Identification and Characterization of Heparin/Heparan Sulfate Binding Domains of the Endoglycosidase Heparanase*

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The endo-β-glucuronidase, heparanase, is an enzyme that cleaves heparan sulfate at specific intra-chain sites, yielding heparan sulfate fragments with appreciable size and biological activities. Heparanase activity has been traditionally correlated with cell invasion associated with cancer metastasis, angiogenesis, and inflammation. In addition, heparanase up-regulation has been documented in a variety of primary human tumors, correlating with increased vascular density and poor postoperative survival, suggesting that heparanase may be considered as a target for anticancer drugs. In an attempt to identify the protein motif that would serve as a target for the development of heparanase inhibitors, we looked for protein domains that mediate the interaction of heparanase with its heparan sulfate substrate. We have identified three potential heparin binding domains and provided evidence that one of these is mapped at the N terminus of the 50-kDa active heparanase subunit. A peptide corresponding to this region (Lys158–Asp171) physically associates with heparin and heparan sulfate. Moreover, the peptide inhibited heparanase enzymatic activity in a dose-responsive manner, presumably through competition with the heparan sulfate substrate. Furthermore, antibodies directed to this region inhibited heparanase activity, and a deletion construct lacking this domain exhibited no enzymatic activity. NMR titration experiments confirmed residues Lys158–Asn162 as amino acids that firmly bound heparin. Deletion of a second heparin binding domain sequence (Gln194–Lys206) yielded an inactive enzyme that failed to interact with cell surface heparan sulfate and hence accumulated in the culture medium of transfected HEK 293 cells to exceptionally high levels. The two heparin/heparan sulfate recognition domains are potentially attractive targets for the development of heparanase inhibitors.

Heparan-sulfate proteoglycans (HSPGs) are members of the glycosaminoglycan family, a class of molecules that consists of unbranched, repeated disaccharide units attached to a core protein. Proteoglycans are present essentially in every tissue compartment, localized in the extracellular matrix (ECM), on the cell surface, intracellularly in granules, and even in the nucleus (1–3). Virtually all cells express at least one proteoglycan on their surface. Membrane-associated proteoglycans are mostly HS that can be either transmembrane (syndecan) or glycosylphosphatidylinositol-anchored (glypican). From mice to worms, embryos that lack HS die during gastrulation (3), suggesting a critical developmental role for HSPGs. HSPGs play key roles in numerous biological settings, including cytokinetization, cell/cell, and cell/ECM interactions (4–6).

For biological function, HSPGs exert their multiple functional repertoire via several distinct mechanisms that combine structural, biochemical, and regulatory aspects. By interacting with other macromolecules such as laminin, fibronectin, and collagen IV, HSPGs contribute to the structural integrity, self-assembly, and insolubility of the ECM and basement membrane. ECM components are, however, only one class of HSPG-binding proteins. In fact, numerous enzymes, growth factors, cytokines, and chemokines are sequestered by HSPGs on the cell surface and ECM, most often as an inactive reservoir (7, 8). Cleavage of HSPGs would ultimately release these polypeptides and convert them into bioactive mediators, thus ensuring rapid tissue response to local environmental alterations. The protein core of HSPGs is susceptible to cleavage by several classes of proteases (9–11). A more delicate way to modify HSPGs is provided by the endo-β-glucuronidase, heparanase, an enzyme that cleaves HS at specific intra-chain sites, yielding HS fragments with appreciable size and biological activities (12–15).

Heparanase activity has been correlated traditionally with cell invasion associated with cancer metastasis, angiogenesis, and inflammation (16–19). A proof of concept for this notion has been provided recently by applying short interfering RNA and ribozyme technologies (20). In addition, heparanase up-regulation has been documented in a variety of primary human tumors correlating with increased vascular density and poor postoperative survival, in some cases (21–25). Heparanase overexpression has also been noted in several pathologies other than cancer and inflammation (26–29), suggesting a broader pathological repertoire than originally thought. The heparanase cDNA encodes for a protein of 543 amino acids that undergoes proteolytic processing at two potential cleavage sites, Glu109–Ser110 and Gln157–Lys158, yielding an 8-kDa

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‡ The abbreviations used are: HSPGs, heparan sulfate proteoglycans; HS, heparan sulfate; ECM, extracellular matrix; WT, wild type; Tricine, [2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; CHO, Chinese hamster ovary; CM, conditioned medium; PBS, phosphate-buffered saline; HA, hyaluronic acid; GS, glycol-split.

