Gene Ther Mol Biol Vol 8, 523-538, 2004

Molecular insight into human heparanase and tumour progression

Review Article

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Key words: human heparanase, angiogenesis, tumour progression, metastasis, angiogenic factors, molecular modeling

Abbreviations: basic fibroblast growth factor, (bFGF); chinese hamster ovary, (CHO); complex extracellular matrix, (ECM); connective-tissue-activating peptide III, (CTAP III); endothelial cells, (ECs); glycosaminoglycan, (GAG); heparan sulfate proteoglycans, (HSPGs); heparan sulfate, (HS); human heparanase 1, (Hpa 1); human heparanase 2, (Hpa2); matrix metalloproteinase, (MMP); platelet-derived growth factor, (PDGF); transforming growth factor-β, (TGF-β); tumour necrosis factor-α, (TNF-α); vascular endothelial growth factor, (VEGF)

Received: 13 December 2004; Accepted: 10 January 2005; electronically published: January 2005

Summary

The human heparanase is a key enzyme in tumour vascularisation and metastasis. Here we review the current molecular knowledge on this protein and present a model of its active domain.

I. Introduction

A. Angiogenesis-A key event in tumour progression from its cellular aspects

Angiogenesis or neovascularization is denoted as the process of the formation of new capillaries and vessels from preexisting blood vessels during the development as well as during the maintenance of all organ systems. This is in clear contrast to arteriogenesis or vasculogenesis, which is characterised by the assembly of new vessels from endothelial precursor cells. On the one hand angiogenesis is known to occur in selected physiological processes during the development of the vasculature, e.g. ovulation or wound healing, but on the other hand it also plays a significant role in pathophysiology, for example by mediating the vascularization of tumours. In both cases it is a very tightly regulated and complex cascade of multiple interrelating processes involving endothelial cell activation and migration, proliferation, extracellular proteolysis, multicellular organisation and differentiation including final branching and stabilisation (Nicosia and Madri, 1987; Buschmann and Schaper, 1999; Jain 1999). A delicate balance between positive angiogenic stimuli and endogenous inhibitors (Folkman, 1995) has to exist since observations of new ("*de novo*") blood vessel formation initiated in vivo by a local application of an exogenous angiogenesis factor, showed abnormal rapid involution due to the discontinuation of the angiogenic stimulus (interruption of the exogenous factor) (Liotta et al, 1991; Benett and Stetler-Stevenson, 2001). Thus, the angiogenic response associated with many pathological phenomena (e.g. cancer metastasis, Kaposi`s sarcoma, rheumatoid arthritis, psoriasis) probably involves both the continuous release of potent angiogenic signals, as well as down-regulation or even the removal of natural antiangiogenic effectors.

Angiogenesis takes place in a structurally heterogenous and complex extracellular matrix (ECM) environment and is therefore strongly influenced by the ECM organisation and composition. Remodelling of the extracellular matrix in terms of modulating endothelial and vascular cell behaviours (Kalluri, 2003) is a major prerequisite for the growth (formation) of new blood vessels. This involves an initial breakdown of the subendothelial basement membrane, an amorphous, dense, sheet-like structure, which is 50 to 100 nm thick (Kalluri, 2003), as well as the turn over of the intercellular matrix components during new vessel outgrowth. These modifications, which obviously necessitate a finely controlled interplay of proteinases and proteinase inhibitors, remove physical barriers (e.g. basement membrane, ECM macromolecules) and prepare states that may stimulate endothelial cell migration (Iozzo and San Antonio, 2001; Cleaver and Melton, 2003) **(Figure 1)**.

The series of tissue-cell-matrix interactions of all invasive cell types is generally divided into three phases (Stetler-Stevenson, 1993): (i) modification of cell-cell contacts and establishing new cell-matrix contacts (Sasisekharan et al, 2002; Sanderson, 2001); (ii) proteolytic modification of the ECM that removes barriers,

restructures cell-matrix contacts, and prepares the matrix to facilitate cell movement (Sharma et al, 1998; Iozzo and San Antonio, 2001); (iii) migration of the invasive cell through the proteolysed matrix to establish new matrix contacts (Carmeliet and Jain, 2000). This cycle is repeated until the new blood vessel is fully developed (Seftor et al, 1992; Ray and Stetler-Stevenson, 1994).

