rabbit anti-mouse and FITC-conjugated hamster anti-goat polyclonal antibodies were from Zymed (San Francisco, CA). Recombinant 65 kDa and 50 kDa human heparanases were provided by InSight Ltd (Rabin Science Park, Rehovot, Israel).

Heparanase activity

Cell-associated heparanase activity was determined as previously described (Vlodavsky et al., 1992; Vlodavsky et al., 1994; Vlodavsky et al., 1999a). Briefly, metabolically sulfate ($Na_2[^{35}S]O_4$)-labeled ECM was prepared as described previously (Vlodavsky, 1999b) and incubated (4 hours, 37°C) with the cells at pH 6.6. In order to evaluate the occurrence of heparan sulfate degradation products, the incubation medium was collected and applied for gel filtration on Sepharose 6B columns. Degradation fragments of HS side chains were eluted at $0.5<k_{av}<0.8$, peak II. Nearly intact HSPGs were eluted next to the V_0, ($k_{av}<0.2$, peak I) (Vlodavsky et al., 1992; Vlodavsky et al., 1994; Vlodavsky et al., 1999a). Each experiment was performed at least three times, and the variation of elution position (k_{av} values) did not exceed ±15%.

Indirect immunofluorescence

Cultured primary human foreskin fibroblasts were stained by indirect immunofluorescence, as previously described (Katz et al., 1999). Cells were fixed with either acetone (–20°C, 5 minutes) or 4% formaldehyde in PBS for 20 minutes and permeabilized with 0.5% Triton X-100 in PBS for 5 minutes. The cells were then incubated with the indicated first (45 minutes) and second (45 minutes) antibodies in PBS. Anti-heparanase antibodies were used at 10 μg/ml followed by staining with secondary Alexa- or Cy3-conjugated goat anti-mouse antiserum diluted 1:200.

Labeling of intracellular organelles

HFF were incubated with 50 nM LysoTracker™ DNA– 99, Texas-red-conjugated dextran or TRITC-conjugated ConA (Molecular Probes, Eugene, OR) in growth medium containing recombinant heparanase for 2 hours at 37°C. The cells were then fixed and permeabilized with acetone (–20°C, 5 minutes), followed by immunofluorescent staining for heparanase, as described above.

Digital fluorescence imaging analysis of heparanase subcellular distribution

Quantitative fluorescence microscopy was performed using the DeltaVision system (Applied Precision Inc., Issaqua, WA) attached to an inverted Zeiss Axiovert microscope using a 100×/1.4 Plan-APOCHROMAT objective (Zeiss, Oberkochen, Germany). The image processing and analysis methods used here were described in details previously (Levkowitz et al., 1998; Zamir et al., 2000). Briefly, these methods include: (a) image filtration; original images of fluorescently labeled cells were subjected to high-pass filtration, which subtracts the local average intensity surrounding each vesicle, using the Priism software as described elsewhere (Levkowitz et al., 1998; Zamir et al., 2000); (b) segmentation and quantitation; vesicles in fluorescently labeled cells were segmented using the 'water' software (Levkowitz et al., 1998; Zamir et al., 2000) in order to measure the area and the average fluorescence intensity of each individual vesicle. The parameters of the water software were adjusted to the dimensions of the vesicles as described (Levkowitz et al., 1998; Zamir et al., 2000); (c) 'Spectral' presentation of fluorescence intensities: in order to compare fluorescence intensities visually, filtered images were presented using a blue-to-red linear spectrum scale; (d) fluorescence ratio imaging; to analyze the relationships between heparanase-labeled structures and LysoTracker- or TRIT-ConA-labeled vesicles, ratio images (heparanase:LysoTracker or heparanase:TRIT-ConA) were calculated as described previously (Levkowitz et al., 1998; Zamir et al., 2000) and presented in a spectral, log scale color look-up table that ranged from blue for low heparanase:fluorescent label ratios (≤0.1) to red for high heparanase:fluorescent label ratios (≥10). To utilize this two-order of magnitude range optimally and to compensate for the differences in photon yields of different fluorescent labels, all the ratios were normalized linearly by a constant that shifted their average ratio toward a value around 1.

Results

Human foreskin fibroblasts (HFF) were incubated (37°C) with the 65 kDa heparanase precursor for 30, 60 or 120 minutes and then extensively washed. At each time point, heparanase activity associated with the cells was examined by measuring the release of low molecular weight labeled

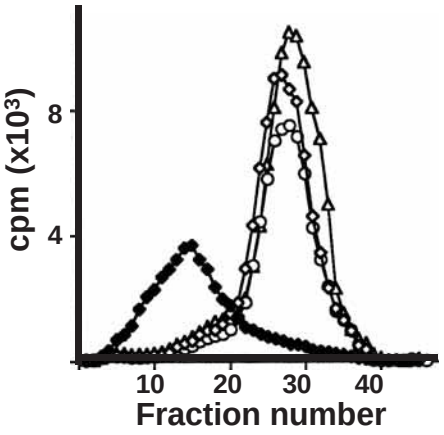

Fig. 1. Acquisition of heparanase activity by primary human fibroblasts. Human foreskin fibroblasts were incubated for two hours in the absence (◆) or the presence of 10 μg/ml recombinant 65 kDa heparanase for 30 (◇), 60 (○) and 120 (△) minutes. Cells (2.5×10^6 cells/ml) were then washed and incubated (4 hours, 37°C, pH 6.6) with sulfate-labeled ECM. Labeled degradation fragments released into the incubation medium were analyzed by gel filtration over Sepharose 6B as described in the Materials and Methods.

degradation products from metabolically labeled ($Na_2{}^{35}SO_4$) ECM (Vlodavsky et al., 1992; Vlodavsky et al., 1994; Vlodavsky et al., 1999a). As shown in Fig. 1, HFF do not express any endogenous heparanase activity. However, a marked heparanase enzymatic activity was determined in HFF lysates prepared after 30 minutes or more of incubation of the cells with the recombinant 65 kDa heparanase precursor (Fig. 1).