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polypeptide at the N terminus and a 50-kDa polypeptide at the C terminus that heterodimerize to form an active heparanase enzyme (30–33). The present study was undertaken to identify functional domains that would serve as targets for drug development. Based on published consensus sequences that mediate the interaction between polypeptides and heparin, we have identified three potential heparin binding domains mapped at Lys158–Asp171, Glu270–Lys280, and Lys411–Arg432 of the 50-kDa heparanase subunit. Here we provide evidence that the Lys158–Asp171 N-terminal peptide physically associates with heparin and HS more strongly than the other two peptides. Moreover, the peptide inhibits heparanase enzymatic activity in a dose-responsive manner, presumably through competition with the HS substrate. Furthermore, antibodies directed to this region inhibit heparanase activity (34), and a deletion construct lacking this domain exhibits no enzymatic activity. NMR titration experiments performed with a synthetic pentasaccharide confirmed residues Lys158–Asn162 as the amino acids important for heparin binding, identifying this recognition domain as a potentially attractive target for the development of heparanase inhibitors.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents—**The rabbit anti-heparanase antibody (1435) recognizing the 65- and 50-kDa heparanase proteins and the rabbit anti-8-kDa (810) antibody have been characterized previously (31, 34). The anti-Myc epitope tag (sc-40) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). 10–20% Tris-Tricine gels were prepared, and 30 % polyacrylamide gels were run. The gels were then fixed and probed with the appropriate antibody followed by horse- radish peroxidase-conjugated antibody (Pierce) and visualized by chemiluminescence (ECL, Amersham Biosciences). A 65-kDa polypeptide at the N terminus and a 50-kDa polypeptide at the C terminus that heterodimerize to form an active heparanase enzyme (30–33). For inhibition studies, heparanase (20 ng) was incubated (2 h, 37 °C) with 35S-labeled ECM in the presence of the indicated concentration of peptides. For heparanase inhibition studies with intact cells, B16 melanoma cells (2 × 106) were resuspended in RPMI medium and incubated (18 h, 37 °C) with 35S-labeled ECM in the presence of the indicated peptide concentration.

To evaluate heparanase activity in cell extracts, heparanase-depleted cells were incubated (2 h, 4 °C) with 8-kDa (0.1 mg/ml) heparanase and ovalbumin (compound ST1514) was kindly provided by Claudio Pisano (Signalling Genetics). Bovine lung HS was kindly provided by Dr. Jin-Ping Li (Biomedical Sciences, University of California, Los Angeles). DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) was purchased from Sigma (St. Louis, MO). Bovine serum albumin (fraction V) and T麻辣oo (M) were purchased from Sigma. 10,000 transfectant pools were obtained after 2–3 weeks and used for further experiments.

**Heparanase Activity Assay—**Preparation of ECM-coated 35-mm dishes and determination of heparanase activity were performed as described (14, 31). For inhibition studies, heparanase (20 ng) was incubated (2 h, 37 °C) with 35S-labeled ECM in the presence of the indicated concentration of peptides. For heparanase inhibition studies with intact cells, B16 melanoma cells (2 × 106) were resuspended in RPMI medium and incubated (18 h, 37 °C) with 35S-labeled ECM in the presence of the indicated peptide concentration. For inhibition studies with intact cells, B16 melanoma cells (2 × 106) were resuspended in RPMI medium and incubated (18 h, 37 °C) with 35S-labeled ECM in the presence of the indicated peptide concentration. For inhibition studies with intact cells, B16 melanoma cells (2 × 106) were resuspended in RPMI medium and incubated (18 h, 37 °C) with 35S-labeled ECM in the presence of the indicated peptide concentration.
Identification of putative heparin binding domains in the heparanase protein. Schematic diagram of heparanase structure and processing and the localization of the heparin-binding candidate sequences. The sequence Lys\(^157\)–Asn\(^{162}\) and Pro\(^{271}\)–Met\(^{278}\) matches the XBBXXB and XBBBXXBX consensus sequences, respectively, and the Lys\(^{411}\)–Arg\(^{422}\) sequence is enriched with basic amino acids. s.p., signal peptide.