B. Proteoglycans-Bridging macromolecules in cell-cell communication and cell-growth

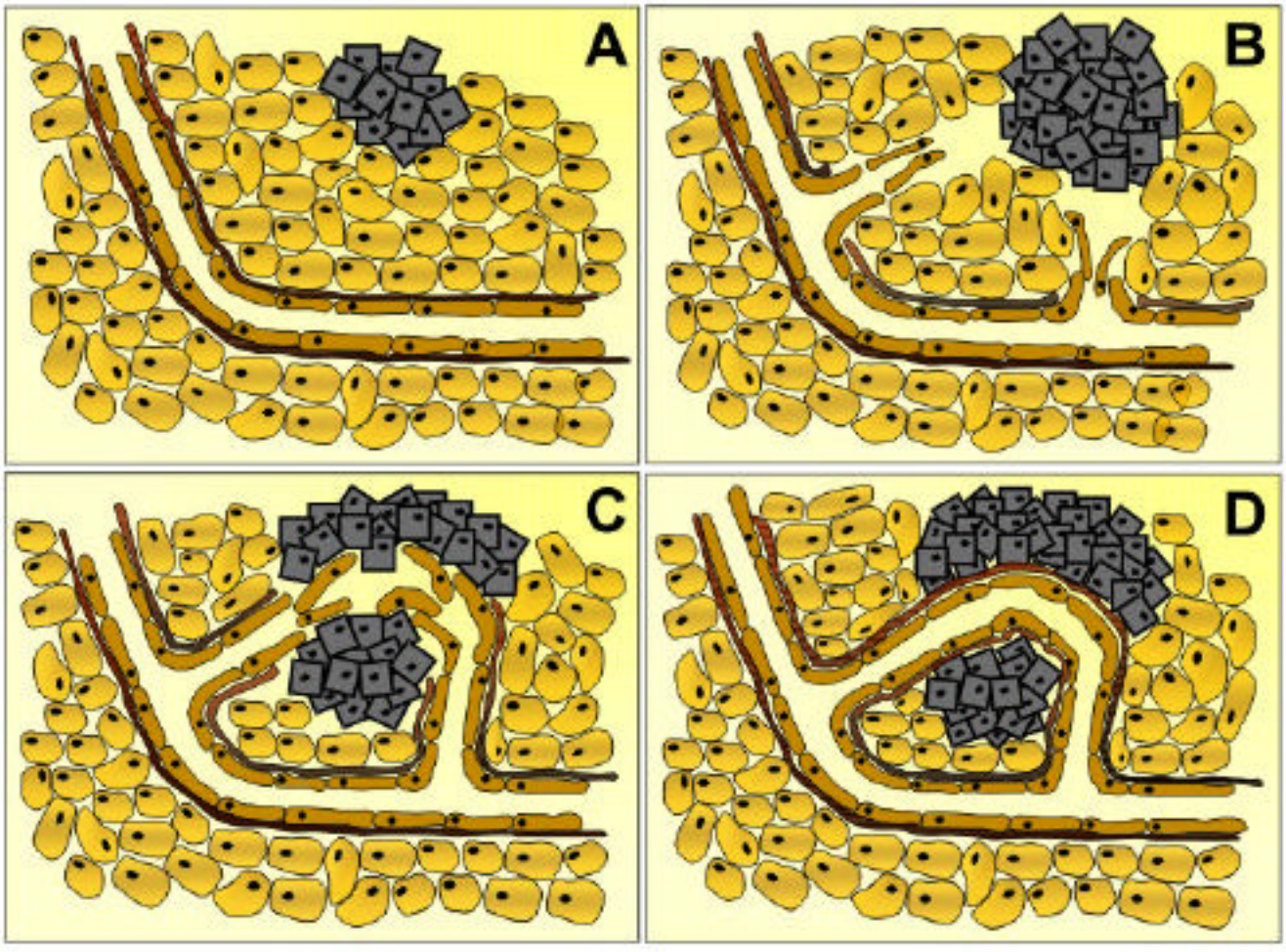
The enormous heterogeneity of the extracellular matrix is probably one of its most important properties and therefore responsible for its functional diversity in relationship to angiogenesis (Mecham, 1998). Some components are designed to be rigid (e.g. collagens), others elastic (e.g. elastin); some wet, others sticky. These diverse modular designs impart diverse roles, yet allow for highly specialized functions. Beside collagen-proteins, which are designed to provide structure and resilience to tissues, and the microfibrillar proteins like elastin and fibrillin, that ensure the structural integrity and function of tissues in which reversible extensibility or deformability are crucial, proteoglycans complete the complexity of the ECM.

Proteoglycans - found in most mammalian cells and tissues - are composed of glycosaminoglycan (GAG) chains covalently linked to a core protein. While the protein part determines the localisation of the proteoglycan in the cell membrane or in the ECM, the GAG component mediates the broad functional interactions with a great variety of ligands (Guimond et al, 1993; Walker and Gallagher, 1996).

GAGs are complex, linear polysaccharides consisting of a disaccharide repeat unit of glucosamine linked to either an iduronic or a glucuronic acid. Further modification of the individual backbone introduces additional structural complexity. The variations can occur at the 2-O position of the uronic acid and the 6-O and 3-O positions of the glucosamine. The N-position can either be sulphated or acetylated but can also stay unmodified (Sasisekharan et al, 2002).

The polysaccharide chains are flexible in a certain way, but cannot fold up into the compact globular structures that polypeptide chains typically form. Moreover, they are highly negatively charged and strongly hydrophilic. Thus GAGs tend to adopt extended conformations that occupy a huge volume and enable cell-cell-interactions over extensive regions inside of tissues.