Although untreated HFF do not express the heparanase protein (Fig. 2A), western blot analysis revealed that the 65 kDa heparanase precursor became associated with the cells and was already partially converted to a 50 kDa form after 30 minutes (Fig. 2A). Analysis of longer incubation time points indicated that although the amount of heparanase precursor associated with the cells remained constant, the amount of 50 kDa processed form increased significantly (Fig. 2A).

To investigate whether the enzyme is associated with the cells, and/or undergoes internalization, the subcellular localization of heparanase was determined by immunofluorescent staining. For this purpose, HFF incubated for 30, 60 and 120 minutes with the heparanase precursor were fixed, permeabilized and stained with anti-heparanase mAb 130. Intracellular accumulation of heparanase was clearly observed in a granular pattern after 60 minutes of incubation and was significantly increased after 120 minutes (Fig. 2B-D). In contrast, non-permeabilized cells exhibited only low levels of diffuse heparanase staining at the different time points (data not shown), confirming internalization of cell-associated heparanase and accumulation in the cytoplasm. Interestingly, only weak immunofluorescent staining of the cells was observed at the same time points (30-120 minutes) when a mAb directed against the N-terminus of heparanase, which is present only in the precursor protein, was utilized (not shown). These findings indicate that the heparanase precursor is proteolytically cleaved, followed by internalization and cytoplasmic accumulation of the processed enzyme. Heparanase-containing cytoplasmic granules were observed even after 16 hours following internalization, suggesting a prolonged cytoplasmic storage (not shown).

The subcellular heparanase-containing granules were characterized using either LysoTracker (an acidic granules marker) or TRITC-conjugated ConA that marks endosomes

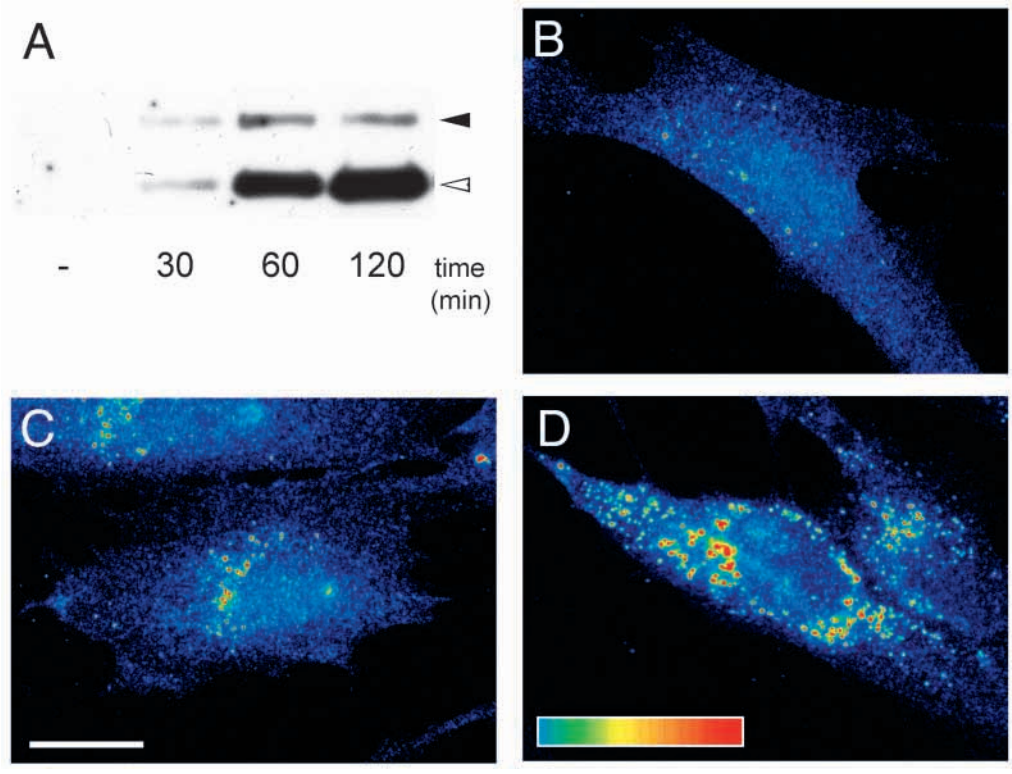

Fig. 2. Uptake and processing of extracellular heparanase pro-enzyme. (A) Western blot analysis of cell-associated heparanase. Human foreskin fibroblasts were incubated with 10 μg/ml recombinant 65 kDa heparanase for 30, 60 and 120 minutes. At each time point, the cells were lysed and subjected to western blot analysis using anti-heparanase mAb 130. (B-D) Subcellular localization of cell-associated heparanase. Human foreskin fibroblasts were incubated with 10 μg/ml recombinant 65 kDa heparanase for 30 (B), 60 (C) and 120 (D) minutes. The cells were fixed, permeabilized and subjected to immunofluorescent staining with anti-heparanase mAb 130. Note the accumulation of heparanase in granules at 60 (C) and 120 (D) minutes. Bar, 20 μm.

