Heparanase and a Synthetic Peptide of Heparan Sulfate-interacting Protein Recognize Common Sites on Cell Surface and Extracellular Matrix Heparan Sulfate*

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Dario Marchetti,§ Shouchun Liu¶, William C. Spohn¶, and Daniel D. Carson¶

From the Departments of §Tumor Biology and ¶Biochemistry and Molecular Biology, University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

Heparanase is an endo-β-D-glucuronidase that degrades the glycosaminoglycan chains of heparan sulfate (HS) proteoglycans at specific sites. Elevated levels of heparanase are associated with the metastatic potential of melanoma and other types of tumor cells. We previously reported heparanase degradation of cell surface HS subpopulations of the human adenocarcinoma cell line RL95. In the present study, heparanase activity was examined on RL95 cell surface HS subpopulations in the presence of a synthetic peptide (CRPKA{AKAKAKA-DQTK) of heparin/heparan sulfate-interacting protein (HIP; Liu, S., Smith, S. E., Julian, J., Rohde, L. H., Karin, N. J., and Carson, D. D. (1996) J. Biol. Chem. 271, 11817–11823). Heparanase digestion generated HS fragments from cell surface- or extracellular matrix-derived HS of approximately 25 and 9 kDa, respectively. In contrast, HS of various size classes isolated from proteoglycans secreted or released by RL95 and endothelial cells in culture were not susceptible to heparanase digestion. Incubation of heparanase-containing melanoma cellular extracts or partially purified heparanase preparations with cell surface- or ECM-derived HS and HIP peptide, but not a scrambled sequence of this peptide or other HS-binding proteins present in ECM, completely inhibited heparanase action. Conversely, predigestion of cell surface HS with either heparanase-containing cellular extracts or with secreted or partially purified heparanase destroyed binding to HIP peptide. Preincubation of HS with HIP peptide prevented subsequent heparanase digestion. Collectively, these data demonstrate that HIP peptide and heparanase recognize specific, common motifs within HS chains at cell surfaces and in ECM and may mutually modulate HS-dependent activities.

Metastasis occurs via a sequential and complex series of interactions between tumor cells and normal host cells and tissues (1, 2). During the formation of metastases, migrating cells are confronted by natural tissue barriers, such as connective tissue stroma and basal lamina (2, 3). The ability of malignant cells to penetrate these barriers depends upon the presence of enzymes capable of degrading extracellular matrix (ECM) components (1–5). For these reasons, considerable effort has been focused on the study of tissue-degradative enzymes produced and secreted by metastatic tumor cells as well as normal cells of the tissue being invaded.

Important ECM targets for degradation by invading melanoma cells are the heparan sulfate (HS) chains found on proteoglycans (3, 6, 7). HS are highly negatively charged linear polysaccharides consisting of alternating residues of uronic acids and glucosamine. Proteins containing one or more covalently attached HS chains are called HS proteoglycans (HSPGs). The dynamic role of HSPGs in biology has become increasingly apparent (8–23). As a result of characterizing heparin (HP) and HS binding sites related to the initial attachment of trophoblast cells to uterine epithelial cells of murine and human origin, we recently reported the cell surface expression and molecular cloning of a novel HP/HS-interacting protein (HIP) of human epithelial and endothelial cells (24–26). HIP not only recognizes HS and HP in a highly specific fashion, but it also binds a subset of HP and HS enriched at cell surfaces and in ECM. In contrast, HIP does not bind intracellular or secreted forms of HS. Furthermore, HIP also appears to bind the anticoagulantly active species of HP efficiently and with high affinity. HP octasaccharides, but not hexasaccharides, are large enough to bind HIP with high affinity. Thus, HIP appears to recognize a motif that at least overlaps the anticoagulant motif in HP and HS chains.