On the basis of their structural composition GAG chains are classified into different groups, i.e. heparan sulfate (HS)/heparin, keratan sulfate, chondroitin sulfate and some more (Esko and Lindahl, 2001). Most important for angiogenesis are heparan sulfate proteoglycans (HSPGs) as they are predominantly found on cell surfaces and in the ECM. Particular sulfation patterns in their GAG



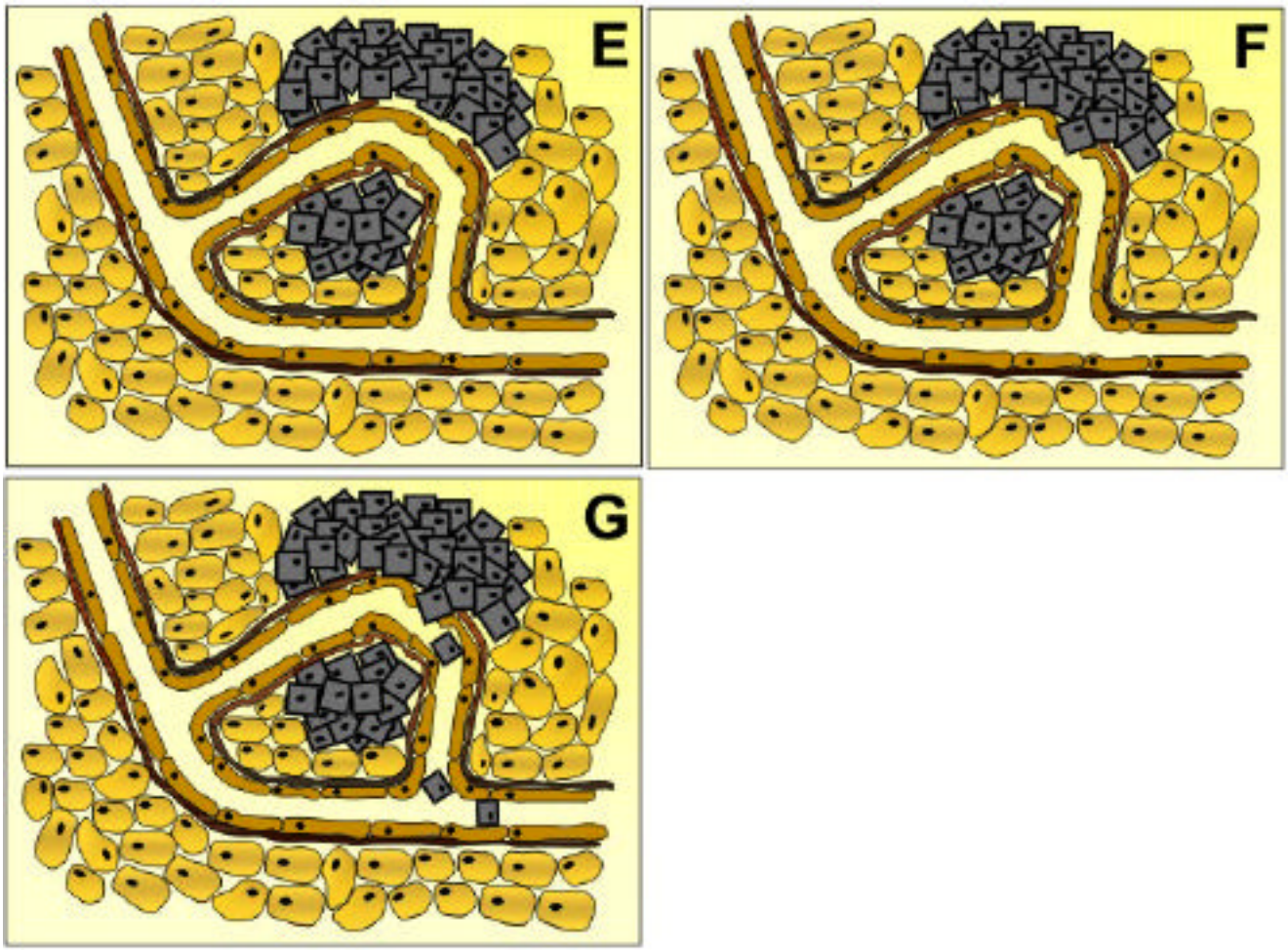


Figure 1. **A rough scheme of angiogenesis (I) and tumour metastasis (II)**
(I) (**A**) Most tumours start growing as nodules provided with nutrients by diffusion processes until they reach a steady-state size of proliferating and apoptosing cells. Massive tumour growth however necessitates the process of angiogenesis. In the first steps, pericytes detach from the vessel, the blood vessel dilates to a limited extent (**B**) before the basement membrane and the extracellular matrix undergo degradative and structural changes as a result of the release of matrix metalloproteinases (MMPs) and growth factors. The growth factors, for example vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and platelet-derived growth factor (PDGF) are released from the basement membrane, but also produced by tumour cells, fibroblasts and immune cells. This induces the endothelial cells to emigrate into the intercellular space towards the angiogenic stimuli triggered by the tumour-nodule. (**C**) The endothelial cells proliferate and start forming the sprout into a new blood vessel, which is guided and supported by pericyte-cells. At the same time new intermediate basement membranes are built up. (**D**) Finally, the endothelial cells adhere tightly to each other to strengthen the new lumen, the intermediate basement membrane matures and pericytes attach again to the blood vessel (not shown in the picture). Thus the new blood vessel is in working order and is integrated in the circulatory system by flooding. The blood-vessel formation will continue as long as the tumour grows and the hypoxic and necrotic areas of the tumour are provided with essential nutrients and oxygen.
(II) (**E**) The metastasis of tumour cells starts with the destruction of heparan sulfate rich basement membrane by the release of heparanase into the microenvironment of the tumour. This is the prerequisite for the tumour cells for invading the underlying extra cellular space towards the endothelial cells. The extracellular matrix is degraded and heparan sulfate chains are presented on cell surfaces. The resulting fragments of the heparan sulfate chains activate growth and motility factors in the surrounding area of the tumour. (**F**) Prior to "invasion", the entry of tumour cells in the blood vessel, the basement membrane has to be fully disrupted and in addition the tight junctions between the endothelial cells have to be loosened. (**G**) Finally the tumour cells pass into the blood vessel and are transported by the blood-system through the whole organism.

chains allow interactions with a series of bioactive molecules, such as growth factors, chemokines, morphogenes, lipoproteins and enzymes. These interactions are mainly driven by electrostatic forces of the HS-sulfate groups with the basic amino acids, like lysine, arginine or histidine of the protein counterpart. Van-der-Waals- and hydrophobic forces may also affect the process of ligand binding to a significant extent (Thompson et al, 1994).