**RESULTS**

Identification of Heparin Binding domains—Sequence alignment of heparin binding domains from several proteins had led to the characterization of two consensus sequences, XBBXXB and XBBBXXBX, where B is basic and X is hydrophilic (neutral and hydrophobic) amino acid (43). Analysis of the primary protein sequence of heparanase revealed the existence of two domains that match the consensus sequence for heparin binding (Fig. 1). These are mapped at Lys\(^{158}\)–Asp\(^{171}\) (KKDC) peptide or its scrambled control peptide. Following incubation (1 h, 4 °C), cells were washed, lysed, and counted with y-counter.

**NMR Spectra**—NMR samples of the unlabeled Lys\(^{158}\)–Asp\(^{171}\) peptide (KKDC) and the corresponding scrambled sequence were dissolved in 20 mM sodium phosphate buffer, pH 5.8, supplemented with 0.15 M NaCl and 10% D\(_2\)O. All NMR spectra were recorded on a Bruker Avance 600 spectrometer equipped with a quadrupole resonance proton-carbon-nitrogen-deuterium 5-mm probe (Bruker TCI cryo-probe\©), with cooled coil and preamplifier and using a self-shielded z gradient coil. Spectra were acquired nonspinning at temperatures of 295 K. For two-dimensional homonuclear \(^1\)H experiments, DQF-COSY, TOCSY, and ROESY were performed according to the Bruker library pulse sequences. For ROESY, mixing times of 200 ms and a spin-lock of 150 ms were used. COSY/TOCSY and ROESY experiments were acquired using 8 and 16 scans per series of 1Kx512 data points, respectively. Data were zero filled in F1, and a shifted squared cosine function was applied prior to Fourier transformation. Water suppression was carried out using excitation sculpting sequence with gradients (42). Two-dimensional \(^1\)H–\(^15\)N HSQC experiments were acquired with 96 scans for each of 320 increments on F1 dimension. The matrix size 1Kx512 was zero filled to 4Kx2K by application of a squared cosine window. Spectra were acquired nonspinning at temperatures of 295 K.

**Titrations**—For titrations of both the KKDC and scrambled control peptide with the synthetic pentasaccharide: GlcNS\(_o\), 6SO\(_o\), GlcA-GlcNS\(_o\), 3,6SO\(_o\), IdoA2SO\(_o\), GlcNS\(_o\), 6SO\(_o\), OMe (AGA\(^\ast\)A) (Sanofi-Synthelabo\©), peptides concentration were 1.3 mM in the buffer described above. To minimize dilution, titration steps were carried out by adding small quantities (8 \(\mu\)l) of a concentrated (58 mM) solution of AGA\(^\ast\)A pentasaccharide to reach a peptide/pentasaccharide molar ratio of 1:5 (asterisk indicates a trisulfated saccharide). Proton and nitrogen chemical shifts of the amide cross-peaks of HSQC spectra were measured. Values were referred to trimethylsilyl propionate sodium salt and urea for proton and nitrogen, respectively. For each NH cross-peak, the composite chemical shift variation vector \(\Delta CS = [\Delta CS_{HN} + \Delta CS_{N}]/2\) \((\mu \text{Hz})\) for both peptides was calculated.

**DISCUSSION**

**The KKDC (Lys\(^{158}\)–Asp\(^{171}\)) Peptide Physically Interacts with HS**—In order to study the relevance of the above sequences for heparanase/HS interaction, we synthesized three peptides that contained the putative heparin binding domain (Fig. 2A). Peptides (50 \(\mu\)M), or their scrambled (Scr) counterparts, were incubated with heparin-Sepharose beads (2 h, 4 °C), and the bound peptides were visualized by Tris-Tricine gel electrophoresis and Coomassie Blue staining. The KKDC peptide was found to associate specifically with the heparanase beads, whereas no such binding was observed with its control scrambled peptide (Fig. 2B, heparin). In contrast, peptides KKLR (Fig. 2B) and QPLK (data not shown) exhibited only very weak interaction with heparin. As the QPLK peptide is shorter and spans only 10 amino acids, we synthesized peptides containing additional amino acids, N-KLYGPDVGGPRRKTAKMLK or C-terminal (QPRRKTAKMLKSFLKA, QPKA) (the boldface letters represent amino acids added to the N and C terminus core peptide QPLK in Fig. 2A) to the core 10-amino acid QPLK sequence. The latter 16 amino acids peptide exhibited low but detectable interaction with heparin (Fig. 2B). Addition of the C-terminal cysteine residue shown to promote peptide dimerization and to improve the interaction with heparin (44) only slightly enhanced heparin binding (Fig. 2B, QPKAC). These results suggest that only the KKDC peptide exhibits high affinity to and physically interacts with heparin. Because heparin contains a higher number of sulfate groups and iduronic acid residues and its structure does not fully reflect the organization of HS, it was crucial to examine further the ability of the KKDC peptide to interact with HS. As demonstrated in Fig. 2B, the KKDC peptide specifically binds to HS-Sepharose beads, indicating that the peptide is capable of interacting with both heparin and HS to a similar extent.

