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

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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 of 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

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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.

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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 performed 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 suspension in Life Technologies, Grand Island, NY supplemented with β-mercaptoethanol (5 × 10−3 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 cultures 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 × 105 cells/ml and cultured as described above, except that 4% dextran T-40 was included in the growth medium (26, 28). Na2SO4 (25 μC/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 7, the cultures were returned to normal growth medium.

**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 sulfated 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 content counted in a scintillation counter (10, 11, 15, 26). Degradation fragments of HS side chains were eluted from Sepharose 6B at 0.5 < Kav < 0.8 (peak II) (11, 15, 26). Each experiment was performed at least 3 times and the variation of elution positions (Kav values) did not exceed ±15%.

**Cloning of Chicken Heparanase cDNA**—The cDNA sequence of human heparanase (15, 16) was used to screen EST data base 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 PolyAttractmRNA Isolation System (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)12–18 as primer. The resulting cDNAs were extended by 3’ C-tailing, using terminal deoxynucleotidyl transferase (Promega). Expand High Fidelity enzyme (Boehringer, Mannheim, Germany) was applied for PCR amplification. The first step applied the AP1 primer and the gene specific primer ChkL1: 5’-GACCTCTCAGCATTCCCTCAG-3’ and the second step used the nested 5’ primer AP2 and a nested gene specific 3’ primer ChkL2: 5’-AGGCTGTGACTTCTGCGTGC-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 sequence analyzer (ABI PrismTM 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/SPP, 5’-CGGGGTACCCAGATCTGTGTCG-3’; SPL, 5’-AGGTTCGACGACTTCTGCGTCCTGGCCTCG-3’). The H-hpa region extending from the first amino acid downstream the H-hpa signal peptide was amplified using the specific primers (HU, 5’-CAAGGCGACGCACAGCGACTGTGGA-3’; H/BHIL, 5’-CACCATTGAGGTAGTGGATC-3’). The PCR products were used in a mixture of primers extension PCR and amplification. The resulting fragment was then cloned into-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 is replaced by 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 ClustalW 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. Be cells were grown in suspension in RPMI 1640 medium (Life Technologies, Grand Island, NY) containing 10% fetal calf serum (15, 26) at a split ratio of 1:10. Cells were transfected by the DEAE-dextran method (26, 28). After a selection step at 30 μg/ml G418 was used for selection of transfected cells. Several independent clones were selected with 350 μg/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-hpa) plasmids were also applied for stable transfection of C6 rat glioma cells. With these cells, 600 μg/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 μg of total RNA by oligo(dT) priming, the resulting single stranded cDNA was amplified using TaqDNA polymerase (Promega). Oligonucleotide primers Chk-U (5’-GTGGACACGTAAGATATTCTCT-3’) and Chk-L (5’-AATCCCTCCCTGCTGACT-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 at 1 min at 72°C (33 cycles). Aliquots (15 μl) of the amplified cDNA were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining (15).

**Immunostaining**—C6 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 with PBS and intracellular fluorescence was detected with 50 μm NH4Cl for 5 min. Cells were then washed (×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 μg/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 incubation...
tion with mAb 130, cells were washed (×5) with PBS and incubated with Cy-3-conjugated goat anti-mouse IgG (1:100, Jackson, Bar-Harbor, ME) for 1 h at 4° C. Slides were then washed 8 times with PBS, mounted with 90% glycerol in PBS, and visualized with a Zeiss LSM 410 confocal microscope. Chicken embryos were dissected, fixed, and processed, as described (29). Sections (5 μm) were deparaffinized, rehydrated, and subjected to immunofluorescence staining (mAb 130 followed by Cy-3 conjugated goat anti-mouse IgG) and visualization, as described above.

**Western Blot Analysis**—Cells transfected with either Chk-hpa or H-hpa were maintained for 24 h in serum-free RPMI medium. The conditioned media (∼500 ml) were applied onto a 5-ml fast-flow SP Sepharose column (Amersham Pharmacia Biotech, Stockholm, Sweden) and eluted with 20 mM phosphate-citrate buffer, pH 6.2, containing 0.5 M NaCl, 1 mM dithiothreitol, and 0.1% CHAPS (Sigma). After desalting using a microcentrifuge filter (NMWL 5,000; Sigma), 0.5 μg of protein was separated on 10% SDS-PAGE and the proteins transferred onto Immobilon-P membrane (Millipore, Bedford, MA). The membrane was subjected to successive incubations with block solution, polyclonal anti-heparanase antibodies (1:2500) in 1% bovine serum albumin, 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.05% Tween-20, and horseradish peroxidase-conjugated anti-mouse antibodies (Jackson) as described (15, 21). The anti-heparanase antibodies were raised in rabbit against KLH-conjugated synthetic peptide (residues 322–330 of the human heparanase) and kindly provided by Dr. Robert Heinrikson (Upjohn-Pharmacia, Kalamazoo, MI) (19). Immunoreactive bands were detected by the enhanced chemiluminescence reagent using luminol and p-cumary acid (Sigma). The light emitted by the chemical reaction was detected by exposure to medical x-ray film green CP-G Plus (Agfa, Belgium) for 30–60 s.

