

Expression Pattern and Secretion of Human and Chicken Heparanase Are Determined by Their Signal Peptide Sequence*

Received for publication, March 19, 2001, and in revised form, May 21, 2001
Published, JBC Papers in Press, May 31, 2001, DOI 10.1074/jbc.M102462200

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Cleavage of heparan sulfate (HS) proteoglycans affects the integrity and function of tissues and thereby fundamental phenomena, involving cell migration and response to changes in the extracellular microenvironment. The role of HS-degrading enzymes, commonly referred to as heparanases, in normal development has not been identified. The present study focuses on cloning, expression, and properties of a chicken heparanase and its distribution in the developing chicken embryo. We have identified a chicken EST, homologous to the recently cloned human heparanase, to clone and express a functional chicken heparanase, 60% homologous to the human enzyme. The full-length chicken heparanase cDNA encodes a 60-kDa proenzyme that is processed at the N terminus into a 45-kDa highly active enzyme. The most prominent difference between the chicken and human enzymes resides in the predicted signal peptide sequence, apparently accounting for the chicken heparanase being readily secreted and localized in close proximity to the cell surface. In contrast, the human enzyme is mostly intracellular, localized in perinuclear granules. Cells transfected with a chimeric construct composed of the chicken signal peptide preceding the human heparanase exhibited cell surface localization and secretion of heparanase, similar to cells transfected with the full-length chicken enzyme. We examined the distribution pattern of the heparanase enzyme in the developing chicken embryo. Both the chicken heparanase mRNA and protein were expressed, as early as 12 h post fertilization, in cells migrating from the epiblast and forming the hypoblast layer. Later on (72 h), the enzyme is preferentially expressed in cells of the developing vascular and nervous systems. Cloning and characterization of heparanase, the first and single functional vertebrate HS-degrading enzyme, may lead to identification of other glycosaminoglycan degrading enzymes, toward elucidation of their significance in normal and pathological processes.

Heparan sulfate proteoglycans (HSPGs)¹ are ubiquitous macromolecules associated with the cell surface, extracellular matrix (ECM), and basement membranes (BM) of a wide range of cells of vertebrate and invertebrate tissues (1–4). The basic HSPG structure consists of a protein core to which several linear heparan sulfate (HS) chains are covalently *O*-linked (1). HSPGs play a key role in the self-assembly and integrity of the multimolecular architecture of BM and ECMs (1–5). Hence, their enzymatic degradation is likely to affect diverse processes associated with cell migration, including embryonic morphogenesis, angiogenesis, metastasis, inflammation, neurite outgrowth, and tissue repair (5–12). Mammalian endoglycosidases, capable of partially depolymerizing HS chains and commonly referred to as heparanases, have been identified in a variety of cell types and tissues (9–14). Interestingly, only a single heparanase cDNA sequence encoding an active enzyme was identified, indicating that this enzyme is the dominant endo- β -D-glucuronidase in mammalian tissues (15–19). The heparanase mRNA and protein are preferentially expressed in metastatic cell lines and specimens of human tumors (13–16, 20, 21). Moreover, treatment with heparanase inhibitors markedly reduced the incidence of metastasis in experimental animals (9, 10, 12). The enzyme is also expressed by activated cells of the immune system, and participates in inflammation and autoimmunity (11, 22, 23). Apart from its involvement in the egress of cells from the vasculature, heparanase is tightly involved in angiogenesis, primarily by means of releasing heparin-binding angiogenic factors sequestered by HS in BM and ECM (24, 25). The human heparanase cDNA contains an open reading frame of 1629 bp which encodes for a latent 61.2-kDa polypeptide of 543 amino acids (15–19). The mature 50-kDa active enzyme has its N terminus 157 amino acids downstream from the initiation codon (15–19). The proteolytic activity responsible for the post-translational processing of the enzyme has not been characterized.

The involvement of heparanase in cell migration associated with pathological processes such as tumor metastasis, angiogenesis, and autoimmunity led us to investigate its pattern of expression and role in normal developmental processes. The present study focuses on the cloning, expression, and properties of a chicken heparanase and its distribution pattern in the highly characterized developmental program of the chicken

* This work was supported by grants from the Cooperation program in Cancer Research of the Deutsches Krebsforschungszentrum (DKFZ) and the Israeli's ministry of Science, the Science Foundation funded by the Israel Academy of Sciences and Humanities, the Israel Cancer Research Fund, and the Association for International Cancer Research, United Kingdom. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY037007.

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¹ The abbreviations used are: HSPGs, heparan sulfate proteoglycans; HS, heparan sulfate; ECM, extracellular matrix; BM, basement membranes; MMP, matrix metalloproteinase; bp, base pair(s); EST, expressed tag sequence; PBS, phosphate-buffered saline; RT-PCR, reverse transcriptase-polymerase chain reaction; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

embryo. We have used the human heparanase cDNA sequence to screen EST data base for homology to a chicken unidentified mRNA sequences. A single related chicken EST was identified, leading to the cloning and expression of a 60-kDa chicken heparanase, exhibiting 58–61% identity to the human, mouse, and rat enzymes. Interestingly, the chicken cDNA encodes for a highly hydrophobic signal sequence, exhibiting 39% similarity to that of the human enzyme. Unlike the human heparanase, the chicken enzyme is preferentially localized in proximity to the cell surface and is readily secreted as a 45-kDa active protein. Immunolocalization studies preformed in chicken embryos revealed that the heparanase gene and protein are preferentially expressed in cells migrating from the epiblast and forming the hypoblast layer, as early as 12 h post-fertilization. Later on, the protein is highly expressed in the developing nervous and vascular systems.

