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Structure-function approach identifies a C-terminal domain that mediates heparanase signaling

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Abstract

Heparanase is an endo- β -D-glucuronidase capable of cleaving heparan sulfate, activity that is strongly implicated in cellular invasion associated with tumor metastasis, angiogenesis, and inflammation. In addition, heparanase was noted to exert biological functions apparently independent of its enzymatic activity, enhancing the phosphorylation of selected protein kinases and inducing gene transcription. A predicted three dimensional structure of constitutively active heparanase clearly delineates a TIM-barrel fold previously anticipated for the enzyme. Interestingly, the model also revealed the existence of a C-terminal domain (C-domain) apparently not being an integral part of the TIM-barrel fold. We provide evidence that the C-domain is critical for heparanase enzymatic activity and secretion. Moreover, the C-domain was found to mediate non-enzymatic functions of heparanase, facilitating Akt phosphorylation, cell proliferation, and tumor xenograft progression. These findings support the notion that heparanase exerts enzymatic activity-independent functions, and identify, for the first time, a protein domain responsible for heparanase-mediated signaling. Inhibitors directed against the C-domain, combined with inhibitors of heparanase enzymatic activity, are expected to neutralize heparanase functions and to profoundly affect tumor growth, angiogenesis and metastasis.

Keywords

heparanase; three-dimensional model; secretion; Akt; phosphorylation

Introduction

Heparanase is an endo- β -D-glucuronidase capable of cleaving heparan sulfate (HS) side chains at a limited number of sites, thus facilitating structural alteration of the extracellular matrix (ECM) and basement membrane underlying epithelial and endothelial cells (1,2). Heparanase activity has long been detected in a number of cell types and tissues. Importantly, heparanase activity correlated with the metastatic potential of tumor-derived cells, attributed to enhanced cell dissemination as a consequence of HS cleavage and remodeling of the ECM barrier (3, 4), a notion that is supported experimentally (5) and, moreover, clinically (6–8).

Similar to several other classes of enzymes, heparanase is first synthesized as a latent enzyme that appears as a ~65 kDa protein when analyzed by SDS-PAGE. The protein undergoes proteolytic processing that removes a 6 kDa linker segment, yielding an 8 kDa polypeptide at

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the N-terminus and a 50 kDa polypeptide at the C-terminus that heterodimerize to form the active heparanase enzyme (9–11). Latent heparanase secretion is directed by an N-terminal signal peptide (Met¹-Ala³⁵). The secreted protein interacts rapidly and efficiently with cell surface heparan sulfate proteoglycan (HSPG), low density lipoprotein receptor-related protein (LRP), and mannose 6-phosphate receptor (MPR) (12–14), followed by internalization and processing, collectively defined as heparanase uptake (6,12,13). Following uptake, heparanase was noted to reside primarily within endocytic vesicles identified as late endosomes and lysosomes (15,16). This observation led to the identification of lysosome as the heparanase processing organelle (17), and cathepsin family members, mainly cathepsin L, as the heparanase activating protease (18,19). The strict requirement of protein secretion for proteolytic activation of heparanase (12–14,20) has been shown to be experimentally bypassed by a single chain gene construct (21). In this protein variant, the linker segment was replaced by three glycine-serine repeats (GS3), resulting in constitutively active heparanase enzyme (21).

Apart of the well studied catalytic feature of the enzyme, heparanase was noted to exert biological functions apparently independent of its enzymatic activity. Non enzymatic functions of heparanase include enhanced cell adhesion (22–24), and induction of p38 and Src phosphorylation (24) associated with vascular-endothelial growth factor (VEGF) (25) and tissue factor (TF) (26) gene induction. Moreover, we have demonstrated that exogenous addition of latent heparanase or its over expression in tumor-derived cells stimulate Akt phosphorylation and PI3-kinase-dependent cell invasion and migration (27), likely supporting endothelial and tumor cell survival (28). Protein domains other than amino acid residues critical for heparanase catalysis (Glu²²⁵ and Glu³⁴³) (29) and heparin/HS binding regions (20) have not been so far identified in the heparanase molecule, primarily because the crystal structure of heparanase has not been resolved yet. The present study was undertaken to identify functional domains that would advance the basic understanding of this molecule and, possibly, serve as targets for rational drug development. A predicted three dimensional structure of active heparanase clearly delineated a TIM-barrel fold previously anticipated for the enzyme. Notably, the model also revealed the existence of a C-terminal domain (C-domain) apparently not being an integral part of the TIM-barrel fold. We provide evidence that the C-domain is critical for heparanase enzymatic activity and secretion. Moreover, the C-domain was found to mediate non-enzymatic functions of heparanase, facilitating Akt phosphorylation, cell proliferation, and tumor xenograft progression.

Materials and Methods

A model of heparanase structure

A three dimensional structure of constitutively active single chain heparanase enzyme (GS3) (21) was generated by a protein structure prediction server* (30). The server combines template-based and *de novo* structure prediction methods. The software first screened for confident match to a protein of known structure using PSI-BLAST, FFAS03, or 3D-Jury software (31). The "significant hit" (the closest match) was found to be α -L-arabinofuranosidase isolated from *Geobacillus stearothermophilus* T-6. The three dimensional structure of this protein was then used as a template for comparative modeling of heparanase.

Heparanase gene constructs

Plasmids and viral gene constructs that were used in this study are listed in Supplementary Table 1.