**The KKDC Peptide Inhibits Heparanase Enzymatic Activity**—Giving the specific interaction of the KKDC peptide with HS, we hypothesized that it may compete with heparanase for interaction with HSPGs and thus inhibit its enzymatic activity. In order to test this possibility, the KKDC, or control scrambled (Scr) peptides, were applied onto \(^35\)S-labeled HS-containing ECM substrate and incubated (2 h, 37 °C) with active mouse (mHpa, Fig. 2C) or human (hHpa, Fig. 2D) heparanase. The KKDC peptide was noted to inhibit significantly the enzymatic activity (i.e., release of HS degradation fragments from ECM) of both the

**Fig. 1.** Identification of putative heparin binding domains in the heparanase protein.
mouse and human heparanase in a dose-dependent manner, whereas the control scrambled peptide did not. Moreover, the peptide efficiently neutralized endogenous heparanase enzymatic activity in B16 melanoma cells (Fig. 2 E). In contrast, the QPLK and KKLK peptides had no such inhibitory effect on heparanase activity (data not shown), correlating with the very low heparin binding abilities of these peptides (Fig. 2 B, heparin).

The KKDC Peptide Interferes with Heparin Binding to HS—Next, we evaluated the ability of the KKDC peptide to interfere with heparanase binding to HS. For this purpose, active heparanase (20 ng), which exhibits a high affinity toward its HS substrate, was incubated (2 h, 4 °C) with HS-Sepharose beads in the absence or presence of increasing concentrations of the KKDC or control scrambled (Sc) peptide. Following incubation (1 h, 4 °C), cells were washed, lysed, and counted. Note that the KKDC peptide significantly inhibits heparanase binding to intact cells.

Fig. 2. The peptide KKDC physically interacts with heparin and HS and inhibits heparanase enzymatic activity. A, peptide sequences synthesized to contain each of the putative heparin binding regions. The peptides are named according to the first and last two amino acids (boldface). A represents the deletion construct of the corresponding sequence. B, heparin/HS binding. Peptides or their control scrambled (Scr) peptides were incubated (2 h, 4 °C) with heparin- (left) or HS-Sepharose (right) beads and washed with 0.35 M NaCl to reduce nonspecific binding. Sample buffer was then added, and following boiling for 5 min, samples were loaded on Tris-Tricine gel, and bound peptides were detected with Coomassie Blue staining. Note the high affinity and physical interaction of the KKDC peptide with heparin and HS. C–E, enzymatic activity. Mouse (C) and human (D) heparanase (20 ng) or B16 melanoma cells (2 × 10⁶, E) were incubated (2 h, 37 °C) with 35S-labeled ECM in the presence of the indicated concentrations of the KKDC peptide or its control scrambled (Sc) peptide. Labeled degradation products released into the incubation medium were analyzed by gel filtration, as described under “Experimental Procedures.” Note the inhibition of heparanase activity by the KKDC peptide in a dose-responsive manner. F, inhibition of heparanase binding to HS-conjugated beads. Purified mouse heparanase (20 ng) was incubated (2 h, 4 °C) with HS beads in the absence (0) or presence of the indicated concentrations of the KKDC peptide or its control scrambled (Sc) peptide. Following 2 washes with 0.6 M NaCl, bound heparanase was detected by immunoblotting with anti-heparanase antibodies. Note inhibition of heparanase binding to HS in the presence of the KKDC peptide. G and H, binding of iodinated heparanase to CHO cells. G, iodinated heparanase was added to CHO cells in the absence (0) or presence of the indicated concentration of heparin (μg/ml). Following incubation (1 h, 4 °C), cells were washed, lysed, and counted. H, iodinated heparanase was added to CHO cells together with the indicated concentrations (μM) of KKDC or its control scrambled peptide. Following incubation (1 h, 4 °C) cells were washed, lysed, and counted. Note that the KKDC peptide significantly inhibits heparanase binding to intact cells.
competing peptide (0 μM). The KKDC peptide at a concentration of 25 μM or higher markedly inhibited the association of heparanase with HS (Fig. 2F, KKDC), whereas the scrambled peptide had no such effect at the same concentrations (Fig. 2F, sc). Previously, we have demonstrated that binding of the 65-kDa latent heparanase to cell surface HS is followed by uptake and processing into a 50-kDa active enzyme (41). In order to verify the possibility that the KKDC peptide will inhibit heparanase binding to cell surface HS, we employed 125I-labeled heparanase. We first confirmed that heparanase iodination did not harm its ability to interact with heparin/HS. To this end, iodinated heparanase was added to CHO cells in the absence or presence of the indicated concentrations (micromolar) of heparin. Thus, iodination did not harm its ability to interact with heparin/HS. To this end, iodinated heparanase was added to CHO cells in the absence or presence of the indicated concentrations (micromolar) of heparin. Thus, iodination did not harm its ability to interact with heparin/HS.