In light of the above findings, activities that mediate HSPG degradation are expected to have significant regulatory consequences. Indeed, HSPG catabolism is observed in inflammation, wound repair, diabetes, and cancer metastasis, suggesting that enzymes that degrade the HS chains play important roles in pathologic processes (3, 4). Furthermore, malignant cells are capable of modulating cellular interactions with HSPGs by producing and releasing a HS-degrading enzyme, heparanase (3, 7, 27, 28). Recently, we reported that purified, high Mr, subpopulations of cell surface HS were more sensitive to heparanase action than secreted HS (29). In the present study, we have investigated the relationship between tumor (melanoma) heparanase activity and HIP binding in HS subpopulations whether on the cell surface, secreted, or deposited in the ECM. By use of sensitive heparanase assays that separate

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1 The abbreviations used are: ECM, extracellular matrix; CHAPS, 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; ddE3O, doubly distilled water; GAG, glycosaminoglycan; HIP, heparin/heparan sulfate-interacting protein; HP, heparin; HS, heparan sulfate(s); HSPG, heparan sulfate proteoglycan; SAL, d-saccharic acid 1,4-lactone; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

2 S. Liu and D.D. Carson, submitted for publication.

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Heparanase and Heparin Recognition of Heparan Sulfate

Materials—Heparin and heparan sulfate were acquired from Sigma. [3H]Heparin (0.44 mCi/mg) and [35S]sulfate (43 Ci/mg) were purchased from DuPont NEN and ICN Biochemical (Irvine, CA), respectively. Fetal bovine serum, Dulbecco's phosphate-buffered saline (PBS) and Dulbecco's minimum essential medium were purchased from Life Technologies, Inc. HEPES, RPMI 1640 medium and DMEM were purchased from GIBCO-BRL. [3H]-5-bromo-2′-deoxyuridine (BrdU) and [3H]thymidine (specific activity 2.0 Ci/mmol) were obtained from New England Nuclear. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma-Aldrich. ECL Plus Western blotting detection reagents were from Amersham Biosciences. Anti-BrdU antibody was purchased from Dako. All other chemicals used were reagent grade or better.

EXPERIMENTAL PROCEDURES

Preparation of Heparan-Sulfate-Containing Cellular Extracts and Partial Purification of Heparanase—Subconfluent cells (2 × 10^6) were harvested and solubilized in 50 ml of 50 mM Tris-HCl, pH 7.5, containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5% (w/v) sodium azide, 0.1% (v/v) Triton X-100, and 0.05% (w/v) sodium azide at 4 °C for 30 min. The cell extract was centrifuged at 12,000 g for 30 min, and the supernatant was loaded onto a concanavalin A-Sepharose column (Pharmacia Biotech, Inc.) equilibrated with buffer (7). The heparan-Sepharose column was sequentially washed with buffer 1/20 mM sodium acetate containing 0.5% (v/v) Triton X-100, and 0.05% (w/v) sodium azide at 4 °C for 1 h. The supernatant containing approximately 50 mg of protein was passed through a column of heparin-Sepharose CL-6B (Pharmacia Biotech, Inc.) equilibrated with buffer 2. The heparan-Sepharose column was sequentially washed with buffer 2/10 mM sodium acetate containing 0.2% (v/v) Triton X-100, pH 6.0 (buffer 2) and 0.15 M sodium chloride, 20 mM sodium acetate, pH 6.0 (buffer 3). Heparin-binding proteins were eluted with a linear sodium chloride gradient (0.15–1.5 M) in 20 mM sodium acetate, pH 6.0. The eluted materials were monitored by measuring absorbance at 280 nm, and the heparanase activity was measured as described below. Fractions containing heparanase activity after this step contained approximately 5 mg of protein and 90% of total heparanase activity. Following dialysis against buffer 3, the heparanase-containing fractions were centrifuged for 30 min, and the supernatant was loaded onto a concanavalin A-Sepharose column (Pharmacia) equilibrated with buffer 3. After the column had been washed with buffer 3, concanavalin A-binding proteins were eluted with 1.0 M α-methyl-d-mannopyranoside in buffer 3. The eluate, containing approximately 0.3 mg of protein and 72% of total heparanase activity, was collected, extensively dialyzed against buffer 3, and concentrated with Centricon 30 concentrators (Amicon, Beverly, MA). Thus, these preparations were approximately 120-fold enriched for heparanase activity over the initial 30,000 × g soluble cell extracts.

