

## Heparanase Induces Endothelial Cell Migration via Protein Kinase B/Akt Activation\*

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**Heparanase is a mammalian endoglycosidase that degrades heparan sulfate (HS) at specific intra-chain sites. Blood-borne neutrophils, macrophages, mast cells, and platelets exhibit heparanase activity that is thought to be stored in specific granules. The degranulated heparanase is implicated in extravasation of metastatic tumor cells and activated cells of the immune system. Degranulation and heparanase release in response to an inflammatory stimulus or platelet activation would facilitate cellular extravasation directly, by altering the composition and structural integrity of the extracellular matrix, or indirectly, by releasing HS-bound proinflammatory cytokines and chemokines. We hypothesized that in addition to such indirect effect, the released heparanase may also locally affect and activate neighboring cells such as endothelial cells. Here, we provide evidence that addition of the 65-kDa latent heparanase to endothelial cells enhances Akt signaling. Heparanase-mediated Akt phosphorylation was independent of its enzymatic activity or the presence of cell membrane HS proteoglycans and was augmented by heparin. Moreover, addition of heparanase stimulated phosphatidylinositol 3-kinase-dependent endothelial cell migration and invasion. These results suggest, for the first time, that heparanase activates endothelial cells and elicits angiogenic responses directly. This effect appears to be mediated by as yet unidentified heparanase receptor.**

Heparanase is an endo- $\beta$ -D-glucuronidase capable of cleaving heparan sulfate (HS)<sup>1</sup> side chains at a limited number of sites, yielding HS fragments of still appreciable size (~5–7 kDa) (1–3). Participating in extracellular matrix (ECM) degradation and remodeling, heparanase activity has been traditionally correlated with the metastatic potential of tumor-derived cells

(4–7). Similarly, heparanase has been shown to facilitate cell invasion associated with angiogenesis, autoimmunity, and inflammation (6–9). Among the few cell types that express heparanase under normal physiological conditions, platelets possess a high heparanase activity and were used as a source for heparanase purification (2, 10). In fact, serum heparanase is mainly derived from activated platelets (11). Heparanase was localized to tertiary granules of neutrophils (12, 13) and mast cells (7) and was released upon tumor necrosis factor- $\alpha$  and calcium ionophore treatments, respectively. Heparanase release by degranulation has been implicated in diapedesis and extravasation of a number of immune cells, including neutrophils, macrophages, and lymphocytes (8, 14, 15), while heparanase inhibitors exhibited an anti-inflammatory activity (15). Cleavage of HS side chains by degranulated heparanase during inflammation may facilitate the passage of blood-borne normal and malignant cells into tissues by altering the composition and structural integrity of the subendothelial ECM (1, 8, 14). In addition, heparanase may facilitate the release of a multitude of HS-bound growth factors, cytokines and chemokines that would, in turn, amplify the immune reaction or activate the vascular endothelium (16, 17). We hypothesized that in addition to this indirect effect mediated by HS-bound growth factors, degranulated heparanase may also activate endothelial cells directly. To study this hypothesis, human (HUVEC) and bovine (BAEC) endothelial cells (EC) were incubated with the latent 65-kDa heparanase protein. As noted for primary fibroblasts (18) and tumor-derived cells (19), the exogenously added heparanase rapidly interacts with EC, followed by internalization and processing into the 50-kDa active form. Interestingly, heparanase uptake by EC was accompanied by Akt phosphorylation that preceded heparanase processing and exhibited time and dose dependence. Heparanase-mediated Akt phosphorylation was independent of its enzymatic activity and the presence of membrane HS proteoglycans (HSPG) and was augmented by heparin. Moreover, addition of heparanase stimulated phosphatidylinositol 3-kinase (PI3K)-dependent endothelial cell migration and invasion. These results suggest, for the first time, that heparanase activates EC and elicits angiogenic responses directly. This effect appears to be mediated by as yet unidentified heparanase receptor.

### MATERIALS AND METHODS

**Cells and Cell Culture**—HUVEC were kindly provided by Dr. Neomi Lanir (Rambam Medical Center, Haifa, Israel) and were cultured in gelatin-coated dishes, as described previously (20). Adult BAEC were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 1 ng/ml basic fibroblast growth factor and antibiotics, as described previously (21). Mutant Chinese hamster ovary (CHO) cells (pgsA-745) deficient in xylosyltransferase and unable to initiate glycosaminoglycan synthesis, were kindly provided by Dr. J. Esko (Uni-