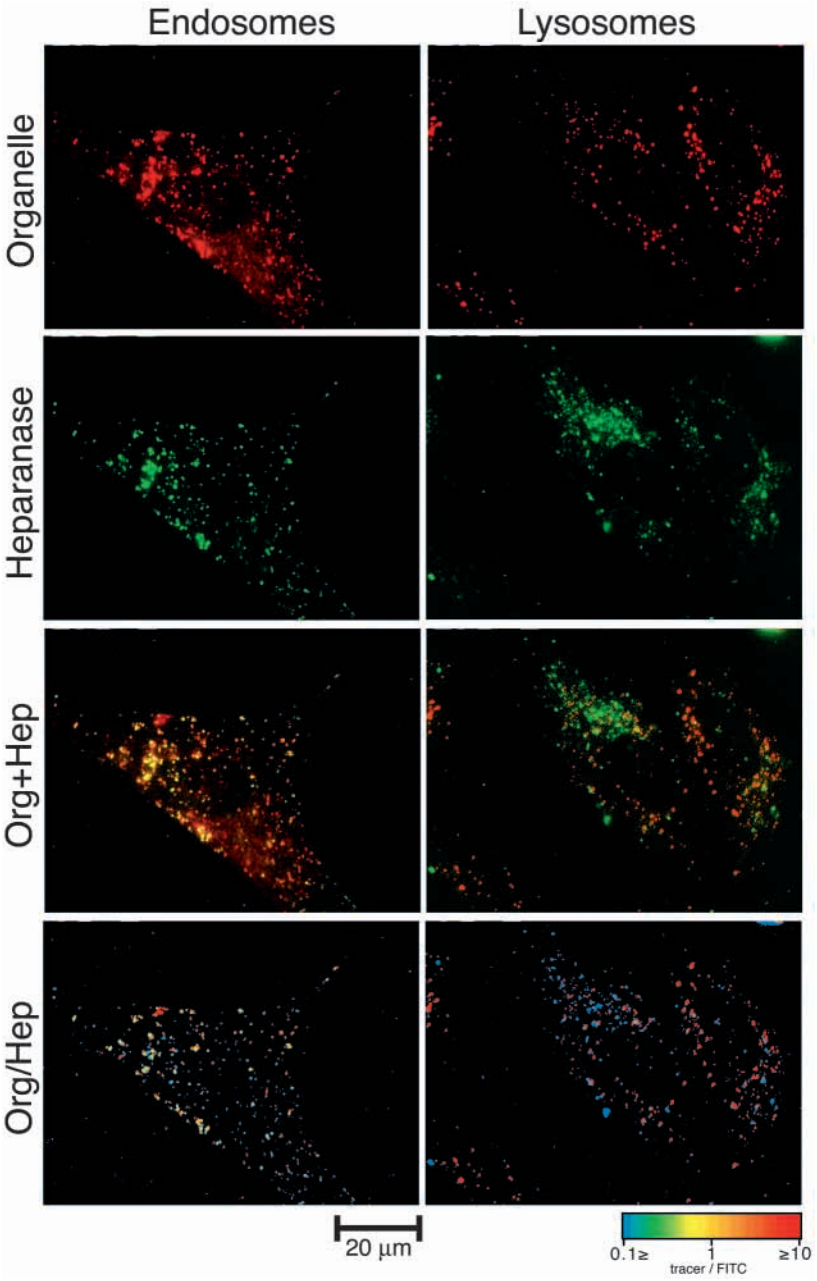

Fig. 3. Endosomal localization of heparanase. Human foreskin fibroblasts were incubated with 10 μg/ml recombinant 65 kDa heparanase, and labeled for endosomes (TRITC-labeled ConA) and lysosomes (LysoTracker) for 120 minutes. Following fixation and permeabilization, the cells were stained by immunofluorescence with anti-heparanase mAb 130. Note the colocalization of the endosomal staining (red) and the heparanase-containing granules (green). The overlap is represented in yellow when the images are superimposed (third panel from top) and in fluorescent ratio image analysis (bottom panel).

(Barzu et al., 1996). These dyes were applied to living HFF, which were simultaneously incubated with the 65 kDa heparanase precursor for 120 minutes. Following staining with anti-heparanase mAb 130, we observed colocalization of heparanase-containing granules with the endosomal (Fig. 3, left panels) but not lysosomal (Fig. 3, right panels) labels.