Preparation of HS Fractions—RPMI 1640 medium was used as the basal medium for [35S]sulfate metabolic labeling of RL95 cell HS subpopulations as described previously (27, 28). Briefly, nearly confluent RL95 cells were rinsed several times with serum-free RPMI 1640 medium supplied with 3.3 mM MgCl₂, 1.2 g/liter NaHCO₃, 15 mM Hepes, pH 7.2, 2.5 units/ml penicillin, and 2.5 μg/ml streptomycin sulfate. Streptomycin sulfate (Irvine Scientific, Santa Ana, CA) served as the sole source of nonradioactive sulfate in this medium (final concentration 0.625 μM). The cultures were incubated overnight in the same low sulfate medium described above containing 0.5 μCi/ml [35S]sulfate. Anion exchange chromatography demonstrated that [35S]HS synthesized by RL95 in low sulfate media had an elution profile identical to that of [35S]HS synthesized in sulfate-replete medium, indicating that HS was sulfated to a similar degree under both conditions. The medium was collected and used as a source of secreted [35S]HASGs. Cell monolayers then were rinsed several times with ice-cold PBS. The cell monolayers then were incubated for 30 min on ice with PBS containing 50 μg/ml trypsin to release cell surface proteoglycans. Cells did not detach from the tissue culture surfaces under these conditions, nor was cell viability compromised as assessed by trypan blue dye exclusion. The material released into the “trypsinate” was collected and placed in a boiling water bath for 5 min immediately before inactivation by trypsin. The trypsinate was extensively dialyzed against ddH₂O and aliquots of the dialysis buffer were examined after each dialysis step until no background levels of radioactivity were detected. Samples of both RL95 cell surface and secreted HSPsGs then were either incubated overnight with Pronase (10 ng/ml; Refs. 33 and 34) or subjected to alkaline borohydride treatment (β-elimination) at 45 °C in the presence of 0.05 M NaOH, 1 mM sodium borohydride for 24 h, followed by neutralization with acetic acid (35). Precipitates were collected, and supernatants were dialyzed extensively with ddH₂O. Aliquots were removed and radioactivity determined.

Sensitivity to nitric acid degradation (35) was used to confirm the identity of HS. Briefly, samples for digestion were dissolved in a 0.5:1:10 (v/v/v) ratio of 20% (v/v) N-butyl-nitrite in 100% ethanol.1.4 HCl. The 20% (v/v) N-butyl-nitrite was immediately formed a turbid precipitate. Samples were dialyzed and gel permeation chromatography (34). The size of HS chains was estimated by liquid chromatography by Superose column chromatography (2.0 × 30 cm; Pharmacia). The resin was eluted with 4 M guanidine hydrochloride, 0.5% (w/v) CHAPS, 50 mM sodium acetate, pH 6.0, at a flow rate of 0.40 ml/min, and 0.4 ml fractions were collected. Aliquots were taken for determination of radioactivity by scintillation counting. Molecular size estimates for GAG chains were based on the method of Westman (30) using chondroitin sulfate chains of known molecular mass as calibration standards (27, 37).