Among the signalling molecules which influence normal and pathologic processes like tissue repair, neurite outgrowth, inflammation and autoimmunity, there are growth factors including fibroblast growth factor 1 and 2 (FGF1 and 2), vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β) and other factors, which have not been identified yet (Sasisekharan and Venkataraman, 2000; Turnbull et al, 2001), that are important for tumour development and angiogenesis.

Binding to HS can modulate a tethered molecule's biological activity or protect it from proteolytic cleavage and inactivation. Due to the multifaceted roles of HSPGs in cell physiology, their cleavage is likely to alter the integrity and functional state of tissues and to provide a mechanism by which cells can respond rapidly to changes in the extracellular environment. It is a fact that cancer cells, as part of the transformation process, do not only alter their HSPGs profile, including differential expression of particular proteoglycan protein-core sequences, as well as alter the heparan sulfate glycosaminoglycan fine structure of given proteoglycans, but also change their protein expression levels. Thus higher amounts of angiogenic differentiation and development factors (Burnfield et al, 1999; Tumova et al, 2000; Esko and Lindahl, 2001) are available and increased enzymatic degradation of such HSPGs plays a crucial role at the beginning stages of angiogenesis.

Invading cells, particularly metastatic tumour cells and leukocytes, traverse ECM barriers and basement membranes by liberating masses of degradative enzymes. A large number of proteases (e.g. matrix metalloproteinases, serine, cysteine and aspartatic protease families) have been described that can disassemble the extracellular matrix (Vlodavsky et al, 1999; Parish et al, 2001). However, for efficient degradation of the extracellular environment, a cooperative action of proteases and HSPG cleaving enzymes is indispensable. The crucial enzyme for HS degradation is the human heparanase.

II. Human heparanase–Molecular biology and structure

A. Genetic organisation of the human heparanase

The cloning of only one single human heparanase cDNA sequence was independently published by several groups, resulting in an identical sequence being obtained from a human placental cDNA library and a human T- cell lymphoma cell line (Hulett et al, 1999; Kussie et al, 1999; Toyoshima and Nakajima, 1999; Vlodavsky et al, 1999). The human gene of the heparanase-enzyme is located on the chromosome 4q21.3, contains 50 kilo base pairs and encircles 14 exons and 13 introns. As a consequence of alternative splicing the gene-information may either be translated as 2,0 kb or as 4,4 kb mRNA (Hulett et al, 1999). While the 50 kb species contains 14 exons and 13 introns, the 1,7 kb form is created with the first and fourteenth exon remaining untranslated. Nonetheless both transcripts contain the same open reading frame, producing the single heparanase enzyme, which is also abbreviated as Hpa1 (Dong et al, 2000; Parish et al, 2001).

