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Cloning, expression, and characterization of an alternatively spliced variant of human heparanase

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Abstract

Heparanase is an endoglycosidase that cleaves heparan sulfate in the extracellular matrix (ECM) and hence participates in ECM degradation and remodeling. Heparanase is involved in fundamental biological processes such as cancer metastasis, angiogenesis, and inflammation. Alternative splicing in the coding region of human heparanase was not reported. Here, we report the cloning of a splice variant of human heparanase that lacks exon 5 and is missing 174 bp compared to the wild-type cDNA. Splice 5 is expressed as a 55 kDa protein compared to the 65 and 50 kDa latent and active wild-type enzyme. Splice 5 was not detected in the incubation medium of tumor cells as opposed to the wild-type latent heparanase. Splice 5 escaped proteolytic cleavage, was devoid of HS degradation activity and exhibited diffused rather than granular cellular localization.

Keywords: Heparan sulfate; Extracellular matrix; Heparanase; Alternative splicing

Heparan sulfate proteoglycans (HSPGs) are macromolecules associated with the cell surface and extracellular matrix (ECM) of a wide range of cells [1–3]. The basic HSPG structure consists of a protein core to which several linear heparan sulfate (HS) chains are covalently O-linked. The polysaccharide chains are typically composed of repeating hexuronic and D-glucosamine disaccharide units that are modified at various positions by sulfation, epimerization, and *N*-acetylation, yielding clusters of sulfated disaccharides separated by low or non-sulfated regions [1–3]. Heparan sulfate binds to and assembles ECM proteins, including fibronectin, laminins, and interstitial collagens, and is playing important roles in cell–cell and cell–ECM interactions [1–3].

Mammalian heparanase capable of HS cleavage was cloned by several groups [4–7]. Despite of earlier reports on the existence of several distinct mammalian HS degrading endoglycosidases (heparanases), cloning of the same single gene suggests that mammalian cells express primarily a single dominant functional heparanase enzyme. Recently, we cloned heparanase of the blind subterranean mole rat (*Spalax*) and characterized a related splice variant originating from skipping of exon #7 [8]. Splice 7 was shorter by 48 bp compared to the wild-type cDNA, with no frameshift. Despite, the fact that splice 7 possesses the same signal peptide as that of the wild-type enzyme, it was not detected in the incubation medium, escaped processing, and activation and hence was devoid of enzymatic ability [8].

Alternative splicing in the coding region of human heparanase was, to the best of our knowledge, not reported. On average, each human gene generates three alternatively spliced mRNAs [9,10]. This fact and the cloning of splice 7 heparanase of *Spalax*, led us to the search for human splice variants of the heparanase gene. Here, we describe the cloning and characterization of a unique splice variant of human heparanase which results from skipping of exon #5.

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Materials and methods

RNA and cDNA preparation. RNA of human kidney excised from a patient suffering from renal cell carcinoma, was kindly provided by Prof. Shimon Meretyk (Urology Department, Rambam Health Care Campus, Haifa, Israel). cDNA was prepared by reverse transcription (M-MLV reverse transcriptase, Promega, Madison, WI) of 1 μg total RNA, using oligo(dT)15 and random primers [4].

Gene cloning. For cloning splice variants of human heparanase, we performed PCR utilizing the following primer pair designed according to the published human heparanase sequence [4,5]: sense 5′-CCA GAG GCT TGT CTC CTG CGT AC-3′, anti-sense 5′-CAT AAA GCC AGC TGC AAA GGT G-3′; cDNA of kidney was used as a template.

DNA sequencing. DNA sequencing was performed using vector-specific and gene-specific primers, with an automated DNA sequencer (ABI Prism™ model 310 Genetic Analyzer, Perkin-Elmer, Foster city, CA).