### Deletion Mutants Lacking Heparin Binding Domains Exhibit No Enzymatic Activity

In order to study further the importance of the sequences identified to contain the heparin-binding motif, we have undertaken a different approach and created deletion mutants lacking the relevant sequences (Fig. 3). Deletion mutants were created for each of the three regions in the full-length 65-kDa heparanase, and the gene constructs were stably transfected into HEK 293 cells. 293 cells transfected with the wild type (WT) construct exhibited high levels of heparanase enzymatic activity (Fig. 3A, 65WT). By contrast, no enzymatic activity of heparanase was detected in each of the deletion mutants (Fig. 3A, 65Δ15, 65Δ10, and 65Δ20). Cell lysates were next subjected to immunoblot analysis with anti-heparanase antibodies. High levels of the 50-kDa heparanase protein were detected in 293 cells transfected with the full-length WT heparanase gene construct (Fig. 3B, 65WT, Lysate), in agreement with high levels of heparanase activity in these cells (Fig. 3A, 65WT). Most interestingly, however, the 50-kDa heparanase protein was not detected in lysates of cells transfected with the deletion constructs (Fig. 3B, 65Δ15; 65Δ10; 65Δ20, Lysate). Instead, the 65-kDa form of heparanase was highly abundant, suggesting that the protein failed to be processed into the 50- and 8-kDa active heterodimer form (Fig. 3B, Lysate), which explains the lack of enzymatic activity in cells transfected with these deletion constructs (Fig. 3A).

### QPLK Deletion Mutant Accumulates in the Culture Medium of Stably Transfected HEK 293 Cells—Next, we evaluated heparanase secretion by exposing the cell conditioned medium (CM) to immunoblot analysis with anti-heparanase (Fig. 3B, upper panel) and anti-Myc tag antibodies (lower panel). Low levels of the 65-kDa heparanase were detected in the CM of cells transfected with the WT construct (Fig. 3B, 65WT, Me-
Heparin/HS Binding Domains of Heparanase

dium), in agreement with the notion that heparanase is subjected to rapid uptake mediated by cell surface HSPGs and thus does not accumulate to high levels extracellularly (41). By contrast, exceptionally high levels of heparanase secretion were detected in the CM of cells transfected with the 65Δ10 gene construct (Fig. 3B, 65Δ10, Medium), whereas cells transfected with the 65Δ15 and 65Δ20 variants did not secrete detectable amounts of heparanase (Fig. 3B, Medium). The lack of heparanase activity in cells transfected with the deletion constructs is most likely due to lack of heparanase processing which, in turn, seems to result from a defect in heparanase secretion (65Δ15; 65Δ20) and uptake (65Δ10) (see below). Moreover, we confirmed similar synthesis levels of heparanase by employing metabolic labeling and immunoprecipitation analysis (Fig. 3B, right), indicating that different secretion and activity levels are not due to differences in heparanase synthesis. In order to overcome the lack of processing, deletion mutants were similarly generated in the 50-kDa heparanase subunit.