EXPERIMENTAL PROCEDURES

Cells—The methylcholanthrene-induced nonmetastatic Eb (L5178Y) T-lymphoma cells were kindly provided by Dr. V. Schirmacher (Deutsches Krebsforschungszentrum, Heidelberg, Germany). The cells were grown in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with β -mercaptoethanol (5×10^{-5} M) and 10% fetal calf serum (15, 26). C6 rat glioma cells were obtained from Dr. E. Keshet (Department of Molecular Biology, The Hebrew University School of Medicine) (27). Cells were cultured in DMEM (4.5 g of glucose/liter) containing 10% fetal calf serum. Cultures of bovine corneal endothelial cells were established from steer eyes and maintained in Dulbecco's modified Eagle's medium (1 g of glucose/liter) supplemented with 5% newborn calf serum and 10% fetal calf serum, as described (28). Recombinant human basic fibroblast growth factor (kindly provided by Dr. Peter Bohlen, Imclone Systems, New York, NY) was added (1 ng/ml) every other day during the phase of active cell growth. Confluent cells were maintained at 37 °C in a 10% CO₂ humidified incubator. Cells were dissociated with 0.05% trypsin and 0.02% EDTA and subcultured at a split ratio of 1:10.

Preparation of Dishes Coated with ECM—Bovine corneal endothelial cells (second to fifth passage) 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 (26, 28). Na₂³⁵SO₄ (25 μ Ci/ml) (Amersham Pharmacia Biotech, Buckinghamshire, UK) was added on days 2 and 5 after seeding and the cultures were incubated with the label without medium change. On day 12, the subendothelial ECM was exposed by dissolving the cell layer with PBS containing 0.5% Triton X-100 and 20 mM NH₄OH, followed by four washes with PBS (10, 11, 15, 26, 28). The ECM remained intact, free of cellular debris and firmly attached to the entire area of the tissue culture dish. Nearly 80% of the ECM radioactivity was incorporated into HSPGs (26, 28).

Heparanase Activity—Cell lysates, intact cells, or serum-free conditioned medium were incubated (24 h, 37 °C, pH 6.2–6.6) with ³⁵S-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 \times 30 cm). Fractions (0.2 ml) were eluted with PBS and their radioactivity counted in a β -scintillation counter (10, 11, 15, 26). 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 V₀ ($K_{av} < 0.2$, peak I) (10, 11, 15, 26). Each experiment was performed at least 3 times and the variation of elution positions (K_{av} values) did not exceed $\pm 15\%$.

Cloning of Chicken Heparanase cDNA—The cDNA sequence of human heparanase (15, 16) was used to screen EST data bases for homology to a chicken unidentified mRNA sequences. A single heparanase related chicken EST (number AI980994) was identified, sharing 60.5% sequence homology with 276 bp at the 3' end of the human heparanase coding sequence. The full-length chicken heparanase cDNA was derived from chicken kidney mRNA isolated from fresh chicken kidneys using PolyATtract™ mRNA Isolation System III (Promega, Madison, WI). Amplification of 5' ends was performed with the 5' rapid amplification of cDNA ends system of Life Technologies, Inc. (15). Briefly, chicken kidney mRNA was reverse transcribed (RT) using SuperScript II (Life Technologies, Inc.) and oligo(dT)₁₅ as primer. The resulting cDNAs were extended by 3' C-tailing, using terminal deoxynucleotidyl transferase (Promega). Expand High Fidelity enzyme (Boehringer, Mannheim, Ger-

many) was applied for PCR amplification. The first step applied the AP1 primer and the gene specific primer ChkL1: 5'-GACTCCTCAAG-CATTCCTCAG -3'. The second step used the nested 5' primer AP2 and a nested gene specific 3' primer ChkL2: 5'-AGCCCTGTTACTCT-GCGTGCTC-3'. The gene-specific primers ChkL1 and ChkL2 were selected according to the sequence of the EST. After an initial denaturation step of 3 min at 94 °C, the samples were incubated for 30 cycles at 94, 64, and 72 °C for 30 s, 1 min, and 3 min, respectively, followed by an extension step at 72 °C for 7 min (15). The resulting 1.8-kilobase PCR product was cloned into the pGEM-T Easy vector (Promega), sequenced, and found to correspond to the full-length chicken heparanase cDNA (Chk-hpa).

DNA Sequencing—Sequence determination was performed using vector-specific and gene-specific primers, with an automated DNA sequencer (ABI Prism™ model 310 Genetic Analyzer, PerkinElmer Life Sciences, Foster City, CA). Several independent clones were sequenced to confirm the primary structure of the gene.

Generation of a Chimeric Chicken-Human Heparanase Gene—The N-terminal end of the human hpa cDNA containing the signal peptide was replaced by the corresponding sequence of the chicken hpa cDNA. For this purpose, the Chk-hpa signal peptide was amplified using specific primers (KPN/SPU, 5'-CGGGGTACCCGATGCTGGTCT-3'; SPL, 5'-AGGTCCACGACGTCCTGTGCCGTCCGCCTCG-3'). The H-hpa region extending from the first amino acid downstream the H-hpa signal peptide to the BamHI restriction site was amplified, using H-hpa specific primers (HU, 5'-CGAGGCGGACGGACACAGGACGTCGTGGACCT-3'; H/BHIL, 5'-CCACATCAGGAGGGATGGATCC-3'). The PCR products were combined by means of primer extension and PCR amplification. The resulting fragment was then cloned in-frame into a pcDNA3 plasmid (Invitrogen, NV Leek, Netherlands) containing the H-hpa cDNA downstream the BamHI site, generating a chimeric construct in which the Chk-hpa signal peptide precedes the H-hpa. The chimeric gene was validated by sequencing.

Computer Analysis of Sequence—Data base searches for sequence similarities were performed using the NCBI Blast network service. Sequence analysis and alignment of DNA and protein sequences were performed using the DNA sequence analysis software package developed by the Genetic Computer Group (GCG) at the University of Wisconsin. Multiple sequence alignment was analyzed using the Clustal-W alignment program (www.ibcp.fr/clustalw.html).