*<http://www.robetta.org>

Antibodies and reagents

Anti-Myc-tag (sc-40), anti-Akt (sc-5298), anti-syndecan-4 (sc-12766) and anti-calnexin (sc-11397) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-Akt (Ser473) antibody was purchased from Cell Signaling Technologies (Beverly, MA). Anti mouse platelet endothelial cell adhesion molecule (PECAM)-1 (CD31) polyclonal antibody was kindly provided by Dr. Joseph A. Madri (Yale University, New Haven, CT) (25). Bromodeoxyuridine (BrdU) was purchased from GE Healthcare (Buckinghamshire, England) and anti-BrdU monoclonal antibody-HRP conjugated was purchased from Roche (Mannheim, Germany). Hsp90 inhibitor 17-Allylamino-17-demethoxygeldanamycin (17-AAG) was purchased from Alomone Labs (Jerusalem, Israel) and was dissolved in DMSO as stock solution. DMSO was added to the cell culture as a control. Fluorescein wheat germ agglutinin was purchased from Vector Laboratories Inc (Burlingame, CA).

Cells and cell culture

HEK 293, human choriocarcinoma JAR, cervical adenocarcinoma HeLa, U87-MG glioma, A549 lung carcinoma, and Chinese hamster ovary (CHO) K1 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). FaDu human pharynx carcinoma cells were kindly provided by Dr. Eben L. Rosenthal (University of Alabama at Birmingham, Birmingham, AL) (32). Cells were grown in Dulbecco's modified Eagle's medium (Biological Industries, Beit Haemek, Israel) supplemented with 10% fetal calf serum and antibiotics. Mutant CHO cells (pgs A-745) deficient of xylosyltransferase and unable to initiate glycosaminoglycan synthesis, were kindly provided by Dr. J. Esko (University of California, San Diego) and grown in RPMI 1640 medium (Biological Industries) supplemented with 10% FCS and antibiotics (27). Human umbilical vein endothelial cells (HUVEC) were kindly provided by Dr. Neomi Lanir (Rambam Health Care Campus, Haifa, Israel) and were grown essentially as described (27).

Transfection and recombinant proteins

Transient and stable transfections were performed using FuGENE 6 reagent, according to the manufacturer's (Roche) instructions, essentially as described (10,13,24,25). Recombinant wild type heparanase and heparanase C-domain proteins were purified from the conditioned medium of stably transfected or infected HEK 293 cells, essentially as described (24).

Cell lysates and protein blotting

Preparation of cell lysates, protein blotting, and measurement of heparanase enzymatic activity were carried out as described previously (13,24,25,27).

Binding and cross-linking

Binding experiments were carried out essentially as described (12). Briefly, recombinant C-domain or 8-C proteins were iodinated to a high specific activity by the chloramine T method. Cells were grown in 24-well multidishes and incubated (2 h on ice) with binding buffer (RPMI 1640, 10 mM HEPES, 0.2% BSA) containing increasing concentrations of ^{125}I -C-domain in the absence or presence of 400–2000 nM unlabeled heparanase or C-domain proteins. Cells were then washed with ice-cold PBS, solubilized with 200 μl of 1 M NaOH, and counted in a γ -counter. Binding parameters (K_d , B_{max}) were obtained by the Prism 4 software (GraphPad Software, San Diego, CA) (12). For Cross-linking experiments, cells were grown in 60-mm dishes and incubated for 2 h on ice with binding buffer containing 4 nM iodinated C-domain or 8-C proteins in the absence and presence of heparin (10 $\mu\text{g/ml}$) or 400 nM unlabeled heparanase or C-domain proteins. The cross linker sulfo-EGS (Pierce; 0.2 mM) was then added for 10 min, followed by quenching with 50 mM Tris-HCl, pH 7.5. Cells were then washed

with PBS, scraped and collected in an eppendorf tube, lysed, and subjected to SDS-PAGE followed by autoradiography.

Tumorigenicity and immunohistochemistry

Cells from exponential cultures of control-, heparanase-, C-domain- and TIM-barrel-transfected U87 human glioma cells were detached with trypsin/EDTA, washed with PBS and brought to a concentration of 5×10^7 cells/ml. Cell suspension ($5 \times 10^6/0.1$ ml) was inoculated subcutaneously at the right flank of 5-weeks old female Balb/C nude mice (n=7). Xenograft size was determined twice a week by externally measuring tumors in two dimensions using a caliper. At the end of the experiment, mice were sacrificed and xenografts were resected, weighted and fixed in formalin. Paraffin-embedded 5 micron sections were subjected to immunostaining with anti-phospho-Akt and anti-PECAM-1 antibodies, using the Envision kit, according to the manufacturer's (Dako, Glostrup, Denmark) instructions, as described (24, 25). All animal experiments were approved by the Animal Care Committee of the Technion, Haifa, Israel.

Statistics

Data is presented as mean $\pm$ SE. Statistical significance was analyzed by two-tailed Student's t test. The value of $p < 0.05$ was considered significant. All experiments were repeated at least three times with similar results.