**Deletion Mutants Lacking the KKDC or QPLK Sequences Are Capable of Heterodimer Formation**—We have demonstrated previously that the 50-kDa heparanase protein lacks enzymatic activity and that co-expression of the 50- and 8-kDa subunits is necessary and sufficient for heparanase enzymatic activity (31). Indeed, co-transfection of the 50- and 8-kDa proteins into 293 cells resulted in a high level of heparanase activity (Fig. 3C, 8+50). In striking contrast, co-transfection of the 8-kDa subunit with the 50-kDa deletion mutants yielded no enzymatic activity (Fig. 3C, 50Δ15, 50Δ10, and 50Δ20). Given the necessity of heterodimer formation between the 50- and 8-kDa subunits for obtaining active heparanase, it is conceivable that the deletions harmed regions that are required for this interaction. Subjecting total cell lysates to immunoblot analysis revealed similar expression levels of the 50- (Fig. 3D, upper panel) and 8-kDa (Fig. 3D, middle panel) subunits in the transfected cell lines. Next, lysate samples were immunoprecipitated with antibody (810) directed against the 8-kDa subunit followed by immunoblotting with anti-Myc tag antibodies that recognize the 50-kDa protein. Both the 50Δ15 and 50Δ10 constructs appeared to be associated with the 8-kDa subunit to levels comparable or even higher than the control 50-kDa protein (Fig. 3D, lower panel). Thus, the lack of enzymatic activity in these co-transfectants is most probably due to a reduced affinity of the heparanase protein to the HS substrate. By contrast, the 50Δ20 failed to interact with the 8-kDa subunit (Fig. 3D, 8+50Δ20), suggesting that this region may be involved in heterodimer formation, resulting in a lack of enzymatic activity in this co-transfection.

**QPLK Deletion Mutant Fails to Bind Cell Surface HS**—
Studying the biosynthesis and trafficking route of heparanase, we have demonstrated recently that secretion of the 65-kDa heparanase precursor is followed by a rapid uptake mediated by cell surface HSPGs, which can be competed by soluble heparin. This efficient uptake mechanism maintains extracellular levels of heparanase, tightly regulated (41). Accumulation of the 65Δ10 protein in the culture medium to exceptionally high levels (Fig. 3B, Medium) may indicate that this protein variant was not subjected to cellular uptake. In order to explore this possibility, 293 cells stably transfected with the different deletion mutants were grown in the absence or presence of heparin, HS, or HA, and lysate (Fig. 4A, lower panels) and medium (Fig. 4A, upper panels) samples were analyzed by immunoblotting. Only low levels of heparanase were detected in the CM of cells expressing the WT 65-kDa protein in the absence of a competitor (Fig. 4A, 65WT, Con). A marked increase in heparanase accumulation in the CM was evident in the presence of heparin or HS, whereas HA had no such effect. These results indicate that sulfation is absolutely necessary for the interaction of heparanase with heparin/HS, in agreement with our previous findings (41). Only marginal accumulation of the 65Δ15 heparanase was detected in the presence of heparin or HS (Fig. 4A, 65Δ15), supporting the critical role of this protein domain in the interaction with heparin/HS. Furthermore, the 65Δ10 heparanase did not respond to heparin or HS, and the protein levels in the CM appeared unchanged (Fig. 4A, 65Δ10), suggesting that the 65Δ10 heparanase mutant lost its ability to bind heparin or HS. Similar findings were obtained by employing metabolic labeling and immunoprecipitation analysis (data not shown). In contrast, the 65Δ20 heparanase was not detected to accumulate to high levels in the presence of heparin or HS (Fig. 4A, 65Δ20), clearly indicating that this domain was not involved in heparin/HS interaction. Most interestingly, the 65Δ20 protein was not detected upon re-blotting the membrane with anti-Myc tag antibodies (data not shown), suggesting that this protein variant lost its tag prior to its secretion (see also Fig. 3B, lower panel).
QPLK Deletion Mutant Is Not Subjected to Cellular Uptake—
Next, the 65-kDa WT and the 65Δ10 mutant proteins were purified from the CM and exogenously added to CHO K1 cells for the indicated time, and protein uptake was examined by immunoblotting with anti-heparanase antibodies (Fig. 4B). Uptake of the WT 65-kDa heparanase was noted already 30 min after its addition, followed by processing into the 50-kDa protein that continued to accumulate by 2 and 4 h (Fig. 4B, WT). In striking contrast, uptake of the 65Δ10 mutant heparanase could not be detected at all, even at 4 h of incubation (Fig. 4B, 65Δ10), supporting the notion that the QPLK sequence is critically important for the interaction of heparanase with heparin/HS.