Plasmids and Transfections—Chicken and human heparanase cDNAs were subcloned into the eukaryotic expression plasmid pcDNA3 (Invitrogen) at the EcoRI site. The chimeric cDNA was subcloned into pcDNA3 plasmid at a KpnI site. Eb cells were grown in suspension (0.5×10^6 cells/ml) and incubated (48–72 h, 37 °C) with a total of 1–2 μ g of DNA and 6 μ l of FuGene transfection reagent (Roche Molecular Biochemicals) in 94 μ l of Opti-MEM (Life Technologies, Inc.). Transfected cells were selected with 350 μ g/ml G418 and stable populations of heparanase expressing cells were obtained (15). Expression of heparanase was evaluated by RT-PCR and measurements of enzymatic activity. The pcDNA3 Chk-hpa, human-hpa (H-hpa), and chimeric-hpa (Chk-sp/H-hpa) plasmids were also applied for stable transfection of C6 rat glioma cells. With these cells, 600 μ g/ml G418 was used for selection.

RNA Isolation and RT-PCR—RNA was isolated with Tri-Reagent (MRC, Cincinnati, OH) according to the manufacturers instructions and was quantitated by ultraviolet absorption. After reverse transcription of 2 μ g of total RNA by oligo(dT) priming, the resulting single stranded cDNA was amplified using TaqDNA polymerase (Promega). Oligonucleotide primers Chk-U (5'-GTGGCACCAGTACAGATTTTCCT-3') and Chk-L (5'-AATCCTCCTCGTTGCACTT-3') were used. The PCR conditions were an initial denaturation at 94 °C for 2 min, denaturation at 94 °C for 15 s, annealing for 45 s at 60 °C, and extension for 1 min at 72 °C (33 cycles). Aliquots (15 μ l) of the amplified cDNA were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining (15).

Immunostaining—C-6 glioma cells transfected with Chk-hpa, H-hpa, or chimeric-hpa were seeded for 24 h on round glass coverslips in 4-well plates, washed twice with PBS and fixed with 100% chilled (–20 °C) methanol for 3 min. Following fixation, cells were washed ($\times 5$) with PBS and intrinsic fluorescence was blocked with 50 mM NH₄Cl for 5 min. Cells were then washed ($\times 3$) with PBS, incubated (30 min, 24 °C) with 5% goat serum, and washed twice with PBS. Slides were incubated (2 h, 24 °C) with monoclonal anti-human heparanase antibodies (mAb 130, 10 μ g/ml) directed against the C terminus of the 50-kDa active enzyme (15). This mAb cross-react with the chicken heparanase. The preparation and specificity of mAb 130 were previously described and demonstrated (15). Following incuba-

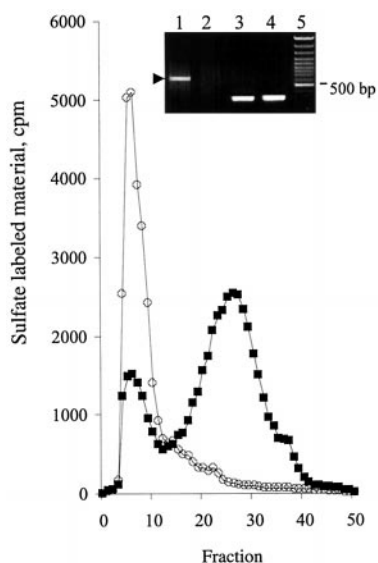


FIG. 3. Chicken heparanase activity in transfected C6-glioma cells. C6 rat glioma cells transfected with *Chk-hpa* (■), or with control pcDNA3 plasmid alone (○) were incubated (24 h, 37 °C, pH 6.6) in serum-free medium in contact with sulfate-labeled ECM. Labeled degradation fragments released into the incubation medium were analyzed by gel filtration on Sepharose 6B. Nearly intact HSPGs elute next to V_0 (peak I, fractions 1–10) (○), whereas heparan sulfate degradation products elute toward the V_i of the column (peak II, fractions 20–35) (■). *Inset*, RT-PCR. Total RNA isolated from a pooled population of C6 glioma cells stably transfected with *Chk-hpa* (lanes 1 and 3), or with control plasmid alone (lanes 2 and 4) were tested for expression of the *Chk-hpa* mRNA (lanes 1 and 2, arrow), using primers specific for *Chk-hpa* (amplifying a 611-bp cDNA fragment), or β -actin (lanes 3 and 4). Lane 5, 100-bp DNA molecular weight markers.

bands corresponding to the 60- and 45-kDa forms of the chicken enzyme (Fig. 4C, *inset*, lane 1) versus the 65- and 50-kDa latent and active forms of the human heparanase (Fig. 4C, *inset*, lane 2). The heparanase protein was not detected in the mock transfected cells (lane 3). Similar results were obtained with *hpa*-transfected C-6 glioma cells (not shown).

Next, we assessed the ability of the chicken heparanase to degrade HS in intact ECM. For this purpose, *Chk-hpa*- and mock transfected C-6 glioma cells were seeded on intact naturally produced sulfate-labeled ECM. Labeled degradation fragments released into the incubation medium were then analyzed by gel filtration on Sepharose 6B (26). Material released by the mock transfected cells eluted just after the void volume (V_0) (peak I, fractions 1–10, $K_{av} < 0.2$) and consisted almost entirely of intact, high-molecular weight HSPGs. In contrast, incubation of the ECM with *Chk-hpa*-transfected C-6 glioma cells resulted in release of low-molecular weight labeled degradation fragments, eluted toward the V_i of the column (peak II, fractions 20–35, $0.5 < K_{av} < 0.75$) (Fig. 3). Release of these fragments was abolished in the presence of heparin, an alternative substrate of the heparanase enzyme (10, 15). Similar results were obtained with *Chk-hpa* versus mock transfected Eb lymphoma cells (Fig. 4A). Labeled fragments eluted in peak II were shown to be degradation products of HS, as they were 5–6-fold smaller than intact HS side chains, resistant to further digestion with papain or chondroitinase ABC and susceptible to deamination by nitrous acid (26).

