Heparanase mediates cell adhesion independent of its enzymatic activity

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ABSTRACT **Heparanase is an endo-β-D-glucuronidase that cleaves heparan sulfate and is implicated in diverse physiological and pathological processes. In this study we report on a novel direct involvement of heparanase in cell adhesion. We demonstrate that expression of heparanase in nonadherent lymphoma cells induces early stages of cell adhesion, provided that the enzyme is expressed on the cell surface. Heparanase-mediated cell adhesion to extracellular matrix (ECM) results in integrin-dependent cell spreading, tyrosine phosphorylation of paxillin, and reorganization of the actin cytoskeleton. The surface-bound enzyme also augments cell invasion through a reconstituted basement membrane. Cell adhesion was augmented by cell surface heparanase regardless of whether the cells were transfected with active or point mutated inactive enzyme, indicating that heparanase functions as an adhesion molecule independent of its endoglycosidase activity. The combined feature of heparanase as an ECM-degrading enzyme and a cell adhesion molecule emphasizes its significance in processes involving cell adhesion, migration, and invasion, including embryonic development, neovascularization, and cancer metastasis.—Goldshmidt, O., Zcharia, E., Cohen, M., Aingorn, H., Cohen, I., Nadav, L., Katz, B.-Z., Geiger, B., Vlodavsky, I. Heparanase mediates cell adhesion independent of its enzymatic activity. *FASEB J.* 17, 1015–1025 (2003)**

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CELL ADHESION to the extracellular matrix (ECM) is a tightly regulated process that plays a key role in the control of cell migration, proliferation, and differentiation associated with diverse physiological and pathological processes (1–3). The predominant integrin receptor family mediates cell adhesion and provides a physical link between the ECM and cytoskeletal elements (4–6). Integrins also initiate and regulate a variety of signaling responses, including the activation of mitogen activated protein kinase (MAPKs) and tyrosine phosphorylation of cytoplasmic molecules (e.g., paxillin) (7–9). Nonetheless, cell adhesion is a multistep process that involves membrane constituents other than integrins (10). For example, leukocyte adhesion to the endothelium involves a rapid, low-affinity, non-integrin-mediated adhesion, followed by prolonged integrin-mediated, high-affinity adhesive interactions (11, 12). Non-integrin mechanisms may play a key role in the formation of early cell–ECM adhesions occurring before the formation of specific integrin-mediated interactions of cells with the ECM. It was shown, for example, that hyaluronan might support the formation of such early adhesive cell–ECM interactions in adherent cells (10). Other cell surface, non-integrin molecules such as heparan sulfate proteoglycans (HSPGs) may also be involved in the regulation of early stages of cell–ECM interactions (13–16). Heparan sulfate proteoglycans play a major role in diverse cellular processes and are regarded as key molecules in the self-assembly, insolubility, and barrier properties of basement membranes (BM) and the ECM (13–17). Mammalian endoglycosidase (heparanase), capable of partially depolymerizing heparan sulfate (HS) chains, has been identified in highly invasive normal and malignant cells including cytotrophoblasts, activated cells of the immune system, lymphoma, melanoma, and carcinoma cells (18–22). Expression of heparanase has long been correlated with the metastatic potential of tumor cells, and treatment with heparanase inhibitors markedly reduced the incidence of experimental metastasis and autoimmunity (20–24). Moreover, there is a significant correlation between enhanced heparanase mRNA expression and shorter postoperative survival of cancer patients (25, 26). Apart from its involvement in the egress of cells from the vasculature, heparanase is tightly involved in normal and pathological angiogenesis, primarily by means of releasing heparin binding angiogenic factors sequestered by HS in BM and ECM (21, 27–29). The human heparanase cDNA encodes a

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latent enzyme of 543 amino acids, which is then cleaved at the amino terminus, yielding the mature highly active 50 kDa enzyme (20, 30). Only a single heparanase cDNA sequence encoding a functional enzyme was identified (31–34), indicating that this enzyme is the dominant HS-degrading endoglycosidase in mammalian tissues. The direct role of heparanase in these processes has been further emphasized by demonstrating that a surface-associated and secreted form of the enzyme (chicken heparanase or chimeric heparanase composed of the chicken heparanase signal sequence fused to the human enzyme) (35) markedly promotes tumor angiogenesis and metastasis (29).

In a previous study it was hypothesized that depending on the local pH, heparanase may function as an ECM-degrading enzyme (pH<6.8) or as a T cell adhesion molecule (pH>7.0) (36). Our recent identification of a cell surface-associated form of heparanase (35) led us to investigate and characterize the involvement of heparanase in cell adhesion. Nonadhesive Eb mouse lymphoma cells were stable transfected with the human enzyme (H-*hpa*, localized primarily in perinuclear granules) or with the chicken and chimeric heparanase cDNAs (Chk-*hpa* and chimeric *hpa*, respectively) expressed predominantly on the cell surface (35). The transfected cells were then compared for their adhesiveness to a naturally produced subendothelial ECM and to a confluent vascular endothelial cell monolayer. Our results indicate that surface-associated heparanase promotes adhesion of otherwise nonadherent lymphoma cells. Heparanase-stimulated cell adhesion was accompanied by tyrosine phosphorylation and reorganization of paxillin in ECM adhesions. Cell adhesion was augmented by cell surface heparanase whether the cells were transfected with active or point mutated inactive enzyme, indicating a novel adhesion feature of the heparanase molecule.

MATERIALS AND METHODS

Cells

The methylcholanthrene-induced nonmetastatic mouse Eb (L5178Y) T lymphoma cells were kindly provided by Dr. V. Schirrmacher (DKFZ, Heidelberg, Germany). The cells were grown in RPMI 1640 medium (Life Technologies, Grand Island, NY, USA) supplemented with β-mercaptoethanol (5×10^{-5} M) and 10% fetal bovine serum (FCS), as described (29, 35). Cultures of bovine corneal endothelial cells were established from steer eyes and maintained in DMEM (1 g glucose/L) supplemented with 5% newborn calf serum, 10% FCS, and 1 ng/mL bFGF, as described (37). Confluent cells were dissociated with 0.05% trypsin and 0.02% EDTA and subcultured at a split ratio of 1:10 (37). Adult bovine aortic endothelial cells were established from bovine aorta and maintained in DMEM (1 g glucose/L) supplemented 10% FCS and 1 ng/mL bFGF, as described (37).

Plasmids

pcDNA3 plasmids (Invitrogen, NV Leek, Netherlands) containing the different *hpa* cDNA sequences encoding chicken, human, and chimeric heparanase cDNAs (Chk-*hpa*, H-*hpa* and chimeric *hpa*, respectively) or an empty pcDNA3 plasmid, all under the control of the CMV promoter, were prepared and subcloned as described previously (35). A construct encoding mutated chimeric heparanase (Mut-chimeric *hpa*) was prepared as described (35) except that a point mutation was first introduced in the H-*hpa*, replacing the proton donor Glu^{225} with alanine (38).