Results

Three dimensional model of active heparanase

A three dimensional structure of active heparanase (GS3; Suppl. Fig. 1B) was obtained based on sequence and structure resemblance to α -L-arabinofuranosidase enzyme from *Geobacillus stearothermophilus* T-6 (31) (Fig. 1A). The structure clearly illustrates a TIM-barrel fold that has previously been predicted for the enzyme (19, 29). In addition, conserved glutamic acid residues critical for heparanase catalysis (Glu²²⁵ and Glu³⁴³) (Fig. 1A, red), as well as heparin/HS binding regions (Lys¹⁵⁸-Asp¹⁷¹ and Gln²⁷⁰-Lys²⁸⁰; Fig. 1A, cyan and green, respectively) (20) were situated in close proximity to the micro-pocket active site, corroborating the relevance of the model (Fig. 1A, left panel). Notably, the structure also delineates a C-terminal fold positioned next to the TIM-barrel fold. The C-terminal domain (C-domain) appears to comprise of 8 β strands arranged in two sheets (Fig. 1A, right panel), as well as a flexible, unstructured loop (Fig. 1A, right, arrow) that lies in-between. The two sheets are packed against each other and are stabilized by hydrophobic interactions between the upper and lower β sheets. Notably, one of the C-domain β strands is contributed, apparently, by the 8 kDa protein subunit (Fig. 1A, right panel, yellow), suggesting that the protein N-terminus plays a structural role in the establishment of the TIM-barrel (29) and C-domain folds.

The C-terminal domain is essential for heparanase enzymatic activity

Apart of the well documented catalytic activity of the enzyme, heparanase was also noted to exert enzymatic activity-independent functions, facilitating the phosphorylation of selected protein kinases and inducing gene transcription (22,24–27,33,34). We hypothesized that the seemingly distinct protein domains observed in the three dimensional model, namely the TIM-barrel and C-domain regions, mediate enzymatic and non-enzymatic functions of heparanase, respectively. In order to examine this possibility, we designed gene constructs carrying Myc-tagged wild type (aa 36–543), TIM-barrel (aa 36–417), and C-domain (aa 413–543) heparanase variants (Suppl. Fig. 1A), and heparanase enzymatic activity was evaluated in stably transfected human choriocarcinoma JAR cells. Release of sulfate-labeled HS degradation fragments was readily detected in JAR cells transfected with wild type heparanase. In striking

contrast, cells transfected with the TIM-barrel construct failed to display heparanase enzymatic activity (Suppl. Fig. 2A). We suspected that the lack of enzymatic activity is due to impaired protein secretion shown previously to be required for the delivery of latent heparanase to late endosomes/lysosomes and its subsequent processing and activation by lysosomal cathepsins (12–14,20). In order to overcome impaired trafficking, we applied the constitutively active GS3 gene construct to generate the TIM-barrel variant, thus bypassing the requirement for protein secretion. JAR cells transfected with the GS3-TIM-barrel construct failed to yield heparanase activity (Fig. 1B, TIMB), while the full length GS3-heparanase was highly active (Fig. 1B, Hepa). These results thus imply that the C-domain is required for the establishment of an active heparanase enzyme, possibly by stabilizing the TIM-barrel fold. Deletion and site directed mutagenesis approaches were next employed to identify regions and amino acids critical for this function of the C-domain. Notably, deletion of the last C-terminus 17 amino acids (Phe⁵²⁷-Ile⁵⁴³; Δ 17, Suppl. Fig. 2A) completely abolished heparanase enzymatic activity, and a similar result was observed when the deletion was constructed in the GS3 backbone [Fig. 1B, Δ 17(GS3)]. Likewise, deletion of the unstructured loop (Leu⁴⁸³-Pro⁵⁰⁹, Suppl. Fig. 1C, red box) resulted in an inactive heparanase enzyme (Suppl. Table 2, Suppl. Fig. 2A). Moreover, point mutations of evolutionary conserved amino acid residues Phe⁵³¹, Val⁵³³, Ile⁵³⁴, Ala⁵³⁷, and Cys⁵⁴² (Suppl. Fig. 1C, red labeled aa) resulted in inactive heparanase (Fig. 1D, Suppl. Table 2), thus supporting a critical role of the C-domain in the establishment of active heparanase enzyme.

The C-domain is critical for heparanase secretion

We have, next, examined the expression, secretion, and cellular localization of the heparanase variants. All heparanase variants were readily detected applying anti-Myc immunoblotting, yet the C-domain construct appeared to be expressed at lower levels (Fig. 2A, upper panel). The relatively low signal observed in the lysate of cells transfected with wild type heparanase (Fig. 2A, upper panel, Hepa) is due to efficient secretion of the latent 65 kDa protein (Fig. 2A, third panel) and accumulation of the processed, 50 kDa protein subunit in the cell lysates (Fig. 2A, second panel). In striking contrast, the TIM-barrel protein variant appeared unprocessed (Fig. 2A, second panel, TIMB) and was undetected in the cell conditioned medium even in the presence of heparin which enhances the accumulation of heparanase extracellularly (Fig. 2A, lower panel, TIMB, +) (13). Likewise, deletion of 17 amino acid residues from the heparanase C-terminus markedly attenuated protein secretion (Fig. 2A, Δ 17, lower panel), and point mutations (Phe⁵³¹, Val⁵³³, Ile⁵³⁴, Ala⁵³⁷, Cys⁵⁴²) that yielded proteins devoid of enzymatic activity (Fig. 1D) also failed to get processed (Fig. 2B, second panel) or secreted (Fig. 2B, lower panel). In contrast, the C-domain protein variant was noted to be secreted, albeit less efficient than wild type heparanase (Fig. 2A, lower panel). These findings imply that intact C-domain is critical for the establishment of active heparanase enzyme (Fig. 1) and for heparanase secretion.