In view of the differences between the chicken and human heparanase cDNAs and particularly between sequences of the predicted signal peptides, we compared cells transfected with these heparanase species for their ability to secrete the enzyme and degrade intact ECM. For this purpose, Eb lymphoma cells

were maintained for 24 h in serum-free RPMI medium at a density of 2×10^6 cells/ml. The cells were then centrifuged and both the conditioned medium (1 ml) and respective intact cells were incubated (37 °C, 24 h) in contact with sulfate-labeled ECM coating the surface of 35-mm culture dishes. Cells were also subjected to three cycles of freezing and thawing and lysates of 2×10^6 cells were similarly incubated with the labeled ECM. Degradation fragments released into the incubation medium were then analyzed by gel filtration. As shown in Fig. 4, Eb cells transfected with the *Chk-hpa* exhibited a higher heparanase activity than H-*hpa*-transfected Eb cells. This difference was observed with intact cells (2–3-fold) (Fig. 4A) and even more so (4–5-fold) with their conditioned media (Fig. 4B). Similar results were obtained with C-6 glioma cells transfected with the chicken versus the human-*hpa* cDNAs. Cells transfected with control plasmid alone failed to express heparanase activity (Fig. 4). Unlike the results with intact cells (Fig. 4A) and their conditioned media (Fig. 4B), there was no apparent difference in heparanase activity determined in lysates of the chicken- and human-*hpa*-transfected cells (Fig. 4C). To investigate whether the two heparanase species differ in specific activity, serum-free medium (500 ml) conditioned by Eb cells transfected with the chicken or human *hpa* cDNAs were subjected to partial purification on SP-Sepharose. The enzymes were eluted from the column with 0.5 M NaCl in citrate phosphate buffer, pH 6.2, and equal amounts of total protein were tested for heparanase activity. Both enzymes exhibited a similar apparent specific activity, indicated by the almost identical amount and elution pattern of ECM-derived HS degradation fragments (not shown). We have recently developed a quantitative enzyme-linked immunosorbent assay specific for the active 50-kDa form of the human heparanase. Using this assay, it was found that serum-free medium conditioned for 24 h by H-*hpa*-transfected Eb cells (2.5×10^6 cells/ml) contains 1 ± 0.2 ng of heparanase protein per ml. Based on measurements of heparanase activity (Fig. 4B), it is estimated that medium conditioned by the *Chk-hpa*-transfected cells contains 4–5 ng/ml of the heparanase enzyme. Altogether, these results indicate that the chicken enzyme is more readily secreted into the incubation medium and/or retained on the cell surface, as compared with the human enzyme, most likely due to the marked difference between the respective signal peptide sequences. In order to clarify this assumption, we generated a chimeric construct composed of the chicken signal peptide fused to the human cDNA downstream nucleotide 105. Briefly, chicken-specific primers were used to amplify the chicken signal sequence which was then fused by means of primer extension to the human *hpa* sequence, replacing its signal peptide, as described under “Experimental Procedures.” The chimeric construct, subcloned into pcDNA3 plasmid, was applied to transfect Eb mouse lymphoma and C-6 rat glioma cells. Serum-free medium conditioned for 24 h by Eb cells stably transfected with the chimeric construct (chimeric-*hpa*) was tested for heparanase activity. As shown in Fig. 5A, cells transfected with the chimeric enzyme were comparable to cells transfected with *Chk-hpa* in their ability to secrete the heparanase enzyme into the culture medium. In contrast, little or no heparanase activity was detected in medium conditioned by H-*hpa* transfected cells (Fig. 5A), indicating that secretion of the enzyme is in fact driven by the chicken signal peptide sequence. Similar results were obtained with C-6 glioma cells (not shown).

Cellular Localization of the Chicken Versus Human Heparanase Enzymes—The observed differences between the chicken and human enzymes in the sequence and length of their signal peptides and secretion properties, led us to investigate their cellular localization pattern. For this purpose, C-6 glioma cells