Transfection

Eb mouse lymphoma cells constitutively overexpressing the various heparanase constructs (Chk-*hpa*, H-*hpa*, chimeric *hpa*, Mut-chimeric *hpa*) or the pcDNA3 vector alone were generated as described previously (35). Eb cells 0.5×10^6 cells/mL were incubated (48–72 h, 37°C) with a total of 1–2 μg DNA and 6 μL FuGene transfection reagent (Boehringer, Mannheim, Germany) in 94 μL Optimem (Gibco-BRL, Invitrogen) (35). Transfected cells were selected with 350 μg/mL G418 (Gibco-BRL) and stable populations of heparanase-expressing cells were obtained and maintained in growth medium containing 150 μg/mL G418 to avoid the overgrowth of nontransfected cells. Expression of heparanase was evaluated by RT-PCR, activity measurements, and immunostaining (35).

Preparation of dishes coated with ECM

Bovine corneal endothelial cells were plated into 35-mm tissue culture dishes at an initial density of 2×10^5 cells/mL and cultured as described above except that 4% dextran T-40 was included in the growth medium (37). On day 12, the subendothelial ECM was exposed by dissolving the cell layer with PBS containing 0.5% Triton X-100 and 20 mM NH_4OH, followed by four washes with PBS (37). The ECM remained intact, free of cellular debris and firmly attached to the entire area of the tissue culture dish. To produce sulfate-labeled ECM, $Na_2{}^{35}SO_4$ (25 μCi/mL) (Amersham, Buckinghamshire, UK) was added on days 2 and 5 after seeding, and cultures were incubated with the label without medium change and processed as described (37). Nearly 80% of the ECM radioactivity was incorporated into HSPGs.

Heparanase activity

Hpa-transfected Eb cells were incubated (24 h, 37°C, pH 6.6) with ^{35}S-labeled ECM. The incubation medium was centrifuged and the supernatant containing sulfate-labeled degradation fragments was analyzed by gel filtration on a Sepharose CL-6B column (0.9×30 cm). Fractions (0.2 mL) were eluted with PBS and their radioactivity counted in a β-scintillation counter. Degradation fragments of HS side chains were eluted from Sepharose 6B at $0.5 < K_{av} < 0.8$ (peak II). Nearly intact HSPGs were eluted just after the V_0 ($K_{av} < 0.2$, peak I) (23, 31). The experiment was performed at least three times and the variation in elution positions (K_{av} values) did not exceed ± 15%.

Cell adhesion

Hpa-transfected Eb cells were grown (1×10^6 cells/mL, 48 h, 37°C) in complete medium in the presence of [^{3}H]-thymidine (1 μCi/mL) (Amersham). The labeled cells were washed (×3) free of unincorporated thymidine and incubated (37°C, pH 7.2) in complete medium for various periods on either intact naturally produced ECM (15 min–8 h) or confluent vascular endothelial cell monolayers (2–24 h). After incubation, cells were washed (×3) with serum-free

medium; the remaining firmly attached cells were solubilized (2 h, 0.2 M NaOH, 37°C) and counted in a β-scintillation counter. In some experiments the ECM was pretreated with ECM-degrading enzymes (heparanase, Chondroitinase ABC, hyaluronidase). Cell adhesion was also performed in the presence of 1 mg/mL RGD or RAD peptides (Calbiochem, La Jolla, CA, USA).

Immunoprecipitation and Western immunoblot

Eb cells were incubated on naturally produced ECM (2×10^6 cells, 20 and 60 min, RPMI complete medium, 37°C), rinsed with PBS, and lysed with RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris, pH 8.0, 20 μg/mL aprotinin, 2 μg/mL leupeptin, 1 μg/mL pepstatin, 2 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate), followed by immunoprecipitation (IP) with anti-paxillin monoclonal antibodies (Transduction Laboratories, Lexington, KY, USA), and protein-G Sepharose beads (Sigma, St. Louis, MO, USA). Cell extracts were then subjected to 10% SDS-PAGE and Western blot analysis using the anti-paxillin or anti-phosphotyrosine monoclonal antibodies (4G10, Upstate Biotechnologies, Lake Placid, NY, USA), followed by secondary peroxidase-conjugated goat anti-mouse antibodies. For phospho-FAK detection, cells were incubated on ECM, processed as described above, and cell lysates were subjected to immunoblotting with anti-phospho-FAK polyclonal antibodies (Cell Signaling Technology, Beverly, MA, USA) and HRP-conjugated goat anti-rabbit antibodies.

Matrigel invasion assay

Eb cells were grown for 48 h in the presence of [^{3}H]-thymidine (1 μCi/mL) (Amersham) and assayed (37°C, 5% CO_2 incubator, 6 h) for invasion through Matrigel-coated filters using blind-well chemotaxis chambers and polycarbonate filters, as described (29). Medium conditioned by 3T3 fibroblasts was applied as a chemoattractant and placed in the lower compartment of the Boyden chamber (29). After incubation, the upper surface of the filter was wiped free of cells and filters were counted in a β-scintillation counter.

Indirect immunofluorescence

Eb cells were permeabilized for 3 min in PBS containing 0.5% Triton X-100 and 4% formaldehyde, followed by fixation with 4% formaldehyde in PBS for 20 min. The cells were then incubated for 1 h with anti-paxillin monoclonal antibodies in PBS, washed, and further incubated (45 min, 24°C) with Cy3-conjugated goat anti-mouse antibodies and FITC-conjugated phalloidin. Eb cells were also double stained with anti-paxillin monoclonal antibodies (Transduction Laboratories) and with anti-phosphotyrosine polyclonal antibodies (Upstate Biotechnologies), applying same fixation and incubation conditions as described above. After extensive washes, coverslips were mounted in Elvanol and images of the double-stained cells were acquired using an Axioscope microscope (Zeiss, Oberkochen, Germany) equipped with a charged-coupled device camera (1024×1024 pixel chip readout generating 12-bit digital data). Heparanase staining was performed on either permeabilized (methanol, –20°C, 5 min) or nonpermeabilized (4% freshly prepared paraformaldehyde in PBS, 20 min, 24°C) cells. Cells were incubated with anti-heparanase rabbit polyclonal antibodies (p9), kindly provided by Insight Ltd. (Rehovot, Israel), diluted to 2 μg/mL. These antibodies are directed against a specific peptide: PGKKVWLGETSSAYGGGAP of the human heparanase enzyme. Cy2-conjugated goat anti-mouse antibodies (Jackson Immunoresearch Laboratories, Bar Harbor, MA, USA) diluted 1:200 were used as secondary antibodies.