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<sup>1</sup> The abbreviations used are: HS, heparan sulfate; BAEC, bovine aortic endothelial cells; CHO, Chinese hamster ovary; EC, endothelial cells; ECM, extracellular matrix; HSPG, heparan sulfate proteoglycans; HUVEC, human umbilical vein endothelial cells; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; LS, laminar sulfate; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

versity of California, San Diego, CA) and cultured in RPMI medium supplemented with 10% fetal calf serum and antibiotics. CHO K1 and HT-29 colon carcinoma cells (also known as WiDr) were purchased from the ATCC and cultured in Dulbecco's modified Eagle's medium, as above. HT-29 cells synthesize perlecan, a secreted proteoglycan, but no other proteoglycans (22) and are therefore a useful model for cell surface HS-deficient cells. *In vitro* tube-like structure formation on Matrigel-coated plates was performed as described (23). HEK 293 cells, stably transfected with the human heparanase cDNA construct in the mammalian pSecTag vector (Invitrogen), were kindly provided by Dr. H-Q. Miao (ImClone Systems Inc., New York). This plasmid vector provides the IgGκ signal peptide to ensure efficient protein secretion, together with Myc and His tags at the C terminus of the enzyme to enable easy detection and purification (19).

**Antibodies and Reagents**—The following antibodies were purchased from Santa Cruz Biotechnology: anti-Erk 2 (sc-154), anti-phospho-ERK (sc-7383), anti-Akt (sc-5298), and anti-Myc epitope tag (sc-40). Anti-phospho-Akt antibody was purchased from Cell Signaling Technology (Beverly, MA). Anti-heparanase antibody (number 1453) was raised against the entire 65-kDa heparanase precursor isolated from the conditioned medium of heparanase-transfected 293 cells. This antibody was affinity-purified on immobilized bacterially expressed 50-kDa heparanase-glutathione *S*-transferase fusion protein (24). Laminaran sulfate (LS) was purchased from Qingdao Third Pharmaceutical Company (Qingdao, China) (25). The PI3K inhibitor LY 294002 was purchased from Sigma and was dissolved in Me<sub>2</sub>SO. Equivalent volume of the vehicle was always included as a control.

**Heparanase Uptake Studies**—For uptake studies, the 65-kDa Myc-tagged heparanase precursor was added to confluent cell cultures at a concentration of 1 μg/ml, under serum-free conditions. At the indicated time points, the medium was aspirated, cells were washed twice with ice-cold PBS, and total cell lysates were prepared as described below. Heparanase uptake and processing were analyzed by immunoblotting with anti-Myc and anti-heparanase (number 1453) antibodies.

**Cell Lysates, Immunoprecipitation, and Protein Blotting**—Cell cultures were pretreated with 1 mM orthovanadate for 10 min at 37 °C, washed twice with ice-cold PBS containing 1 mM orthovanadate, and scraped into lysis buffer containing a mixture of proteinase inhibitors (Roche Applied Science). Lysate samples were subjected to immunoblotting as described previously (19, 20).

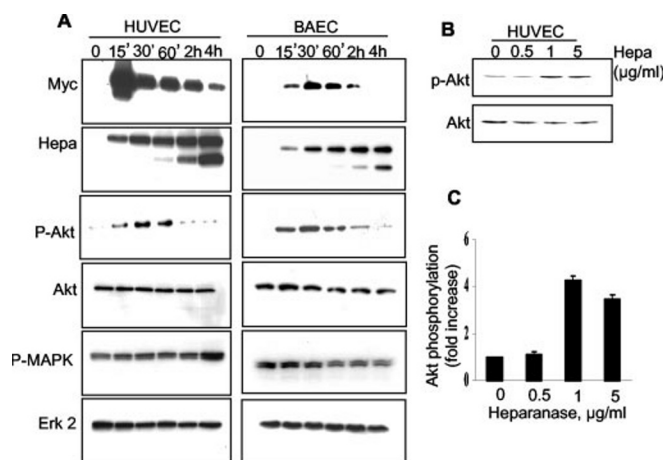
**Cell Migration Assay**—HUVEC were allowed to grow in tissue culture plates until confluence and were then scraped with the wide end of a 1-ml tip (time 0). Plates were washed twice with PBS to remove detached cells and incubated with complete growth medium, and cell migration into the wounded area was followed for 7 days in the presence or absence of exogenously added heparanase (1 μg/ml).

**Matrigel Invasion Assay**—Invasion assay was performed using modified Boyden chambers with Matrigel-coated polycarbonate Nucleopore membrane (Corning Glass), essentially as described previously (19). Migration assay was similarly performed, except that inserts were coated with fibronectin.