Lysine Residues 158 and 161 Are Critically Important for Heparanase/HS Interaction—Unlike 65Δ10, the 65Δ15 protein exhibited very low secretion levels (Figs. 3B and 4A) that may result from the presence of a glycosylation site (Asn162) in the KKDC sequence, a site that has been shown to be important for heparanase secretion (45). In order to verify this possibility and to better define basic amino acids within the KKDC sequence that are responsible for HS binding, we point-mutated lysine 158 or both lysine 158 and lysine 161 to alanine. Stable transfected 293 cells were then examined for their secretion levels. In addition, we examined heparanase accumulation in response to the addition of HS or glycol-split (GS)-modified heparin, a most potent heparanase inhibitor (46). Only low levels of heparanase were detected in the culture medium of WT heparanase-transfected cells as opposed to vigorous accumulation noted in the presence of HS- or GS-modified heparanase (Fig. 4C, WT). Secretion of the K158A point mutant was similar to that of the WT heparanase in the absence of competitor. In contrast, no further accumulation was observed upon addition of HS, whereas GS-modified heparin yielded a 2-fold increase in heparanase accumulation (Fig. 4C, K158A), suggesting that the GS heparin has a higher affinity toward heparanase than HS. Moreover, mutating both lysine 158 and 161 abolished the ability of heparanase to interact with HS and even with the GS-modified heparin (Fig. 4C, KK,AA), pointing to these two basic amino acid residues as critically important for heparanase interaction with HS. These results further suggest that the low secretion levels of the 65Δ15 protein is in fact because of the lack of Asn162 glycosylation sites.

A Specific Region of the KKDC Peptide (Lys158–Asp172) Physically Interacts with AGA*IA Pentasaccharide—In order to define further the amino acids important for heparin/HS recognition, the KKDC or its scrambled control peptide were subjected to NMR analysis. The backbone NMR resonances of the peptide and scrambled control sequence were assigned for all amino acid residues and side chains by analysis of the COSY, TOCSY, and ROESY spectra (data not shown). To identify the heparin-binding site of the peptide KKDC, we studied the chemical shifts perturbation of NMR resonances in the presence of the pentasaccharide AGA*IA (asterisk indicates a trisulfated saccharide). This synthetic pentasaccharide mimics the high affinity binding of heparin/HS and contains the minimal sequence cleavable by heparanase (47, 48). Moreover, AGA*IA is susceptible to limited cleavage by heparanase and is thus most relevant and suitable for NMR studies because of its low molecular weight. The chemical shift perturbation of HN and N resonances of KKDC induced by addition of AGA*IA are shown in Fig. 5A. Most chemical shift changes were the continuous function of the amount of added pentasaccharide, indicating a fast exchange regime on the NMR time scale. The residues showing the largest shift are Phe160–Lys161–Asn162 located in the N-terminal region of the peptide. Moreover, signals of Lys158 and Lys161 not detectable in the free peptide, appeared upon binding to AGA*IA with larger shift changes for increasing addition of the ligand. During titration their line broadening decreased with a consequent increase in signal intensity because of the slower exchange of NH proton with the solvent. The interaction of AGA*IA with the peptide reached saturation at the stoichiometric ratio of 4:1. In order to verify the specificity of the binding, the same experiment was repeated with the scrambled control peptide (Fig. 5B).

Although some resonance perturbations could be observed after addition of the AGA*IA pentasaccharide, their random occurrence and their small signal shifts are indicative of nonspecific interaction. Clearly, although for the scrambled peptide no linkage region can be identified, the Lys158–Phe160–Lys161–Asn162 N-terminal sequence of the KKDC peptide appears to mediate high affinity binding to the AGA*IA pentasaccharide.

DISCUSSION

Heparanase as a Target for the Development of Anticancer Drugs—The mammalian endoglycosidase heparanase is the predominant enzyme responsible for degradation of HS, an activity that is thought to play a decisive role in cellular invasion associated with cancer metastasis, angiogenesis, and inflammation. Recently, this notion gained further support by employing specific anti-heparanase short interfering RNA and ribozyme strategies (20), providing a proof-of-concept for the pro-metastatic and pro-angiogenic functions of heparanase. Moreover, heparanase up-regulation has been documented in an increasing number of primary human tumors, including cancers of the bladder, colon, stomach, breast, pancreas, esophagus, multiple myeloma, and acute myeloid leukemia (20, 49). These findings and the occurrence of a single functional heparanase enzyme position heparanase as an attractive target for the development of anticancer drugs. Currently available heparanase inhibitors are the various sulfated poly- and oligosaccharides such as modified species of heparin, laminaran sulfate, and Pl-88 (33, 50, 51). These compounds were shown to inhibit heparanase activity and exert anti-metastatic and anti-angiogenic effects (52–54). Nevertheless, the lack of specificity makes interpretation questionable when using these and other polysulfated reagents (52, 53).