RESULTS

Cell surface heparanase increases cell adhesion to ECM and endothelial cells

Mouse Eb lymphoma cells are anchorage independent, grow in suspension, and exhibit no attachment or cell spreading when plated on regular or ECM-coated dishes. We have noticed that Eb cells stably transfected with a cDNA-encoding, surface-associated and secreted form of heparanase (chimeric *hpa*) (35) became firmly attached to the tissue culture plastic, exhibiting an adherent phenotype and spreading characteristic of anchorage-dependent cells (**Fig. 1*A***). On the other hand, Eb cells overexpressing the human enzyme (H-*hpa*) and exhibiting the heparanase protein predominantly in perinuclear acidic vesicles (35) grew in suspension and remained floating (Fig. 1*A*), similar to mock-transfected cells (not shown). To better elucidate the involvement of cell surface heparanase in cell adhesion and spreading, Eb cells transfected with surface-associated (Chk-*hpa*, chimeric *hpa*) or perinuclear (H-*hpa*) heparanase were compared for their ability to adhere to a naturally produced subendothelial ECM and to a confluent vascular endothelial cell monolayer. The various heparanase constructs are schematically presented in Fig. 1*B*. As demonstrated in Fig. 1*C*, cells expressing surface-associated heparanase (Chk-*hpa*, chimeric *hpa*) adhered to ECM within minutes. Nearly 50% of the added cells attached to the ECM within 15 min of incubation whereas 90–100% of the cells adhered to the ECM within 1 h, remaining firmly bound thereafter (Fig. 1*C*). Cell adhesion was followed by cell spreading (Fig. 1*A*, top-left). In contrast, cells transfected with the human heparanase (H-*hpa*) or mock-transfected cells exhibited very low or no adhesion to ECM even after a prolonged (i.e., 8 h) incubation time (Fig. 1*C*). These experiments indicate that heparanase stimulates rapid and firm cell adhesion in nonadherent cells if the enzyme is expressed on the cell surface and/or secreted.

The various *hpa*-transfected cells were tested for their ability to adhere to confluent, contact-inhibited endothelial cell monolayers. Regardless of the heparanase type expressed, Eb cell adhesion to endothelial cell monolayers was relatively slow (~15% of the added *hpa*-transfected cells adhered within 2 h) and reached a lower level (< 30% cell adhesion within 24 h) compared with cell adhesion to ECM. A three- to fivefold increased adhesion was noted with Chk-*hpa*-transfected cells overexpressing surface heparanase vs. cells expressing the enzyme in perinuclear acidic vesicles (H-*hpa*) or mock-transfected cells, respectively (Fig. 1*D*). Whereas cell adhesion to ECM and endothelial cells was prominent after 15 min and 8 h of incubation, respectively, adhesion to plastic required at least 24 h and occurred exclusively with cells

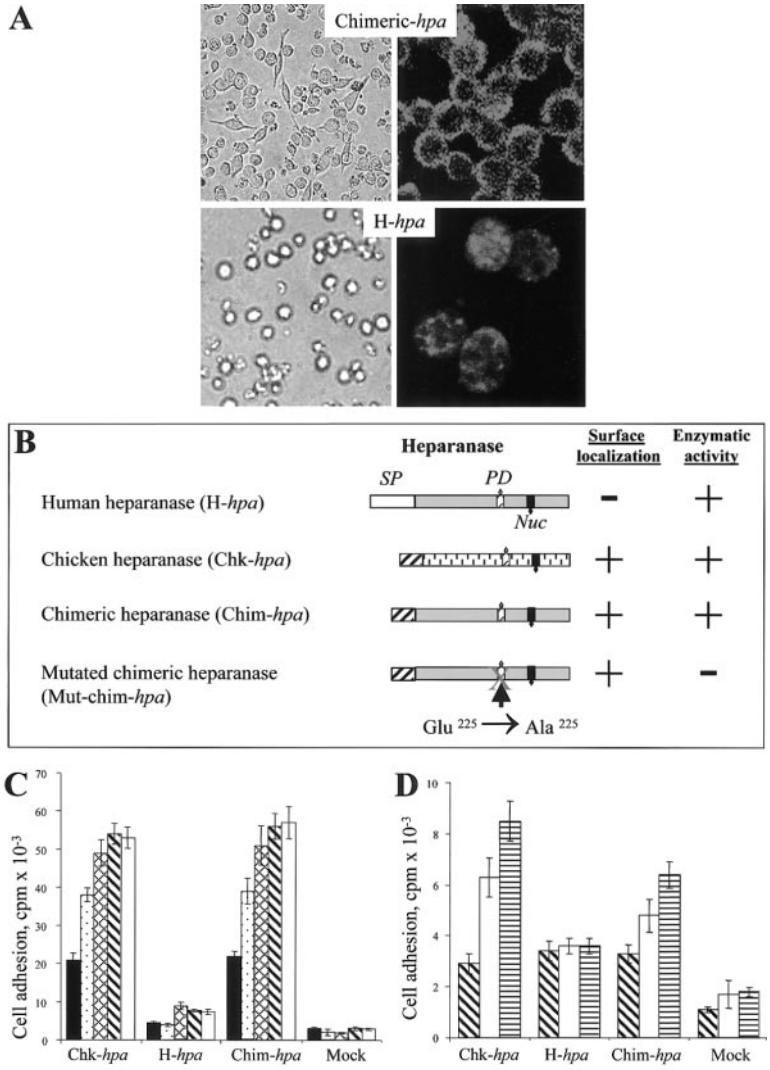

Figure 1. Cell surface heparanase increases cell adhesion to ECM and endothelial cells. *A)* Left panels: morphology of Eb mouse lymphoma cells expressing surface-associated (chimeric *hpa*) vs. perinuclear (H-*hpa*) heparanase. Cells were grown in RPMI medium (pH 7.4) containing 10% fetal calf serum in the presence of 150 μg/mL G418 and photographed (phase-contrast microscope, ×200) 18 h after plating. Cells transfected with H-*hpa* (bottom, left) are floating and hence appear out of focus. Right panels: Immunofluorescent staining of Eb cells transfected with chimeric *hpa* (top) or H-*hpa* (bottom), applying rabbit anti heparanase polyclonal antibodies (p9) and Cy2-conjugated goat anti-rabbit antibodies. *B)* Schematic presentation of the various heparanase constructs used for transfection. *SP,* signal peptide; *PD,* proton donor; *Nuc,* nucleophil; hatched box, Chk-*hpa* signal peptide. *C, D)* Cell attachment. Eb cells expressing the various heparanase forms were prelabeled with ^{3}H-thymidine and seeded on ECM (*C*) or endothelial cell monolayer (*D*). Cells suspended in RPMI medium were allowed to attach for various periods (■ 15 min; ⬚ 30 min; ☒ 1 h; ▨ 2 h; □ 8 h; ⊟ 24 h) at 37°C and the extent of cell adhesion was measured as described in Materials and Methods. Experiments were performed at least 3 times and each data point is the mean ± SD of quadruplicates wells.

expressing the surface-associated and secreted enzyme (not shown).

