Heparanase Uptake Is Mediated by Cell Membrane Heparan Sulfate Proteoglycans

Heparanase is a mammalian endoglycosidase that degrades heparan sulfate (HS) at specific intrachain sites, an activity that is strongly implicated in cell dissemination associated with metastasis and inflammation. In addition to its structural role in extracellular matrix assembly and integrity, HS sequesters a multitude of polypeptides that reside in the extracellular matrix as a reservoir. A variety of growth factors, cytokines, chemokines, and enzymes can be released by heparanase activity and profoundly affect cell and tissue function. Thus, heparanase bioavailability, accessibility, and activity should be kept tightly regulated. We provide evidence that HS is not only a substrate for, but also a regulator of, heparanase. Addition of heparin or xylosides to cell cultures resulted in a pronounced accumulation of heparanase in the culture medium, whereas sodium chlorate had no such effect. Moreover, cellular uptake of heparanase was markedly reduced in HS-deficient CHO-745 mutant cells, heparan sulfate proteoglycan-deficient HT-29 colon cancer cells, and heparinase-treated cells. We also studied the heparanase biosynthetic route and found that the half-life of the active enzyme is ~30 h. This and previous localization studies suggest that heparanase resides in the endosomal/lysosomal compartment for a relatively long period of time and is likely to play a role in the normal turnover of HS. Co-localization studies and cell fractionation following heparanase addition have identified syndecan family members as candidate molecules responsible for heparanase uptake, providing an efficient mechanism that limits extracellular accumulation and function of heparanase.

Heparanase is a mammalian endo-β-D-glucuronidase that cleaves heparan sulfate (HS)1 side chains at a limited number of sites (1–3). Such enzymatic activity is thought to participate in degradation and remodeling of the extracellular matrix and to facilitate cell invasion associated with cancer metastasis and inflammation (1, 4–6). Under normal conditions, heparanase activity is restricted to the placenta and skin tissues and to blood-borne cells such as platelets, neutrophils, monocytes, mast cells, and T lymphocytes (1, 4, 5, 7–12). In these cells, heparanase is thought to be stored in specific granules, and its release by degranulation has been implicated in diapedesis and extravasation of a number of immune cells (4, 5, 11–13). Heparanase up-regulation has been documented in a variety of human tumors correlating, in some cases, with increased vascular density and poor postoperative survival (14–17). Heparanase overexpression has also been noted in several other pathologies such as cirrhosis (18), nephrosis (19), and diabetes (20). In addition to its intimate involvement in the egress of cells from the blood stream, heparanase activity may release a multitude of HS-bound, extracellular matrix-resident growth factors, cytokines, chemokines, and enzymes that might profoundly affect cell and tissue function (1, 21). Thus, heparanase activity and bioavailability should be kept tightly regulated. Mechanisms that dictate heparanase regulation are only poorly understood, but are expected to operate at several distinct levels. Induced heparanase expression under pathological conditions suggests a transcriptional regulation. Heparanase gene expression has been shown to involve promoter methylation (22), eukaryotic initiation factor 4E (23), and the Ets (24) and Egr1 (25) transcription factors. Recently, estrogen has been shown to induce heparanase promoter activation in estrogen receptor-positive breast cancer cells (26). Regulation at the post-translational level, viz. heparanase processing, cellular localization, and secretion, has also been implicated as a major regulatory mechanism (27–30).

We have previously shown that exogenously added heparanase rapidly interacts with primary human fibroblasts (31) as well as with tumor-derived cells (32), followed by internalization and processing into a highly active enzyme, collectively defined as heparanase uptake. The role of heparan sulfate proteoglycans (HSPGs) in heparanase uptake has not been studied in detail. Here, we provide evidence that the addition of heparin to cell cultures results in a pronounced accumulation of heparanase in the culture medium, whereas treatment with sodium chlorate had no such effect. Moreover, cellular uptake of heparanase was markedly reduced in HS-deficient CHO-745 cells, HSPG-deficient HT-29 cells, and heparinase-treated cells. We also studied the heparanase biosynthetic route and estimated the half-life of the active enzyme to be ~30 h. Co-localization and cell fractionation studies following heparanase addition have identified syndecans, rather than glypicans, as candidate HSPGs responsible for heparanase uptake.

Received for publication, February 26, 2004, and in revised form, July 22, 2004
Published, JBC Papers in Press, July 29, 2004, DOI 10.1074/jbc.M402131200

Svetlana Gingis-Velitski‡§, Anna Zetser‡§, Victoria Kaplan‡, Olga Ben-Zaken‡, Esti Cohen‡, Flonia Levy-Adam‡, Yulia Bashenko‡, Moshe Y. Flugelman‡, Israel Vlodavsky‡¶, and Neta Ilan‡

From the ‡Cancer and Vascular Biology Research Center, Bruce Rappaport Faculty of Medicine, Technion, Haifa 31096, Israel and the §Department of Cardiology, Lady Davis Carmel Medical Center, Haifa 34632, Israel

© 2004 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

* This work was supported by Grant 532/02 from the Israel Science Foundation; by United States Public Health Service Grant R01 CA106456-01 from NCI, National Institutes of Health; by the Israel Cancer Research Fund; and by a charitable fund established in memory of Rachel Litvin. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Both authors contributed equally to this work.

¶ To whom correspondence should be addressed: Cancer and Vascular Biology Research Center, Faculty of Medicine, Technion, P. O. Box 9649, Haifa 31096, Israel. Tel.: 972-4-829-5410; Fax: 972-4-852-3947; E-mail: vladavsk@cc.huji.ac.il.