Cells and transfections. Human MCF-7 breast carcinoma and U87 glioma cells were cultured in Dulbecco's modified Eagle's medium (DMEM, 4.5 g glucose/liter) containing 10% fetal calf serum (FCS), and antibiotics, as described [11]. Cells were grown in 60 mm tissue culture dishes and transfected with a total of 1–2 μg plasmid DNA mixed with 6 μl of FuGene transfection reagent (Roche Applied Science, Mannheim, Germany) and 94 μl DMEM. Transiently transfected cells were obtained after 24–48 h incubation at 37 °C. Stable populations of transfected cells were selected with G418 [4,11].

Western blot analysis. Cells (2×10^6) transfected with either insert free pcDNA3 vector alone, or pcDNA3 containing the wild-type human heparanase or its splice variant (splice 5), were lysed in 1 ml lysis buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, and a mixture of protease inhibitors (Roche Applied Science, Mannheim, Germany). Heparanase was concentrated by incubating (4 °C, 1 h) the cell lysate or the incubation medium with ConA beads (Amersham Biosciences, Uppsala, Sweden) and washing (2×) with PBS [12,13]. The beads

```
  1    atgctgctgcgctcgaagcctgcgctgccgccgccgctgatgctgctgctcctggggccg
1      M  L  L  R  S  K  P  A  L  P  P  P  L  M  L  L  L  L  G  P
 61    ctgggtcccctctcccctggcgccctgccccgacctgcgcaagcacaggacgtcgtggac
21     L  G  P  L  S  P  G  A  L  P  R  P  A  Q  A  Q  D  V  V  D
 121   ctggacttcttcacccaggagccgctgcacctggtgagcccctcgttcctgtccgtcacc
41     L  D  F  F  T  Q  E  P  L  H  L  V  S  P  S  F  L  S  V  T
 181   attgacgccaacctggccacggacccgcggttcctcatcctcctgggttctccaaagctt
61     I  D  A  N  L  A  T  D  P  R  F  L  I  L  L  G  S  P  K  L
 241   cgtaccttggccagaggcttgtctcctgcgtacctgaggtttggtggcaccaagacagac
81     R  T  L  A  R  G  L  S  P  A  Y  L  R  F  G  G  T  K  T  D
 301   ttcctaattttcgatcccaagaaggaatcaacctttgaagagagaagttactggcaatct
101    F  L  I  F  D  P  K  K  E  S  T  F  E  E  R  S  Y  W  Q  S
 361   caagtcaaccaggatatttgcaaatatggatccatccctcctgatgtggaggagaagtta
121    Q  V  N  Q  D  I  C  K  Y  G  S  I  P  P  D  V  E  E  K  L
 421   cggttggaatggccctaccaggagcaattgctactccgagaacactaccagaaaaagttc
141    R  L  E  W  P  Y  Q  E  Q  L  L  L  R  E  H  Y  Q  K  K  F
 481   aagaacagcacctactcaaaacctaacagtttccttaagaaggctgatattttcatcaat
161    K  N  S  T  Y  S  K  P  N  S  F  L  K  K  A  D  I  F  I  N
 541   gggtcgcagttaggagaagatttattcaattgcataaacttctaagaaagtccaccttc
181    G  S  Q  L  G  E  D  F  I  Q  L  H  K  L  L  R  K  S  T  F
 601   aaaaatgcaaaactctatggtcctgatgttggtcagcctcgaagaaagacggctaagatg
201    K  N  A  K  L  Y  G  P  D  V  G  Q  P  R  R  K  T  A  K  M
 661   ctgaagagcttcctgaaggctggtggagaagtgattgattcagttacatggcatcactac
221    L  K  S  F  L  K  A  G  G  E  V  I  D  S  V  T  W  H  H  Y
 721   tatttgaatggacggactgctaccagggaagattttctaaaccctgatgtattggacatt
241    Y  L  N  G  R  T  A  T  R  E  D  F  L  N  P  D  V  L  D  I
 781   tttatttcatctgtgcaaaaagttttccaggtggttgagagcaccaggcctggcaagaag
261    F  I  S  S  V  Q  K  V  F  Q  V  V  E  S  T  R  P  G  K  K
 841   gtctggttaggagaaacaagctctgcatatggaggcggagcgcccttgctatccgacacc
281    V  W  L  G  E* T  S  S  A  Y  G  G  G  A  P  L  L  S  D  T
 901   tttgcagctggctttatgtggctggataaattgggcctgtcagcccgaatgggaatagaa
301    F  A  A  G  F  M  W  L  D  K  L  G  L  S  A  R  M  G  I  E
 961   gtggtgatgaggcaagtattctttggagcaggaaactaccatttagtggatgaaaacttc
321    V  V  M  R  Q  V  F  F  G  A  G  N  Y  H  L  V  D  E  N  F
1021   gatcctttacctgattattggctatctcttctgttcaagaaattggtgggcaccaaggtg
341    D  P  L  P  D  Y  W  L  S  L  L  F  K  K  L  V  G  T  K  V
1081   ttaatggcaagcgtgcaaggttcaaagagaaggaagcttcgagtataccttcattgcaca
361    L  M  A  S  V  Q  G  S  K  R  R  K  L  R  V  Y  L  H  C  T
1141   aacactgacaatccaaggtataaagaaggagatttaactctgtatgccataaacctccat