Since Eb murine lymphoma cells are nonadherent and grow in suspension, we examined whether heparanase can affect the adhesive properties of adherent cells as well. We found that human primary foreskin fibroblasts that were first incubated with exogenously added 65 kDa latent heparanase attached faster (4- to 5-fold) to the tissue culture plastic than control untreated cells (not shown). Again, heparanase-stimulated cell attachment was accompanied by increased cell spreading (not shown). Similarly, preincubation (30 min, 37°C) of Eb cells with recombinant heparanase (1–10 μg/mL) resulted in a threefold increase in cell adhesion to ECM vs. control untreated cells (not shown). We have previously demonstrated that recombinant latent heparanase (65 kDa) binds to the cell surface and then is processed into its highly active 50 kDa form (39).

RGD-containing peptides inhibit heparanase-mediated cell spreading, but not adhesion

Heparanase-mediated cell adhesion is likely to involve interaction between the surface-associated enzyme and the ECM HS. To investigate this possibility, ECM was treated with human recombinant heparanase before its incubation with chimeric *hpa*-transfected Eb cells. As demonstrated in **Fig. 2**, exposure of ECM to heparanase had no effect on cell adhesion despite removal of ~65% of the total sulfate-labeled material as measured by release of labeled HS degradation products. Similarly, cell adhesion was not affected by a combined pretreatment of the ECM with heparanase and Chondroitinase ABC, removing 85–90% of the ECM sulfate-labeled material (not shown). Cell attachment also was not inhibited in the presence of 10 μg/mL heparin, again suggesting that cell adhesion mediated by cell surface heparanase does not necessarily involve interaction with the ECM HS. Next we examined the possible involvement of integrin receptors in heparanase-mediated cell adhesion. Chimeric *hpa*-transfected Eb cells were incubated on ECM in the absence or presence of an RGD-containing peptide (i.e., Gly-Arg-Gly-Asp-Ser-Pro). An RAD-containing peptide (i.e., Gly-Arg-Ala-Asp-Ser-Pro) was used as a control. As demonstrated in Fig. 2*A*, neither peptide affected the number of cell adhering to the ECM; nor did the peptides affect cell adhesion to ECM pretreated with

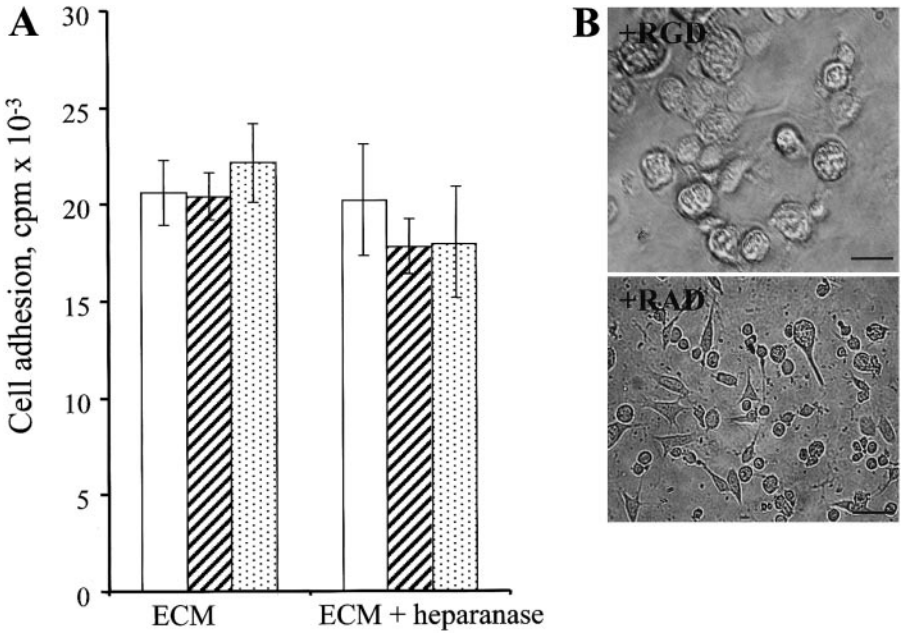

Figure 2. Effect of RGD peptide on heparanase-mediated cell adhesion and spreading. *A)* ^{3}H-thymidine-labeled Eb mouse lymphoma cells overexpressing surface-associated heparanase (chimeric *hpa*) were preincubated (1×10^6 cells/mL, 1 h, RPMI medium, pH 7.4, 37°C) in the absence (□) or presence of 1 mg/mL RGD- (▨) or RAD (▩)-containing peptides. The cells were then incubated (1 h, 37°C, RPMI, pH 7.4) on intact ECM or ECM that was first treated with recombinant human heparanase (2.5 μg/mL, 24 h pH 5.8, 37°C). Unbound cells were washed away and the remaining firmly attached cells were solubilized in 1 M NaOH and counted in a β-scintillation counter, as described in Materials and Methods. Each data point represents the mean ± SD of quadruplicate wells. *B)* Cell spreading. Eb cells were incubated on ECM for 1 h in the presence of 1 mg/mL RGD- (top, bar=20 μm) or RAD- (bottom, bar=50 μm) containing peptides and photographed under phase-contrast microscopy.

active 50 kDa human recombinant heparanase (Fig. 2*A*). However, a marked difference was noted upon microscopic examination of the attached cells. Whereas in the absence (Fig. 1*A*) or presence (Fig. 2*B*, bottom) of the RAD peptide the chimeric *hpa*-transfected cells were spread and exhibited cell processes, cells plated on ECM in the presence of the RGD peptide were firmly attached, but failed to spread and remained round (Fig. 2*B*, top). These results indicate that cell surface heparanase play a role in the initial cell attachment whereas cell spreading involves the subsequent participation of integrin receptors.