Next, we examined the cellular localization of the heparanase variants by confocal microscopy (Fig. 2C). Control (mock; upper panels), heparanase (Hepa; second panels), TIM-barrel (TIMB; third panels), C-domain (fourth panels), Δ 17 (fifth panels), and heparanase mutated at alanine⁵³⁷ (A537K; lower panels) transfected HEK 293 cells were triple stained applying anti-Myc-tag (left, red), anti-calnexin (an ER marker, second left, green), and TO-PRO, which labels the cell nucleus (merge, blue, third left). As expected, all heparanase protein variants were noted to be sorted to the ER, co-localizing with calnexin (third left, yellow). In striking contrast, only the heparanase and C-domain proteins, shown to be secreted (Fig. 2A, lower panel), were noted to co-localize with the Golgi marker, wheat germ agglutinin (right most second and fourth panels; yellow). These results thus indicate that the C-domain and more specifically its C-terminus are critically important for the shuttling of heparanase from the ER to the Golgi apparatus and subsequent secretion.

Like heparanase, the C-domain protein was noted to interact with cultured cells following its exogenous addition (Fig. 3A). Binding of heparanase and C-domain proteins was significantly inhibited by heparin (Fig. 3B), indicating HS-mediated interaction. Indeed, both proteins were noted to reside in typical endocytic vesicles, co-localizing with syndecan-4 (Fig. 3C), a cell membrane HSPG (13,17,34). The observed decrease in C-domain levels 2h following its addition (Fig. 3A, upper and lower panels, orange) suggests that the C-domain protein variant is less stable in the acidic lysosomal environment. Rapid degradation may be envisioned as a mechanism that down regulates C-domain signaling (see below).

Heparanase C-domain induces Akt phosphorylation

We have previously reported that heparanase induces Akt phosphorylation independently of its enzymatic activity (27,33). We hypothesized that the C-domain, seemingly comprising a protein entity which is not an integral part of the TIM-barrel fold, and its apparent unstructured flexible loop (Fig. 1A, arrow), mediate the activation of Akt. To examine this possibility, HEK 293 cells were stably transfected with control empty vector (mock), wild type heparanase (Hepa), TIM-barrel (TIMB), or C-domain gene constructs. Cell lysate (Fig. 3D, left panels) samples were subjected to immunoblotting applying anti-phospho-Akt (p-Akt; Fig. 3D, upper panel) and anti-Akt (Fig. 3D, second panel) antibodies. Akt phosphorylation was stimulated by cells over expressing wild type heparanase (Hepa) 2 ± 0.3 folds as quantified by densitometry (Fig. 3D, left, lower panel), in agreement with our previous findings (13). Notably, Akt phosphorylation was similarly (2.7 folds) stimulated by cells over-expressing the C-domain, as determined by densitometry analysis (Fig. 3D, left, lower panel), while the TIM-barrel protein variant yielded no Akt activation compared with control, mock transfected cells. To further substantiate these findings, conditioned medium was collected from stably transfected cells, applied onto control HEK 293 cells for 30 min and evaluated for stimulation of Akt phosphorylation (Fig. 3D, middle panels). Indeed, medium conditioned by C-domain expressing cells yielded a marked, 4 ± 1.5 fold induction of Akt phosphorylation, while TIM-barrel expressing cells did not (Fig. 3D, middle, lower panel). Furthermore, Akt activation in HS-deficient CHO-745 cells following the addition of purified heparanase or C-domain proteins (Fig. 3D, right panels) appeared comparable in magnitude to Akt activation in HEK 293 and HeLa cells (data not shown), suggesting HS-independent Akt activation, as previously noted for heparanase (27). These results clearly indicate that non-enzymatic, signaling function of heparanase leading to activation of Akt is mediated by the C-domain.

Secretion and signaling properties are enhanced by combining the 8 kDa and C-domain sequences

Since the C-domain gene construct lacks the 8 kDa segment which, according to our model, contributes one beta strand to the C-domain structure (Fig. 1A, right), the resulting protein may render imperfect in terms of secretion, stability, and Akt activation. In order to address this possibility, we constructed a mini gene comprising a segment of the 8 kDa subunit predicted by the model to contribute a beta strand (Gln³⁶-Ser⁵⁵) to the C-domain structure, linked to the C-domain sequence (8-C; Suppl. Fig. 1A). Indeed, secretion of the resulting 8-C protein was markedly enhanced and reached the level of wild type heparanase (Fig. 4A, right). Densitometry analysis revealed approximately 10-fold increase in 8-C protein secretion compared with the C-domain alone. Furthermore, stable transfection of the 8-C gene construct (Fig. 4B, left) or exogenous addition of medium conditioned by 8-C transfected 293 cells markedly enhanced Akt phosphorylation in FaDu pharynx carcinoma (Fig. 4B, left) and primary endothelial cells (HUVEC; Fig. 4B, middle), in a time (Fig. 4B, right) and dose [maximal activation at 1 μ g/ml purified proteins, while lower (0.25 μ g/ml) or higher (5 μ g/ml) concentrations were less effective; not shown] dependent manner. Densitometry analysis revealed that the 8-C protein elicit 6–8 -fold induction of Akt phosphorylation, compared with 2 to 3-fold induction of Akt phosphorylation by the C-domain (Fig. 4B, ,lower panels).