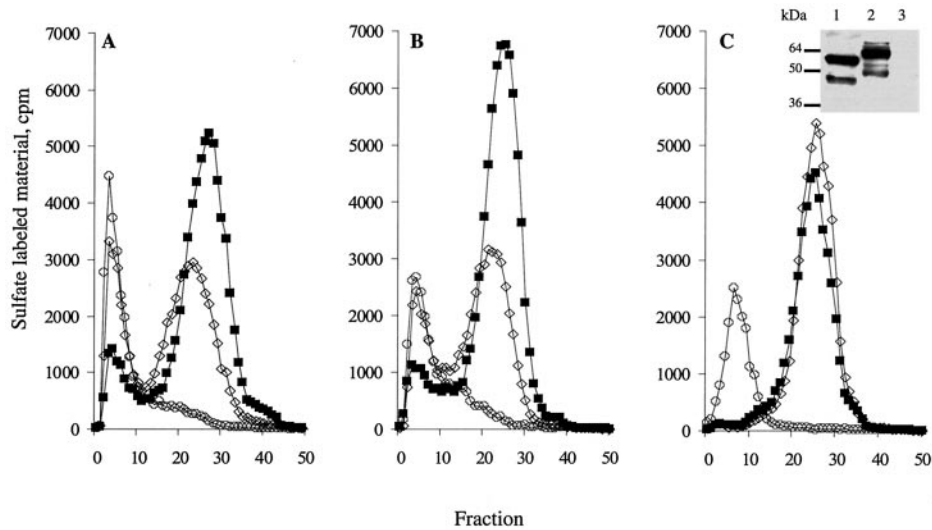


FIG. 4. Heparanase activity in cell lysates, intact cells, and medium conditioned by lymphoma cells transfected with chicken versus human *hpa* cDNA. Eb mouse lymphoma cells transfected with *Chk-hpa* (■) or *H-hpa* (◇) were maintained (24 h, 2×10^6 cells/ml) in serum-free RPMI medium. Intact cells (A), cell lysates (C), or conditioned media (B) corresponding to 2×10^6 cells were then incubated (24 h, 37 °C) in serum-free medium with sulfate-labeled ECM. Labeled degradation fragments released into the incubation medium were analyzed by gel filtration on Sepharose 6B, as described under "Experimental Procedures" and in the legend to Fig. 3. Mock transfected Eb lymphoma cells (○) were used as control. *Inset*, Western blot analysis. Partially purified (SP-Sepharose) heparanase secreted into serum-free medium conditioned by Eb lymphoma cells transfected with *Chk-hpa* (lane 1), *H-hpa* (lane 2), or plasmid alone (lane 3) were subjected to 10% SDS-PAGE and Western blot analysis applying polyclonal rabbit anti-heparanase antibodies (directed against residues 322–330) and ECL visualization, as described under "Experimental Procedures." The molecular weight markers used were: glutamic dehydrogenase (64 kDa), alcohol dehydrogenase (50 kDa), and carbonic anhydrase (36 kDa).

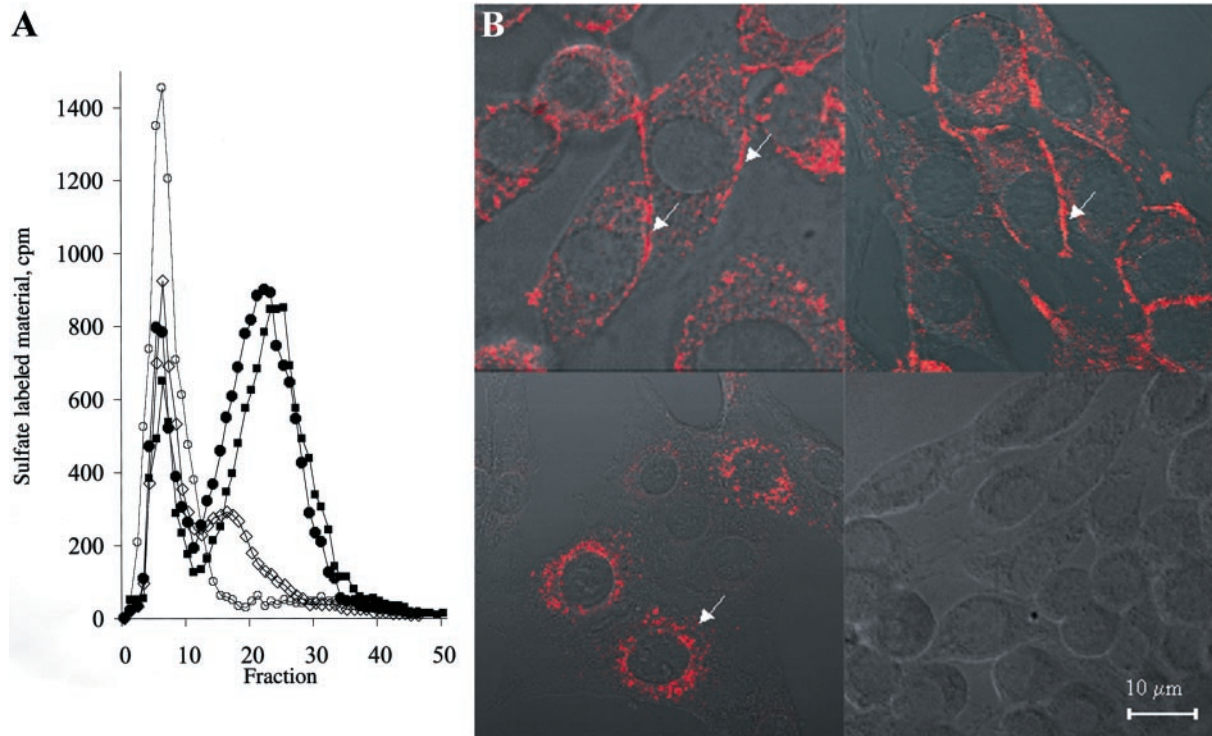


FIG. 5. Secretion and cellular localization of the chicken, human, and chimeric heparanases. A, heparanase activity. Eb mouse lymphoma cells were stably transfected with *Chk-hpa* (■), *H-hpa* (◇), or *chimeric-hpa* (●). Serum-free medium conditioned by these cells and prepared as described in the legend to Fig. 4, was incubated (24 h, 37 °C, pH 6.2) with sulfate-labeled ECM and tested for heparanase activity. Mock transfected Eb lymphoma cells (○) were used as control. B, immunostaining. C6 rat glioma cells were transfected with chicken (upper left), human (lower left), or chimeric (upper right) heparanase cDNAs. Pooled populations of stable transfected cells were subjected to indirect immunofluorescence staining with monoclonal anti-heparanase antibodies (mAb 130) followed by Cy-3-conjugated goat anti-mouse Ab, as described under "Experimental Procedures." Mock transfected C6 glioma cells (lower right) were used as control and showed no staining. *Chk-hpa* (upper left) and *chimeric-hpa* (upper right) transfected cells exhibited intense staining associated mostly with the cell membrane (arrow), while cells transfected with *H-hpa* cDNA (lower left) displayed primarily a perinuclear granular staining (arrow). Bar, 10 μ m.

stable transfected with the chicken, human, or chimeric heparanase cDNAs were grown in 4-well chamber slides and subjected to indirect immunofluorescence staining with the

anti-human heparanase mAb 130 (15). These antibodies cross-react with the chicken enzyme. Confocal fluorescence microscopy revealed that C-6 glioma cells transfected with the *Chk-*