381    N  T  D  N  P  R  Y  K  E  G  D  L  T  L  Y  A  I  N  L  H
1201   aacgtcaccaagtacttgcggttaccctatccttttctaacaagcaagtggataaatac
401    N  V  T  K  Y  L  R  L  P  Y  P  F  S  N  K  Q  V  D  K  Y
1261   cttctaagacctttgggacctcatggattactttccaaatctgtccaactcaatggtcta
421    L  L  R  P  L  G  P  H  G  L  L  S  K  S  V  Q  L  N  G  L
1321   actctaaagatggtggatgatcaaaccttgccacctttaatggaaaaacctctccggcca
441    T  L  K  M  V  D  D  Q  T  L  P  P  L  M  E  K  P  L  R  P
1381   ggaagttcactgggcttgccagctttctcatatagttttttgtgataagaaatgccaaa
461    G  S  S  L  G  L  P  A  F  S  Y  S  F  F  V  I  R  N  A  K
1441   gttgctgcttgcatctga
481    V  A  A  C  I  *
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Fig. 1. Nucleotide and predicted amino acid sequences of splice 5 human heparanase. Nucleotide sequences are shown above the predicted amino acid sequences. Numbers on the left correspond to nucleotides (roman) and amino acid residues (bold italic). The signal peptide is underlined. The catalytic nucleophile Glu residue is in bold with asterisks above. The three potential *N*-glycosylation sites are shaded.

were boiled (3 min) in sample buffer, centrifuged and the supernatant subjected to SDS–PAGE and immunoblot analysis, using polyclonal anti-heparanase antibody #1453, as described [12,13]. Antibody #1453 was raised in rabbit against the entire 65 kDa heparanase precursor [13]. Immunoreactive bands were detected by the enhanced chemiluminescence reagent, as described [4,11–14].

Heparanase activity. Cell lysates prepared from 1×10^6 cells by three cycles of freezing and thawing in heparanase reaction buffer (20 mM phosphate–citrate buffer, pH 6.0, 1 mM dithiothreitol, 1 mM $CaCl_2$, and 50 mM NaCl) were incubated (4 h, 37 °C, pH 6.0) with ^{35}S-labeled ECM, prepared as described [4]. The incubation medium containing ^{35}S-labeled HS degradation fragments was analyzed by gel filtration on a Sepharose CL-6B column [4,11]. Fractions (0.2 ml) were eluted with phosphate-buffered saline (PBS) and their radioactivity counted in a β-scintillation counter. Degradation fragments of HS side chains were eluted from Sepharose 6B at $0.5 < K_{av} < 0.8$ (peak II, fractions 20–30) [4,11]. Each experiment was performed at least three times and the variation in elution positions (K_{av} values) did not exceed ±15% of the mean.

Immunocytochemistry. U87 glioma cells stably transfected with the wild-type heparanase or its splice variant #5 were grown on glass coverslips for 8 h. Staining was then performed essentially as described [12]. Briefly, cells were fixed with cold methanol for 10 min, washed with PBS and subsequently incubated in PBS containing 10% normal goat serum for 1 h at room temperature, followed by 2 h incubation with anti-heparanase monoclonal antibody [15] (kindly provided by Dr. Hua-Quan Miao, Imclone Systems Inc., New York, NY). Cells were extensively washed with PBS, incubated with secondary antibody (Jackson ImmunoResearch) for 1 h, washed and mounted in Vectashield (Vector, Burlingame, CA) [12].

Results

Identification and cloning of a splice variant of human heparanase lacking exon 5

We cloned a novel splice variant (#5) of heparanase from the cDNA of human kidney infiltrated by renal cell carcinoma cells. Sequence analysis revealed that it originates from splicing out of exon 5, yielding a deletion of 174 bp compared to the wild-type cDNA (Fig. 1). The reading frame of the splice variant is conserved compared to that of the wild-type gene, and its predicted amino acid sequence is shorter by 58 residues (485 aa for splice #5 compared to 543 aa of the wild-type enzyme) (Fig. 2). The predicted amino acid sequence of splice 5 heparanase

