Structure-Function Approach Identifies a COOH-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 COOH-terminal domain (C-domain) that apparently is not 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 nonenzymatic 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. [Cancer Res 2009;69(5):1758–67]

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 several 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 the NH₂ terminus and a 50-kDa polypeptide at the COOH terminus that heterodimerize to form the active heparanase enzyme (9–11). Latent heparanase secretion is directed by an NH₂-terminal signal peptide (Met¹-Ala³⁵). The secreted protein interacts rapidly and efficiently with cell surface HS proteoglycan (HSPG), low-density lipoprotein receptor-related protein (LRP), and mannose 6-phosphate receptor (MPR; refs. 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) is 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. Nonenzymatic functions of heparanase include enhanced cell adhesion (22–24), and induction of p38 and Src phosphorylation (24) associated with vascular endothelial growth factor (25) and tissue factor (26) gene induction. Moreover, we have shown that exogenous addition of latent heparanase or its overexpression in tumor-derived cells stimulates Akt phosphorylation and phosphatidylinositol 3-kinase–dependent cell proliferation, 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.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Materials and Methods

A model of heparanase structure. A three-dimensional structure of constitutively active single-chain heparanase enzyme (GS3; ref. 21) was
generated by a protein structure prediction server\(^1\) (30). The server combines template-based and \textit{de novo} structure prediction methods. The software first screened for confident match to a protein of known structure using PSI-BLAST, FFA503, or 3D-jury software (31). The "significant hit" (the closest match) was found to be \(\alpha\)-1-arabinofuranosidase isolated from \textit{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 S1.

**Antibodies and reagents.** Anti-Myc-tag, anti-Akt, anti-syndecan-4, and anti-calnexin antibodies were purchased from Santa Cruz Biotechnology. Anti-phospho-Akt (Ser\(^{373}\)) antibody was purchased from Cell Signaling Technology. 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; ref. 25). Bromodeoxyuridine (BrdUrd) was purchased from GE Healthcare and anti-BrdUrd monoclonal antibody-horseradish peroxidase conjugate was purchased from Roche. Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin was purchased from Alomone Labs 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.

**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. FaDu human pharynx carcinoma cells were kindly provided by Dr. Eben L. Rosenthal (University of Alabama at Birmingham, Birmingham, AL; ref. 32). Cells were grown in DMEM (Biological Industries) supplemented with 10% FCS 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, CA) and grown in RPMI 1640 (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 extracts, 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 mmol/L HEPES, 0.2% bovine serum albumin) containing increasing concentrations of \(^{125}\)I-C-domain in the presence or absence of 400 to 2000 nmol/L of unlabeled heparanase or C-domain proteins. Cells were then washed with ice-cold PBS, solubilized with 200 \(\mu\)L of 1 mol/L NaOH, and counted in a gamma counter. Binding variables \((K_D\) and \(B_{max}\)) were obtained by the Prism 4 software (GraphPad Software; ref. 12). For cross-linking experiments, cells were grown in 60-mm dishes and incubated for 2 h on ice with binding buffer containing 4 nmol/L iodinated C-domain or 8-C proteins in the presence and absence of heparin (10 \(\mu\)g/mL) or 400 nmol/L of unlabeled heparanase or C-domain proteins. The cross-linker sulfo-EGS (0.2 mmol/L; Pierce) was then added for 10 min followed by quenching with 50 mmol/L 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-transfected, heparanase-transfected, C-domain–transfected, and TIM-barrel–transfected U87 human glioma cells were detached with trypsin/EDTA, washed with PBS, and brought to a concentration of \(5 \times 10^6/mL\). Cell suspension (\(5 \times 10^4/0.1\) mL) was inoculated s.c. at the right flank of 5-wk-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-\(\mu\)m sections were subjected to immunostaining with anti-phospho-Akt and anti-PECAM-1 antibodies using the Envision kit, according to the manufacturer's (Dako) instructions, as described (24, 25). All animal experiments were approved by the Animal Care Committee of the Technion (Haifa, Israel).

**Statistics.** Data are presented as mean ± 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 thrice with similar results.