Moreover, C-domain and 8-C protein facilitated endothelial cells organization into tube-like structures (Suppl. Fig. 2C), in line with pro-angiogenic features of inactive heparanase (27). These results critically support our predicted three dimensional models (Fig. 1A), indicating that the C-domain is a valid functional domain.

Heparanase C-domain stimulates cell proliferation and facilitates tumor xenograft development

Having demonstrated a signaling function of the C-domain we, next, examined the cellular consequences of such induction. Stably transfected HEK 293 cells were incubated with BrdU and its incorporation was evaluated by immunocytochemistry as an indication of cellular proliferation (Fig. 5A). Heparanase (Hepa) and C-domain expressing cells were noted to incorporate twice as much BrdU compared with control (mock transfected) cells ($P < 0.005$; Fig. 5A). In order to further substantiate this finding, we examined the progression of tumor xenografts produced by mock-, heparanase-, TIM-barrel-, and C-domain- transfected U87 glioma cells. Tumor xenografts produced by heparanase expressing cells assumed a higher growth rate and generated increasingly bigger tumors compared with tumor xenografts produced by control, mock-transfected cells (Fig. 5B), in agreement with previous studies utilizing this model system (24). At the end of the experiment on day 51, xenograft produced by heparanase-transfected cells were 6.5-fold bigger in volume ($p < 0.017$; Fig. 5B) and 5.5-fold higher in weight ($p < 0.02$; Fig. 5C), compared with control cells (mock). Notably, tumor xenografts produced by C-domain-transfected cells appeared comparable or even slightly bigger than those produced by heparanase-transfected cells, yielding tumors that were 8-fold bigger in volume ($p < 0.0007$; Fig. 5B) and 6.5-fold higher in weight ($p < 0.001$; Fig. 5C) compared with controls. As expected, the progression of tumors produced by TIM-barrel-transfected cells appeared comparable with controls, in agreement with the lack of enzymatic activity, secretion, and signaling capability of this protein variant (Fig. 1–Fig. 3). Notably, phosphorylated Akt was highly abundant in xenografts produced by heparanase- and C-domain- transfected cells (Fig. 5D, left, second and third panels), compared with xenograft produced by mock- and TIM-barrel- transfected cells (Fig. 5D, left, upper and fourth panels). In addition, tumor angiogenesis was markedly stimulated in xenografts produced by heparanase- and C-domain- expressing U87 cells compared with mock and TIM-barrel transfected cells (Fig. 5D, right panels). This is in agreement with the observed induction of Akt and tube-like structures in endothelial cells by the C-domain (Fig. 4B, Suppl. Fig. 2C), and the pro-angiogenic capacity of heparanase noted in several experimental settings (5,25,28,35, 36). These results imply that the pro-tumorigenic and pro-angiogenic properties of heparanase are mediated, at least in part, by the C-domain, clearly supporting a clinical relevance of heparanase enzymatic-independent functions.

The heparanase C-domain interacts with cell surface proteins

The secreted nature of the C-domain and its capacity to elicit signaling cascades and to accelerate tumor growth suggest its interaction with cell membrane protein(s)/receptor(s). In order to explore this possibility, purified recombinant C-domain was iodinated to a high specific activity and binding experiments were performed. Binding of ^{125}I -C-domain to HeLa cells exhibited linearity at low concentrations, and approached saturation at high levels of ^{125}I -C-domain (Fig. 6A). Analyses of C-domain binding by the Prism 4 software suggested the existence of high affinity ($K_d = 7 \text{ nM}$), low abundant ($B_{\text{max}} = 170 \times 10^3$) binding sites. Such affinity is similar to that observed for wild type heparanase ($K_d = 2\text{--}4 \text{ nM}$) (12). We then assessed the existence of C-domain/8-C binding protein(s)/receptor(s) by cross-linking experiments. Cross-linking of the C-domain or 8-C proteins followed by SDS/PAGE revealed the existence of two major cell surface protein(s)/receptor(s) complexes, exhibiting molecular weights of ~ 130 and $\sim 170 \text{ kDa}$ that interact with the heparanase C-domain (Fig. 6C, indicated by arrows). The molecular weight of cell surface proteins shown previously to bind heparanase

[i.e., LRP1 (85 kDa) (12,14); cation-dependent MPR (46 kDa) (12,14); and cation-independent MPR (300 kDa) (37)] is not in accordance with the molecular weights of the protein complexes found by cross-linking. These findings suggest the existence of novel heparanase/C-domain binding proteins/receptors that bind the heparanase/C-domain with high affinity and likely mediate Akt activation.