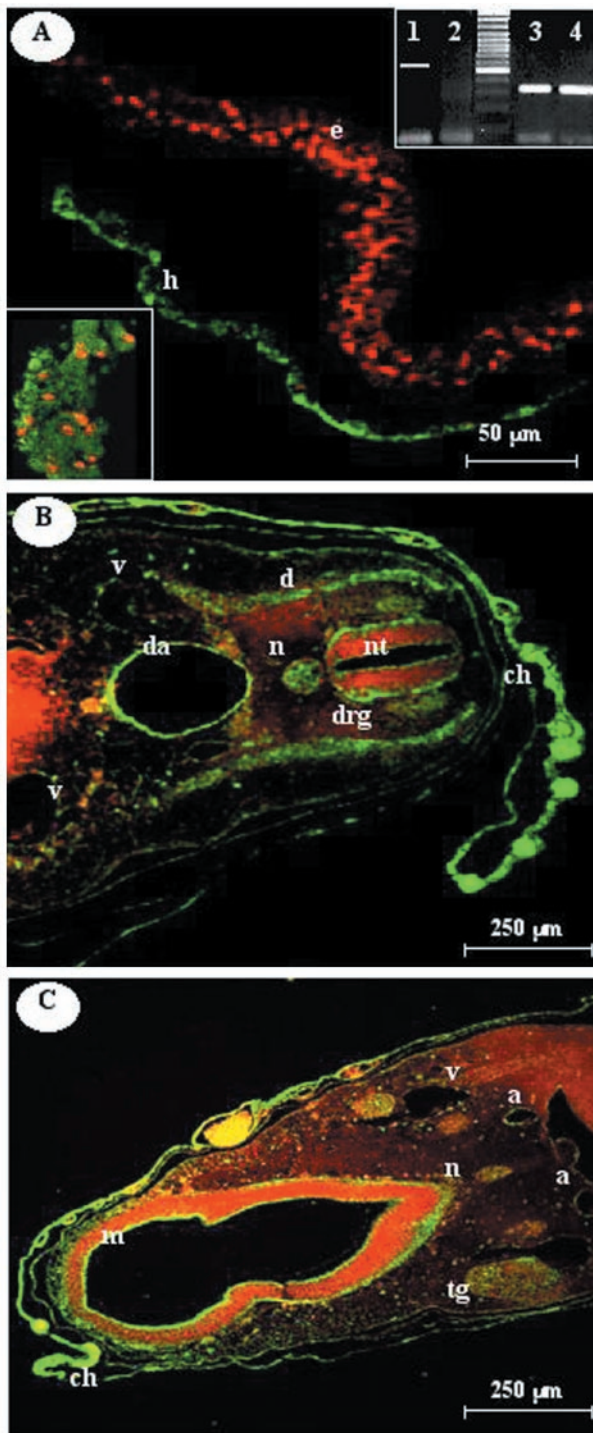


FIG. 6. Heparanase expression in the chicken embryo. Paraffin-embedded sections derived from 12- (stage 2–3) (A) and 72-h (stage 18–19) (B and C) chicken embryos were subjected to indirect immunofluorescence staining (green) with monoclonal anti-heparanase antibodies (mAb 130) and counterstaining with propidium iodide (red) for visualization of nuclei. A, intense expression of the heparanase protein is seen in the hypoblast (*h*) of a 12-h chicken embryo, while cells in the epiblast (*e*) exhibit little or no heparanase staining. Bar, 50 μ m. Insets: upper right, RT-PCR. Total RNA isolated from hypoblasts (lanes 1 and 3) or epiblasts (lanes 2 and 4) of 29 (12 h) chicken embryos was tested for expression of *Chc-hpa* mRNA using primers specific for *Chc-hpa* (amplifying a 611-bp cDNA fragment) (lanes 1 and 2), or β -actin (lanes 3 and 4). Middle lane, 100-bp DNA molecular weight markers. Lower left, magnification ($\times 100$) of the hypoblast area. B and D, 72-h chicken embryo expresses the heparanase protein in the developing vascular (*da*, dorsal aorta; *v*, veins; *a*, arteries) and nervous (*n*, notochord; *nt*, neural tube; *m*, mesencephalon; *g*, trigeminal nerve ganglion; *drg*, dorsal root ganglia; *d*, dermomyotome) systems. An intense staining is observed in the chorioallantoic membrane (*ch*). Bar, 250 μ m.

hpa cDNA exhibited an intense granular staining of the heparanase protein mostly associated with the cell surface, as opposed to a weak and scattered staining in the cell cytoplasm (Fig. 5B). Preferential localization of the chicken heparanase was noted in areas of cell to cell contacts (Fig. 5B, upper left, arrow). Unlike this pattern of immunostaining, C-6 glioma cells overexpressing the human heparanase displayed primarily a perinuclear granular staining pattern with almost no detectable surface localization of the enzyme (Fig. 5B, lower left). Immunostaining of C-6 glioma cells transfected with the chimeric heparanase revealed preferential surface localization pattern (Fig. 5B, upper right), similar to that of cells expressing the chicken heparanase. Mock transfected glioma cells showed no staining (Fig. 5B, lower right). The results of the swapping experiment emphasize that the pronounced difference in cellular localization of the chicken and human heparanases is due primarily to the marked difference in sequence, length, and hydrophobic properties of the respective signal peptides. We have also expressed the chicken and human heparanase cDNAs in homologous cells (*i.e.* QT6 quail fibrosarcoma and Huh7 human hepatocarcinoma cells, respectively), resulting in an immunostaining pattern (not shown) similar to that observed with the transfected C-6 rat glioma cells. The preferential cell surface association of the chicken and chimeric heparanases is in accordance with the higher HS degrading activity expressed by intact cells overexpressing the chicken or chimeric enzymes *versus* the human heparanase.