**Results**

**Three-dimensional model of active heparanase.** A three-dimensional structure of active heparanase (GS3; Supplementary Fig. S1B) was obtained based on sequence and structure resemblance to \(\alpha\)-1-arabinofuranosidase enzyme from \textit{Geobacillus stearothermophilus} T-6 (Fig. 1A; ref. 31). 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 (Glu\(^{223}\) and Glu\(^{270}\); Fig. 1A, red), as well as heparin/HS binding regions (Lys\(^{158}\)–Asp\(^{171}\) and Gln\(^{270}\)–Lys\(^{380}\); Fig. 1A, cyan and green, respectively; ref. 20), were situated in close proximity to the micropocket active site, corroborating the relevance of the model (Fig. 1A, left panel). Notably, the structure also delineates a COOH-terminal fold positioned next to the TIM-barrel fold. The C-domain seems to comprise eight \(\beta\)-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 \(\beta\)-sheets. Notably, one of the C-domain \(\beta\)-strands is contributed, apparently, by the 8-kDa protein subunit (Fig. 1A, right panel, yellow), suggesting that the protein NH\(_3\) terminus plays a structural role in the establishment of the TIM-barrel (29) and C-domain folds.

The C-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 (i.e., the TIM-barrel and C-domain regions) mediate enzymatic and nonenzymatic functions of heparanase, respectively. To examine this possibility, we designed gene constructs carrying Myc-tagged wild-type (amino acids 36–543), TIM-barrel (amino acids 36–417), and C-domain (amino acids 413–543) heparanase variants (Supplementary Fig. S1A, B). 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 (Supplementary Fig. S2A). We suspected that the lack of enzymatic activity is due to impaired protein secretion shown previously to be required for the delivery
Likewise, deletion of 17 amino acid residues from the heparanase COOH terminus (17; GS3) completely abolished heparanase enzymatic activity, and a similar result was observed when the deletion was constructed in the GS3 backbone [Fig. 1B, A17 (GS3)]. Likewise, deletion of the unstructured loop (Leu483-Pro509; Supplementary Fig. S2A) completely abolished heparanase enzymatic activity, and a similar result was observed when the deletion was constructed in the GS3 backbone [Fig. 1B, A17 (GS3)]. Moreover, point mutations of evolutionary conserved amino acid residues Phe531, Val533, Ile534, Ala537, and Cys542 (Supplementary Fig. S1C, red-labeled amino acids) resulted in inactive heparanase activity (Fig. 1D; Supplementary Table S2) and hightlighted the importance of these residues for heparanase enzymatic activity. Heparanase activity was determined as described above. D, point mutations. JAR cells were transfected with control empty vector (Mock), heparanase, or cotransfected 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 described above. Representative activity assays are shown for the IS34R and AS37K mutations.

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 seemed to be expressed at lower levels (Fig. 2A, top panel). The relatively low signal observed in the lysate of cells transfected with wild-type heparanase (Fig. 2A, top panel) 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 seemed unprocessed (Fig. 2A, second panel) and was undetected in the cell conditioned medium even in the presence of heparin, which enhances the accumulation of heparanase extracellularly (Fig. 2A, bottom panel, TIMB; +; ref. 13). Likewise, deletion of 17 amino acid residues from the heparanase

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 (Lys415-Asp420 and Gin469-Lys470; 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 α-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 to D, intact C-domain is critical for heparanase enzymatic activity. B, heparanase (Hepa)–transfected, TIM-barrel (TIMB)–transfected, and heparanase deleted for the COOH-terminal 17 amino acids (A17)–transfected, all as GS3 gene constructs, as well as C-domain–transfected and control-transfected (Mock) JAR cells (2 x 10⁶) 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 was counted in a γ-scintillation counter. Degradation fragments of HS side chains are eluted at 0.5 < Kav < 0.8 (fractions 15–30) and represent heparanase-generated degradation products. C, cotransfection. JAR cells were transfected with control empty vector (Mock) and heparanase or cotransfected 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 described above. Representative activity assays are shown for the IS34R and AS37K mutations.