Discussion

Attempts to inhibit heparanase enzymatic activity were initiated short after its discovery (38), in parallel with the emerging clinical relevance of this activity (3), and heparanase inhibitors are currently under clinical trials (39). It seems, however, that better characterization of the heparanase molecule and its three dimensional structure is required for the development of more efficacious and highly specific inhibitors in a rational manner. Here, we describe, for the first time, a predicted model of active heparanase and its heterodimer nature. Notably, the 8 kDa subunit appears to enfold the 50 kDa subunit (Fig. 1A, middle panel), contributing $\beta/\alpha/\beta$ unit to the TIM-barrel fold, as predicted (21,29) and, moreover, one of the 8 β strands that comprise the C-domain (Fig. 1A, right, yellow). In fact, the N-terminus of the 8 kDa subunit first generates a β strand of the C-domain (Fig. 1A, right, yellow) and then yields the first $\beta/\alpha/\beta$ fold of the TIM-barrel. Thus, it is conceivable that the heparanase molecule and its major constituents (8 kDa, linker, 50 kDa) are assembled and folded properly while being biosynthesized. In this regard, the 8 and 50 kDa proteins are not merely subunits that need to reassemble following their generation, but rather function as one entity.

In addition, the model also underlines the existence of a C-terminal domain (C-domain) that appears not to participate in the TIM-barrel fold. The seemingly distinct protein domains led us to hypothesize that these molecular entities mediate enzymatic (TIM-barrel) and non-enzymatic (C-domain) functions of heparanase. In fact, the C-domain was found to be critically essential for heparanase secretion, enzymatic activity, and Akt activation. Clearly, deletion of the C-domain generates enzymatically inactive heparanase (TIM-barrel), even when constructed as a GS3 protein variant (Fig. 1B). Furthermore, co-transfection of TIM-barrel and C-domain gene constructs failed to produce an active enzyme (Fig. 1C), suggesting that the two domains must be co-assembled while being synthesized, unlike co-transfection of the 8 and 50 kDa subunits that yields an active enzyme (10,11). Thus, the C-domain appears to play an important structural role although not comprising an integral part of the TIM-barrel fold, possibly stabilizing the TIM-barrel conformation. In support of this notion is the lack of heparanase secretion following deletion of the entire C-domain or its C-terminus ($\Delta 17$, Fig. 2). Furthermore, point mutations of conserved amino acids at this region eradicated heparanase secretion (Fig. 2; Supplementary Table S2) (40), suggesting that relatively modest alterations in the structure of the C-domain significantly affect the integrity/conformation of the heparanase molecule. While the C-domain appeared to get properly folded, judged by its localization to the Golgi apparatus, its secretion appeared modest compared with wild type heparanase, and more strictly dependent upon functional HSP-90 chaperon (Suppl. Fig. 2B). Introducing the beta-strand contributed by the 8 kDa subunit to the C-domain fold, nonetheless, dramatically enhanced secretion of the 8-C protein (Fig. 4A). This finding critically supports our predicted model and highlights the C-domain as a valid, functional domain. The 8-C protein variant is expected to significantly advance our effort to obtain a crystal structure of this domain by purifying it in large quantities and thus assists in the development of a new class of small molecule inhibitors. Studies aimed at this direction are currently underway.

Notably, the C-domain not only critically affects heparanase secretion and enzymatic activity, but also appears to mediate its signaling function. This is best exemplified by the 8-C protein variant. Thus, while over expression of the C-domain was accompanied by ~3-fold induction of Akt phosphorylation, over expression of the 8-C protein resulted in ~8-fold increase in

phospho-Akt levels (Fig. 4B), clearly delineating the C-domain as the molecular entity that mediates Akt activation by heparanase (Fig. 3D, 4B). Furthermore, xenografts produced by C-domain expressing cells appeared markedly enlarged compared with xenografts produced by control (mock) or TIM-barrel transfected cells and were similar in size to tumor xenografts produced by heparanase transfected cells (Fig. 5), possibly through enhanced angiogenesis. A pro-angiogenic effect is supported by marked induction of Akt phosphorylation in primary endothelial cells and improved endothelial cell organization into tube-like structures by the C-domain and 8-C proteins (Suppl. Fig. 2). Our tumor xenograft model shows, for the first time, that in some tumor systems (i.e., glioma) heparanase facilitates primary tumor progression regardless of its enzymatic activity.

The molecular mechanism utilized by heparanase to elicit signal transduction has not been resolved yet, but is thought to involve heparanase binding protein(s)/receptor(s). Employing binding studies, we have recently reported the existence of low-affinity, high abundant, as well as high-affinity, low abundant binding sites for heparanase (12). While the low-affinity binding sites of heparanase were identified as HSPGs, high-affinity binding sites were thought to be MPR or LRP, cell surface proteins implicated in heparanase uptake (14). Akt activation by heparanase was noted, however, in MPR-, and LRP-deficient cells (33), suggesting the existence of additional cell surface receptors that mediate the signaling function of heparanase. Indeed, cross-linking experiments consistently revealed the existence of two protein complexes of ~130 and ~170 kDa (Fig. 6), representing binding sites/receptors with molecular weights of ~110 and ~150 kDa, respectively, following subtraction of the labeled 20 kDa C-domain. Such molecular weights are not in accordance with proteins shown to bind heparanase (i.e., MPR, LRP, CD222) (12,14,37), likely representing a novel high affinity heparanase receptor. Enhanced C-domain cross-linking by heparin (Fig. 6) and the ability of heparin to augment Akt phosphorylation by heparanase (27) and C-domain (data not shown) support the possible function of these molecules as heparanase signaling receptors. While Akt is probably one downstream effector, the machinery underlying pro-tumorigenic function of heparanase or C-domain proteins is likely much more complexed. Gene and antibody arrays methodologies are currently underway in order to exploit the full repertoire of signaling pathways/genes being induced or repressed.