‡ The abbreviations used are: HS, heparan sulfate; HSPGs, heparan sulfate proteoglycans; CHO, Chinese hamster ovary; GPI, glycosylphosphatidylinositol; PBS, phosphate-buffered saline.

44084 This paper is available online at http://www.jbc.org
Heparanase Uptake Is Mediated by HSPGs

**Materials and Methods**

**Antibodies and Reagents—**Antibody 1453 was raised against the entire 65-kDa heparanase precursor isolated from the conditioned medium of heparanase-transfected 293 cells (35). This antibody was affinity-purified on immobilized, bacterially expressed, 50-kDa heparanase-glutathione S-transferase fusion protein (30). Anti-heparanase monoclonal antibody, recognizing the 65-kDa latent heparanase (32), was purchased from BD Biosciences. Anti-Myc epitope tag, anti-glypican-3, and anti-syndecan-4 monoclonal and polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and from Chondroitin ABC was purchased from Seikagaku Kogyo Co. (Tokyo, Japan). Totally N-desulfated, N-acetylated heparin and totally O-desulfated heparin were kindly provided by Drs. Benito Casu and Dr. Annamaria Naggi (“Ototally 2-desulfated heparin was used for all experiments.”)

Heparin, heparanase I, sodium chloride, 4-methylumbelliferyl 7-β-D-xyloside, and anti-actin and anti-pan-cadherin monoclonal antibodies were purchased from Sigma. Chondroitin ABC was purchased from Seikagaku Kogyo Co. (Tokyo, Japan). Totally N-desulfated, N-acetylated heparin and totally O-desulfated heparin were kindly provided by Drs. Benito Casu and Dr. Annamaria Naggi (“Ototally 2-desulfated heparin was used for all experiments.”)

**Immunoprecipitation, equal volumes (0.1 ml) of equal trichloroacetic acid plus the indicated concentrations of heparin or chlorate. For immunoprecipitation, equal volumes (0.1 ml) of equal trichloroacetic acid plus the indicated concentrations of heparin or chlorate.**

**Cell Fractionation—**Isolation of plasma membrane and endosomal/lysosomal cell fractions was carried out essentially as described (37). Lysates of each of the cell lines, rapidly interacts with membranous plasma membranes. The supernatant was centrifuged at 100,000 × g for 10 min to sediment endosomes and lysosomes. Pellets were resuspended in lysis buffer containing 1% Triton X-100, and 0.5% deoxycholate-containing lysis buffer and equal amounts of protein were analyzed by immunoblotting as described above.

**Heparanase Purification and Uptake Studies—**The latent Myc-tagged 65-kDa heparanase precursor was purified from the culture medium of heparanase-transfected HEK-293 cells essentially as described (33). 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 above. Heparanase uptake and processing were analyzed by immunoblotting with anti-heparanase (1453) or anti-Myc tag antibody.

**Immunocytochemistry—**Human U87 glioma cells were left untreated or were incubated with exogenously added 65-kDa heparanase (10 μg/ml) for 15 min and subjected to indirect immunofluorescence staining essentially as described (32, 33). Briefly, cells were fixed with cold methanol for 10 min. Cells were then washed with PBS and incubated in PBS containing 10% normal goat serum for 1 h at room temperature, followed by a 2-h incubation with the indicated primary antibodies. Cells were then washed extensively with PBS, incubated with Cy2/Cy3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) for 1 h, washed, and mounted (Vecastash, Vector Laboratories, Burlingame, CA).

**RESULTS**

**Heparanase, but Not Sodium Chlorate, Enhances Heparanase Accumulation in Cell Culture Medium—**To explore the role of cell membrane HS in heparanase uptake, heparanase-transfected cell lines were incubated with increasing concentrations of heparin, and accumulation of the enzyme in the culture medium was evaluated by immunoblotting (Fig. 1, **A–D, upper panels**) with anti-heparanase (C and D) or anti-Myc (A and B) antibodies. Addition of heparin (1–50 μg/ml) resulted in a marked dose-dependent accumulation of heparanase in the culture medium of 293 (Fig. 1A), MDA-435 (Fig. 1B), C6 (Fig. 1C), and U87 (Fig. 1D) cells. In contrast with 293 cells, heparanase was not detected in the culture medium of untreated MDA-435, C6, and U87 cells, and heparanase at concentrations of 5 μg/ml or higher was required for heparanase accumulation in the culture medium. Heparin had no effect on the levels of the 65-kDa heparanase in the cell lysates (Fig. 1, **A–D, upper panels, Lysate**). These results suggest that heparanase, secreted by each of the cell lines, rapidly interacts with membranous HSPGs, which is presumably followed by uptake and internalization (see below) and is competed by the addition of heparin.