```
S5      1 MLLRSKPALPPPLMLLLLGPLGPLSPGALPRPAQAQDVVDLDFFTQEPLH 50
          ||||||||||||||||||||||||||||||||||||||||||||||||||
WT      1 MLLRSKPALPPPLMLLLLGPLGPLSPGALPRPAQAQDVVDLDFFTQEPLH 50
                  .         .         .         .         .
S5     51 LVSPSFLSVTIDANLATDPRFLILLGSPKLRTLARGLSPAYLRFGGTKTD 100
          ||||||||||||||||||||||||||||||||||||||||||||||||||
WT     51 LVSPSFLSVTIDANLATDPRFLILLGSPKLRTLARGLSPAYLRFGGTKTD 100
                  .         .         .         .         .
S5    101 FLIFDPKKESTFEERSYWQSQVNQDICKYGSIPPDVEEKLRLEWPYQEQL 150
          ||||||||||||||||||||||||||||||||||||||||||||||||||
WT    101 FLIFDPKKESTFEERSYWQSQVNQDICKYGSIPPDVEEKLRLEWPYQEQL 150
                  .         .         .         .         .
S5    151 LLREHYQKKFKNSTYSK..............................  167
          |||||||||||||||||:
WT    151 LLREHYQKKFKNSTYSRSSVDVLYTFANCSGLDLIFGLNALLRTADLQWN 200
                  .         .         .         .         .
S5    168 ........................PNSFLKKADIFINGSQLGEDFIQLH 192
                                  ||||||||||||||||||||||||||
WT    201 SSNAQLLLDYCSSKGYNISWELGNEPNSFLKKADIFINGSQLGEDFIQLH 250
                  .         .         .         .         .
S5    193 KLLRKSTFKNAKLYGPDVGQPRRKTAKMLKSFLKAGGEVIDSVTWHHYYL 242
          ||||||||||||||||||||||||||||||||||||||||||||||||||
WT    251 KLLRKSTFKNAKLYGPDVGQPRRKTAKMLKSFLKAGGEVIDSVTWHHYYL 300
                  .         .         .         .         .
S5    243 NGRTATREDFLNPDVLDIFISSVQKVFQVVESTRPGKKVWLGETSSAYGG 292
          ||||||||||||||||||||||||||||||||||||||||||||||||||
WT    301 NGRTATREDFLNPDVLDIFISSVQKVFQVVESTRPGKKVWLGETSSAYGG 350
                  .         .         .         .         .
S5    293 GAPLLSDTFAAGFMWLDKLGLSARMGIEVVMRQVFFGAGNYHLVDENFDP 342
          ||||||||||||||||||||||||||||||||||||||||||||||||||
WT    351 GAPLLSDTFAAGFMWLDKLGLSARMGIEVVMRQVFFGAGNYHLVDENFDP 400
                  .         .         .         .         .
S5    343 LPDYWLSLLFKKLVGTKVLMASVQGSKRRKLRVYLHCTNTDNPRYKEGDL 392
          ||||||||||||||||||||||||||||||||||||||||||||||||||
WT    401 LPDYWLSLLFKKLVGTKVLMASVQGSKRRKLRVYLHCTNTDNPRYKEGDL 450
                  .         .         .         .         .
S5    393 TLYAINLHNVTKYLRLPYPFSNKQVDKYLLRPLGPHGLLSKSVQLNGLTL 442
          ||||||||||||||||||||||||||||||||||||||||||||||||||
WT    451 TLYAINLHNVTKYLRLPYPFSNKQVDKYLLRPLGPHGLLSKSVQLNGLTL 500
                  .         .         .         .
S5    443 KMVDDQTLPPLMEKPLRPGSSLGLPAFSYSFFVIRNAKVAACI* 486
          |||||||||||||||||||||||||||||||||||||||||||||
WT    501 KMVDDQTLPPLMEKPLRPGSSLGLPAFSYSFFVIRNAKVAACI* 544
```