Heparanase-mediated cell attachment is associated with paxillin recruitment and tyrosine phosphorylation

Cell adhesion may result in generation of several types of cell–ECM attachment sites (e.g., focal contacts, fibrillar adhesions) (40). We examined whether cell surface heparanase triggers the formation of characteristic cell–ECM adhesions. Both Chk-*hpa*- (**Fig. 3*A***) and chimeric *hpa* (not shown) -transfected Eb cells, adhere, spread, and form elongated cellular processes when plated on ECM. We observed that the cytoskeletal protein paxillin (red) is organized in defined regions within these processes; however, these regions contained relatively low amounts of phosphotyrosine (Fig. 3*B*, green). Moreover, the actin cytoskeleton (FITC-conjugated phalloidin) of the heparanase-transfected Eb adherent cells was organized in a cortical pattern rather then in elongated stress fibers (Fig. 3*A*). A similar staining pattern was obtained with Chk-*hpa*- (Fig. 3*A*) and chimeric *hpa* (not shown) -transfected cells.

Integrin-mediated cell adhesion often results in the activation of various biochemical responses, including tyrosine phosphorylation of cytoskeletal molecules (e.g., FAK, paxillin) (9). These initial biochemical events may trigger the activation of signaling pathways (e.g., ERK) that affect cellular responses (e.g., cell proliferation). We examined whether cell adhesion stimulated by cell surface-associated heparanase results in activation of signaling events. Chk-*hpa*-transfected cells were seeded on ECM for 30 min and stained for both paxillin and phosphotyrosine. As shown in Fig. 3*B*, paxillin (red) and phosphotyrosine (green) exhibited a low degree of colocalization, as demonstrated by the few yellow spots representing phosphorylated paxillin-containing sites. The same staining pattern was obtained with chimeric *hpa*-transfected lymphoma cells. The occurrence of phosphorylated paxillin is also demonstrated in Fig. 3*C* in which paxillin was first concentrated by immunoprecipitation, then subjected to immunoblotting with anti-phosphotyrosine antibodies. In all the species of *hpa*-transfected Eb cells, paxillin was not phosphorylated on tyrosine when the cells were in suspension (Fig. 3*C*). However, when plated on ECM, tyrosine phosphorylation of paxillin, lasting for at least 1 h, was detected in the adherent Chk-*hpa*- (not shown) and chimeric *hpa* (Fig. 3*C*) -transfected Eb cells. We next evaluated the occurrence of phosphorylated FAK in ECM-adhering Eb lymphoma cells expressing cell surface heparanase. H-*hpa*-transfected and chimeric *hpa*-transfected lymphoma cells were seeded on ECM (37°C, 30 min), collected, and subjected to SDS/PAGE and Western blot analysis using anti-phosphorylated FAK antibodies. Unlike control 3T3 fibroblasts, phosphorylated FAK was hardly detected in chimeric *hpa*-transfected cells that adhere to ECM and there was very little detectable phospho-FAK in the H-*hpa*-transfected

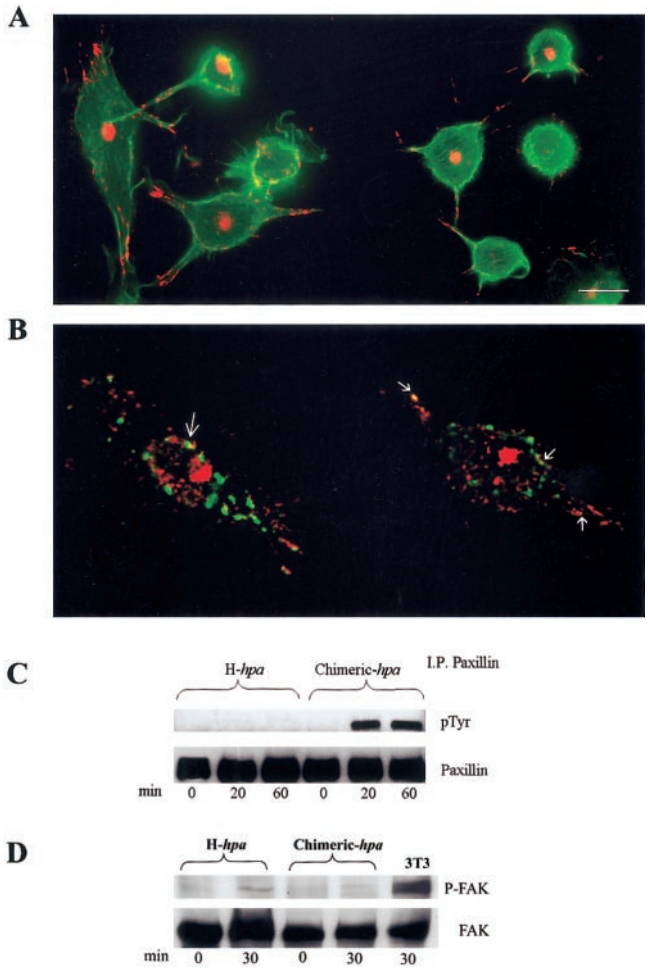

Figure 3. Heparanase-mediated cell adhesion is associated with paxillin tyrosine phosphorylation. *A)* Double staining of paxillin and actin. Chk-*hpa*-transfected Eb cells were seeded (30 min, 37°C) on coverslips coated with ECM and subjected to double immunofluorescence staining with *1)* anti-paxillin monoclonal antibodies, followed by Cy3-conjugated goat anti-mouse antibody (red), and *2)* FITC-conjugated phalloidin (green, bar=20 μm). *B)* Immunostaining of paxillin and phosphotyrosine. Chk-*hpa*-transfected Eb cells were seeded on ECM and subjected to double immunofluorescent staining with *1)* anti-paxillin antibodies (as described in panel *A*) (red) and *2)* anti-phosphotyrosine polyclonal antibodies, followed by Cy2-conjugated goat anti-rabbit antibodies (green). Yellow spots (arrows) indicate paxillin and phosphotyrosine colocalization. *C)* Tyrosine phosphorylation of paxillin. H-*hpa*- and chimeric-*hpa*-transfected Eb cells were seeded (1×10^6 cells/mL) on ECM in complete medium. The cells were collected after 20 and 60 min of incubation, lysed, and subjected to immunoprecipitation (IP) with anti-paxillin mAb. Immunoprecipitated material was bound to protein G-Sepharose and subjected to 10% SDS/PAGE and Western blot analysis using 4G10 anti-phosphotyrosine antibodies (see Materials and Methods). Cells that were not incubated on ECM were used as zero time control. Paxillin phosphorylation was sustained for at least 1 h in the adhering cells. *D)* FAK phosphorylation. Eb lymphoma cells (2×10^6 cells) transfected with H-*hpa* or chimeric-*hpa* were incubated on ECM (37°C, 30 min), collected, and lysed as described in panel *C*. Cell lysates were subjected to Western blot analysis with anti-phospho-FAK polyclonal antibodies. 3T3 fibroblasts were used as a positive control. Cells that were not incubated on ECM were used as zero time control.

cells (Fig. 3*D*). Altogether, these data suggest that heparanase may promote the formation of adhesion sites similar to the recently described 3-dimensional adhesions formed by cells plated on endothelial cell derived ECM (41). In contrast to integrin-mediated signaling activation, we did not detect ERK activation after heparanase-mediated cell adhesion (not shown).