Interaction of a multitude of molecules with HS is determined by the sequence and sulfation level of the sugar moieties (38, 39). Sulfation can be partially inhibited by sodium chloride (40–42). Interestingly, pretreatment with sodium chloride at concentrations up to 50 μM had no effect on heparanase accumulation in the culture medium of all cell lines examined (Fig. 1, **A–D, lower panels**). Heparanase could not be detected in the culture medium of the other cell lines, unlike 293 cells, regardless of chlorate pretreatment. The effect of heparin and chloride on extracellular heparanase accumulation was next eval-
Heparanase accumulates in the culture medium of cells treated with heparin, but not with sodium chlorate. A–D, immunoblot analyses. Heparanase-transfected 293 (A), MDA-435 (B), C6 (C), and U87 (D) cells were left untreated (0) or incubated for 24 h with the indicated concentrations of heparin (µg/ml; upper panels) or sodium chlorate (mM; lower panels). Medium and cell lysate samples were subjected to SDS-PAGE, followed by immunoblotting with anti-Myc (A and B) or anti-heparanase (C and D) monoclonal antibody. E, metabolic labeling. Heparanase-transfected C6 (upper panel) and 293 (lower panel) cells were methionine-starved for 30 min, followed by 20-min pulse with [35S]methionine. Cells were then washed and incubated for 2 h with complete growth medium supplemented with the indicated concentrations of heparin, chlorate (Chl; 50 mM), or heparin (50 µg/ml) plus chlorate (50 mM) (Chl + 50). Medium samples were collected and subjected to immunoprecipitation with anti-heparanase (upper panel) or anti-Myc (lower panel) monoclonal antibody and autoradiography as described under “Materials and Methods.” For chlorate treatment, cells were preincubated with chlorate (50 mM) for 24 h prior to metabolic labeling. Note accumulation of heparanase in the culture medium upon incubation with heparin, but not with sodium chlorate. F, modified heparins. Heparanase-transfected 293 cells were left untreated (control (Con)) or were incubated for 24 h with heparin (Hep), N-desulfated, N-acetylated heparin (N-Ac), or 2-O-desulfated heparin (2-O), all at 10 µg/ml. Medium samples were collected and analyzed for heparanase accumulation by immunoblotting with anti-Myc antibody. G, xyloside. Heparanase-transfected 293 cells were left untreated (0) or were incubated with the indicated concentrations of 4-methylumbelliferyl 7-β-D-xyloside (β-DX). After incubation for 48 h, medium samples were collected and analyzed for heparanase accumulation by immunoblotting with anti-Myc antibody.

Heparanase Uptake Is Mediated by HSPGs

Uptake of heparanase by cells was quantitated by metabolic labeling and immunoprecipitation analysis. C6 (Fig. 1E, upper panel) and 293 (lower panel) cells were pulsed for 20 min with [35S]methionine, followed by a 2-h incubation without or with heparin (1–5 µg/ml), chlorate (50 mM), or both. Medium samples were then collected and subjected to immunoprecipitation with anti-heparanase (Fig. 1E, upper panel) or anti-Myc (lower panel) antibody. Heparanase was readily detected in the medium of untreated 293 cells, and the addition of heparin resulted in further accumulation of the de novo synthesized heparanase. Chlorate treatment had no effect on heparanase accumulation, and the combination of heparin and chlorate was as effective as heparin alone (Fig. 1E, lower panel). Similar qualitative results were obtained with C6 glioma cells (Fig. 1E, upper panel), in agreement with the immunoblotting results (Fig. 1C). Chlorate at a concentration of 50 mM has been shown to inhibit overall O-sulfation by 70%, whereas N-sulfation remains unchanged in Madin-Darby canine kidney cells (40). This suggests that O-sulfation is not involved in HS-heparanase interaction, yet N-sulfation may still be necessary. To test this possibility, heparanase-transfected 293 cells were incubated with chemically modified heparin that was totally N-desulfated, followed by N-acetylation. Modified heparin lacking 2-O-sulfation was also examined for its ability to bind heparanase. Indeed, N-desulfated heparin lost its ability to bind heparanase as revealed by the low levels of heparanase accumulated in the culture medium (Fig. 1F, N-Ac). In contrast, 2-O-desulfated heparin was as efficient as unmodified heparin, further suggesting that N-sulfation is necessary for the interaction of HS with heparanase. Moreover, the addition of 4-methylumbelliferyl 7-β-D-xyloside, a xyloside that substitutes for the linker moiety to the proteoglycan core protein and thus functions as a soluble primer for glycosaminoglycan biosynthesis, resulted in accumulation of heparanase in the culture medium of 293 cells (Fig. 1G), mimicking the effect of heparin.

Reduced Heparanase Uptake by HS-deficient Cells—Next, we examined the uptake of heparanase by HS-deficient cells. To this end, the Myc-tagged 65-kDa heparanase precursor was exogenously added to the culture medium, and uptake was analyzed by immunoblotting. Heparanase binding to wild-type CHO-K1 cells was rapid and appeared maximal by 15 min, followed by a gradual decrease (Fig. 2A, left panels, K1). Interestingly, the anti-Myc tag antibody detected only a single protein band that corresponded in its molecular mass to the added 65-kDa heparanase precursor (Fig. 2A, upper left panel, K1). Heparanase processing into the 50-kDa active enzyme was
clearly detected by the anti-heparanase 1453 antibody (Fig. 2A, middle left panel, K1), suggesting that the heparanase C terminus was subjected to processing that removed the Myc tag sequence. Heparanase processing at the C terminus has not been reported so far. A marked decrease in heparanase binding and uptake was observed at 37 °C (upper left panel) or 4 °C (middle left panel). Cells were then washed, and heparanase uptake was evaluated by subjecting total cell lysates to immunoblotting with anti-Myc antibody (upper panels), anti-heparanase antibody 1453 (Hepa; middle panels), or anti-actin antibody (lower panels). Note the reduced heparanase uptake by heparinase-treated cells, but not chondroitinase-treated cells. CHO-K1 and CHO-745 cells were transfected with the full-length heparanase cDNA cloned into the pSecTag vector. The medium (Med; upper panel) and total cell lysates (Lys.; middle panel) were immunoblotted with anti-Myc (upper panel) or anti-heparanase (middle panel) antibody. Heparanase-transfected CHO-K1 cells were left untreated (0) or were incubated with the indicated concentrations of heparin for 24 h (lower panel). Medium samples were immunoblotted with anti-Myc antibody.