Fig. 2. Comparison of splice 5 (S5) and wild-type (WT) human heparanase amino acid sequences (GCG alignment program). The two catalytic Glu residues, the proton donor and nucleophile, are marked in bold. Note that the proton donor is missing in splice 5. Potential *N*-glycosylation sites are shaded. Note that splice 5 lacks three out of the six *N*-glycosylation sites found in the wild-type protein.

has three potential *N*-glycosylation sites (Fig. 1), compared to six in the wild-type protein (Fig. 2). Gel electrophoresis of PCR products amplified using primers designed around the deletion segment and kidney cDNA as a template, revealed both the wild type and spliced forms. Plasmids containing the coding region of either form were subjected to PCR and used as positive controls (Fig. 3A).

Functional expression of wild-type and splice 5 human heparanases in mammalian cells

We compared the expression pattern of splice 5 and wild-type human heparanases, applying U87 and MCF-7 cells transiently transfected with each form. Western immunoblotting (using anti-heparanase antibody 1453) of cell lysates revealed a single band of about 55 kDa in splice 5 transfected cells compared to 65 and 50 kDa protein bands in cells transfected with the wild-type heparanase. In order to evaluate secretion of splice 5, we cultured (24 h, 37 °C) U87 and MCF-7 cells transfected with human wild-type heparanase, splice 5, or insert free mock plasmid. Western blot analysis of the incubation medium using anti-heparanase antibodies revealed secretion and accumulation of the wild type 65 kDa latent enzyme in the culture medium (Fig. 3B). In contrast, splice 5 was not detected in the incubation medium (Fig. 3B), indicating its inability to be secreted and to accumulate in the culture medium.

Heparanase enzymatic activity

We assessed the ability of heparanase splice variant #5 to degrade HS in intact ECM. For this purpose, lysates of U87 cells stably transfected with the full length human heparanase, splice 5, or mock control were incubated (4 h, 37 °C, pH 6.0) with intact naturally produced sulfate-labeled ECM. Labeled degradation fragments released into the incubation medium were then analyzed by gel filtration on Sepharose 6B. Incubation of the ECM with lysates of cells transfected with the wild-type human heparanase resulted in release of low-molecular weight labeled degradation fragments eluted toward the V_t of the column (fractions 20–30, $0.5 < K_{av} < 0.8$) (Fig. 3C). These fragments were shown to be degradation products of HS as they were (i) 5–6-fold smaller than intact HS side chains; (ii) resistant to further digestion with papain and chondroitinase ABC, and (iii) susceptible to deamination by nitrous acid [16]. In contrast, both splice 5 and mock transfected cells failed to produce HS degradation products, indicating lack or undetectable levels of heparanase enzymatic activity.