**Heparanase Immobilization and Adhesion Studies**—Heparanase (1 μg/ml) was diluted with 50 mM carbonate/bicarbonate buffer, pH 9.6, and applied onto tissue culture dishes (16 h, 4 °C). Dishes were subsequently washed, and the remaining binding sites were blocked with 1% BSA for 1 h at 37 °C. Control dishes were coated with BSA alone. HUVEC ( $2 \times 10^6$ ) were plated onto BSA- or heparanase-coated 35-mm dishes (1 h, 37 °C) and, following several washes, fixed with 4% paraformaldehyde, photographed, and counted. For immunoblot analysis, HUVEC were similarly plated onto heparanase-coated dishes or were left in suspension as control, and cell lysates were prepared as described above.

**Heparanase Activity Assay**—Preparation of ECM-coated dishes and determination of heparanase activity were performed as described in detail elsewhere (12, 26). Briefly, purified heparanase (1 μg) was incubated (4 h, 37 °C, pH 6) with soluble Na<sub>2</sub><sup>35</sup>SO<sub>4</sub>-labeled material extracted from ECM (peak I). The incubation medium was subjected to gel filtration on a Sepharose CL-6B column. Fractions (0.2 ml) were eluted with PBS and their radioactivity counted in a β-scintillation counter. To evaluate the enzymatic activity of immobilized heparanase, soluble ECM-derived sulfate-labeled material (peak I) was applied onto heparanase-coated dishes and following incubation (4 h, 37 °C, pH 6) was subjected to gel filtration, as described above.

All experiments were repeated at least three times with similar results. Akt activation is presented as mean ± S.E. of at least five independent experiments quantified by densitometry analysis.

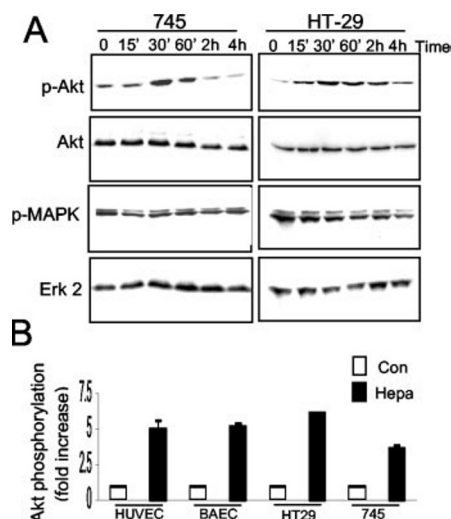


**FIG. 1. Heparanase induces Akt phosphorylation.** A, HUVEC (left panel) and BAEC (right panel) were left untreated (0) or incubated with exogenously added 65-kDa heparanase (1 μg/ml). At the indicated time points, cells were washed, and total cell lysates were subjected to SDS-PAGE followed by immunoblotting with anti-Myc epitope tag (upper panel), anti-heparanase number 1453 (Hepa, second panel), anti-phospho-Akt (p-Akt, third panel), anti-Akt (fourth panel), anti-phospho-MAPK (p-MAPK, fifth panel), or anti-Erk 2 (bottom panel) antibodies. B, dose response. HUVEC were left untreated (0) or incubated with exogenously added latent heparanase at the indicated concentrations (μg/ml). Total cell lysates were prepared after 30 min of incubation, and Akt phosphorylation was evaluated as above. C, densitometry analysis. Akt phosphorylation index was calculated by densitometry analysis of phosphorylated Akt levels divided by the total Akt values. Data are presented as -fold increase of Akt phosphorylation compared with untreated cells, set arbitrary to a value of 1. Note time- and dose-responsive Akt phosphorylation upon heparanase treatment.

## RESULTS

**Heparanase Is Subjected to Processing at the C Terminus**—Under certain physiological conditions vascular EC are likely to be exposed locally to elevated levels of heparanase. To study the effect of heparanase on EC, human (HUVEC) and bovine (BAEC) EC were incubated with exogenously added Myc-tagged latent 65-kDa heparanase precursor, and heparanase uptake was followed by means of immunoblotting. As demonstrated in Fig. 1A, heparanase rapidly interacts with primary EC in culture. Immunoblot analysis with the anti-Myc epitope antibody revealed a strong signal already 15 min (HUVEC, upper panel, left) and 30 min (BAEC, upper panel, right) following heparanase addition. Interestingly, the anti-Myc antibody recognized only a single protein band that corresponded to the added 65-kDa heparanase form, while a lower protein band was not detected even at later time points, when heparanase processing was evident (Fig. 1, second panel, 2h and 4h). Reactivity with the anti-Myc antibody declined rapidly and preceded heparanase processing that became detectable by 60 min and was mainly apparent by 2 h and 4 h (Hepa, second panel). This may suggest that in addition to the processing at Glu<sup>109</sup>-Ser<sup>110</sup> and Gln<sup>157</sup>-Lys<sup>158</sup> that removes the linker domain and ultimately generates the 8- and 50-kDa heparanase subunits (27, 28), heparanase is also subjected to processing at the C terminus, resulting in loss of the tag sequences.