Heparanase Uptake Is Mediated by HSPGs

Fig. 2. Heparanase uptake is HS-dependent. A, heparanase uptake by HS-deficient cells. Wild-type (K1) and mutant HS-deficient (745) CHO cells and HSPG-deficient HT-29 cells (HT29) were left untreated (0) or were incubated with the latent 65-kDa heparanase precursor (1 μg/ml). At the indicated time points, cells were washed, and heparanase uptake was evaluated by subjecting total cell lysates to immunoblot analysis with anti-Myc antibody (upper panels), anti-heparanase antibody 1453 (Hepa; middle panels), or anti-actin antibody (lower panels). Note the pronounced decrease in heparanase uptake by HS-deficient cells. B, heparinase and chondroitinase treatments. CHO-K1 cells were left untreated (Control) or were incubated with bacterial heparinase I (1 unit/ml) or chondroitinase ABC (0.1 unit/ml) for 2 h at 37 °C. Cells were washed and incubated with 1 μg/ml heparanase for the indicated time points at 37 °C (upper left panel) or 4 °C (middle left panel). Cells were then washed, and heparanase uptake was evaluated by subjecting total cell lysates to immunoblotting with anti-Myc antibody (upper panels), anti-heparanase antibody 1453 (Hepa; middle panels), or anti-actin antibody (lower panels). Note the reduced heparanase uptake by heparinase-treated cells, but not chondroitinase-treated cells. C, CHO-K1 and CHO-745 cells were transfected with the full-length heparanase cDNA cloned into the pSecTag vector. The medium (Med; upper panel) and total cell lysates (Lys.; middle panel) were immunoblotted with anti-Myc (upper panel) or anti-heparanase (middle panel) antibody. Heparanase-transfected CHO-K1 cells were left untreated (0) or were incubated with the indicated concentrations of heparin for 24 h (lower panel). Medium samples were immunoblotted with anti-Myc antibody.
heparanase uptake observed with CHO-K1 cells at 37 °C (Fig. 2, A, K1; and B, upper left panel, Control), binding at 4 °C was time-dependent and appeared maximal by 60 min (Fig. 2B, middle left panel, Control). Under these conditions, heparanase binding (4 °C) and uptake (37 °C) were barely detected in the heparanase-treated cells (Fig. 2B, upper and middle left panels, Heparanase I). In contrast, pretreatment with chondroitinase ABC had no effect on heparanase binding and uptake (Fig. 2B, right panels, ABC), supporting the specificity of the HS-heparanase interaction. The pronounced decrease in heparanase uptake by HS-deficient cells (Fig. 2A, left panels, 745; and right panels, HT29) rationalizes that heparanase expression by these cells would result in reduced uptake and increased accumulation of the enzyme in the culture medium. Indeed, large amounts of the 65-kDa heparanase precursor were found in the culture medium of heparanase-transfected CHO-745 cells (Fig. 2C, upper panel, 745). In contrast, heparanase was not detected in the culture medium of wild-type CHO-K1 cells (Fig. 2C, upper panel, K1), unless heparin was supplemented (lower panel). Accumulation of heparanase in the culture medium of transfected CHO-745 cells correlated with reduced amounts of the 50-kDa active enzyme in the cell lysates (Fig. 2C, middle panel). These results imply that the 50-kDa heparanase present in cell lysates originated from the internalized 65-kDa heparanase precursor.

**Heparanase Biosynthetic Route and Half-life**—To further study the ratio between extracellular (65 kDa, latent) and intracellular (50 kDa, active) heparanase forms, we utilized metabolic labeling and immunoprecipitation analysis to follow heparanase biosynthesis. Heparanase-transfected 293 cells, which exhibited the highest enzyme expression and secretion levels (Fig. 1A), were pulsed for 20 min with [35S]methionine and chased for the indicated time periods with complete growth medium supplemented with excess unlabeled methionine. At each time point, the medium (Med) and cell lysate (Lys) samples were collected and subjected to immunoprecipitation with anti-Myc monoclonal antibody (upper panel) or anti-heparanase monoclonal antibody 130 (lower panel), followed by SDS–PAGE and autoradiography. B, densitometry analyses were carried out with the 65-kDa heparanase precursor found in the cell lysate (65 Lys, black diamonds) and medium (65 Med, black triangles) samples as well as the 50-kDa subunit (50 Lys, empty squares) found in the cell lysates. The half-life of the 50-kDa enzyme exceeded 24 h and was calculated to be ~30 h, AU, arbitrary units. C and D, half-life prediction for exogenously added heparanase. C, CHO-K1 cells were left untreated (0) or were incubated with exogenously added heparanase (1 µg/ml) for 4 h. Cells were washed and lysed (4 h) or chased for an additional 24 or 48 h with complete growth medium, and total cell lysates were immunoblotted with anti-heparanase antibody 1453 (Hepa; upper panel) or anti-actin antibody (lower panel). D, densitometry analysis of the 50-kDa heparanase generated upon uptake, internalization, and processing. E and F, inverse correlation between the extracellular 65-kDa and intracellular 50-kDa heparanase forms. E, MDA-MB-435 breast cancer cells were left untreated (0) or were incubated with the indicated concentrations of heparin for 24 h. Medium (Med) and cell lysate (Lys) samples were immunoblotted with anti-Myc antibody (upper panel), anti-heparanase antibody 1453 (middle panel), or anti-actin antibody (lower panel). F, shown are the results from densitometry analysis of the 50-kDa heparanase subunit found in the cell lysates.
agreement with the previous CHO studies presented in Fig. 2A (middle left panel, K1). Densitometry analysis revealed 30 and 80% decreases in the levels of the 50-kDa heparanase protein following 24 and 48 h of chase, respectively (Fig. 3D), and the half-life of the exogenously added heparanase was calculated to be \( \sim 30 \) h. Thus, endogenously expressed heparanase (Fig. 3, A and B) and exogenously added heparanase (Fig. 3, C and D) exhibited similar half-lives, suggesting targeting to the same cellular compartment, most likely late endosomes and lysosomes (32, 43).