Taken together, we have identified a protein domain critical for heparanase enzymatic activity, secretion, and signaling. Pro-tumorigenic properties of the C-domain clearly reveal the biological significance of this domain, and support the notion that heparanase exerts enzymatic activity-independent functions. The C-domain, and particularly its unstructured flexible loop, appears as an attractive target for the development of a new class of heparanase inhibitors. Inhibiting heparanase enzymatic and non-enzymatic functions, applying, for example, N-acetylated glycol-split heparin (8) and C-domain neutralizing antibodies, respectively, is therefore expected to profoundly affect tumor progression and metastasis.

The predicated structure, still, awaits further conformation once the crystal structure of the molecule is resolved. Notably, non-enzymatic functions described here for the heparanase C-domain may be common with other enzymes exhibiting TIM-barrel/ C-terminal domain structure [see for example (41)]. Given the large number of such enzymes, it is conceivable that the function of at least few may be more diverse than currently recognized. Our findings, thus, may open new prospects in the appreciation of the function and biological significance of these enzymes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. A. Three dimensional model of heparanase

The model, including the 8 kDa (yellow) and 50 kDa (gray) protein subunits, amino acids critical for heparanase catalysis (Glu²²⁵ and Glu³⁴³, red), and heparin binding regions (Lys¹⁵⁸-Asp¹⁷¹ and Gln²⁷⁰-Lys²⁸⁰; cyan and green, respectively) is shown in the left and middle panels. A more detailed structure of the C-domain is shown in the right panel. The model illustrates eight β -strands, one of which is contributed by the 8 kDa subunit (yellow), arranged in two sheets (blue and orange) which are connected by an unstructured, flexible loop (arrow). **B-D. Intact C-domain is critical for heparanase enzymatic activity.** **B.** Heparanase (Hepa)-, TIM-barrel (TIMB)-, and heparanase deleted for the C-terminal 17 amino acids ($\Delta 17$)-, all as GS3 gene constructs, as well as C-domain- and control (Mock)- transfected JAR cells (2×10^6) were subjected to three freeze/thaw cycles and cell lysates were applied onto 35 mm dishes coated with ³⁵S-labeled ECM. Release of sulfate-labeled material was evaluated by 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 are eluted at $0.5 < K_{av} < 0.8$ (fractions 15–30) and represent heparanase generated degradation products. **C.** Co-transfection. JAR cells were transfected with control empty vector (Mock), heparanase (Hepa), or co-transfected with TIM-barrel (GS3) and C-domain gene constructs and heparanase activity was determined as described above. **D.** Point mutations. JAR cells were transfected with control empty vector (Mock), heparanase, or heparanase mutated at Phe⁵³¹, Val⁵³³, Ile⁵³⁴, Ala⁵³⁷, or Cys⁵⁴², all as GS3 gene constructs, and heparanase activity was determined as above. Representative activity assays are shown for the I534R and A537K mutations.

Figure 2. Intact C-domain is required for heparanase secretion

A. HEK 293 cells were stably transfected with control, empty vector (mock), or Myc-tagged wild type (Hepa), TIM-barrel (TIMB), and C-domain heparanase gene constructs, or heparanase deleted for its 17 C-terminal amino acids ($\Delta 17$). Cells were incubated (20 h, 37°C) without (–) or with heparin (+, 50 μ g/ml) under serum-free conditions; Total cell lysates were prepared and subjected to immunoblotting applying anti-Myc (upper panel) or anti-heparanase (1453; second panel) antibodies. Conditioned medium was collected from corresponding cultures and medium samples were similarly blotted with anti-Myc antibody (lower panel). **B.** Point mutations. HEK 293 cells were stably transfected with control (mock), heparanase (Hepa), or heparanase gene constructs mutated at isoleucine⁵³⁴ (I534R) or

alanine⁵³⁷ (A537K) and lysate (upper and second panels) and medium (lower panel) samples were similarly blotted with anti-Myc (upper and lower panels) and anti-heparanase (second panel) antibodies. **C.** Cellular localization. Control (Mock, upper panels), heparanase (Hepa, second panels), TIM-barrel (TIMB, third panels), and C-domain (fourth panels) transfected HEK 293 cells, as well as cells transfected with heparanase deleted for its C-terminal 17 amino acids ($\Delta 17$, fifth panels) or mutated at alanine 537 (A537K, lower panels) were triple stained for Myc-tag (left, red), the ER marker calnexin (second left, green), and merged with cell nuclei labeled with TO-PRO (third left, blue). Cells were similarly stained with anti-Myc-tag (third right, red), the Golgi marker wheat germ agglutinin-FITC (second right, green) and merged with cell nuclei labeled with TO-PRO (right most, blue). Note that all heparanase variants are found co-localized with the ER marker (third left, yellow), while only heparanase and the C-domain are co-localized with the Golgi marker (right most, yellow).