Localization of human heparanase and its splice variant in glioma cells

U87 cells stably transfected with the full length human heparanase or its splice variant #5, were immunostained with monoclonal anti-heparanase antibody [15] in order to evaluate the subcellular localization of the wild-type enzyme versus its splice variant. As shown in Fig. 4, wild-type heparanase exhibited predominantly a perinuclear, granular pattern, suggesting lysosomal–endosomal localization, as previously described [17]. In contrast, staining of cells transfected with splice variant #5 yielded a diffused cytoplasmic staining (Fig. 4).

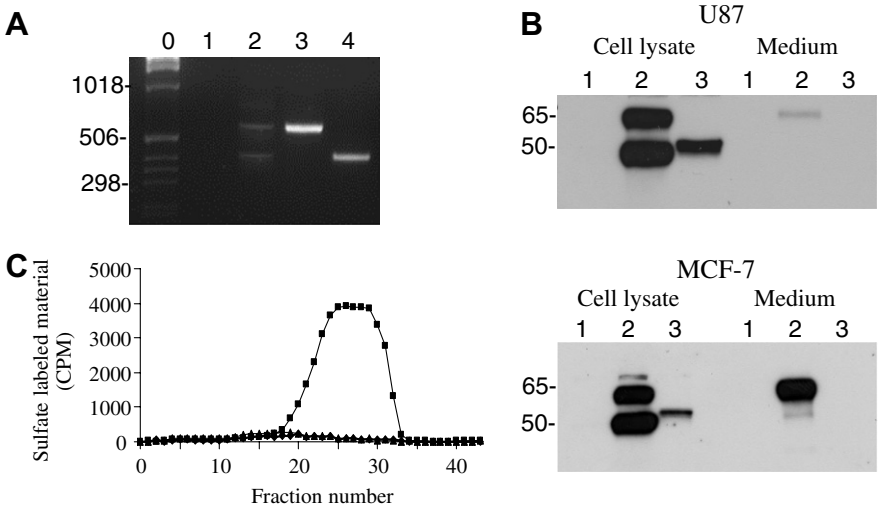

Fig. 3. Cloning and expression of human heparanase splice variant lacking exon 5. (A) Semi-quantitive RT-PCR using primers located around the human heparanase cDNA region encoded by exon 5. Bands of 579 bp represent the wild-type enzyme, while those of 405 bp represent its spliced 5 form. Lane 1, reaction mixture alone; lane 2, cDNA of kidney; lanes 3 and 4, plasmids containing the cDNA sequence corresponding to the wild-type and the splice 5 human heparanases, respectively. Left to the DNA ladder (lane 0) are the respective numbers of base pairs. (B) Western blot analysis utilizing anti-heparanase antibody 1453 on lysates and culture media of U87 and MCF-7 cells transfected with either a mock empty vector (lane 1), or vectors containing the human wild-type (lane 2) or splice 5 (lane 3) heparanases. (C) Heparanase enzymatic activity. Lysates of U87 cells stably transfected with pcDNA3 vectors containing splice 5 (▲) or wild-type (■) heparanases vs. mock, insert free plasmid alone (♦), were incubated (4 h, 37 °C, pH 6.0) with ^{35}S-labeled ECM. Labeled degradation fragments released into the incubation medium were analyzed by gel filtration on Sepharose 6B. Labeled material (fractions 20–30), representing HS degradation products, was obtained only in cells transfected with the wild-type enzyme (■).

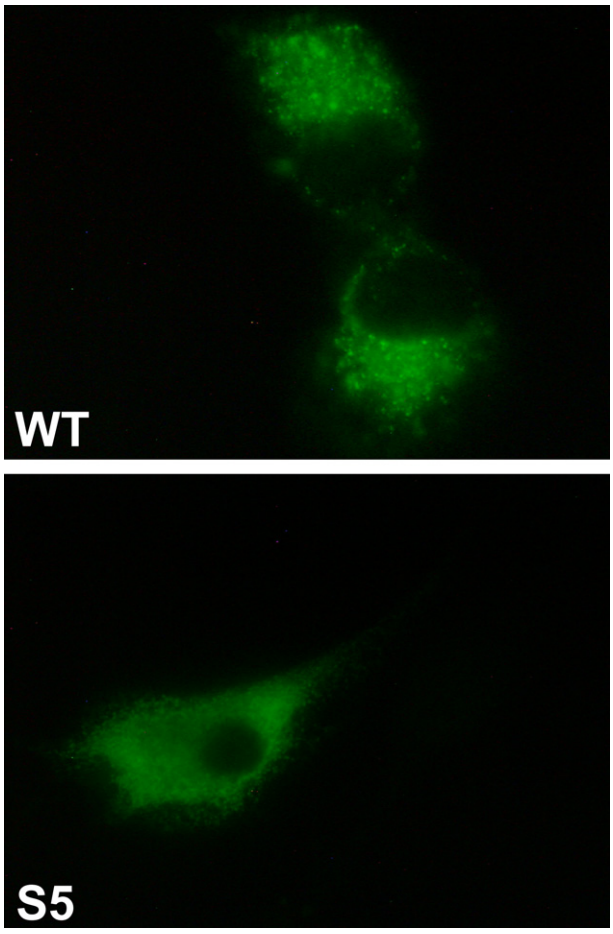

Fig. 4. Localization of human heparanase and its splice variant in glioma cells. U87 cells stably transfected with the full length wild-type (WT) human heparanase or its splice variant #5 (S5), were immunostained with monoclonal anti-heparanase antibody. Note that staining for wild-type heparanase is predominantly of perinuclear, granular pattern, while splice variant #5 exhibits a diffused cytoplasmic staining.

Discussion