If indeed the intracellular active heparanase originates from uptake and internalization of the 65-kDa heparanase precursor, prevention of uptake and accumulation of the 65-kDa enzyme in the culture medium should result in decreased intracellular levels of the 50-kDa heparanase. Addition of heparin to the culture medium of heparanase-transfected MDA-435 cells resulted in a typical accumulation of the 65-kDa heparanase precursor in the cell culture medium (Figs. 1B and Fig. 3E, upper panel), correlating with a proportional decrease in the intracellular levels of the 50-kDa active heparanase (Fig. 3E, middle panel). Densitometry analysis revealed a 60–70% decrease in the intracellular pool of the 50-kDa heparanase in response to heparin treatment (Fig. 3F). This decrease was further confirmed by metabolic labeling analysis (data not shown).

Syndecans as Mediators of Heparanase Uptake—We searched for HSPGs that interact with heparanase and mediate its uptake. Syndecans are transmembrane HSPGs implicated in the uptake of several classes of ligands, including lipoproteins and pathogens (44–47). As expected, syndecan-1 and syndecan-4 staining was largely restricted to the plasma membrane of human U87 glioma cells (Fig. 4A, first panels, red). Interestingly, however, shortly after heparanase addition, syndecan-1 and syndecan-4 were found to be localized mainly in endocytic vesicles (Fig. 4A, second panels), colocalizing to a large extent, with heparanase (fourth panels). This marked change in syndecan localization suggests that syndecan family members and heparanase are subjected to endocytosis and internalization as a complex, resulting in co-localization. Moreover, such rapid internalization would transiently reduce syndecan membrane localization and thus may modify cell behavior. Redistribution of syndecan upon heparanase addition was further examined biochemically by cell fractionation. Since the expression levels of syndecans and glypicans are often altered in tumor-derived cells, we chose primary endothelial cells (human umbilical vein endothelial cells) for the fractionation studies. Heparanase was noted to be highly abundant in the endosomal/lysosomal (E/L) fraction 40 min after its addition (Fig. 4B, upper panel), in agreement with the immunofluorescence staining (Fig. 4A) (31, 32, 43). Moreover, a shift of syndecan-1 from the plasma membrane (M) into the endosomal/lysosomal compartment
upon heparanase addition was observed (Fig. 4B, middle panel), supporting the immunostaining results (Fig. 4A). Interestingly, no significant change in glypic-an-3 distribution was observed upon heparanase addition (Fig. 4B, lower panel), suggesting that syndecans, rather than glypicans, mediate heparanase uptake. The role of glypicans in heparanase regulation was further evaluated by applying GPI-deficient cells that lack glypicans on their cell surface (35). Heparanase uptake by these cells appeared unchanged compared with the control cells (Fig. 4C), supporting the notion that glypicans, unlike syndecans, are not critically important for heparanase uptake.

**DISCUSSION**

Virtually all cells express at least one HS-bound core protein (HSPG) on their surface. From mice to worms, embryos that lack HS die during gastrulation (48), positioning HSPGs as critical regulators of cell-cell signaling during embryogenesis. Such critical function is not restricted to developmental processes. HSPGs are thought to play key roles in numerous biological settings, including cytoskeleton organization, cell migration, wound healing, inflammation, cancer metastasis, and angiogenesis (48–51). HSPGs exert their multiple functions via several distinct mechanisms, combining biochemical, structural, and regulatory aspects. Our results indicate that HS is not only the substrate for, but also a regulator of heparanase. HS is well known for its ability to assemble ligands and receptors into ternary signaling complexes, best exemplified by the fibroblast growth factor-fibroblast growth factor receptor-heparin complex (52). The multitude of polypeptides sequestered and regulated by HS (53) and the ability of heparanase to convert these into bioavailable molecules (1, 21) require that these activities will be kept tightly regulated. Several lines of evidence indicate that HS mediates cellular uptake of heparanase and thus regulates its extracellular retention. Addition of heparin to cell cultures resulted in extracellular accumulation of heparanase (Fig. 2A). This observation suggests that heparanase is being secreted but does not normally accumulate extracellularly unless the cell membrane HS is removed (i.e., by heparanase treatment) (Fig. 2B) or competed with heparin or soluble xyloside primers (Fig. 1). In contrast, chlorate treatment had no such effect on extracellular heparanase accumulation (Fig. 1), suggesting that N- rather than O-sulfate groups mediate the interaction of HS with heparanase, as also indicated by the inability of N-acetylated heparin to promote extracellular accumulation of heparanase (Fig. 1F). Such a sulfation pattern is different from that shown to mediate the uptake of atherogenic lipoproteins, which is significantly inhibited by chlorate treatment (54–56), arguing for sulfation pattern specificity of HS-mediated cellular uptake of ligands. In line with the effect of heparin, heparanase uptake was markedly inhibited in HS-deficient CHO-745 cells and HSPG-deficient HT-29 cells (Fig. 2A). In contrast with the CHO-745 cells, which bear defective xylosyltransferase responsible for assembly of the proteoglycan saccharide linkage region, resulting in cell-surface HS deficiency, HT-29 cells synthesize perlecain, a proteoglycan that is primarily secreted and assembled in the extracellular matrix. However, perlecain can also be found on the cell surface, associated with integrin molecules, and thereby may mediate uptake of specific ligands (42). Heparanase uptake by CHO-745 and HT-29 cells was marginal and appeared similar in terms of kinetics and magnitude (Fig. 2A), indicating that perlecain does not play a significant role in this process. This observation implies that the core protein together with its HS side chains determine the specificity of HSPG interaction with various protein ligands. Analysis of the localization of syndecans and glypicans following heparanase addition further supports this notion. Heparanase was noted to accumulate in perinuclear vesicles already 15 min following its addition to U87 cells (Fig. 4), in agreement with previous reports (31, 32). Interestingly, both syndecan-1 and syndecan-4 were shown to accumulate in endocytic vesicles shortly after heparanase addition, co-localizing with heparanase (Fig. 4A), a redistribution that was further confirmed biochemically (Fig. 4B). This observation suggests that heparanase and syndecans are internalized as a complex and thus co-localize and that both syndecan-1 and syndecan-4 participate in heparanase uptake. Such a rapid and efficient process would ultimately reduce the amounts of syndecans on the cell surface, altering the ability of the cells to respond to extracellular cues (57). In contrast, glypican-3 was not subjected to such redistribution upon heparanase addition (Fig. 4B), and heparanase uptake by GPI-deficient cells that lack glypicans on their surface was as efficient as that by control cells (Fig. 4C). These results suggest that syndecans rather than glypicans are the major mediators of heparanase uptake and regulation. It is important to note that although we have utilized the heparanase precursor in our uptake studies, the active enzyme exhibits an even higher affinity for HS (31) and is therefore likely to be similarly regulated by HS.