Early, rather controversial, developments determined heparanase activity for several proteins ranging from 8 kDa, over 50 kDa up to 134 kDa in molecular mass (Oosta et al, 1982; Freeman and Parish, 1998). There have also been claims that the enzyme is a heat shock protein (Graham et al, 1994) or might even be related to the CXC chemokine, β-thromboglobuline, also known as connective-tissue-activating peptide III (CTAP III) (Hoogewerf et al, 1995). Further findings of a full length rat heparanase cDNA and a partial mouse heparanase (Miao et al 2002) cDNA sequence in combination with reported amino acid sequences in rat and chicken (Goldshmidt et al 2001; Podyma-Inoue et al, 2002; Kizaki et al, 2003) indicate that all these proteins are highly conserved, as confirmed by 80 % identity in the amino acid sequence between the human and murine protein and nearly 93 % identity between mouse and rat sequences (Hulett et al, 1999). The 214 amino acids encoding cDNA fragment of bovine heparanase is to 82 % identical with the human heparanase. Only recently a human cDNA fragment encoding a novel human protein, namely human heparanase 2 (Hpa 2), with significant homology to heparanase was cloned (McKenzie et al, 2000). However, differences in expression profiles, predicted cellular locations and tissue distributions suggest that human heparanase 1 (Hpa 1) and Hpa2 may somehow be related but clearly exhibit distinct biological functions and represent members of two dissimilar mammalian heparanase families (McKenzie et al, 2000). In addition, some more heparanases (C1A, C1B, C2A, C2B) of different molecular weights have been partially purified from chinese hamster ovary cells (CHO cells) and have been preliminarily characterised (Bame et al 1998; Bame, 2001).

Despite the existence of several heparanases the hypothesis of multiple enzymes with similar biological function has never really been established. More likely is the assumption of the Hpa1 enzyme being unique and being the only transcript used by invading cells to degrade heparan sulfate proteoglycans. In summary, its molecular characteristics are described as follows.

The complete human heparanase cDNA contains 1629 bp and encircles an open reading frame that encodes a polypeptide of 543 amino acids with a calculated molecular weight of 61,2 kDa which appears as a ~65 kDa band in the SDS-PAGE analysis.

B. Protein function of the human heparanase

Discussing the features of the heparanase at protein level will give insights into the specific mechanism of its biological function. The hydropathic profile (Vlodavsky et al, 1998) of the heparanase protein indicates a hydrophobic region at the N-terminus (Met^{1} to Ala^{35}) which is assumed to function as signal peptide for secretion. Conversely, the chicken heparanase signal peptide which spans 19 amino acids and which shows only 39 % homology (Goldshmidt et al, 2001) to the human analogue, ensures that the chicken enzyme is readily secreted. These findings suggest that human heparanase is primarily localised in perinuclear acidic endosomal and lysosomal cellular granules before it is secreted/ translocated to the extracellular space (Mollinedo et al, 1997; Bame, 2001; Goldshmidt et al, 2001). The C-terminus is a highly conserved and hydrophobic stretch ranging from Pro^{515} to Ile^{543}. One could argue that this part defines the putative transmembrane domain or a GPI anchor which could be responsible for the enzyme's retention on the cell surfaces (Bartlett et al, 1995; Hulett et

al, 1999; Parish et al, 2001). Further prediction of a hydrophilic region between the amino acids 110 and 170 indicates that this fragment is exposed at the protein's surface and therefore accessible for proteases (Vlodavsky et al, 1999).

The active form of the human heparanase has long been thought to be a 50 kDa polypeptide, isolated and purified from various tissues. However, several attempts to obtain heparanase activity after expression of the 50 kDa subunit in insect cells as well as in mammalian systems (Vlodavsky et al, 1999; McKenzie et al, 2003) failed, suggesting that the N-terminal part including an 8 kDa fragment is important for enzymatic activity. More likely is that the isolated 50 kDa fragment represents a processed form of the native, full-length 65 kDa heparanase (Freeman and Parish, 1998; Toyoshima and Nakajima, 1999) as it always appears together with the 8 kDa peptide when analysing the purified enzyme on a SDS-PAGE gel. This observation supports the hypothesis that the 65 kDa full length protein represents the immature, inactive enzyme, which is subsequently called pro-heparanase (Gln^{36} to Ile^{543}) originating from a pre-pro-form (Met^{1} to Ile^{543}) after removal of the putative signal peptide (Met^{1} to Ala^{35}) **(Figure 2)**. This pro-form undergoes further proteolytic processing which is likely occur within the hydrophilic region at two potential cleavage sites, Glu^{109} to Ser^{110} and Gln^{157} to Lys^{158}, yielding an 8 kDa polypeptide at the N-terminus and a 50 kDa polypeptide at the C-terminus. Subsequent complexation of the two obtained subunits finally forms the active, mature heparanase protein and an intervening 6 kDa linker peptide (Ser^{110} to Gln^{157}) (Fairbanks et al, 1999; Parish et al, 2001; Vlodavsky et al, 2001; Levy-Adam et al, 2003; Nardella et al, 2004). Additionally, the region Glu^{288} – Lys^{417} in the 50 kDa large fragment is believed to facilitate the physical association to the 8 kDa subunit (Levy-Adam et al, 2003).