In order to further characterize the heparanase-containing granules, we performed fluorescent labeling of cells incubated for two hours with extracellular heparanase using antibodies directed against either EEA1 (an early endosome resident molecule), dextran (an endosomal marker), Lamp-1 (a protein associated with lysosomes) or cathepsin D (a proteolytic enzyme located in lysosomes) (Nagamatsu et al., 2001; Thomsen et al., 2000; Reddy et al., 2001; Storrie and Desjardins, 1996). As shown in Fig. 4, the heparanase-containing granules were negative for EEA1 staining. In a similar manner, heparanase-containing granules did not contain Lamp-1 or cathepsin D, both molecules found in lysosomes (Table 1). In contrast, the endosomal marker dextran accumulated in the heparanase-containing granules (Table 1), again indicating that the enzyme is stored primarily in the endosomal compartment.

The most common endocytosis process is relatively rapid, depends on the integrity of the actin cytoskeleton and results in the formation of either clathrin-coated or non-coated membrane pits (Corvera et al., 1999). Another major endocytosis pathway is mediated through caveolae, specialized membrane invaginations previously shown to be involved in HSPGs-mediated uptake (Williams and Fuki, 1997). To test the possibility that heparanase is endocytosed via caveolae we have double-labeled cells for heparanase using mAb 130 and for caveolae using anti-caveolin-1 polyclonal antibodies, and we found no colocalization (Table 1). Incubation with cytochalasin D, which destroys the actin cytoskeleton, completely inhibited heparanase endocytosis (Fig. 5). In contrast, nocodazole, which inhibits microtubules integrity, had no effect on heparanase endocytosis (Fig. 5).

As described above, heparanase-containing endosomes were observed relatively late, only after 60 minutes of incubation of the cells with the heparanase precursor enzyme. Since heparanase processing apparently involves at least two distinct events – proteolytic activation and endocytosis –

Table 1. Characterization of heparanase-containing granules

Molecule	Organelle	Colocalization with heparanase
Caveolin	Caveolae	–
EEA1	Early endosomes	–
ConA	Endosomes	+
Dextran	Endosomes	+
LysoTracker	Acidic vesicles	–
Cathepsin D	Lysosomes	–
Lamp-1	Lysosomes	–

Human foreskin fibroblasts were incubated with 10 μg/ml recombinant 65 kDa heparanase for 120 minutes. The cells were then double labeled for heparanase and each of the indicated markers. The organelle specificity of each marker and its colocalization with heparanase (or the lack of colocalization) are indicated. The endosomal labels ConA and dextran accumulated in heparanase-containing granules.

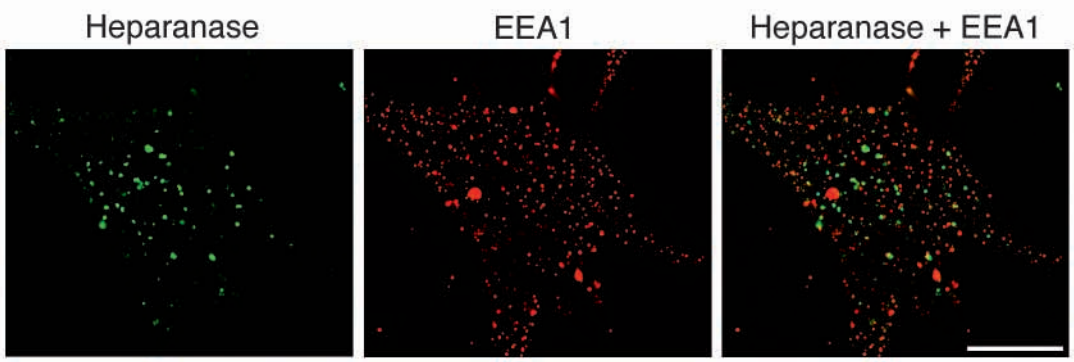

Fig. 4. Heparanase-containing granules do not contain the EEA1 early endosomal protein. Human foreskin fibroblasts were incubated with 10 μg/ml 65 kDa heparanase for 120 minutes. The cells were then fixed, permeabilized and subjected to double immunofluorescent staining of heparanase (green, left image) and EEA1 (red, middle image). The superimposed image (right image) shows no colocalization of heparanase and EEA1. Bar, 20 μm.

we examined whether cleavage of the heparanase N-terminus affects the cytoplasmic accumulation rate of the enzyme. For this purpose, HFF were incubated with the 50 kDa truncated heparanase form for 30, 60 and 120 minutes, followed by staining with anti-heparanase mAb 130, as described above. Accumulation of heparanase-containing granules was already observed after 30 minutes, indicating that uptake of the processed enzyme is significantly more efficient then that of the non-processed precursor (Fig. 6). Unlike the latent enzyme,