Expression of Heparanase in the Developing Chicken Embryo—The involvement of heparanase in cell migration associated with tumor metastasis, angiogenesis, and inflammation, and its high expression in placenta led us to investigate the pattern of its expression during early developmental stages of the chicken embryo. For this purpose, paraffin-embedded sections derived from 12 h (stage 2–3) (Fig. 6A) and 72 h (stage 18–19) (Fig. 6, B and C) chicken embryos (30) were subjected to indirect immunofluorescent staining with the anti-human heparanase mAb 130. Cell nuclei were counterstained with propidium iodide and sections were visualized by confocal microscopy. As shown in Fig. 6A, cells migrating from the epiblast and residing in the newly formed hypoblast layer of a 12-h chicken embryo were intensely stained by the anti-heparanase antibodies. In contrast, cells remaining in the primary ectoderm (epiblast) showed little or no expression of the heparanase protein. Preferential expression of the heparanase mRNA in the hypoblast *versus* epiblast was also noted by RT-PCR of mRNA extracted from each layer (Fig. 6A, inset). A complex immunostaining pattern was revealed in sections derived from a 72-h chicken embryo. Prominent expression of the enzyme was noticed in the developing vascular (dorsal aorta, veins, and arteries) and nervous (notochord, mesencephalon, trigeminal nerve ganglia, dorsal root ganglia, neural tube, and dermomyotome) systems (Fig. 6, B and C). Intense specific staining was observed in the chorioallantoic membrane, particularly in sprouting capillaries, which supply oxygen and nutrients to the developing embryo (Fig. 6B). The lung buds, trachea, pharynx, preoral gut, and pronephric ducts were faintly stained. Little or no expression of the heparanase protein was detected in the atrium, branchial arches, and in the mandibular and maxillary processes (not shown). These results suggest that the heparanase enzyme may play a role in cell migration occurring both at the very early stages of embryogenesis and later on in morphogenesis of the cardiovascular and nervous systems.

DISCUSSION

Discrete species of HS play important roles in normal growth, morphogenesis, and pattern formation during development, primarily through regulation of growth factor-induced pathways (31, 32). For example, the gene for *N*-deacetylase *N*-sulfotransferase enzymes, involved in modification of HS, is required for viability and normal patterning of multiple organs in *Drosophila* (33) emphasizing the involvement of HS-modifying enzymes and their proteoglycan substrates in spatially regulated signaling events during development and growth (31–33). Unlike enzymes involved in the biosynthesis of HS, the role of HS-degrading enzymes (*i.e.* heparanase) in normal development and tissue remodeling has not been investigated, simply because of the lack of appropriate molecular probes and antibodies. The recent cloning and expression, independently by several groups, of a single dominant gene encoding functional heparanase (15–19), led us to investigate its expression pattern in the highly defined chicken developmental program. For this purpose, we have identified a chicken EST homologous to the human heparanase cDNA and applied the rapid amplification of cDNA ends technique to clone and express a functional chicken heparanase, 60% homologous to the human enzyme. Recently, the active site residues of human heparanase were identified and the enzyme was classified as a member of the clan A glycosyl hydrolases (29). Interestingly, the predicted active site involves two highly conserved glutamic acid residues which are the proton donor (Glu²²⁵ and Glu²⁰⁴) and the nucleophile (Glu³⁴³ and Glu³²³), with an asparagine (Asn²²⁴ and Asn²⁰³) preceding the proton donor at the active site of the human and chicken enzymes, respectively. Sixteen out of 17 amino acids flanking the proton donor and 18 out of 19 residues which flank the nucleophile are identical in the human and chicken enzymes (Fig. 2), supporting a common catalytic mechanism. The occurrence of such highly conserved regions surrounding the active site residues of the human, rodents, and chicken enzymes, the identification of the human enzyme as an endo- β -D-glucuronidase (34), and the similar size of HS degradation fragments produced by the chicken and human heparanases, suggest that the newly cloned chicken heparanase is an endo- β -D-glucuronidase.

The most prominent difference between the human and chicken heparanase sequences resides in the predicted signal peptide region, showing 39% homology and a marked difference in hydrophobicity and length (35 *versus* 19 amino acids, respectively). This difference may, among other effects, account for the chicken enzyme being readily secreted into the culture medium of Chk-*hpa*-transfected cells. Moreover, intact cells overexpressing the chicken heparanase-degraded HS in a naturally produced subendothelial ECM to a higher extent than cells transfected with the human enzyme. There was no significant difference, however, in the apparent specific activity of the two species of enzymes. The higher enzymatic activity expressed by intact cells transfected with the chicken *versus* the human heparanase cDNAs was also reflected by a marked difference in cellular localization of the chicken and human heparanase enzymes. Whereas the chicken enzyme was primarily localized in close proximity to the cell membrane, particularly in areas of cell to cell contacts, the human enzyme exhibited a mostly perinuclear granular distribution and almost no surface localization. Our preliminary results using green fluorescent protein-conjugated human heparanase cDNA indicate that the human enzyme is localized predominantly in acidic granules, apparently lysosomes, and in perinuclear endosomal granules associated with the endoplasmic reticulum. In fact, heparanase activity was first isolated from rat liver lysosomes (35) and found to be present in both chloroquine

sensitive (lysosomal) and insensitive (endosomal) compartments in rat and human tumor cells (36). ECM-degrading enzymes may, however, translocate from within lysosomes to the plasma membrane, in correlation with the metastatic potential of cells. Thus, cathepsin B is localized on the surface of invasive breast and bladder cancer cells, but is confined to lysosomes in the respective non-invasive cell variants or normal bladder epithelium (37). In human neutrophils, the enzyme has been found to be readily secreted, co-localized with MMP-9 activity in tertiary granules (38, 39). Degradation enzymes expressed on the cell surface are likely to be more effective than intracellular enzymes in solubilizing the ECM, as was in fact demonstrated in the present study by the markedly increased heparanase activity expressed by intact lymphoma and glioma cells transfected with the chicken *versus* the human cDNAs. Our preliminary studies indicate that cells overexpressing the chicken, or chimeric enzyme also exhibit a higher invasiveness *in vitro* and metastatic potential *in vivo*, as compared with cells overexpressing the human enzyme.² The Chk-*hpa*-transfected cells are also expected to elicit a pronounced angiogenic response, primarily by virtue of a more efficient release of heparin-binding angiogenic factors (*i.e.* basic fibroblast growth factor) sequestered by HS in the ECM (10, 24, 25).