Heparanase-mediated cell adhesion does not require heparanase enzymatic activity

The above-described cell adhesion mediated by heparanase expressed on the cell surface was detected in a physiological pH (~7.4) in which heparanase binds to heparin or HS, but no enzymatic activity was detected (34). It is therefore conceivable that the heparanase-mediated cell adhesion may be independent of its endoglycosidase activity. To investigate this possibility, we examined the adhesive properties of heparanase-transfected cells in the absence or presence of laminaran sulfate, a potent inhibitor of heparanase activity and experimental metastasis (42). Laminaran sulfate is not a substrate for heparanase, but binds to the enzyme and inhibits its hydrolytic activity. As shown in **Fig. 4*A***, laminaran sulfate failed to inhibit adhesion of Chk-*hpa*- and chimeric *hpa*-transfected Eb cells to ECM, although it efficiently inhibited heparanase activity (not shown).

To further investigate the relation between heparanase activity and its involvement in cell adhesion, we have generated a mutated chimeric heparanase (Mut-chimeric *hpa*, Fig. 1*B*) that lacks heparanase activity. The proton donor Glu^{225} in the active site of the human heparanase (38) was point mutated and substituted with alanine in order to abolish heparanase activity (38). Eb cells stable transfected with the mutated *hpa* construct expressed the processed 50 kDa heparanase protein (Fig. 4*B*, inset) but failed to exhibit any heparanase activity (Fig. 4*B*). Cell surface biotinylation (not shown) as well as immunofluorescent staining (Fig. 4*C*, inset) using polyclonal anti-heparanase antibodies revealed intense cell surface expression of the mutated enzyme in nonpermeablized cells, similar to that observed with chimeric *hpa*-transfected Eb cells overexpressing the active cell surface-associated heparanase. Evaluation of cell adhesion revealed that cells expressing the Mut-chimeric *hpa* firmly attached to ECM (Fig. 4*C*) and endothelial cell monolayers (Fig. 4*D*) in a manner similar to chimeric *hpa*-transfected cells. Mock-transfected cells exhibited little or no adhesion. These results clearly indicate that heparanase-mediated cell adhesion is independent of its enzymatic activity provided the enzyme is expressed on the cell surface.

Unlike the adhesion to ECM, Eb cells expressing the cell surface-associated heparanase (Chk-*hpa*, chimeric *hpa*) failed to attach to poly-L-lysine-coated tissue culture plastic. In contrast, cells expressing the enzyme predominantly in perinuclear endosomal/lysosomal granules firmly adhered to poly-L-lysine (Fig. 4*E*) compared with little or no adhesion to ECM (Fig. 1*C* and