Heparanase Assays—A reaction consisting of 10–20 μl of heparanase enzyme source in 200 mM sodium acetate, pH 5.0, was mixed with electrophoresis buffer at a final concentration of 0.1% (w/v) bromphenol blue, 0.025% (w/v) xylene cyanol, 2.5% (w/v) Ficoll (type 400) in ddH₂O as described previously (27). Before electrophoresis, 1% (w/v) SDS final concentration was added to each sample for 15 min at 25 °C to dissociate [35S]labeled digestion products from high molecular weight complexes. Following this step, agarose gel 1.5% (w/v) electrophoresis in the presence of 1% (w/v) SDS, 40 mM Tris acetate (pH 8.3) and 1 mM EDTA was performed at 50 V for 1 h at 25 °C or until the samples migrated approximately two-thirds of the entire gel length as indicated by the tracking dye. Autoradiography was performed on the dried gel by exposure to X-AR 5 film (Eastman Kodak Co., Rochester, NY) for 3–7 days. The direction of electrophoresis shown in all figures is from top to bottom. Alternatively, heparanase activity was determined by high speed gel permeation chromatography (38) with some modifications (27). B16B15 or 70W melanoma cells were used as a source of heparanase activity. We previously showed that 70W metastatic clones possess higher heparanase content than their respective murine (B16F1) or human (MeWo) parental counterparts, and 2) the heparanase activity is indistinguishable in these two cellular sources by the two heparanase assays used in the present studies (7, 27, 29). Briefly, subconfluent cells were harvested and solubilized in 50 mM Tris-HCl, pH 7.5, 0.05% (w/v) sodium azide, 0.5% (v/v) Triton X-100 for
30 min at 4 °C. Cell lysates then were centrifuged at 12,000 × g for 30 min at 4 °C and concentrated by an Amicon-30 microconcentration unit (Amicon, Beverly, MA) according to the manufacturer’s instructions. To perform heparanase digestions, cell lysates (50–70 μg of protein) were incubated with [35S]HS (5 × 10^6 dpm) and 0.2 M sodium acetate, pH 5.0, for 18 h at 37 °C (10–100 μl final reaction volume). The reaction was terminated by heating samples for 15 min at 95 °C. A delipidation step was applied to cell lysates following heparanase incubation and before Superose 6 liquid chromatographic analyses as described above. Alternatively, supernatants (50–100 μl) following the delipidation step were injected into a TSK gel G3000 PWX2 column (7.8 mm × 30 cm; 6-μm particle size from Tosoh (Montgomeryville, PA)) for the high speed gel permeation chromatography. A flow rate of 0.50 ml/min was used, and fractions were collected every 30 s. Radioactivity was determined using a liquid scintillation counter (Pharmacia; 75% efficiency).

Heparanase activity by gel permeation chromatography decreased in the area of the high Mr, half of intact radiolabeled HS peak and correlated with incubation time. Only data within a linear range for relative activity measurements were taken into account, with relative degradation activity determined by the amount of radiolabeled HS degraded/min/μg of protein (7). By performing these heparanase analyses using cellular extracts from subconfluent murine (B16B15b) or human (70W) cells, we have determined heparanase activity, whether from murine or human sources, to be identical toward the substrate as already reported (7, 29).

**Extracellular Matrix Preparation and Characterization of Proteoglycans and Glycosaminoglycans—**For metabolic labeling of endothelial cell monolayers, preconfluent cultures were incubated for 72 h with H[35S]SO4 at 50 μCi/ml. Cloned endothelial (bovine aortic or murine brain) cells were cultured and used for extracellular matrix isolation as described previously (39). Briefly, the endothelial monolayers were rinsed three times with 5 mM Tris-HCl, pH 7.5, and then incubated in the same buffer at 37 °C for 10 min. Cells were swollen in 20 mM NH4OH for 5 min at 25 °C and monitored under a microscope until lysis occurred. Remaining nuclei and cytoskeletons were removed by extensive washes with Dulbecco’s PBS containing 5% (v/v) fetal calf serum to remove residual free isotope. Proteoglycans were extracted from the subendothelial matrix by incubation overnight at 4 °C with extraction buffer in the presence of protease inhibitors. The extraction buffer consisted of 4 M guanidine-HCl, 20 mM Tris-HCl, pH 6.0, 1% (w/v) CHAPS, and 5 mM EDTA. To determine the composition of ECM [35S]HSPGs, the digested material was extensively dialyzed against ddH2O and desalted on a PD-10 column. Only components eluting at V0 of the column were retained for further analyses. These fractions were then digested with chondroitinase ABC (Boehringer Mannheim) at 2 units/ml in a solution containing 50 mM Tris, 50 mM sodium citrate, and 100 μg/ml bovine serum albumin, pH 7.4, for 24 h at 37 °C. Chondroitinase-resistant samples were separated from digested material by Seph- adex G-50 column chromatography and then characterized directly by filtration on a Sepharose CL-6B column (0.7 × 35 cm) with 4 M guanidine-HCl, 0.1 M sodium acetate, pH 5.5, at a flow rate of 0.5 ml/h. V0 and total volume (Vt) were marked by the elution positions of blue dextran and vitamin B12, respectively. Vitamin B12 co-eluted with [35S]HS, and accurately marked Vt for each column. Free glycosaminoglycans were prepared by proteoglycan digestion with Pronase and by mild alkaline hydrolysis as described above. The identity of HS in these fractions was confirmed by nitrous acid degradation.