It was previously reported that complete solubilization of heparanase from human and rat origins required the presence of a detergent during homogenization, indicating that up to 25% of the heparanase activity was membrane bound (16, 40). In accordance with this observation are our recent immunostaining studies showing that the enzyme is found primarily in the cytoplasm, but also on the surface of human colon carcinoma (21), pancreatic carcinoma, and myeloid leukemia cells. Putative transmembrane, highly homologous hydrophobic regions are present at the C terminus of both the human (residues 515–534) (16) and chicken (residues 495–513) enzymes. Cells transfected with chimeric human heparanase bearing the chicken signal sequence closely resembled Chk-*hpa*-transfected cells, indicating that the observed differences between the chicken and human heparanases in cellular localization and secretion properties are due primarily to the unique properties of the chicken heparanase signal peptide sequence, sharing little homology to that of the human, mouse, and rat enzymes. It was proposed that heparanase may, in part, remain associated with the cell surface through interaction with cell surface HSPGs and/or with the 300-kDa mannose 6-phosphate receptor (16). Mannose 6-phosphate was previously reported to displace the enzyme from the surface of T-lymphocytes (22, 23). Our recent experiments indicate that exogenously added latent human heparanase binds to HS on the surface of cells. At physiological pH the HS is not degraded, but rather facilitates uptake of the bound enzyme into intracellular granules. Endocytosis of exogenously added heparanase is accompanied by processing and activation of the enzyme.³

Most studies emphasize the involvement of heparanase in pathophysiology. Little is known, however, about the enzymes contribution to normal cell and tissue function during embryogenesis and in the adult. Heparanase may, for example, play a role in embryo implantation, involving invasive properties and interaction between HS-binding proteins and HSPGs (41). Subsequently, the enzyme may function in embryonic cell migration, proliferation, and differentiation, in a manner similar to its involvement in tumor metastasis, angiogenesis, and inflammation (10–14). We have recently generated transgenic

² O. Goldshmidt, E. Zcharia, S. Metzger, T. Chajek-Shaul, E. Mitrani, and I. Vlodaysky, manuscript in preparation.

³ L. Nadav, O. Yacoby-Zeevi, E. Zamir, I. Pecker, B. Geiger, A. Eldor, I. Vlodaysky, and B. Katz, unpublished results.

mice overexpressing the heparanase cDNA and protein in all tissues. Mammary glands of heparanase overexpressing virgin females showed precocious alveolar development and ductal branching, again demonstrating the involvement of heparanase in morphogenesis and tissue remodeling.⁴

Using monoclonal anti-heparanase antibodies directed against the C terminus of the chicken heparanase, we examined the distribution pattern of the newly cloned heparanase enzyme in the developing chicken embryo. Both the chicken heparanase mRNA and protein were specifically expressed, as early as 12 h post-fertilization, in cells migrating from the epiblast (primary ectoderm) and forming the hypoblast layer. At this point of time, there was virtually no expression of the heparanase mRNA and protein in the epiblast layer. Later on (72 h), the enzyme is preferentially expressed in cells of the developing vascular and nervous systems. Intense expression of the enzyme was observed in dorsal root ganglia, notochord, neural tube, dorsal aorta, and the highly vascularized chorioallantoic membrane. Early expression of heparanase in the nervous system suggests a role for the enzyme in the regulation of neuronal cell migration, proliferation, and differentiation. Interestingly, HSPGs stimulate the proliferation of Schwann cells and their removal prevents the cells from proliferating around the axons (6, 7). Also, nerve growth factor, neurotrophin-3, and astrocytes were reported to stimulate melanoma cell invasion and heparanase activity, both *in vitro* and *in vivo* (42). A novel MT-MMP, cloned from cultured chicken embryo fibroblasts, is expressed in the head and body of 8- and 9-day-old chicken embryos (43), suggesting that MT-MMP plays a role during early stages of development, when large scale cell migration occurs. A high expression of the heparanase protein was noted in the chicken extra embryonic chorioallantoic membrane, characterized by an extensive capillary network that supports the developing embryo. Early expression of both heparanase and MMP-2 (44) in the chicken chorioallantoic membrane when expansion of its vasculature is maximal, suggests a role of these ECM-degrading enzymes in neovascularization. Heparanase, similar to its involvement in pathological angiogenesis, may contribute to normal development of the vascular system through an effect on endothelial cell migration and sprouting, as well as on the bioavailability and mitogenic activity of HS-bound angiogenic factors (24, 25). The enzyme also produces HS degradation fragments that promote receptor binding, dimerization, and signaling of heparin-binding growth factors, primarily basic fibroblast growth factor (45). We have recently observed a profound angiogenic response elicited by heparanase-transfected cells embedded in Matrigel and implanted subcutaneously, *versus* a small or no response to mock-transfected cells (46). Similarly, MMP-9 is regarded as a specific component of the angiogenic switch by rendering vascular endothelial growth factor more available to its receptors (47). Taking into account the involvement of HS (*i.e.* glypican) in growth factor (*i.e.* BMP-2 and BMP-4) signaling during development and pattern formation (31–33, 48), it is conceivable that HS-degrading enzymes will affect developmental processes primarily by virtue of an effect on cell migration and the bioavailability of HS-bound growth- and differentiation-promoting factors, and through an effect on the rate of HS turnover. The development of mice with targeted disruption of the heparanase gene will better elucidate its normal roles in embryonic development and in the mature individual. Heparanase is the first and single functional vertebrate

HS-degrading enzyme that has been cloned, expressed, and characterized. This may pave the way for identification and cloning of other members of a putative family of mammalian glycosaminoglycan-degrading enzymes (*e.g.* chondroitinase, dermatanase, and keratanase), toward a better understanding of the function and biological significance of both the enzymes and their polysaccharide substrates in normal and pathological processes.

Acknowledgments—We are grateful to Tamara Sun, Mark Tarshis, Tamar Kahan, and Yael Shimoni for helpful suggestions and excellent technical assistance.

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