**RESULTS**

**Cloning of the Chicken Heparanase cDNA**—The amino acid sequence of the human heparanase cDNA was used to screen EST data bases for homology to a chicken unidentified mRNA sequences. A single homologous chicken EST, derived from the cDNA of chicken activated T lymphocytes (accession number AI980994), was identified which shared 60.5% identity with 276 bp at the 3′ end of the human heparanase coding sequence. This sequence encodes a truncated open reading frame of 91 amino acids, 74% homologous to the human heparanase, followed by 325 nucleotides of a 3′-untranslated region. Chicken kidney mRNA was isolated and subjected to rapid amplification of cDNA ends (15), using gene-specific PCR primers (ChkL1 and ChkL2) designed according to the above described EST. A DNA fragment of ∼1600 bp was obtained, partially overlapping the identified 3′ encoding EST clone. The entire cDNA, cloned in pGEM-T Easy vector, was designated Chk-hpa. The full-length cDNA (Chk-hpa) is 1,609 bp long. It contains an open reading frame that encodes a polypeptide of 532 amino acids (Fig. 1) with a calculated molecular mass of 58,842 daltons. Analysis of the amino acid sequence and the hydro-
pathic profile of the protein indicated a hydrophobic amino acid sequence at the N terminal (Fig. 1, underlined). This sequence is predicted (signal p program) to comprise a signal peptide spanning the N-terminal 19 amino acids (versus 35 residues in the human enzyme) and showing only a small homology (39%) to the signal peptide of the human heparanase. A second hydrophobic, possibly transmembrane region, was identified at the C terminus (residues 495–513) (Fig. 2, boxed). The identity between the chicken and human hpa coding sequences is 62%, with 61.3% amino acid sequence identity and 68.8% similarity. The chicken heparanase is synthesized as a latent, ~60-kDa precursor protein. Taking into account its similarity to the human heparanase, the latent chicken enzyme is then processed into active, mature 37-kDa form through cleavage between Trp 136 and Lys 137 (Fig. 2). It was proposed that the human heparanase precursor protein is cleaved at the N-terminal amino acids 495–513 and 515–534, respectively (Fig. 2, boxed). Similarly, the putative two catalytic glutamic residues (29), the proton donor (H-, Glu204) and nucleophile (Lys323), as well as the amino acids flanking these residues are highly conserved in the chicken, human, and rodent enzymes (Fig. 2, shadow). The predicted amino acid sequence of the chicken enzyme has 4 potential N-glycosylation sites (Fig. 1), three of which are conserved in the isolated human and chicken heparanases, as indicated by Western blot analysis of the respective recombinant enzymes (Fig. 4C, inset).

Functional Expression of Recombinant Chicken Heparanase in Mammalian Cells—The ability of the Chk-hpa gene product to catalyze degradation of heparan sulfate (HS) in vitro was assessed by expressing the entire open reading frame of the Chk-hpa gene in mammalian cells lacking heparanase activity. The cells were then subjected to RT-PCR (Fig. 3, inset), Western blot analysis (Fig. 4C) and measurements of heparanase activity (Figs. 3 and 4). Rat C6-glioma and mouse Eb-lymphoma cells were then subjected to RT-PCR (Fig. 3, inset). Rat C6-glioma cells were then subjected to RT-PCR (Fig. 3, inset) and Western blot analysis (Fig. 4C). Rat C6-glioma cells were transfected with pcDNA3 plasmid containing the chicken heparanase cDNA (Chk-hpa), or mock transfected with a control plasmid alone. Stable transfected cells were obtained following selection with G418. RT-PCR performed on mRNA isolated from Chk-hpa transfected and mock-transfected C6-glioma cells revealed expression of the Chk-hpa mRNA in the transfected, but not the mock transfected cells. There was no difference in expression of the β-actin gene (Fig. 3, inset). RT-PCR primers designed to detect the Chk-hpa failed to amplify the H-hpa, and vice versa. Western blot analysis of partially purified heparanase secreted by hpa-transfected Eb cells revealed protein

| species     | accession | sequence | length | identity |
|-------------|-----------|----------|--------|----------|
| human       | M17658    | NM_012001.1 | 589    |          |
| chicken     | AF575536  | AF575536  | 589    |          |

Fig. 2. Comparison of the chicken and human heparanase amino acid sequences. The aligned sequences reveal 61.3% identical and 68.8% chemically similar amino acids in the chicken and human heparanase sequences. The conserved amino acids are shown in the third line. Conserved differences are marked by double or single dots, generated by the Clustal-W alignment program. The putative two catalytic Glu residues, the proton donor, and the nucleophile, are bold and their flanking amino acids are shadowed. The predicted signal peptides are underlined. The cleavage sites generating the mature enzymes are marked by an arrow. The boxed sequences shows the hydrophobic potential membrane-spanning domain of 19 amino acids.
were maintained for 24 h in serum-free RPMI medium at a density of $2 \times 10^6$ 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 $2 \times 10^6$ 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 $\times 10^6$ 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 transfected 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...
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-
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 chorionicallantoic 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 (Glu341 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 mannos 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

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² O. Goldshmidt, E. Zcharia, S. Metzger, T. Chajek-Shaul, E. Mitrani, and I. Vlodavsky, manuscript in preparation.
³ L. Nadav, O. Yaooby-Zeevi, E. Zamir, I. Pecker, B. Geiger, A. Eldor, I. Vlodavsky, 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, HS-degrading enzymes will affect developmental pathological processes.

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

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