of latent heparanase to late endosomes/lysosomes and its subsequent processing and activation by lysosomal cathepsins (12–14, 20). 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 (GS3)], whereas the full-length GS3-heparanase was highly active [Fig. 1B, Hepa (GS3)]. 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 used to identify regions and amino acids critical for this function of the C-domain. Notably, deletion of the last COOH terminus 17 amino acids (Phe531–Ile534; A17; Supplementary Fig. S2A) completely abolished heparanase enzymatic activity, and a similar result was observed when the deletion was constructed in the GS3 backbone [Fig. 1B, A17 (GS3)]. Likewise, deletion of the unstructured loop (Leu483-Pro509; Supplementary Fig. S1C, red box) resulted in an inactive heparanase enzyme (Supplementary Table S2; Supplementary Fig. S2A).
COOH terminus markedly attenuated protein secretion (Fig. 2A, A17, bottom panel), and point mutations (Phe531, Val533, Ile534, Ala537, and Cys542) that yielded proteins devoid of enzymatic activity (Fig. 1D) also failed to get processed (Fig. 2B, second panel) or secreted (Fig. 2B, bottom panel). In contrast, the C-domain protein variant was noted to be secreted, albeit less efficient than wild-type heparanase (Fig. 2A, bottom 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-transfected (mock; top panels), heparanase-transfected (second panels), TIM-barrel–transfected (third panels), C-domain–transfected (fourth panels), Δ17-transfected (fifth panels), and heparanase mutated at alanine537–transfected (A537K; bottom panels) HEK 293 cells were triple stained applying anti-Myc-tag (left, red), anti-calnexin [an estrogen receptor (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, colocalizing with calnexin (third left, yellow). In striking contrast, only the heparanase and C-domain proteins, shown to be secreted (Fig. 2A, bottom panel), were noted to colocalize 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 COOH 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).

Figure 3. C-domain binding, localization, and Akt induction. A, binding. U87 human glioma cells were left untreated (0) or incubated with purified heparanase (top 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; top panel) and anti-Myc (second panel) antibodies. Densitometry analysis of protein levels is shown graphically at the bottom panel. B, cells were incubated with purified proteins for 2 h 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 bottom panel. C, localization. U87 cells were incubated with purified C-domain (top panels) and heparanase (bottom panels) proteins for 2 h. Culture medium was then aspirated; cells were washed, fixed with cold methanol, and stained with anti-Myc (left panels, green) and anti-syndecan-4 (Syn4, red) antibodies. Right panels, merge images including nuclear labeling with TO-PRO (blue). Colocalization of syndecan-4 and heparanase or C-domain protein appears yellow. D, Akt induction. HEK 293 cells were stably transfected with control (Mock), heparanase, C-domain, and TIM-barrel gene constructs and lysate samples were subjected to immunoblotting applying anti-phospho-Akt (p-Akt, top 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 (top panel) and anti-Akt (second panel) antibodies (middle panels). Right panels, purified heparanase and C-domain proteins were applied to HS-deficient CHO-745 cells for 30 min and Akt phosphorylation levels were analyzed as above. Akt phosphorylation index was calculated by densitometry analysis of phosphorylated Akt levels divided by the total Akt values (bottom panels). Data are presented as fold increase of Akt phosphorylation compared with control, mock-transfected cells, set arbitrary to a value of 1.

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 that 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 (Mock), heparanase, C-domain, and TIM-barrel gene constructs and lysate samples were subjected to immunoblotting applying anti-phospho-Akt (p-Akt, top 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 (top panel) and anti-Akt (second panel) antibodies (middle panels). Right panels, purified heparanase and C-domain proteins were applied to HS-deficient CHO-745 cells for 30 min and Akt phosphorylation levels were analyzed as above. Akt phosphorylation index was calculated by densitometry analysis of phosphorylated Akt levels divided by the total Akt values (bottom panels). Data are presented as fold increase of Akt phosphorylation compared with control, mock-transfected cells, set arbitrary to a value of 1.