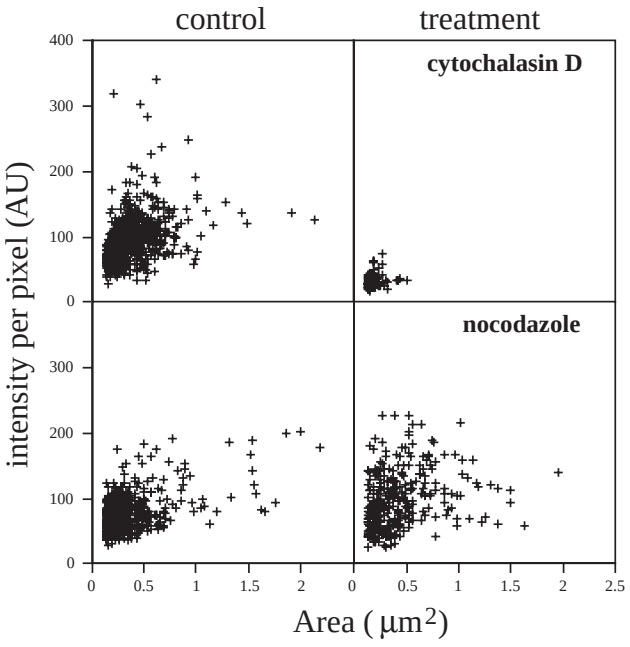

Fig. 5. Involvement of the actin cytoskeleton, but not microtubules, in heparanase endocytosis. Human foreskin fibroblasts were incubated with 10 μg/ml 65 kDa heparanase for 120 minutes in the absence (control) or presence of cytochalasin D or nocodazole. After fixation and permeabilization, immunofluorescent staining was performed with anti-heparanase mAb 130, followed by Cy3-conjugated goat anti-mouse antibody. Heparanase-containing granules were detected and quantified as described in the Materials and Methods. There were no granules containing heparanase in the cytochalasin D treated cells, whereas nocodazole had no apparent effect on heparanase endocytosis.

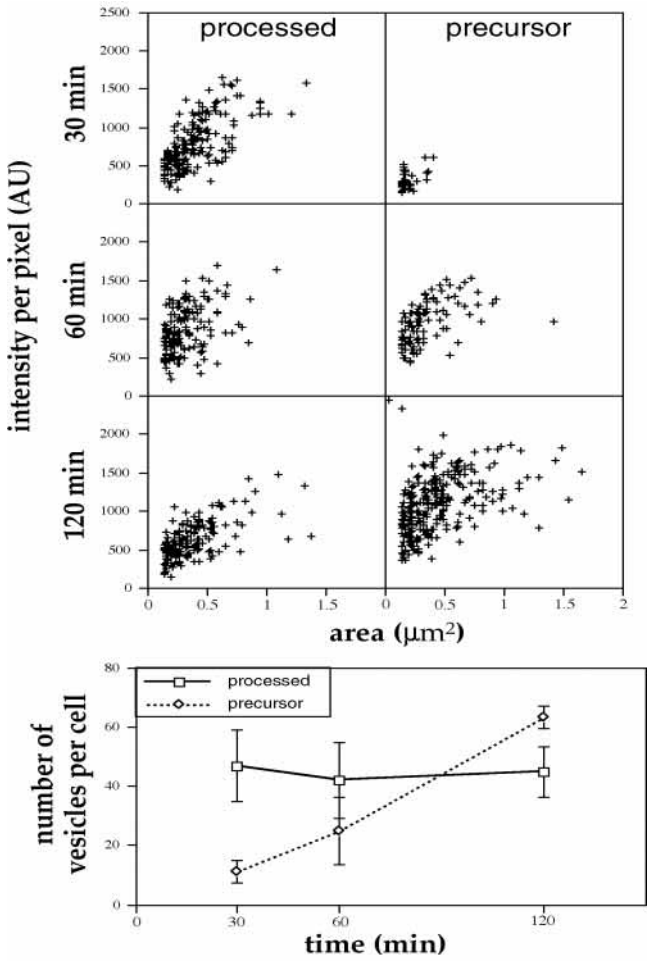

Fig. 6. Cellular endocytosis of truncated versus latent heparanase. Human foreskin fibroblasts were incubated with 10 μg/ml 65 kDa heparanase (right panels) or with 10 μg/ml processed 50 kDa heparanase (left panels) for 30, 60 and 120 minutes. After fixation and permeabilization, immunofluorescent staining was performed with anti-heparanase mAb 130. Heparanase-containing granules were detected and quantified as described in the Materials and Methods. Uptake of the processed enzyme was significantly faster. The intensity of the immunoflourescent staining, size and the number of processed heparanase-containing granules reached saturation after 30 minutes.