**Heparanase Induces Akt Phosphorylation**—We have recently reported that heparanase expression in stably transfected U87 glioma cells induced a marked increase in Akt phosphorylation levels that correlated with an enhanced cellular motility (19). Addition of the latent 65-kDa heparanase protein resulted in a similar Akt phosphorylation in EC. Akt activation appeared maximal 30–60 min after heparanase addition and subsequently declined to basal levels (Fig. 1A, third panel). Moreover, heparanase-induced Akt phosphorylation was dose dependent (Fig. 1B), reaching a maximal effect at 1 μg/ml

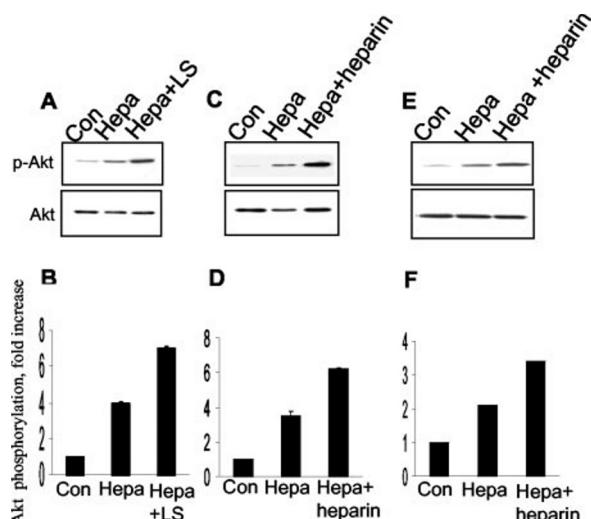


**FIG. 2. Heparanase-induced Akt phosphorylation is HSPG-independent.** A, CHO-745 (left panel) and human colon tumor-derived HT-29 (right panel) cells were left untreated (0) or incubated with exogenously added heparanase (1  $\mu$ g/ml). At the indicated times, total cell lysates were prepared and immunoblotted with anti-phospho-Akt (p-Akt, upper panel), anti-Akt (second panel), anti-phospho-MAPK (p-MAPK, third panel), and anti-Erk 2 (bottom panel) antibodies, as described. B, densitometry analysis of Akt phosphorylation levels was determined following 30-min incubation of heparanase with HUVEC, BAEC, HT-29, and CHO 745 cells. Note comparable Akt phosphorylation levels upon heparanase addition to HSPG-positive (HUVEC, BAEC) and HSPG-negative (745, HT29) cells.

(~5-fold increase as judged by densitometry analysis) (Fig. 1C). This Akt activation was specific as the phosphorylation of other signaling molecules such as MAPK was not induced or even reduced (Fig. 1A, fifth panel). Similar results were obtained with mouse (EOMA) EC (not shown).

**HSPG Are Not Required for Heparanase-induced Akt Activation**—The rapid and efficient uptake of heparanase by various cell types is thought to be mediated, at least in part, by the cell membrane HSPG (18). To evaluate the possible involvement of HSPG in Akt activation, heparanase was added to CHO 745 cells that exhibit minimal HS and chondroitin sulfate synthesis (29). Addition of heparanase to these HS-deficient cells resulted in Akt activation, similar in magnitude and kinetics to that observed in EC (Fig. 2A, left upper panel). This apparent HSPG-independent heparanase function was further confirmed by performing the same experiment with HT-29 cells. This human colon tumor-derived cell line synthesizes perlecan, a secreted HSPG residing mainly in the ECM, but no other species of HSPG (22). Addition of heparanase to HT-29 cells resulted in time-dependent Akt activation (Fig. 2A, right upper panel), further supporting the notion that heparanase-induced Akt activation does not involve membranous HS. As was noted for EC (Fig. 1A), Akt activation appeared specific, and MAPK phosphorylation was not affected by heparanase addition (Fig. 2A, third panel). Densitometry analysis revealed a comparable, 4–5-fold increase in Akt phosphorylation upon heparanase addition to EC and to HS-deficient cells (Fig. 2B). Furthermore, Akt activation was also evident following addition of heparanase to heparitinase-treated EC (data not shown). These findings suggest that cell surface molecules other than HSPG are involved in heparanase-mediated Akt activation.