Activation of the latent 65-kDa heparanase precursor involves proteolytic cleavage at two potential sites located at the N terminus of the molecule (Glu<sup>109</sup>-Ser<sup>110</sup> and Gln<sup>157</sup>-Lys<sup>158</sup>), resulting in the formation of two subunits that heterodimerize and form the active heparanase enzyme (27, 29, 30). Interestingly, when the uptake process was studied with the anti-Myc tag antibody, we consistently observed a single protein band that corresponded in its molecular mass to the added 65-kDa heparanase precursor. The 50-kDa protein was not detected by the anti-Myc antibody even at later time points, when heparanase processing was evident, suggesting that the Myc tag was removed. Such processing at the protein C terminus has not been recognized so far. Moreover, processing at the C terminus preceded the N-terminal processing (36), raising the possibility that cleavage at the C terminus is a prerequisite for further processing events. Studies exploring this possibility are currently under way.

The half-life of heparanase has not been elucidated so far. By employing metabolic labeling and immunoprecipitation analyses, we followed heparanase biosynthesis, secretion, and activation. A single 65-kDa protein band appeared following 20 min of pulse and practically disappeared after 2 h of chase. This rapid decrease correlated with the appearance of a Myc-tagged 65-kDa protein in the culture medium (Fig. 3A). The secreted protein continued to accumulate by 4 h of chase, followed by a gradual decrease. Several lines of evidence suggest that the intracellular active heparanase originates from uptake, internalization, and processing of the extracellular 65-kDa heparanase precursor. The appearance of a 50-kDa active heparanase in the cell lysates correlated with accumulation of <i>de novo</i> synthesized 65-kDa heparanase in the culture medium (Fig. 3). Direct intracellular processing of the 65-kDa precursor into its active form would have been expected sooner, parallel to the decrease in the 65-kDa band. Furthermore, overexpression of heparanase by HS-deficient CHO-745 cells resulted in high levels of heparanase in the culture medium, correlating with reduced amounts of the intracellular 50-kDa enzyme. Similarly, heparanase accumulation in the culture medium upon addition of heparin was accompanied by a comparable decrease in the amount of the intracellular processed enzyme. Collectively, these findings suggest that the intracellular active
Heparanase Uptake Is Mediated by HSPGs

Fig. 5. Schematic representation of a proposed model for heparanase biosynthesis and trafficking. Preproheparanase is first targeted to the endoplasmic reticulum (ER) lumen via its own signal peptide (Met–Ala130) (step 1). The 65-kDa proheparanase is then shuttled to the Golgi apparatus and subsequently secreted via vesicles that is specifically inhibited by brefeldin A (BFA) (step 2). Once secreted, heparanase rapidly interacts with cell membrane HSPGs such as syndecan family members (step 3), followed by rapid endocytosis of the heparanase-HSPG complex (step 4), which appears to accumulate in endosomes (31). This step is inhibited by cytochalasin D (Cyto.D) (31), heparinase (Fig. 2), or heparin (Fig. 1). Conversion of endosomes to lysosomes results in heparanase (Hepa) processing and activation (step 5), which, in turn, participate in the turnover of HS side chains in the lysosome. Heparanase processing and activation are specifically inhibited by chloroquine and bafilomycin A, inhibitors of lysosomal proteases (32). Typically, heparanase appears at perinuclear lysosomal vesicles (step 6). Such a trafficking route may be bypassed by several potential ways, such as direct conversion of exocytosed vesicles to endosomes (broken arrow).

heparanase originates, at least in part, from uptake of the extracellular heparanase precursor, possibly reflecting the in vivo trafficking route.

The half-life of the newly formed processed heparanase exceeded 24 h and was estimated to be ~30 h. A similar value was calculated for the half-life of the 50-kDa subunit generated upon processing of exogenously added latent heparanase. Thus, endogenously secreted or exogenously added latent 65-kDa heparanase appears to follow similar routes, resulting in generation of the active enzyme, which, in turn, exhibits a relatively long half-life. The attended stability of the processed active heparanase is of special significance given its lysosomal localization (32, 43) and stands in contrast with the relatively short half-life (2–6 h) of HSPGs with a transmembrane domain or the even shorter t½ (~25 min) for GPI-anchored HSPGs (58).