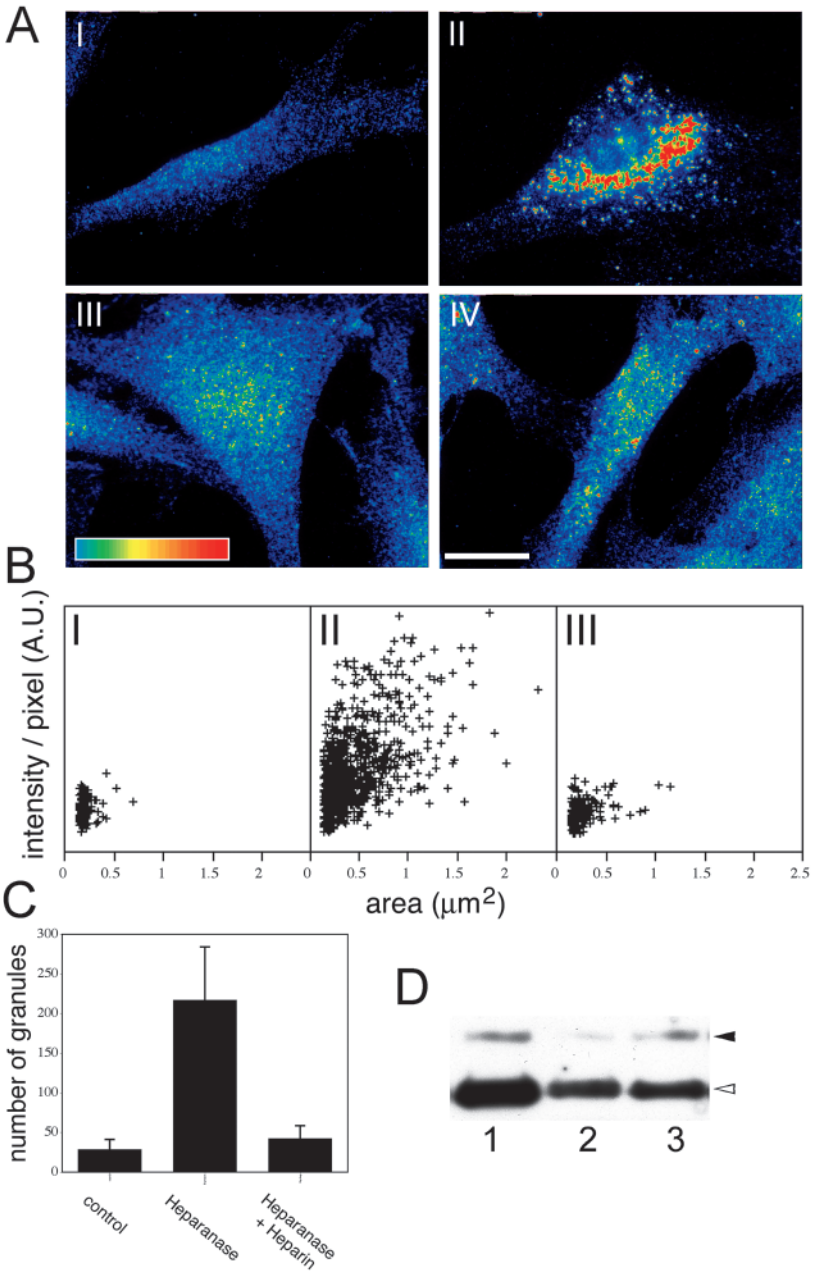

Fig. 7. Inhibitory effect of heparin on heparanase endocytosis. (A) Human foreskin fibroblasts were incubated with 10 μg/ml 65 kDa heparanase for 120 minutes without (II) or with 30 minutes pre-incubation with 0.1 μg/ml (III) or 10 μg/ml (IV) heparin. Untreated cells (I) were used as control. After fixation and permeabilization, immunofluorescence was performed with anti-heparanase mAb 130. Cells that were incubated with heparanase and heparin showed no heparanase-containing granules. (B) Intensity (in arbitrary units) and area (in μm^2) of heparanase-containing granules and (C) number of granules per cell were significantly lower in cells incubated with 1 μg/ml heparin (BIII and C) compared with control cells incubated with heparanase alone (BII and C) and with untreated cells (BI and C). (D) Western blot analysis of extracellular 65 kDa heparanase binding and processing in cells incubated for 120 minutes at 37°C with 10 μg/ml heparanase alone (lane 1) or with 10 μg/ml heparanase plus 1 μg/ml heparin (lane 2) or with 10 μg/ml heparanase at 4°C (lane 3). Note that heparanase binding was only partially inhibited by heparin, and its processing was not affected by heparin. Filled arrowhead, 65 kDa heparanase precursor; empty arrowhead, 50 kDa processed heparanase.

endocytosis of processed heparanase reaches saturation after 30 minutes, with respect to the number and size of endosomes, as well as their heparanase content (Fig. 6). Taken together, the data indicate that proteolytic activation of the enzyme on the cell surface is a rate-limiting step in heparanase endocytosis. Also, the experiment demonstrates that uptake of the enzyme does not involve the N-terminal portion of the protein but rather recognizes the C-terminal region, containing both the heparin-binding domain and the active site of heparanase (Hulett et al., 2000).

Previous studies have shown that at a neutral pH heparanase can bind but not degrade HSPGs (Gilat et al., 1995). Thus, cell-surface HSPGs might function as receptors that mediate the uptake of extracellular heparanase. In fact, cell-surface HSPGs were shown to facilitate the binding and endocytosis of a variety of extracellular ligands, including growth factors (bFGF), lipoproteins (LDL) and various pathogens (Colin et al., 1999; David et al., 1993; Datta et al., 2000; Fuki et al., 2000; Ji et al., 1998; Sperinde and Nugent, 2000; Shukla et al., 1999; Summerford et al., 1999). We therefore examined whether cell association with heparanase and its subsequent endocytosis depend on cell surface HSPGs. For this purpose, the 65 kDa heparanase precursor was pre-incubated for 30 minutes at 37°C with various concentrations of heparin (0.5-200 μg/ml). The pretreated enzyme was then incubated with the cells for 120 minutes in the continuous presence of heparin, followed by immunofluorescent staining of heparanase. As shown in Fig. 7A-C, heparanase endocytosis was completely blocked in the presence of 10 μg/ml heparin. Similarly, pre-treatment of the cells with bacterial heparinase III inhibited heparanase endocytosis (not shown). Previous studies indicated that mannose-6-phosphate may mediate heparanase association with cells (Bartlett et al., 1995). We did not observe any inhibition of heparanase endocytosis in the presence of mannose-6-phosphate (not shown).