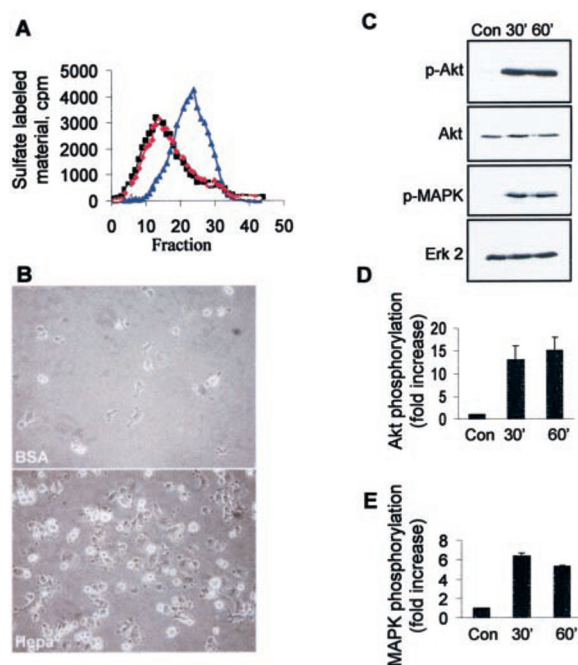
**Heparanase-induced Akt Activation Is Independent of Its Enzymatic Activity**—In terms of kinetics, maximal Akt activation was observed 30 min following heparanase addition and preceded its processing into the 50-kDa active form that was first detected 60 min after its addition (Fig. 1A), suggesting that Akt



**FIG. 3. Heparanase-induced Akt phosphorylation is augmented by heparin.** A and B, CHO-745 cells were left untreated (Con) or incubated with heparanase in the absence (Hepa) or presence (Hepa + LS) of the heparanase inhibitor LS (10  $\mu$ g/ml). Total cell lysates were prepared after 30 min of incubation, and Akt phosphorylation was analyzed by immunoblotting (A). Densitometry analysis is shown in B. Note a 4-fold increase of Akt phosphorylation levels upon heparanase treatment and a further ~2-fold increase when LS was added together with heparanase. C and D, HUVEC were left untreated (Con) or incubated with heparanase (Hepa) or with heparanase and heparin (10  $\mu$ g/ml). Akt phosphorylation was analyzed (C) and quantified (D) 30 min following heparanase addition, as described above. E and F, CHO K1 cells were left untreated (Con) or incubated with heparanase (Hepa) or with heparanase and heparin, and Akt phosphorylation was analyzed and quantified as described above.

activation is independent of heparanase enzymatic activity. To further confirm this notion, CHO 745 cells were incubated with heparanase in the presence or absence of the heparanase inhibitor LS (Fig. 3, left panel). We have previously reported that this chemically sulfated polysaccharide inhibits both heparanase activity and experimental metastasis (25). Addition of heparanase to CHO 745 cells resulted in a 4-fold increase of Akt activation, in agreement with our previous results (Fig. 2A, left panel). Interestingly, addition of heparanase to CHO 745 cells together with LS at 10  $\mu$ g/ml, a concentration that completely abolished heparanase enzymatic activity (25), did not prevent Akt phosphorylation (Fig. 3, A and B). On the contrary, combined addition of heparanase and LS resulted in a further nearly 2-fold induction of Akt phosphorylation (Fig. 3, A and B). It is well documented that several HS-bound growth factors prototyped by members of the fibroblast growth factor family, require HS or heparin to establish a stable complex with their receptor and exert a maximal biological effect (30–32). Being a sulfated polysaccharide, we questioned whether the augmentation of Akt activation by LS could be reproduced by commercial heparin. To this end, HUVEC (Fig. 3, C and D) cells were left untreated or incubated with heparanase without or with heparin. As shown in Fig. 3C, Akt activation by heparanase was augmented by heparin in a manner and magnitude comparable with LS (Fig. 3, C and D), while heparin alone had no effect (not shown). Similarly, heparanase induced Akt activation in wild type CHO K1 cells (Fig. 3, E and F), albeit to a lower magnitude due to high endogenous Akt phosphorylation levels in these cells (not shown). Nevertheless, the addition of heparin augmented Akt phosphorylation by heparanase nearly 2-fold (Fig. 3, E and F), as was noted for primary endothelial cells (HUVEC, Fig. 3, C and D). These findings support the notion that Akt activation is mediated by a putative heparanase receptor and that heparin may be required for maximal receptor activity.