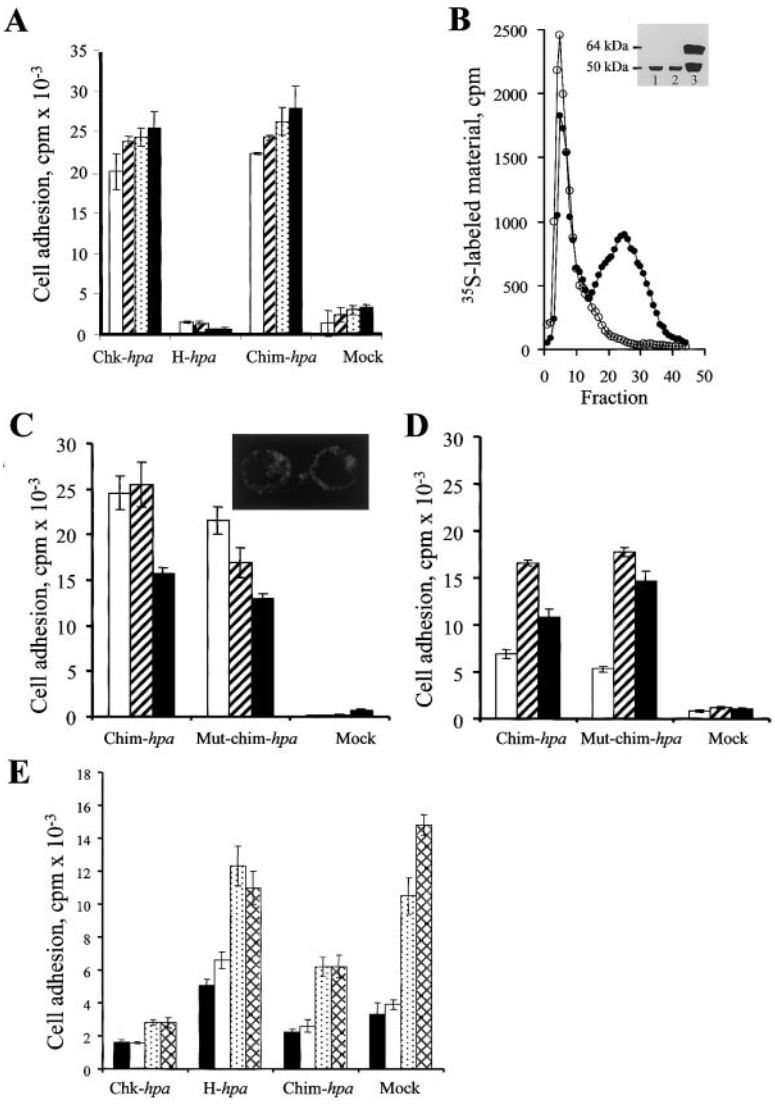

Figure 4. Heparanase-mediated cell adhesion is independent of heparanase activity. *A)* Effect of laminaran sulfate. The various *hpa*-transfected cells were prelabeled with ^{3}H-thymidine and incubated (RPMI complete medium, pH 7.4, 37°C) on ECM for 30 min (□, ▨) or 6 h (▩ , ■) in the absence (□, ▩) or presence (▨ , ■) of 10 μg/mL laminaran sulfate. The extent of cell adhesion was determined as described in the legend of Fig. 2. *B)* Heparanase activity. Eb cells stable transfected with chimeric *hpa* (●) or the mutated *hpa* construct (Mut-chimeric *hpa*) (○) were incubated with ^{35}S-labeled ECM for 24 h at 37°C (pH 6.2). ^{35}S-labeled degradation fragments released into the incubation medium were analyzed by gel filtration on Sepharose 6B, as described in Materials and Methods. Inset: Western blot analysis. Lysates of chimeric *hpa*- (lane 1) and of Mut-chimeric *hpa* (lane 2) -transfected cells were subjected to Western immunoblot analysis as described in the text. Recombinant heparanase preparation containing both the 50 and 65 kDa forms was used as control (lane 3). The 50 kDa heparanase protein is similarly expressed by the chimeric *hpa* and mut-chimeric *hpa*-transfected cells. *C, D)* Inactive heparanase promotes cell adhesion. ^{3}H-thymidine-labeled cells chimeric *hpa* and Mut-chimeric *hpa*-transfected cells were incubated for various periods (□ 30 min; ▨ 2 h; ■ 24 h) on ECM (*C*) or endothelial cell monolayers (*D*) and evaluated for cell adhesion, as described in Fig. 2. Mock-transfected cells were used as control and exhibited little or no adhesion. Inset (*C*): Immunofluorescent staining. nonpermeabilized Mut-chimeric *hpa*-transfected cells were immunostained with polyclonal anti-heparanase antibodies (p9), as described in Materials and Methods. Cell surface staining of the mutated enzyme, was similar to that observed with Eb cells transfected with chimeric *hpa* and expressing an active cell surface-associated heparanase. *E)* Cell adhesion to poly-L-lysine. ^{3}H-thymidine-labeled Eb cells transfected with the various *hpa* constructs were incubated (1×10^{6} cells/mL, RPMI complete medium, 37°C) on poly-L-lysine-coated tissue culture plastic for 15 min (■), 30 min (□), 2 h (▩), and 6 h (☒). Cell adhesion was determined as described in the legend to Fig. 2.

Fig. 4*C*). These results suggest that heparanase-mediated cell adhesion may be attributed in part to an effect on the net cell surface charge. Cell surface heparanase may interact with adjacent cell surface HS, reducing the net negative charge in this specific microenvironment and thereby accelerating interactions with the negatively charged ECM.

Heparanase-mediated cell adhesion promotes cell invasion

Previous studies revealed a correlation between heparanase expression and the metastatic potential of malignant cells (20, 22, 29) as well a as correlation between tumor metastasis and cell adhesion (43–46). We have recently demonstrated that cell surface expression and secretion of heparanase markedly promote cell invasion both in vitro (Matrigel invasion) and in vivo (metastatic dissemination) (29). Since cell adhesion is a prerequisite for cell invasion, we examined the invasive properties of Eb cells overexpressing the enzymatically inactive, surface-associated heparanase (Mut-chimeric *hpa*). Eb cells stable transfected with H-*hpa*, chimeric *hpa*, or Mut-chimeric *hpa* were compared for their ability to invade a reconstituted basement membrane (Matrigel). As demonstrated in **Fig. 5**, cells overexpressing the surface-associated, active chimeric heparanase exhibited the highest degree of Matrigel invasion (4-fold higher than cells transfected with H-*hpa*). Cells expressing the mutated, enzymatically inactive heparanase invaded the Matrigel to a significantly higher extent (P=0.002) than that exhibited by H-*hpa*-transfected cells, albeit lower than the invasion capacity of chimeric *hpa*-transfected cells overexpressing active heparanase (Fig. 5). The various *hpa*-transfected cell types did not differ in their motility on filters coated with collagen type IV, a process that does not involve enzymatic degradation, nor in their gelatinolytic activ-

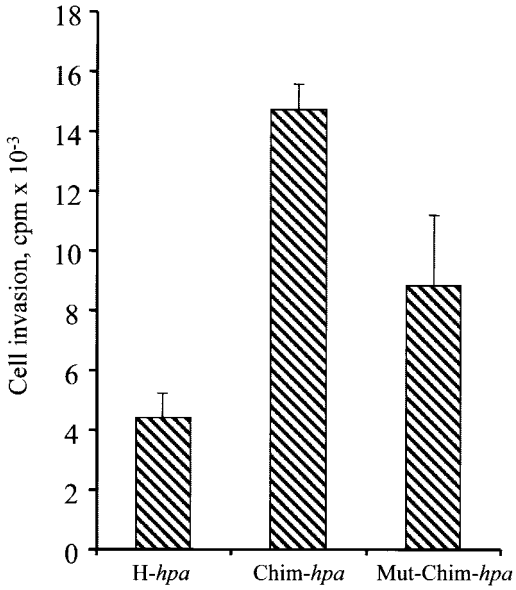

Figure 5. Invasion through Matrigel of cells expressing active vs. inactive heparanase. ^{3}H-thymidine-labeled Eb cells transfected with H-*hpa*, chimeric *hpa*, or Mut-chimeric *hpa* were incubated (1×10^{6} cells/mL, 6 h, 37°C, pH 7.4) in RPMI medium supplemented with 0.1% BSA on top of Matrigel-coated filters. After incubation, the upper surface of the filter was wiped free of cells and the extent of cell invasion was detected by counting in a β-scintillation counter (Materials and Methods). Each data point is the mean ± SD of triplicate filters.

ity, as evaluated by zymography (not shown). These results indicate that cell surface heparanase facilitates cell invasion through its combined effect on cell adhesion and HS degradation.

DISCUSSION

Heparan sulfate and its cleavage by heparanase participate in diverse normal and pathological processes such as development, morphogenesis, tissue repair, inflammation, metastasis, and angiogenesis. Heparanase activity and localization must therefore be tightly regulated (20, 30, 35, 39). For example, the enzyme is highly sensitive to changes in local pH, exerting a high enzymatic activity under acidic conditions that exists in the vicinity of tumors and in inflammatory sites vs. little or no activity at a physiological pH (34, 38). Since the enzyme degrades primarily an extracellular component, regulation by the cell microenvironment is highly probable (36). The occurrence of heparanase on cell surfaces (35) and its secretion into the extracellular space within tissues (39, 47) suggest that at a physiological pH (pH>7.2) heparanase may exert functions other then enzymatic degradation of heparan sulfate (36). To investigate this possibility, we transfected anchorage-independent mouse Eb lymphoma cells with various constructs containing heparanase and examined their adhesion to ECM or to endothelial cell monolayers. Our results demonstrate that surface-associated heparanase promotes a firm adhesion of otherwise nonadherent lymphoma cells compared with cells overexpressing the intracellular form of the enzyme, which remain floating. Clearly, however, the effect of soluble recombinant heparanase on cell adhesion was minor relative to the fast and firm cell adhesion obtained with cell expressing the cell surface-associated enzyme. A possible explanation to the inefficiency of the soluble enzyme in promoting cell adhesion is its low binding and retention on the cell surface. In fact, we observed very little binding of recombinant heparanase to the Eb lymphoma cells. It is also possible that the endogenous cell surface heparanase adopts a conformation that is more favorable to mediating cell adhesion and/or elicits signaling events that promote early cell adhesion. It should be noted that both immunostaining (48, 49) and biochemical studies (20, 32), indicate that heparanase is occasionally detected on cell surfaces whereas it may normally also function in promoting cell adhesion.