Assumptions that the active heparanase enzyme is a noncovalently linked heterodimer were confirmed by several cloning- and expression attempts in mammalian systems and insect cells showing that neither the 8 kDa nor the 50 kDa fragment on their own were able to digest substrate (Vlodavsky et al, 1999; McKenzie et al, 2003). Additional attempts to obtain active protein by reconstituting the small and large units after expressing them separately failed, proposing that active folding needs

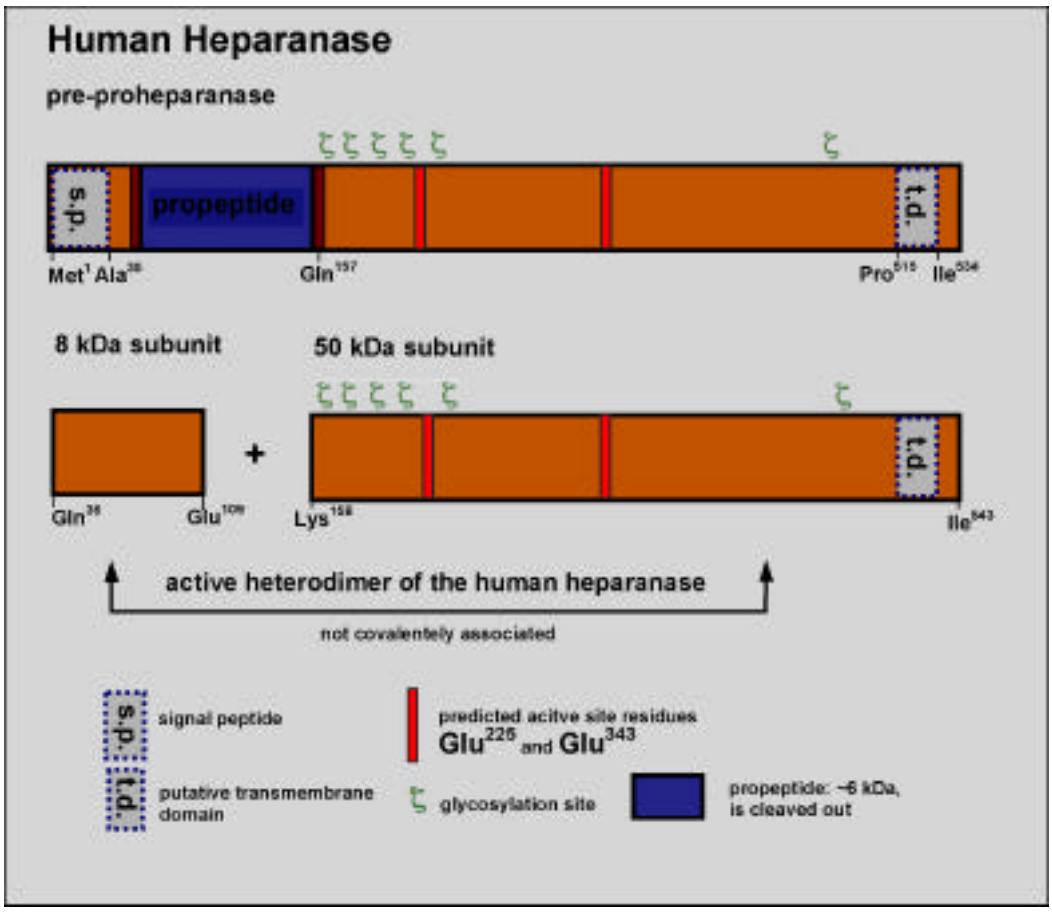


Figure 2. **The processing of the human heparanase.** A scheme of the predicted domain structure of the human heparanase and the processing procedure towards the active form of the enzyme: the non covalent association of the 8 kDa and 50 kDa subunit after the processing of an intervening 6 kDa propeptide. The pre-proheparanase is believed to be translated, first, and is subsequently processed by removal of the propeptide (from amino acid 110 to 157) and by the removal of the signal peptide at the N-terminus of the protein from the residue 1 to 35. Final cleavage results in the 8 kDa subunit from Gln^{36} to Glu^{109} and the 50 kDa subunit from Lys^{158} to Ile^{543}. The six putative N-linked glycosylation sites (N 162, N 178, N 200, N 217, N 238, N 459) are located on the large subunit, from which five cluster in the first 80 amino acids, and the putative catalytic proton donor on Glu^{225} and proton acceptor on Glu^{343}.

a cellular environment. In contrast co-expressed 8 kDa and 50 kDa polypeptides showed high levels of activity. Also notable is the fact that insect expression of the unprocessed 65 kDa precursor form produced little or no active enzyme, respectively, concluding that the mammalian cell facilities are needed to process the human heparanase to its active heterodimer (McKenzie et al, 2003). The involvement of one or even several proteases for this activation-degradation process is highly likely but yet not confirmed as so far they have not been isolated. Deglycosylation of the sugar-residues attached to the six putative N-glycosylation sites, five of which cluster within the first 80 amino acids of the 50 kDa mature protein, had no detectable effect on the enzymatic activity (Vlodavsky et al, 1999). Nevertheless they seem to be responsible for proper translocation and secretion of the enzyme (Simizu et al, 2004).