**FIG. 4. Heparanase-induced Akt phosphorylation is independent of heparanase enzymatic activity.** A, heparanase activity assay. The gel filtration profile of intact soluble ECM derived sulfate-labeled HSPG (peak I, black squares) incubated (1 h, 37 °C) with heparanase before (blue triangles) and after (red diamonds) immobilization is shown. Note complete loss of heparanase activity following exposure to high pH conditions during immobilization. B, immobilized heparanase facilitates cell adhesion. HUVEC ( $2 \times 10^5$ ) were plated onto BSA- or heparanase-coated dishes, and following incubation (1 h, 37 °C), adhering cells were fixed with 4% paraformaldehyde and photographed (B). C–E, immobilized, inactive heparanase induces Akt phosphorylation. HUVEC were plated onto heparanase-coated dishes or were left in suspension (Con). After incubation for 30 and 60 min, total cell lysates were subjected to immunoblotting, applying anti-phospho-Akt (p-Akt, upper panel), anti-Akt (second panel), anti-phospho-MAPK (p-MAPK, third panel), or anti-ERK 2 antibodies (C). Akt (D) and MAPK (E) phosphorylation levels were quantified by densitometry, as described. Note a marked phosphorylation of both Akt and MAPK upon HUVEC adhesion to the immobilized, inactive heparanase protein.

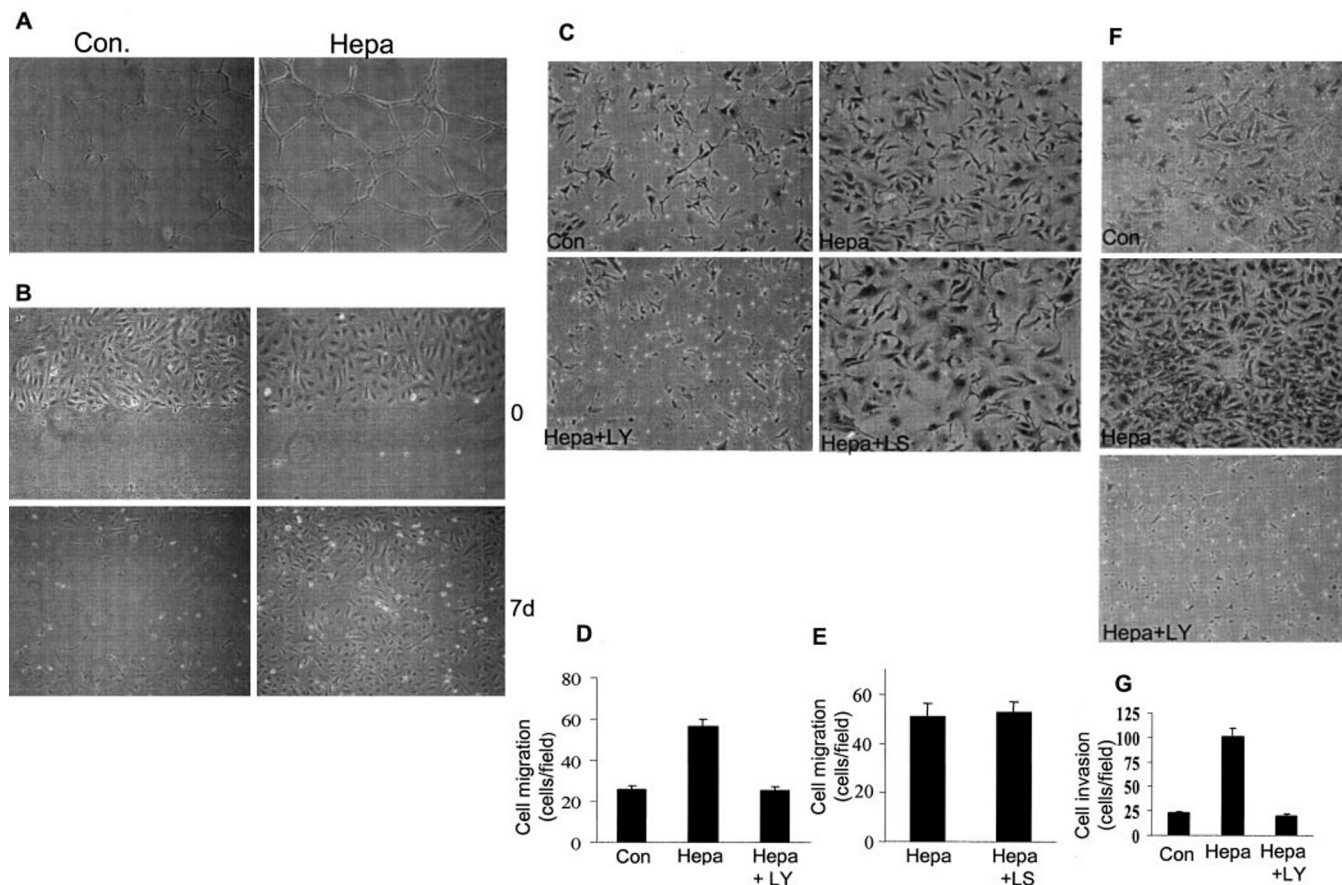
Although the 50-kDa active heparanase enzyme was not detected in our 65-kDa heparanase preparation by means of immunoblotting (not shown), a low heparanase activity was evident applying the highly sensitive sulfate-labeled ECM assay (Fig. 4A). It is therefore possible that Akt activation was induced by this low level of the active 50-kDa heparanase form. To further rule out the necessity of heparanase activity for Akt activation, heparanase was bound to the tissue culture plastic under high pH conditions (carbonate/bicarbonate buffer, pH 9.6). Exposure of active heparanase to pH 9.6 irreversibly inactivated the enzyme (Fig. 4A). In addition, and in contrast to the soluble protein, the immobilized inactive heparanase protein is not available to uptake and processing, providing an experimental system in which inactive heparanase can only function from outside the cell. The immobilized heparanase protein induced an 8-fold increase in EC adhesion compared with control BSA-coated dishes (Fig. 4B), supporting the notion that under certain physiological conditions heparanase may mediate cell adhesion (19, 33, 34). HUVEC adhesion to the immobilized inactive heparanase resulted in a marked 10-fold induction of Akt phosphorylation (Fig. 4C, upper panel, and D), again implying that Akt activation is independent of heparanase activity. Interestingly, under these experimental conditions, MAPK was also activated (Fig. 4C, third panel, and E), suggesting that additional adhesion related pathways were engaged.