**Heparin Peptide Binding Assays—**[3H]HP and [35S]HS binding to HIP peptide was performed in 96-well microassay plates (Corning, New York, NY). Briefly, 2 mg of HIP peptide was conjugated to 2 mg of maleimide-activated BSA (Pierce) as per the manufacturer’s instructions, and the HIP-BSA conjugate was isolated by chromatography on Sephadex G-50 equilibrated and eluted with PBS. Fifty μl of a 100 μg/ml solution of HIP peptide-BSA in PBS was added to each well and dried at 37 °C overnight. The next day, each well was rinsed with 200 μl of PBS three times, and 100 μl of 0.1% (w/v) heat-denatured BSA (90 °C for 20 min) was added and incubated for 1 h to block residual protein-binding sites. Afterward, wells were rinsed three times with 200 μl of PBS. Binding was performed in 50 μl of binding buffer (PBS containing 0.1% (w/v) heat-denatured BSA), and [3H]HP ([2 × 104 cpm]) or [35S]HS ([2 × 104 cpm]) pretreated or not pretreated with heparinase, as indicated, were added and incubated at 37 °C for 2 h. Unbound radioactivity then was removed by rinsing three times with 200 μl of PBS. Bound radioactivity was extracted from each well by overnight incubation at 37 °C with 100 μl of 4 M guanidine-HCl, 25 mM Tris-HCl, pH 8.0, 2.5 mM EDTA, and 0.02% (w/v) sodium azide. Radioactivity in half of the extract was determined by liquid scintillation counting.

**RESULTS**

Heparin inhibits heparanase action on cell surface and ECM forms of HS. We found previously that in the absence of trophic or growth factor stimulation, highly brain-metastatic murine and human melanoma cells express high levels of heparanase compared with poorly metastatic melanoma cells (7, 27, 29). Consistent with these earlier studies, heparanase converted almost all RL95 cell surface-derived forms of HS to smaller sized fragments detectable by either molecular exclusion chromatography or agarose gel electrophoresis (Fig. 1, A and B). The median Mr of the intact HS populations was estimated to be 70,000, while that of the heparanase products was 25,000, or approximately one-third of the undigested original substrate. These values were obtained by running in parallel radiolabeled chondroitin sulfate chains of known median Mr (36). However, because only chains of similar composition to HS are available as standards, the assigned molecular mass must be considered a minimum estimate of a GAG population. These observations suggested that heparanase preferentially hydrolyzed sites infrequently distributed in cell surface HS chains.

As shown in Fig. 2 (A and B), four size classes of HS could be isolated from secreted HSPGs with Mr ranging from 9,000 to 70,000; however, none of the secreted HS fractions were susceptible to
heparanase action. We selected several HS fractions on the basis of size to determine whether they were susceptible to heparanase digestion. It was considered that larger HS chains might be more susceptible to heparanase due to the increased probability of containing an appropriate site; however, none of the four size classes of secreted HS were susceptible to heparanase. For simplicity, only the data for the largest size class, HS-1, are shown (Fig. 2C).