C. Regulation of the human heparanase expression

Due to the fact that inadvertent cleavage and modeling of heparan sulfate causes potential tissue damage, it is obvious that the expression of the heparanase enzyme has to be tightly regulated. However, very little is known about factors influencing its expression and activity in normal and in malignant cells. Inflammatory cytokines, endothelial cells, leukocytes, tumour necrosis factor α (TNF α) are known to enhance the expression-levels (Bartlett et al, 1995; Parish et al, 1998). Latest experiments showed effects of fatty acids, especially oleic acid on the activation of the Sp1 binding site, which is located 192 to 201 bp upstream from the initial ATG codon (Cheng et al, 2004) of the heparanase coding sequence. In the context of breast cancer, putative estrogen response elements in the regulatory sequence of the heparanase gene were identified. Estrogen- induced mRNA transcription could be demonstrated in estrogen-receptor positive, but not in estrogen-receptor negative breast cancer cells, confirming this finding (Elkin et al, 2003).

D. Putative substrate recognition sites for the human heparanase

Characterising the heparanase interaction with its natural glycosaminoglycan substrates, the human heparanase, which constitutes a β-endoglucuronidase, cleaves glycosidic bonds with a hydrolase mechanism and is thus distinct from bacterial heparinases which depolymerise heparin and heparan sulfate by eliminative cleavage generating unsaturated bonds. Secondary structure predictions suggest that the heparanase enzyme consists of a $(\alpha/\beta)_8$-TIM-barrel architecture. This fold is frequently observed in glycosylases and is also proposed for this protein. As the 50 kDa subunit on its own forms 6 α/β units, the missing structural elements have to be completed by the 8 kDa polypeptide, showing a predicted secondary structure of a β/α/β element (Nardella et al, 2004), thereby generating the native fold. The heparanase exhibits the common catalytic mechanism typical for the family of glycosylhydrolases, involving two conserved amino acid residues, the putative proton donor Glu^{225} and the putative proton acceptor (nucleophile) Glu^{343}. Conserved basic residues are found in proximity to the proposed catalytic proton donor (KK residues 231 and 232) and nucleophile (KK residues 337 and 338) responsible for additional, adhesive interactions with GAGs, i.e. HS (Hulett et al, 2000).

The heparan sulfate glycosaminoglycans are cleaved by the enzyme at only a few sites, creating fragments of 10 to 20 sugar units. This observation confirms the thesis that the heparanase enzyme recognizes particular and quite rare heparan sulfate motifs (Freeman and Parish, 1998; Pikas et al, 1998). On the one hand, it has been shown that a 6-O-sulfate group on a glucosamine residue, located two monosaccharide units away from the cleavage site at its non-reducing end, and a 2-sulfated glucosamine-structure on the reducing side are essential for substrate recognition **(Figure 3)**. Substrate cleavage, on the other hand, was found to require a hexuronic carboxyl group (Bai et al, 1997; Vlodavsky and Friedmann, 2001; Okada et al, 2002) and heparan sulfate comprising unsubstituted glucosamine residues is not processed (Parish et al, 1999; Dempsey et al, 2000). Structurally related heparin, however, has a high inhibitory effect on the enzyme's activity (Bar-Ner et al, 1987; Vlodavsky et al, 1994) due to the predominant existence of the $[IdoUA(2\text{-}OSO_3)\text{-}GlcNSO_3(6\text{-}OSO_3)\text{-}]_n$ repeat structure. In contrast to the proposed endolytic cleavage mechanism, it has recently been postulated that human heparanase can also cleave defined oligosaccharide structures in an exolytic action (Gong et al, 2003). Thus the precise localisation of sulfation patterns and the sequences of heparan sulfate residues required for recognition as well as for subsequent cleavage are yet uncertain.

IV. Human heparanase – Involvement in physiological and patho-physiological processes and its inhibition

A. Physiology

Most studies particularly underline the involvement of the heparanase enzyme in pathophysiology with a strong leaning towards cancer. Although only little is known about the enzyme`s contribution to normal cell and tissue function it is strongly suggested that the heparanase plays a crucial role in embryo implantation, which involves invasive cell immigration and interaction between HS-binding proteins (i.e. growth factors) and heparan sulfate proteoglycans in order to ensure normal development (Selleck, 1999; Dempsey et al, 2000; Reiland et al, 2004). In many facets the embryonic cell migration, proliferation and differentiation is similar to its involvement in tumour metastasis, angiogenesis and inflammation. There have been lines of transgenic mice generated which ubiquitously overexpress the human heparanase (Zcharia et al, 2004). Biochemical analysis of isolated heparan sulfate oligosaccharides of transgenic mice revealed a decrease in the size of the HS chains compared to HS from control mice. This is interpreted as an enhanced heparanase cleavage activity in almost every tissue (Zcharia et al, 2004).