**Heparanase Induces Akt-dependent EC Migration**—To evaluate the possible effect of exogenously added heparanase on EC behavior, HUVEC were plated on top of Matrigel-coated dishes. Under these conditions, EC rearrange themselves into lumen-containing tube-like structures (23) in a process that resembles several features of angiogenesis. Control cultures exhibited only a limited network formation (Con, Fig. 5A). In contrast, addition of heparanase resulted in a well organized EC structures composed of interconnected, elongated EC (Hepa, Fig. 5A). EC network formation on Matrigel-coated dishes was rapid and completed within 24 h. Thus, EC migration rather than EC proliferation is likely to be involved in this assay. To evaluate the effect of heparanase on EC migration, confluent HUVEC cultures were scraped with the wide end of a 1-ml pipette tip, and cell migration into the wounded area was examined over 7 days in culture, without or with heparanase addition. As shown in Fig. 5B, addition of heparanase stimulated a significant migratory response compared with untreated cultures. Enhanced migration rates in response to heparanase addition were further confirmed and quantified by migration assay on fibronectin-coated inserts. Under these conditions, addition of heparanase resulted in a 2.5-fold increase in HUVEC migration rate (Fig. 5, C and D). Importantly, addition of heparanase together with the PI3K inhibitor LY 294002 abolished the promigratory effect of heparanase (Fig. 5, C and D), while the heparanase inhibitor LS had no effect on EC migration (Fig. 5, C and E), in agreement with our previous finding that heparanase enzymatic activity is not required for Akt activation (Fig. 3, A and B). Moreover, heparanase stimulated a 5-fold increase in EC invasion through Matrigel-coated inserts, and this effect was similarly prevented by LY 294002 (Fig. 5, F and G). These findings clearly indicate that heparanase directly stimulates EC motility. This effect is PI3K-dependent, is likely mediated by heparanase-induced Akt activation, and may involve a putative heparanase receptor.

## DISCUSSION

Heparanase activity has long been attributed to metastatic and immune cells capable of penetrating the ECM barrier (5, 7, 8). Blood-borne neutrophils, macrophages, mast cells, and platelets exhibit heparanase activity that is thought to be stored in specific granules (1, 4–6, 8, 9, 16). The degranulated heparanase was implicated in cellular extravasation (7, 8). Degranulation and heparanase release in response to a proper inflammatory stimulus, or upon platelet activation, may facilitate cellular extravasation directly by altering the composition and insolubility of the ECM or indirectly by releasing HS-bound proinflammatory cytokines and chemokines (16). In addition, the released heparanase may also locally affect and activate neighboring cells such as endothelial cells. Indeed, exogenously added heparanase stimulated Akt phosphorylation in human, bovine, and mouse EC (Figs. 1–4), while other signaling pathways such as the MAPK were not induced (Figs. 1A and 2A). The rapid and efficient cellular binding and uptake of heparanase appear to be mediated by the cell membrane HSPG (18). Nevertheless, elevated Akt phosphorylation levels, comparable in magnitude and kinetics, were observed in HS-deficient CHO 745 and HT-29 cells (Fig. 2), implying that heparanase can interact with cell surface molecules other than HSPG. The augmentation of Akt phosphorylation levels by heparin (Fig. 3, C and D) may suggest the existence of a specific heparanase receptor that, similar to several growth factor receptors, requires heparin to exert maximal activities (31, 35, 36). Studies aiming to identify this putative heparanase receptor are currently in progress.