The unexpected low number of genes identified in the human and mouse genomes [9,10,18] raise the question of how mammalian organisms could be maintained with 25,000–35,000 genes? Through alternative splicing, the sequence of one gene can yield a variety of RNAs and thus different proteins [9,10,18–27]. On average, each human gene generates three alternatively spliced mRNAs [9,10]. This takes place by several mechanisms including exon skipping, skipping part of an exon, or intron retention [9,24]. Some of the alternatively spliced mRNA's are degraded by nonsense mediated mRNA decay. The distinction of functionally important variants from aberrant splicing remains a challenging issue [27].

The genomic organization of human heparanase and the existence of a splice variant in the untranslated regions of the gene were described by Dong et al. [28]. Recently, we cloned the *Spalax* heparanase and identified several splice variants in its coding region, of which splice variant #7 was described [8]. Alternative splicing in the coding region of human heparanase was not reported.

Here, we identified a splice variant in the coding region of human heparanase (skipping exon 5) which maintained the reading frame of the wild-type gene. Splice variant #5 lacks 174 bp encoding for 58 amino acids, including the active site proton donor (Glu^{225}). Unlike the wild-type enzyme, splice 5 was not detected in the medium of transfected cells, suggesting a defect in its secretion (Fig. 3B). This defect may be due to: (i) conformational change that hinders exit of the spliced enzyme out of the cell, (ii) lack of an epitope in splice 5 that is critical for heparanase recognition by a potential receptor and/or active transporter, and (iii) very efficient reuptake of splice #5 into the cells, leaving undetectable levels of the splice variant in the culture medium.

Whereas, the two proteolytic sites described for processing of the wild-type heparanase [4,11] are conserved in splice #5, processing of the splice variant (i.e., conversion of pro-enzyme into its active form) could not be detected (Fig. 3B). Recently, cathepsin L was implicated in processing and activation of latent heparanase [29]. It is conceivable that the lack of processing of splice #5 is due to a defect in its interaction with cathepsin L, found in lysosomes [12] and the incubation medium [30,31] of some cells. Lack of proteolytic processing may account for the lack of heparanase enzymatic activity in splice 5 (Fig. 3C). Heparanase is implicated in a variety of non-enzymatic functions (i.e., signal transduction, cell adhesion, and survival) [13,32] which may still be conserved in splice 5. These functions are currently being investigated. Several other splice variants of heparanase were identified, resulting in expression of truncated forms (our unpublished results) whose biological significance and function remain to be elucidated.

Acknowledgments

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