The insensitivity of relatively large secreted HS chains to heparanase suggested that heparanase-sensitive motifs were not preserved in secreted forms of HS. The identity of the secreted $^{35}$S-labeled glycosaminoglycans as HS was confirmed by demonstrating quantitative hydrolysis by nitrous acid (Fig. 3A). Previous studies have demonstrated the identity of the cell surface-derived $^{35}$S-labeled glycosaminoglycans from RL95 cells as >95% HS (29, 32). The resistance of secreted HS classes to heparanase action was not due to possible enzyme inactivation processes. This result was consistently obtained in the presence of several preparations of melanoma heparanase independently prepared or after altering assay conditions, i.e. by increasing the incubation times or heparanase doses in the enzyme reaction. In addition, parallel digestions with cell surface $[^{35}S]HS$ demonstrated that heparanase was active in these assays. Furthermore, cell surface $[^{35}S]HS$ was efficiently digested in the presence of unlabeled secreted HS preparations (Fig. 4). These observations indicated that lack of sensitivity of secreted HS to heparanase was not due to the presence of a heparanase inhibitor. Moreover, secreted $[^{35}S]HS$ chains from vascular and brain endothelial cells also were insensitive to heparanase action (data not shown). Collectively, these data indicated that secreted HS from different cell types was insensitive to heparanase digestion.

HIP is a recently described cell surface HP/HS-binding protein (25, 26) and contains a peptide motif that is capable of discriminating between secreted, cell surface, and ECM forms of HS. Therefore, it was considered that heparanase and HIP peptide might recognize similar motifs in HS. As shown in Fig. 1, coincubation of HS with HIP peptide inhibited heparanase in a HIP peptide dose-dependent fashion. In contrast, a peptide with the same amino acid composition, but altered sequence, as

![Fig. 2. Heparanase does not degrade secreted $[^{35}S]HS$. A, agarose gel electrophoretic separation of $[^{35}S]HS$ from secreted HSPGs. The migration positions of four size classes are indicated. B, molecular exclusion chromatography of separated size classes of secreted $[^{35}S]HS$ on Superose 6. The four HS subpopulations fractionated by agarose gel electrophoresis as in A were individually chromatographed: O, HS-1; ●, HS-2; ▲, HS-3; △, HS-4. The elution positions of chondroitin sulfate standards of 60, 42, 20, and 17 kDa are indicated. C, TSK G 3000 PWX2 chromatography of the HS-1 fraction before (O) and after (●) heparanase digestion. This was found to be valid for both murine and human heparanase-containing cellular extracts (B16F15b and 70W, respectively). Preparation and characterization of secreted HS, molecular exclusion chromatography, and agarose gel electrophoresis were performed as described under “Experimental Procedures.”](image-url)

![Fig. 3. Characterization of $[^{35}S]HS$ fractions. A, Sepharose CL-6B chromatography of $[^{35}S]HS$ secreted by endothelial cells (O), subjected to alkaline borohydride hydrolysis (●), or after nitrous acid degradation (△). B, Superose CL-6B chromatography of ECM $[^{35}S]HS$ from endothelial cell monolayers before (O) and after alkaline borohydride hydrolysis (●) or nitrous acid degradation (△).](image-url)
the HIP peptide did not inhibit heparanase action in degrading purified high $M_\text{r}$ RL95 cell surface HS subpopulations (Fig. 5). In addition, other ECM HS-binding proteins such as laminin fibronectin and basic fibroblast growth factor did not prevent heparanase from digesting HS. This was found to be valid at similar mass concentrations, as tested (data not shown). Thus, it appeared that HIP peptide can specifically compete with heparanase for interaction with similar or identical motifs enriched in cell surface HS.