Cell adhesion was not affected by laminaran sulfate, a potent inhibitor of heparanase activity and experimental metastasis (42). This result indicates that heparanase-mediated cell adhesion does not necessarily require its enzymatic activity. To further investigate this feature, we have generated a mutated heparanase, using the chimeric *hpa* cDNA as a template for a point mutation, replacing the active site proton donor Glu^{225} with Ala. Examination of the mutated enzyme's cellular localization and enzymatic activity confirmed the generation of a surface-associated, but enzymatically inactive form of heparanase. Evaluation of cell adhesion revealed, however, that cells overexpressing the mutated heparanase adhere to ECM to the same extent as cells expressing the surface-associated active enzyme. These results clearly indicate that heparanase-mediated cell adhesion is independent of its HS-degrading activity provided that the enzyme is expressed on the cell surface.

Cell adhesion often results in activation of various biochemical responses, including tyrosine phosphorylation of cytoskeletal elements such as FAK and paxillin (9). We therefore examined whether cell surface heparanase triggers the formation of characteristic cell–ECM adhesions. We observed that lymphoma cell spreading on ECM was accompanied by organization of paxillin within cellular processes formed by the adhering cells. These regions, however, contained relatively low amounts of phosphotyrosine, exhibiting little or no colocalization with paxillin. Also, there was little or no phosphorylated FAK in ECM-adhering Eb lymphoma cells expressing surface-associated heparanase. Moreover, the actin cytoskeleton of the adherent Eb cells was organized in a cortical pattern rather then in elongated stress fibers. Altogether, these results suggest that

heparanase may promote the formation of adhesion sites similar to the recently described 3-dimensional matrix adhesions, typical for cell adhering to 3-dimensional ECM, rather then focal contacts characteristic of cell attachment to 2-dimensional surfaces (41). Unlike integrin induced cell signaling and activation, we did not detect ERK activation in response to heparanase-mediated cell adhesion, nor was the heparanase-mediated early cell adhesion inhibited by RGD-containing peptides. These peptides inhibited, however, spreading of the same *hpa*-transfected cells, indicating that a non-integrin primary attachment phase is first stimulated by cell surface heparanase, followed by integrin-dependent cell spreading.

The exact mechanism(s) by which heparanase promotes rapid cell adhesion is still unclear. We found that cells expressing the various forms of cell surface-associated heparanase, including the inactive enzyme, attached to HS-coated beads whereas cells expressing intracellular heparanase or mock-transfected cells failed to attach to these beads (not shown). This result suggests that cell surface heparanase may bind to HS on the adhesion surface, whether ECM or endothelial cells, thereby forming a bridge between circulating cells and the blood vessel wall. Preincubation of the cells with excess exogenous heparin did not inhibit their binding to ECM, however. Also, pretreatment of the ECM with heparanase, hyaluronidase, Chondroitinase ABC, or their combination did not inhibit the adhesion of cells expressing surface-associated heparanase to ECM. It is therefore conceivable that the ECM counterpart on the adhering cells may not necessarily be a glycosaminoglycan (i.e., heparan sulfate, hyaluronic acid, chondroitin sulfate, dermatan sulfate). On the other hand, cells expressing membrane-associated heparanase attached poorly to surfaces coated with poly-L-lysine whereas mock- or H-*hpa*-transfected cells attached quiet well to this positively charged substrate. Thus, heparanase may alter the cell surface charge, thereby modulating early cell adhesion. This possibility is primarily relevant to cells that exhibit little or no HS on their surface, such as Eb lymphoma cells.

Cell surface HS and their core proteins may promote or inhibit cell adhesion and invasion (15, 17, 50). Inhibition occurs via HSPG binding and/or masking of integrin receptors or through a direct interaction of adhesion molecules with HS. In view of this, binding of cell surface heparanase to HS may liberate the inhibitory effect of HS, thus promoting subsequent cell adhesion. This effect may be more relevant to cells that express large amounts of surface HS.

An effect of heparanase on the surface properties of cells may trigger the observed reorganization of cytoskeletal molecules (e.g., F-actin, paxillin) and tyrosine phosphorylation of paxillin. Actin reorganization and tyrosine phosphorylation are associated with cell adhesion and motility (51).

We found that heparanase-mediated cell adhesion also promoted cell invasion through a reconstituted BM (Matrigel). Inactive heparanase was found to stimulate cell invasion, possibly due to its effect on cell adhesion and cytoskeletal organization, both critically involved in cell invasion (44). The increased invasion of cells expressing inactive mutant heparanase was best demonstrated with nonadhering cells, such as the lymphoma cells used in this study, relative to a small effect observed with firmly adherent cells.

Our results indicate that apart from its well-established role as a HS-degrading enzyme, heparanase may function as a proadhesive molecule independent of its endoglycosidase activity. The combined feature of heparanase as an enzyme and cell adhesion molecule further emphasizes its potential significance in processes involving cell adhesion, migration, and invasion such as tumor metastasis, neovascularization, inflammation, and autoimmunity. In fact, we recently demonstrated that lymphoma cells overexpressing cell surface heparanase elicit a markedly increased angiogenic response and metastatic dissemination in vivo (29). The significance of cell surface localization and secretion of ECM-degrading enzymes in cancer metastasis was previously demonstrated by showing a correlation between the metastatic potential of breast and bladder carcinoma cells and translocation of cathepsin D and B from within lysosomes to the plasma membrane (52). Clearly, enzymes expressed on the cell surface and/or secreted are more effective then intracellular enzymes in mediating cell adhesion and invasion. Studies are under way to determine the regulation of heparanase translocation from within cytoplasmic vesicles to the cell membrane. Once localized on the cell surface, heparanase may function as an endoglycosidase or as a cell adhesion molecule, depending on the local pH, as also suggested by Gilat at al. (36). At relatively acidified pH conditions, heparanase performs as a HS-degrading enzyme, whereas at the physiological pH of a quiescent tissue it may function primarily as an adhesion molecule. Thus, the local state of a tissue can regulate the activities of heparanase and can determine whether it will function as an enzyme and/or as a proadhesive molecule.

Our results ascribe a new function for heparanase, paving the way for studies focusing on nonenzymatic activities of the heparanase molecule, similar to those exerted by other ECM-degrading enzymes (i.e., thrombin, plasminogen activator, MMPs) (53, 54). We have shown that latent heparanase readily binds to the cell surface, followed by processing, activation and internalization (39). Studies are under way to elucidate the nature of the respective cell surface receptor, processing enzyme, and the possible involvement of heparanase in signal transduction and cell behavior. FJ

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