Figure 3. C-domain binding, localization, and Akt induction

A. Binding. U87 human glioma cells were left untreated (0) or incubated with purified heparanase (upper panel) and C-domain (second panel) proteins. Cell lysate were prepared at the time indicated and lysate samples were subjected to immunoblotting applying anti-heparanase (1453, upper panel) and anti-Myc (second panel) antibodies. Densitometry analysis of protein levels is shown graphically at the lower panel. **B.** Cells were incubated with purified proteins for 2h without (0) or with the indicated concentration of heparin (μg/ml) and lysate samples were analyzed as above. Densitometry analysis of protein levels is shown graphically at the lower panel. **C.** Localization. U87 cells were incubated with purified C-domain (upper panels) and heparanase (lower panels) proteins for 2h. Culture medium was then aspirated;

cells were washed, fix with cold methanol, and stained with anti-Myc (left panels, green) or anti-syndecan-4 (Syn4, red) antibodies. Merge images including nuclear labeling with TO-PRO (blue) are shown in the right panels. Co-localization of syndecan-4 and heparanase or C-domain protein appears yellow (right panels). **D.** Akt induction. HEK 293 cells were stably transfected with control (mock), heparanase (Hepa), C-domain, and TIM-barrel (TIMB) gene constructs and lysate samples were subjected to immunoblotting applying anti-phospho-Akt (p-Akt, upper panel) and anti-Akt (second panel) antibodies (left panels). Conditioned medium was collected from corresponding cultures and applied to parental HEK 293 cells for 30 min. Cell lysates were then prepared and subjected to immunoblotting applying anti-phospho-Akt (p-Akt, upper panel) and anti-Akt (second panel) antibodies (middle panels). Purified heparanase (Hepa) and C-domain proteins were applied to HS-deficient CHO-745 cells for 30 min and Akt phosphorylation levels were analyzed as above (right panels). Akt phosphorylation index was calculated by densitometry analysis of phosphorylated Akt levels divided by the total Akt values. Data is presented as fold increase of Akt phosphorylation compared with control, mock transfected cells, set arbitrary to a value of 1 (bottom panels).

Figure 4. Combining the 8 kDa and C-domain sequences markedly enhance protein secretion and signaling

A. Protein expression and secretion. HEK 293 cells were stably transfected with an empty vector (Mock), or Myc-tagged heparanase (Hepa), 8-C, or Cdomain gene constructs and cell lysates (left) and medium (right) samples were subjected to immunoblotting applying anti-Myc-tag antibody. **B.** Akt induction. FaDu pharynx carcinoma cells were stably transfected with control (Mock), heparanase (Hepa), or 8-C gene constructs and cell lysates were subjected to immunoblotting applying phospho-Akt (left, upper panel) and Akt (left, second panel) antibodies. Medium conditioned by stably transfected 293 cells (A) were applied to primary endothelial cells (HUVEC, middle panel) and Akt phosphorylation was evaluated as above. Kinetics. FaDu cells were left untreated (0) or were incubated with purified heparanase (Hepa) and 8-C proteins (1 μ g/ml) for the time indicated. Cell lysates were then prepared and Akt phosphorylation was evaluated as above (right panels). Akt phosphorylation index was calculated by densitometry analysis of phosphorylated Akt levels divided by the total Akt values. Data is presented as fold increase of Akt phosphorylation compared with control, mock transfected cells, set arbitrary to a value of 1 (bottom panels).

Figure 5. Heparanase C-domain facilitates cell proliferation and tumor xenograft progression
A. Cell proliferation. Control (mock), heparanase, and C-domain transfected HEK 293 cells were incubated with BrdU for 2 h and BrdU incorporation was evaluated by immunostaining. Shown are representative photomicrographs of BrdU incorporation in control (Mock, left panel), heparanase (Hepa, middle panel), and C-domain (second right) transfected cells. BrdU incorporation was quantified by counting BrdU-positive cells as percentage of total cells in at least eight different microscopic fields. At least 1000 cells were counted for each cell type (right panel). **B–C.** Tumor xenograft progression. Control (Mock), heparanase (Hepa), C-domain, and TIM-barrel (TIMB) transfected U87 cells were inoculated (5×10^6) subcutaneously at the flank of Balb/C nude mice ($n=7$). Xenograft development was measured

with a caliper and tumor volume was calculated as described under "Materials and Methods" (**B**). At the end of the experiment on day 51 animals were sacrificed, xenografts were harvested and weighted (**C**). **D**. Immunohistochemistry. Five micron sections of tumor xenograft produced by control (Mock, upper panels), heparanase (Hepa, second panels), C-domain (third panels), and TIM-barrel (TIMB, fourth panels) transfected U87 glioma cells were subjected to immunostaining applying anti-phospho-Akt (p-Akt, left) or anti-PECAM-1 (CD31, right) antibodies. The total number of blood vessels and blood vessels with lumen diameter above 40 microns was quantified by counting PECAM-1-positive vessels in at least eight different high power fields in each tumor xenograft (right most panels).

Figure 6. The C-domain/8-C proteins interact with high affinity binding site(s)/receptor(s)
A. C-domain binding. HeLa cells were incubated (2 h, 4°C) with increasing concentrations of ^{125}I -labeled C-domain protein without or with 100-fold excess of unlabeled heparanase and binding parameters were obtained by the Prism 4 software. **B.** Cross-linking. ^{125}I -labeled C-domain or 8-C proteins were added to the indicated cell line in the absence or presence of heparin (10 $\mu\text{g/ml}$) and 400 nM unlabeled heparanase protein. The cross linker sulfo-EGS (Pierce; 0.2 mM) was then added for 10 min, cells were washed with PBS, lysed, and subjected to SDS-PAGE followed by autoradiography. Note, the formation of two major protein complexes exhibiting molecular weights of ~130 and 170 kDa (arrows).