Heparanase was also active on HS derived from subendothelial extracellular matrices. HS isolated from ECM produced by endothelial cell monolayers migrated as two major size classes with $M_\text{r}$ of approximately 45,000 and 27,000 (Fig. 6). The identity of the $^{35}\text{S}$-labeled components of these preparations as HS was confirmed by nitrous acid degradation (Fig. 3$B$). Digestion of ECM HS preparations with heparanase-containing metastatic melanoma cell extracts resulted in conversion to products with a median $M_\text{r}$ of $\approx 9,000$ (Fig. 6). Incubation of endothelial ECM $^{35}\text{S}$HS with heparanase and suramin, a potent melanoma heparanase inhibitor (41) or HIP peptide completely inhibited HS digestion. In contrast, inclusion of the exo-$\beta$-$D$-glucuronidase inhibitor SAL (27) or chondroitin 4-sulfate or chondroitin 6-sulfate did not inhibit digestion of endothelial ECM $^{35}\text{S}$HS (Fig. 7). We concluded that HIP peptide effectively inhibited heparanase in its degradation of ECM HS and was comparable with suramin action in this regard.

**Heparanase Digestion Destroys HS Binding to HIP Peptide**—As noted above, heparanase degraded HS to fragments with a median $M_\text{r}$ of $\approx 9,000$. We considered that the highly positively charged HIP peptide might recognize and bind to many HS regions, including heparanase-sensitive domains. In this event, heparanase-insensitive regions of HS should still exist following heparanase digestion supportive of HIP peptide binding. As shown in Fig. 8, inclusion of soluble HIP inhibited $>95\%$ of total $^{35}\text{S}$HS binding. Predigestion of these samples with heparanase (from total melanoma cell extracts, partially purified, or cell-secreted) reduced binding to 5% or less of control values in each case considered. Heparanase inactivated by boiling did not reduce $^{35}\text{S}$HS binding, indicating that this effect required enzymatic activity (data not shown). Consequently, the infrequent sites present within HS chains sensitive to heparanase also appeared to be critical for HIP peptide binding.

**DISCUSSION**

Microvascular endothelial cells are the major cellular source of ECM-type HSPGs in normal brain (43, 50, 51), and recent reports have highlighted the relevance of glycosaminoglycans in nerve injury and regeneration with low doses supplementing the quantity of neurotrophic factors required to promote neurite outgrowth (40, 52). Metastatic tumor cells interact with various ECM glycoproteins including HSPGs (1–5, 44). Previous studies have demonstrated that 1) highly invasive and metastatic human and murine melanoma cells degrade labeled HS chains faster than do sublines of lower metastatic potential (7, 27, 29); 2) selected members of a family of neurotrophic factors, the neurotrophins, augment invasion of brain-metastatic melanoma cells, of both human and murine origin (7, 29); and 3) heparanase is a key enzyme responsible for the observed increased invasive activity and HS degradation in brain-metastatic cells (7). In this regard, heparanase production and activity are stimulated by selected neurotrophins (29). High heparanase content and neurotrophin up-regulation of its activity were found for both murine and human brain-metastatic melanoma cells with heparanase activity indistinguishable by the two heparanase assays developed and used for these studies (7, 27, 29). The time dependence of HS degradation into...
particular $M_r$ fragments indicated that melanoma heparanase cleaves HS at specific intrachain sites (3, 27, 38). Melanoma heparanase was found to produce 12–20 oligosaccharide fragments from bovine lung or kidney HS (3, 27, 38). In contrast, platelet heparinase was reported to produce mainly di- and tetrasaccharides just as bacterial heparitinases (12, 23, 45, 46). Heparanase was identified as an endo-$\beta$-$\mathrm{D}$-glucuronidase that cleaves $\beta$-$\mathrm{D}$-glucuronosyl-$\mathrm{N}$-acyethylglucosaminyl linkages of the HS molecule (27). Additionally, by development of a sensitive heparanase assay that separates purified cell surface [35S]HS species by agarose gel electrophoresis, we have reported that melanoma heparanase preferentially degrades specific cell surface HS species (29).