Heparin Binding Domains as Valid Targets for the Development of Heparanase Inhibitors—In an attempt to apply a more rational approach, we sought for functional domains that would serve as targets for drug development. Most interestingly, functional domains other than the basic heterodimer structure (30–32) and amino acids (Glu225 and Glu343) critical for the enzyme catalytic activity (55) have not been elucidated so far in the heparanase protein. In this study, we have identified and characterized two protein domains that mediate the interaction of heparanase with its HS substrate. A third domain (Lys411–Arg430) seems not to be involved in heparin/HS binding but rather to mediate the interaction of the 50- and 8-kDa subunits to establish an active heterodimer (Fig. 3D). The KKDC peptide exhibits high affinity and physically interacts with immobilized heparin and HS. Moreover, the peptide was able to inhibit significantly the heparanase enzymatic activity (Fig. 2, C–E), an effect attributed to inhibition of heparanase interaction with the heparin/HS substrate (Fig. 2F). This aspect was further confirmed by inhibition of iodinated heparanase binding to CHO cells by the KKDC peptide (Fig. 2H). Thus, the KKDC peptide not only inhibits heparanase interaction with heparin-Sepharose or ECM deposited by...
cells in vitro but also inhibits the interaction of heparanase with cell surface HS. Site-directed mutagenesis (Fig. 4C) and NMR studies (Fig. 5) clearly identified Lys$^{158}$, Lys$^{159}$, and Lys$^{161}$ as important mediators of the heparanase-heparin/HS interaction, as predicted. Most interestingly, NMR studies have also identified Phe$^{160}$ and Asp$^{162}$ as amino acids residues important for this interaction, suggesting that nonbasic amino acids may be involved in heparin/HS binding. In agreement with these biochemical analyses, deletion of the KKDC sequence resulted in a loss of enzymatic activity (Fig. 3A and C) without affecting proper heterodimer formation (Fig. 3D), suggesting that the loss of activity is due primarily to reduced affinity of heparanase to the HS substrate. We have demonstrated previously that a polyclonal antibody raised against the KKDC peptide inhibits heparanase enzymatic activity (34), indicating that this protein domain is a promising target for the development of neutralizing monoclonal antibodies. By contrast, the QPLK peptide exhibited low affinity to heparin or HS (Fig. 2B), and it did not inhibit heparanase enzymatic activity (data not shown). Synthesizing peptides containing additional amino acids, N-(KLYGPDVGQPRRKTAKMLK) or C-terminal (QRRRTAKMLKSFSLKA) to the core 10-amino acid QPLK sequence only slightly improved its interaction with heparin (Fig. 2B). Nevertheless, deletion of this protein domain resulted in a loss of heparanase enzymatic activity (Fig. 3A), although heterodimer formation appeared intact (Fig. 3D), and accumulation of the protein in the conditioned medium reached exceptionally high levels (Fig. 3B). We have demonstrated recently that heparanase does not normally accumulate extracellularly due to rapid and efficient cellular uptake mediated by cell surface HS (41). The high levels of the 65Δ10 protein detected in the CM (Fig. 3B) therefore suggest that this protein variant failed to interact with HS on the cell surface. Indeed, unlike the WT heparanase, addition of heparin or HS to the CM had no effect on the levels of this protein (Fig. 4A), and the addition of purified 65Δ10 protein to CHO cells resulted in no detectable uptake (Fig. 4B). Thus, deletion of one of the HS binding domains (65Δ15 or 65Δ10) resulted in practically complete loss of the ability of heparanase to interact with HS. This situation may imply that the two domains cooperate with one another to establish a single functional binding domain.

Predicted Three-dimensional Heparanase Model—A crystal structure of heparanase has not been resolved yet. Thus, localization of protein domains in a three-dimensional context cannot be performed. In order to overcome the lack of authentic three-dimensional structure, we adopted a predicted model based upon heparanase resemblance to β-1,4-endoxylanase from Thermoanaerobacterium saccharolyticum, an approach that has been undertaken recently to investigate the structural
requirements for proheparanase processing and activation (56). This xylosidase exhibits the best alignment with heparanase and was crystalized together with its substrate, providing a more reliable structure (57). Most interestingly, the KKDC

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Heparin/HS Binding Domains of Heparanase
Identification and Characterization of Heparin/Heparan Sulfate Binding Domains of the Endoglycosidase Heparanase
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