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Heparanase C-Domain

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, 8-C, or C-domain gene constructs and cell lysates (left) or 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, or 8-C gene constructs and cell lysates were subjected to immunoblotting applying phospho-Akt (left, top panel) and Akt (left, second panel) antibodies. Medium conditioned by stably transfected 293 cells (A) was 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 and 8-C proteins (1 μg/mL) for the time indicated (right panels). Cell lysates were then prepared and Akt phosphorylation was evaluated as above. Akt phosphorylation index was calculated by densitometry analysis of phosphorylated Akt levels divided by the total Akt values (bottom panels). Data are presented as fold increase of Akt phosphorylation compared with control, mock-transfected cells, set arbitrary to a value of 1.
control (mock transfected) cells ($P < 0.005$; Fig. 5A). To further substantiate this finding, we examined the progression of tumor xenografts produced by mock-transfected, heparanase-transfected, TIM-barrel–transfected, 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 control, mock-transfected cells (Fig. 5B), in agreement with previous studies using this model system (24). At the end of the experiment on day 51, xenograft produced by heparanase-transfected cells was 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 controls. Notably, tumor xenografts produced by C-domain–expressing U87 cells seemed comparable or even slightly bigger than those produced by heparanase-transfected cells, yielding tumors that were 8-fold bigger in volume ($P < 0.007$; 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 seemed comparable with controls, in agreement with the lack of enzymatic activity, secretion, and signaling capability of this protein variant (Figs. 1–3). Notably, phosphorylated Akt was highly abundant in xenografts produced by heparanase-transfected and C-domain–transfected cells (Fig. 5D, left, second and third panels) compared with xenograft produced by mock-transfected and TIM-barrel–transfected cells (Fig. 5D, left, top and fourth panels). In addition, tumor angiogenesis was markedly stimulated in xenografts produced by heparanase-expressing and C-domain–expressing U87 cells compared with mock-transfected and TIM-barrel–transfected cells (Fig. 5D, right panels). This agrees with the observed induction of Akt and tube-like structures in endothelial cells by the C-domain (Fig. 4B; Supplementary Fig. S2C) and the proangiogenic capacity of heparanase noted in several experimental settings (5, 25, 28, 35, 36). These results imply that the protumorigenic and proangiogenic 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). To explore this possibility, purified recombinant C-domain was iodinated to a high specific activity and binding experiments were performed. Binding of [125I]-labeled C-domain to HeLa cells exhibited linearity at low concentrations and approached saturation at high levels of [125I]-labeled C-domain (Fig. 6A). Analyses of C-domain binding by the Prism 4 software suggested the existence of high-affinity ($K_d = 7 \text{ nmol/L}$), low-abundant ($B_{\text{max}} = 170 \times 10^3$) binding sites. Such affinity is similar to that observed for wild-type heparanase ($K_d = 2–4 \text{ nmol/L}$; ref. 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 $\sim 130$ and $\sim 170$ kDa that interact with the heparanase C-domain (Fig. 6C, arrows). The molecular weight of cell surface proteins shown previously to bind heparanase [i.e., LRPI (85 kDa; refs. 12, 14), cation-dependent MPR (46 kDa; refs. 12, 14), and cation-independent MPR (300 kDa; ref. 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/receptor(s) 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 seems to enfold the 50-kDa subunit (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 NH$_2$ terminus of the 8-kDa subunit first generates a β-strand of the C-domain (Fig. 1A, right, yellow) and then yields the first β/α/βfold 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 only 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-domain that seems 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 nonenzymatic (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, cotransfection of TIM-barrel and C-domain gene constructs failed to produce an active enzyme (Fig. 1C), suggesting that the two domains must be coassembled while being synthesized, unlike cotransfection of the 8- and 50-kDa subunits that yields an active enzyme (10, 11). Thus, the C-domain seems 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 COOH terminus (Δ17; Fig. 2). Furthermore, point mutations of conserved amino acids at this region eradicated heparanase secretion (Fig. 2; Supplementary Table S2; ref. 40), suggesting that relatively modest alterations in the structure of the C-domain significantly affect the integrity/conformation of the heparanase molecule. Although the C-domain seemed to get properly folded, judged by its localization to the Golgi apparatus, its secretion seemed modest compared with wild-type heparanase, and more strictly dependent on functional Hsp90 chaperon (Supplementary Fig. S2B). Introducing the β-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 under way.

Notably, the C-domain not only critically affects heparanase secretion and enzymatic activity but also seems to mediate its signaling function. This is best exemplified by the 8-C protein variant. Thus, whereas overexpression of the C-domain was accompanied by ~3-fold induction of Akt phosphorylation, overexpression 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 (Figs. 3D and 4B). Furthermore, xenografts produce by C-domain-expressing cells seemed markedly enlarged compared with xenografts produced by control-transfected (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 proangiogenic 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 (Supplementary Fig. S2). 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 used by heparanase to elicit signal transduction has not been resolved yet but is thought to involve heparanase binding protein(s)/receptor(s). Using 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). Whereas 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, and CD222; refs. 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. Although Akt is probably one downstream effector, the machinery underlying protumorigenic function of heparanase or C-domain proteins is likely much more complex. Gene and antibody array methodologies are currently under way 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. Protumorigenic 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, seems as an attractive target for the development of a new class of heparanase inhibitors. Inhibiting heparanase enzymatic and nonenzymatic 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 predicted structure, still, awaits further conformation once the crystal structure of the molecule is resolved. Notably, nonenzymatic functions described here for the heparanase C-domain may be common with other enzymes exhibiting TIM-barrel/C-domain structure (see for example ref. 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Heparanase C-Domain
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