We have expanded these previous observations and determined whether HS found in locales accessible to heparanase, i.e. secreted or associated with subendothelial ECM, are targets of heparanase action. Moreover, we have determined whether the HS motifs recognized by heparanase are the same as those recognized by a peptide motif of the recently discovered cell surface HS-binding protein, HIP (25, 26). HIP is a novel protein found on the cell surface and is expressed in a cell type-specific fashion (25, 26). The intact protein binds HIP with high affinity (25, 26). Additional studies have demonstrated that HIP peptide recognizes a subset of HIP and HS (49).

To successfully colonize the brain microenvironment, metastatic melanoma cells must be able to negotiate with the blood-brain-barrier and its thick subendothelial basement membrane and synthesize a battery of specific enzymes targeted toward each of the various components of the matrix. In the present study, we have utilized HS synthesized by microvascular and brain endothelial cells as a heparanase substrate. Interestingly, we have found that brain-metastatic melanoma cellular extracts induce enhanced subendothelial matrix degradation concomitant with invasion of this structure in a process dependent on heparanase presence and action at the level of HS chains of subendothelial ECM HSPGs. Indeed, incubation of HS derived from subendothelial ECM with metastatic melanoma cellular extracts or partially purified preparations of tumor heparanase resulted in solubilization of sulfated glycosaminoglycans with the appearance of a $M_r$ ~ 9,000 degradation product. In contrast, several other agents failed to have any effect in this regard. Furthermore, HS degradation to lower but still relatively large $M_r$ products was inhibited by incubation of these extracts with the potent melanoma heparanase inhibitor suramin (41), being unaffected by the presence of excess amounts of chondroitin sulfates, indicating specificity for HS chains. This digestion appears to proceed by the cleavage of HS at intrachain sites, as expected from previous studies (27). Importantly, coincubation of heparanase with HIP peptide totally inhibited digestion of subendothelial matrix HS in a dose-dependent fashion. This was found to be unique to HIP peptide, since other HP/HS-binding proteins present in the ECM (fibronectin, laminin, basic fibroblast growth factor) did not affect heparanase activity, at least where used in equivalent mass amounts of HIP. All of these findings can be related to heparanase digestion of cell surface, but not secreted HS, compared with parallel binding capabilities of HIP peptide to these GAGs. Heparanase digestion of HS from the subendothelial matrix generated intermediate size fragments. However, this was observed only for ECM and not secreted HS. Heparanase sensitivity of HS was paralleled by specific HIP interactions with these HS subpopulations. We suggest that heparanase and HIP peptide recognize the same sequences within HS subpopulations. This may prove to be very important as more information is added regarding the biological and functional relevance of proteoglycans and their dynamic roles to provide the essential microenvironment for successful interactions between cells and their extracellular surroundings. In the cleavage of HS by melanoma heparanase the recognition of $\mathrm{N}$-acyetyl groups as well as sulfate groups can be important, since the enzyme can distinguish HS from heparin and cleaves HS at specific intrachain sites. Interestingly HS produced by cultured vascular endothelial cells has been reported to have higher $\mathrm{N}$-sulfate content than HS from other sources (47, 48). These findings may provide an explanation as to why HS in the endothelial ECM was degraded into relatively large fragments. Because of its block-type structure of low $\mathrm{N}$-acyetyl group content (heparin-like structures), vascular endothelial matrix HS may have only a few linkages susceptible to melanoma heparanase. Certainly, HIP’s ability to bind specific oligosaccharides within HS chains located in the ECM or at the cell surface, but not in secreted HS, make the hypothesis of a specific and restrictive interaction of heparanase with...
defined domains of HS molecules all the more plausible and consistent with the results obtained. Therefore, in light of the reported results, heparanase has to be regarded not simply as a nonselective HS degradative enzyme but instead as an enzyme recognizing these defined HS "domain" regions as selected by other, biologically active proteins. It is entirely possible that once the $M_r \approx 9,000$ fragments are produced, they are released immediately into the culture medium, where they are no longer susceptible to further digestion by tumor cell heparanase. It will be of interest to compare the pattern of HIP expression during tumor progression with that of heparanase to determine if these proteins modulate aspects of HS-dependent processes.

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