Several lines of evidence suggest that Akt activation is independent of heparanase enzymatic activity. In terms of kinetics,



**FIG. 5. Heparanase stimulates EC migration.** A, tube-like structure formation on Matrigel-coated dishes. HUVEC ( $5 \times 10^4$ ) were plated onto Matrigel-coated dishes in the absence (Con) or presence of heparanase (Hepa,  $1 \mu\text{g/ml}$ ). EC organization was evaluated after 24 h. B–E, heparanase stimulates EC migration. Confluent HUVEC cultures were scraped with the wide end of a 1-ml pipette tip, and cell migration into the wounded area was evaluated after 7 days in the absence (Con) or presence (Hepa,  $1 \mu\text{g/ml}$ ) of heparanase (B). HUVEC ( $2 \times 10^5$ ) were plated onto fibronectin-coated inserts and were left untreated (Con) or incubated with heparanase (Hepa,  $1 \mu\text{g/ml}$ ) alone, heparanase plus the PI3K inhibitor LY 294002 ( $10 \mu\text{g/ml}$ ; Hepa+LY), or heparanase plus laminaran sulfate (Hepa+LS). Cells migrating into the lower compartment were visualized by crystal violet staining (C) and quantified by counting of at least six random fields (D and E). Note  $\sim 2.5$ -fold increase in cell migration upon heparanase treatment and inhibition of this effect by LY 294002 (D) but not LS (E). F and G, heparanase stimulates EC invasion. HUVEC ( $2 \times 10^5$ ) were plated onto Matrigel-coated inserts and were left untreated (Con) or incubated with heparanase in the absence (Hepa) or presence (Hepa+LY) of LY 294002. Cell invasion was visualized (F) and quantified (G) after 5 h.

elevated Akt phosphorylation levels were evident already 15 min following heparanase addition (Figs. 1A and 2A) and preceded heparanase processing detected 60 min following heparanase application (Fig. 1A). In addition, enhanced Akt phosphorylation in response to heparanase was not prevented by the heparanase inhibitor LS but was rather augmented (Fig. 3, A and B). This finding is in agreement with the contribution of heparin to Akt activation by heparanase (Fig. 3, C and D), arguing, perhaps, for a general stimulation mediated by sulfated polysaccharides. Exposure of heparanase to pH 9.6 resulted in inactivation of the enzyme. Immobilizing heparanase under high pH conditions (pH 9.6) not only ensures complete inactivation of heparanase enzymatic activity (Fig. 4A) but also prevents heparanase uptake, thus forcing response mediated by cell membrane proteins. The immobilized heparanase markedly facilitated HUVEC adhesion (Fig. 4B). This phenomenon is in agreement with our recent findings that heparanase-mediated Eb lymphoma (28) and U87 glioma (19) cell adhesion is independent of heparanase enzymatic activity (34). Importantly, HUVEC adhering to the immobilized heparanase responded by a marked elevation of Akt phosphorylation levels (Fig. 4, C and D), strongly arguing for Akt activation independent of heparanase enzymatic activity. Interestingly, the MAPK pathway was also activated (Fig. 4, D and E), suggesting that

under these experimental conditions, other signaling pathways were engaged. Integrins, possibly co-operating with syndecan family members such as syndecan-4 (37, 38), are likely to be involved and are currently under investigation.

The ability of EC to migrate and form capillary-like structures is essential for *in vivo* angiogenesis (39, 40), and the PI3K-Akt pathway was implicated in vascular endothelial growth factor- and endothelial differentiation gene-stimulated EC migration (41–44). In agreement with these reports, Akt phosphorylation by heparanase correlated with enhanced PI3K-dependent EC migration (Fig. 5, B–D) and invasion (Fig. 5, F and G). These findings may provide a possible mechanistic explanation for the strong angiogenic response induced by heparanase under experimental (17, 19, 27) and clinical (45–47) settings. Akt phosphorylation by exogenously added heparanase may not be restricted to EC but similarly occurs in tumor-derived cells as exemplified by HT-29 cells (Fig. 2). This observation may suggest that heparanase overexpression in primary human tumors (1, 6, 45–47) not only supports tumor vascularity and metastasis but also tumor cell survival, a function long attributed to phosphorylated, active Akt (48). Studies examining the potential function of heparanase as a survival factor for endothelial and tumor-derived cells are currently under way.

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