Heparanase endocytosis is accompanied by its processing (Fig. 2). We examined whether association of the 65 kDa heparanase with the cells and/or its processing are inhibited by heparin. As shown in Fig. 7D (lane 2), a significant reduction (~ 50% as evaluated by densitometry) in the amount of heparanase associated with the cells was observed when the cells were incubated for 120 minutes with the enzyme in the presence of 1 μg/ml heparin, compared with control cells that were incubated with the enzyme in the absence of heparin (Fig. 7D, lane 1). However, a significant amount of heparanase was still associated with the cells following heparin treatment (Fig. 7D, lane 2). A similar partial inhibition of binding was observed when the cells

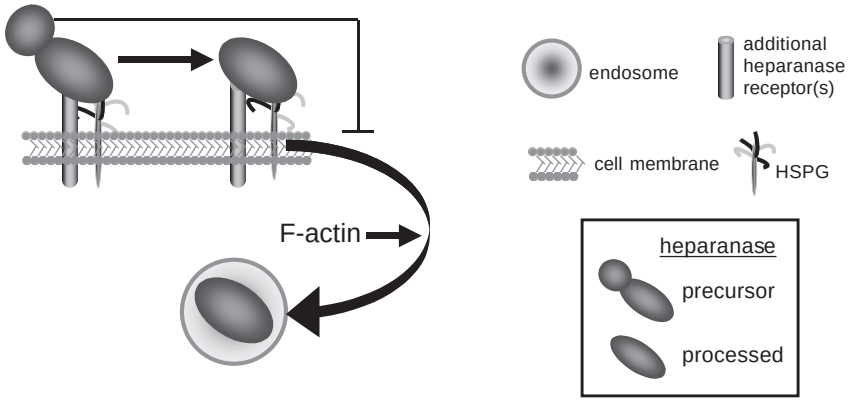

Fig. 8. Uptake, activation and trafficking of extracellular heparanase precursor. Heparanase precursor binds to the plasma membrane via HSPGs and additional putative receptor(s). The 65 kDa enzyme is processed on (or adjacent to) the cell surface, independently of HSPGs. Uptake occurs within 60 minutes, with accumulation of the processed 50 kDa form in endosomes. Heparanase endocytosis depends on the actin cytoskeleton and HSPGs, and may be inhibited by the N-terminal pro-peptide. Apparently, heparanase endocytosis and processing are two independent events. Following endocytosis, heparanase-containing granules are stored within the cell for a prolonged period. These events may control the activation, clearance and storage of extracellular heparanase.

were incubated with heparanase at 4°C (Fig. 7D, lane 3) rather then 37°C. Both heparin (Fig. 7A-C) and incubation at 4°C (not shown) almost totally inhibited endocytosis but did not affect the cleavage of the latent heparanase into an active form. The ratio of cell-associated heparanase precursor to the processed form was similar in control cells, cells incubated with heparin or cells kept at 4°C (Fig. 7D). In a similar manner, cytochalasin D, which totally inhibited heparanase endocytosis (Fig. 5), had no effect on its extracellular processing (not shown).

Discussion

Previous studies have shown that heparanase is released by activated platelets, neutrophils and lymphocytes (Dempsey et al., 2000a; Vlodavsky et al., 1992; Vlodavsky et al., 1993; Wight 1992). Secretion of the non-processed 65 kDa heparanase precursor was also observed in insect cells infected with baculovirus containing the heparanase cDNA and by some clones of heparanase-transfected CHO cells (Vlodavsky et al., 1999a). Activation of human heparanase requires a post-translational modification, namely proteolytic cleavage at the N-terminus (Fairbanks et al., 1999; Vlodavsky et al., 1999a).

We have demonstrated that extracellular heparanase is both activated and endocytosed by primary human fibroblasts. The amount of heparanase precursor associated with the cells reaches saturation within 30 minutes and remains steady, indicating a relatively limited precursor-binding capacity. On the other hand, the cellular content of the processed enzyme significantly increases during 120 minutes of incubation, apparently because of cytoplasmic accumulation of heparanase in endosomes.

Heparanase endocytosis seems to share some of the diverse properties of HSPG-mediated endocytosis pathways (Bernfield et al., 1999; David et al., 1993). Heparanase internalization occurs through an endocytosis pathway, involving the actin cytoskeleton and is not mediated by caveolae. This internalization mechanism differs from that previously suggested for HSPG-mediated uptake of bFGF or LDL via caveolae (Williams et al., 1997) or the perlecan HSPG-mediated endocytosis that is not inhibited by cytochalasin D (Fuki et al., 2000). Moreover, endocytosed heparanase did not translocate into the lysosomal compartment, even after an extended incubation period. This finding is in agreement with a previous study indicating a relative late entry of bFGF into lysosomes during HSPG-mediated endocytosis (Gleizes et al., 1995). Also, heparanase endocytosis was not blocked by mannose-6-phosphate, which may facilitate lysosomal targeting. These results indicate that any heparanase activity exerted by the cells during the 2 hour incubation period originates either from the cell surface or endosomes.