These studies suggest that heparanase may normally function in the turnover of endosomal/lysosomal HSPGs, whereas heparanase secretion (i.e., by atherogenic agents or inflammatory cytokines) (59) is responsible for its pathological actions. Fig. 5 summarizes our proposed model for heparanase biosynthesis and trafficking. Altogether, our results indicate that HSPGs efficiently limit the bioavailability of extracellular heparanase and hence protect cells and tissues from undesirable effects of the enzyme.

Acknowledgment—The anti-human heparanase antibody (monoclonal antibody 130) was kindly provided by InSight Pharmaceuticals. CHO cells deficient in GPI-anchored proteins were kindly provided by Dr. F. Giuso van der Goot, Department of Biochemistry, University of Geneva, Switzerland.

REFERENCES

1. Vlodavsky, I., and Friedmann, Y. (2001) J. Clin. Investig. 108, 341–347
2. Pikas, D. S., Li, J.-P., Vlodavsky, I., and Lindahl, U. (1998) J. Biol. Chem. 273, 18770–18777
3. Freeman, C., and Parish, C. R. (1998) Biochem. J. 330, 1341–1350
4. Vlodavsky, I., Eldor, A., Haimovitz-Friedman, A., Matzner, Y., Ishai-Michaeli, R., Lider, O., Naparstek, Y., Cohen, I. R., and Fuks, Z. (1992) Invasion Metastasis 12, 112–127
5. Bartlett, M. R., Underwood, P. A., and Parish, C. R. (1995) Inflamm. Cell Biol. 73, 123–124
6. Nakajima, M., Irimura, T., and Nicolson, G. L. (1998) J. Cell. Biochem. 68, 157–167
7. Dempsey, L. A., Brunn, G. T., and Platt, J. L. (2000) Trends Biol. Sci. 25, 349–351
8. Vadali, G. G., and Lider, O. (2000) J. Leukocyte Biol. 67, 149–159
9. Bernard, D., Mehul, B., Delattre, C., Simonetti, L., Thomas-Collignon, A., and Schmidt, R. (2001) J. Investig. Dermatol. 117, 1266–1273
10. Parish, C. R., Freeman, C., and Hulett, M. D. (2001) Biochim. Biophys. Acta 1517, M99–M108
11. Matzner, Y., Bar-Ner, M., Yahalom, J., Ishai-Michaeli, R., Fuks, Z., and Vlodavsky, I. (1985) J. Clin. Investig. 76, 1306–1316
12. Molinolo, F., Nakajima, M., Lorens, A., Barbossa, E., Callejo, S., Gajate, C., and Fabra, A. (1997) Biochem. J. 327, 917–923
13. Parish, C. R., Hindmarsh, E. J., Bartlett, M., Staykova, M. A., Cowden, W. B., and Willenborg, D. O. (1998) Inflamm. Cell Biol. 76, 104–112
14. Eil-Assaf, O. N., Yamani, A., Inoue, T., Kohno, H., and Nagase, N. (2001) Clin. Cancer Res. 7, 1299–1305
15. seed, K., Hirano, H., Okamoto, M., Kitazawa, S., Toyoshima, M., Dong, J., Katsuoka, Y., and Nakajima, M. (2001) Int. J. Cancer 95, 295–301
16. Kielopanos, A., Fries, H., Kleeff, J., Shi, X., Liao, Q., Pecker, I., Vlodavsky, I., Zimmermann, A., and Buchler, M. W. (2001) Cancer Res. 61, 4655–4659
17. Kuloff, J., Zinke, J., Schoppmeyer, K., Tannapfel, A., Witzgall, H., Mosnier, J., Wittekind, C., and Caca, K. (2002) Br. J. Cancer 86, 1270–1275
18. Xiao, Y., Kleeff, J., Shi, X., Buchler, M. W., and Friess, H. (2003) Hepatol. Res. 26, 192–198
19. Levivlah, V., Kanelis, J., Jerino, F. L., and Power, D. A. (2001) Kidney Int. 60, 1287–1296
20. Katz, A., Van Dijk, D. J., Aingerm H., Erman, A., Davies, M., Darmon, D., Hurvitz, H., and Vlodavsky, I. (2002) Isr. Med. Assoc. J. 4, 996–1002
21. Elkin, M., Ilan, N., Ishai-Michaeli, R., Friedmann, Y., Papo, O., Pecker, I., and Vlodavsky, I. (2001) FASEB J. 15, 1661–1663
22. Shalapar, P. Z., Zehara, A., Ashhab, Y., Peretz, T., Vlodavsky, I., and Ben-Yehuda, D. (2003) Oncogene 22, 7737–7749
23. Yang, Y. J., Zhang, Y. L., Li, X., Dan, H. L., Lai, Z. S., Wang, J. D., Wang, Q.Y., Cui, H. H., Sun, Y., and Wang, Y. D. (2003) World J. Gastroenterol. 9, 1707–1712
24. Lu, W. C., Liu, Y. N., Kang, B. B., and Chen, J. H. (2003) Oncogene 22, 919–923
25. De Mestre, A. M., Khachigian, L. M., Santiago, F. S., Staykova, M. A., and Hulett, M. D. (2003) J. Biol. Chem. 278, 50377–50385
26. Elkin, M., Zehara, E., Orgel, A., Cohen, I., Guatta-Rangini, Z., Peretz, T., Vlodavsky, I., and Kleinman, H. (2003) Cancer Res. 63, 8821–8826
27. Fairbanks, M. B., Mildner, A. M., Leone, J. W., Cavey, G. S., Mathews, W. R., Drong, R. F., Slighm, J. L., Biewienski, M. J., Smith, C. W., Bannow, C. A., and Heinrikson, R. L. (1999) J. Biol. Chem. 274, 29587–29590
Heparanase Uptake Is Mediated by HSPGs