While pre-incubation of heparanase with heparin completely abolished endocytosis, binding of heparanase to the cells was only partially inhibited, suggesting an involvement of non-heparan-sulfate binding sites to heparanase on the cell surface. Conversion of heparanase from the latent precursor form to an active enzyme was not inhibited by heparin. Since heparanase proteolytic processing took place even when its endocytosis was completely blocked, we conclude that proteolytic activation of the enzyme is a cell surface or juxta-surface event. Heparanase processing may be essential, but not sufficient to induce its endocytosis, which apparently depends on a proper interaction with cell-surface HSPGs (Fig. 8). Previous studies showed that cell-surface HSPGs serve as co-receptors, partially responsible for surface binding of various heparin-binding extracellular molecules (Colin et al., 1999; Datta et al., 2000; Fuki et al., 2000; Ji et al., 1998; Sperinde and Nugent, 2000). Moreover, HSPGs may also be critical for the internalization of these molecules into the cells. For example, HSPGs mediate the uptake and degradation of LDL (Fuki et al., 2000) and bFGF by fibroblasts. Also, HSPGs serve as a primary attachment receptor for herpes simplex type 1 virus and adeno-associated virus type 2 (Shukla et al., 1999; Summerford et al., 1999).

Endocytosis of the processed enzyme was found to be significantly more efficient then that of its precursor protein, already reaching saturation after 30 minutes. This may stem from the fact that the precursor enzyme is proteolytically cleaved outside the cell, which may be the rate-limiting step. Another possible explanation is that binding of the processed enzyme is more efficient in terms of affinity and/or capacity, in other words, more enzyme can bind to HSPGs or other putative receptor(s). Once endocytosed, heparanase can accumulate within the cytoplasm for a prolonged period of time (at least 16 hours).

Our results indicate that the presence of active heparanase

in cells is not necessarily regulated at the gene expression level. Thus, cells that lack endogenous heparanase can acquire heparanase activity by surface binding, activation and internalization of exogenous heparanase. Previous studies have shown that heparanase is excreted by activated platelets and cells of the immune system (Parish et al., 2001; Vlodavsky et al., 1992) and by highly metastatic tumor cells (Dempsey et al., 2000a; Irimura et al., 1986; Parish et al., 2001; Vlodavsky et al., 1990; Vlodavsky et al., 1994; Vlodavsky and Friedman, 2001). Interestingly, cell surface binding, activation and endocytosis of exogenous heparanase appears to alter the metastatic potential of malignant cells from a low metastatic into a highly metastatic phenotype (O.Y.-Z., unpublished). Thus, cancer cells may acquire a highly invasive phenotype by endocytosis and storage of heparanase secreted from stromal cells (Marchetti et al., 2000).

Heparanase endocytosis by normal cells may regulate the course of physiological processes such as wound healing and angiogenesis. During the initial stages of tissue remodeling and repair, the damaged tissue is populated by platelets, neutrophils and macrophages, which release lysosomal enzymes. In these inflammatory, slightly acidic conditions, the extracellular latent heparanase is readily converted to the active form. We show here that heparanase may also be activated on the surface of primary human cells and not necessarily under inflammatory conditions. The active enzyme degrades heparan sulfate and thereby may contribute to the inflammatory process by enabling neutrophils and lymphocytes to migrate from the vasculature into the target tissue (Parish et al., 2001; Vlodavsky et al., 1992). However, at later stages, migration of fibroblasts and endothelial cells occurs, followed by deposition of ECM macromolecules (Eckes et al., 2000). Heparanase activity may therefore constrain this later response (e.g. ECM deposition and assembly) via degradation of the ECM scaffold. Thus, an efficient uptake of the active form of heparanase (and potentially other ECM-degrading enzymes) may accelerate the late stages of wound healing by enabling tissue remodeling and reorganization to occur. In agreement with this concept, we found that endocytosis of processed, highly active heparanase by primary human skin fibroblasts is significantly more efficient than that of the non-processed precursor.

Unlike matrix metalloproteases involved in tumor cell metastasis and angiogenesis, heparanase is a well conserved protein that exists as a single functional endoglycosidase, utilized by normal and malignant cells to degrade HSPGs (Dempsey et al., 2000a; Fairbanks et al., 1999; Hulett et al., 1999; Kussie et al., 1999; Parish et al., 2001; Toyoshima et al., 1999; Vlodavsky et al., 1999a; Vlodavsky and Friedman, 2001). Thus, heparanase represents an attractive target for the development of anti-tumor and anti-inflammatory drugs. Understanding the intracellular localization of heparanase and its endocytotic mechanisms under normal and pathological conditions may thus provide novel therapeutic approaches. In fact, heparanase inhibitors (e.g. non-anticoagulant species of heparin, suramine, castanospermine and phosphomannopenpaose sulfate) were previously shown to inhibit autoimmune disorders (i.e. EAE and adjuvant arthritis) and cancer metastasis in animal models (Bartlett et al., 1995; Parish et al., 1999; Parish et al., 2001; Vlodavsky et al., 1992; Vlodavsky et al., 1994; Vlodavsky and Friedman, 2001). Our study suggests that modulators of heparanase uptake and/or its proteolytic activation should be looked for as such compounds may be utilized as regulators of tissue repair, vascularization and immune surveillance, as well as for pathological conditions such as autoimmunity and metastasis.

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