28. Goldshmidt, O., Zcharia, E., Abramovitch, R., Metzger, S., Aingorn, H., Friedmann, Y., Schirrmacher, V., Mitra, E., and Vlodavsky, I. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 10031–10036
29. McKenzie, E., Young, K., Hirsch, M., Bennett, J., Bhanam, M., Felix, R., Turner, P., Stamp, A., McMillan, D., Saville, G., Ng, S., Mason, S., Snell, D., Schiff, D., Gong, H., Townsend, R., Gallagher, J., Parekh, R., and Sussberg, C. (2003) Biochem. J. 373, 423–435
30. Levy-Adam, F., Miao, H.-Q., Vlodavsky, I., and Ilan, N. (2003) Biophys. Res. Commun. 308, 885–891
31. Nadav, L., Eldor, A., Yacoby-Zeevi, O., Zamir, E., Pecker, I., Ilan, N., Geiger, B., Vlodavsky, I., and Katz, B. Z. (2001) J. Cell Sci. 115, 2179–2187
32. Zetser, A., Levy-Adam, F., Kaplan, V., Gingis-Velitski, S., Bashenko, Y., Schubert, S., Flugelman, M. Y., Vlodavsky, I., and Ilan, N. (2004) J. Cell Sci. 117, 2249–2258
33. Zetser, A., Bashenko, Y., Miao, H.-Q., Vlodavsky, I., and Ilan, N. (2003) Cancer Res. 63, 7733–7741
34. Vlodavsky, I., Friedmann, Y., Elkin, M., Aingorn, H., Atzmon, R., Ishai-Michaeli, R., Bitan, M., Pappo, O., Peretz, T., Michal, I., Spector, L., and Pecker, I. (1999) Nat. Med. 5, 793–802
35. Abumi, I., Fivas, M., Kobayashi, T., Kinoshita, T., Parton, R. G., and van der Geest, F. (2001) J. Biol. Chem. 276, 30729–30736
36. Gingis-Velitski, S., Zetser, A., Flugelman, M. Y., Vlodavsky, I., and Ilan, N. (2004) J. Biol. Chem. 279, 23536–23541
37. Schroeter, C. J., Braun, M., Englert, J., Beck, H., Schmid, H., and Kahlbacher, H. (1999) J. Immunol. Methods 227, 161–168
38. Esko, J. D. (1991) Curr. Opin. Cell Biol. 3, 895–816
39. Esko, J. D., and Selleck, S. B. (2002) Annu. Rev. Biochem. 71, 435–471
40. Saliyana, F., Kolset, S. O., Prydz, R., Gottfridsson, E., Lindahl, U., and Salminen, V. (1999) J. Biol. Chem. 274, 36267–36273
41. Belting, M. (2003) Trends Biochem. Sci. 28, 145–151
42. Fuki, I. V., Iozzo, R. V., and Williams, R. J. (2000) J. Biol. Chem. 275, 25742–25750
43. Goldshmidt, O., Nadav, L., Aingorn, H., Cohen, I., Feinstein, N., Ilan, N., Zamir, E., Geiger, B., Vlodavsky, I., and Katz, B. Z. (2002) Exp. Cell Res. 281, 50–62
44. Fuki, I. V., Kuhn, K. M., Lomazov, I. R., Rothman, V. L., Tuszyński, G. P., Iozzo, R. V., Swenson, T. L., Fisher, E. A., and Williams, R. J. (1997) J. Clin. Investig. 100, 1611–1622
45. Williams, K. J., and Fuki, I. V. (1997) Curr. Opin. Lipidol. 8, 253–262
46. Zeng, B.-J., Mortimer, B.-C., Martins, I. J., Seydel, U., and Redgrave, T. G. (1998) J. Lipid Res. 39, 845–860
47. Freissler, E., auf der Hyde, A. M., David, G., Meyer, T. F., and Dehio, C. (2000) Cell Microbiol. 2, 69–82
48. Kramer, K. L., and Yost, H. J. (2003) J. Cell Sci. 117, 687–695
49. Saoncella, S., Schekman, R., Denhez, F., Nolte, J. K., Meshi, D. F., Robinson, S. D., Holtzer, R. O., and Gotte, M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 2805–2810
50. Bass, M. D., and Humphries, M. J. (2002) Biochem. J. 368, 1–15
51.Gotte, M. (2003) J. Biol. Chem. 278, 575–591
52. Ornitz, D. M. (2000) BioEssays 22, 108–112
53. Berenfield, M., Gotte, M., Park, P. W., Reizes, O., Fitzgerald, M. L., Lincecum, J., and Zako, M. (1999) J. Biol. Chem. 274, 729–737
54. See, T., and St. Clair, R. W. (1997) J. Biol. Chem. 272, 2249–2258
55. Ho, Y. Y., Al-Haideri, M., Mazzone, T., Vogel, T., Presley, J. F., Sturley, S. L., and Deckelbaum, R. J. (2000) Biochemistry 39, 4746–4754
56. See, T., Al-Haideri, M., Treskova, E., Wargall, T. S., Kako, Y., Goldberg, I. J., and Deckelbaum, R. J. (2000) J. Biol. Chem. 275, 30355–30362
57. Liu, B. Y., Kim, Y. C., Leatherberry, V., Cowin, P., and Alexander, C. M. (2003) Oncogene 22, 9243–9253
58. Egelberg, M., Kjeken, K., Kolset, S. O., Berg, T., and Prydz, K. (2001) Biochim. Biophys. Acta 1541, 135–149
59. Chen, G., Wang, D., Viskunamadhyan, R., Yagyu, H., Saxena, U., Pillarisetti, S., and Goldberg, I. J. (2004) Biochemistry 43, 4971–4977