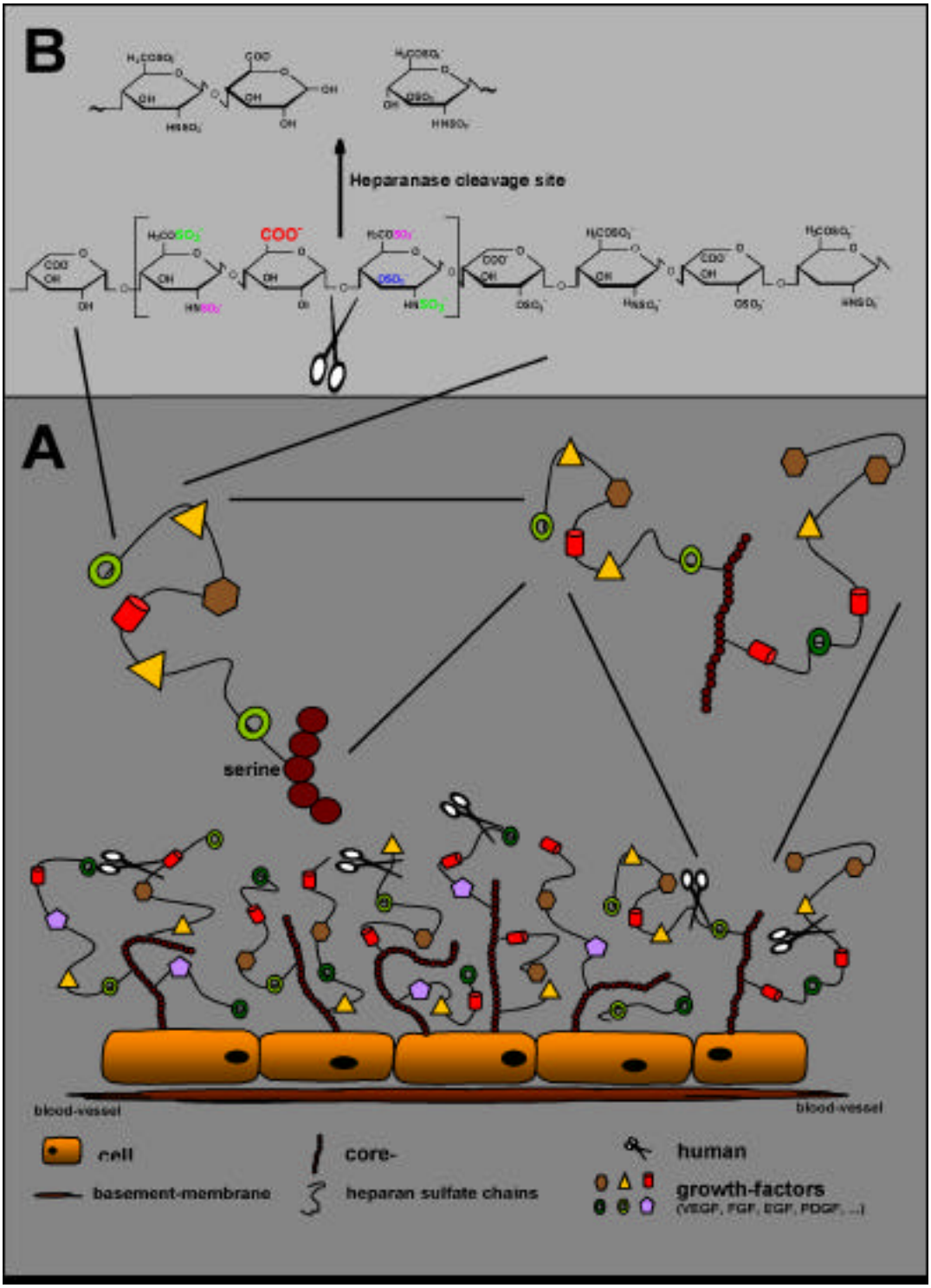


Figure 3. **Substrate of the human heparanase**

(A) The lower part of this figure encircles schematically the localisation and role of heparan sulfate proteoglycans in vascularisation and metastasis. HSPGs are predominantly found on cell surfaces and everywhere in the extracellular-matrix. With their core-proteins they are anchored in the cell membrane. The number and composition of the covalently linked heparan sulfate chains varies enormously depending on the tissue and cell functions, respectively, and the state of cell differentiation - different heparan sulfate expression patterns. Particular the sulfation patterns in the carbohydrate-chains allow interactions with a series of bioactive molecules (e.g. growth factors). Heparanase secreted by the surrounding tumour cells is responsible for the degradation of this heparan sulfate chains and for the release of tethered molecules by cleaving at certain sites. **(B)** The upper part of this figure summerizes the structural characteristics of the minimal human heparanase cleavage site, known so far. It is composed of the trisaccharide sequence between the brackets and occurs quite rarely in HS chains. The arrow indicates the glucuronidic bond cleaved by the heparanase. Highly sulfated structures seem to be essential for the enzymes activity. The N-sulfation on the reducing side, the 6-O-sulfation of the glucosamine on the non reducing side, both indicated in green, and the carboxy-group (hold in red font) on the glucuronic-acid seem to be essential for the enzyme's activity. The additional 2-N-sulfate group on the nonreducing GlcN and the 6-O-sulfate group on the reducing GlcN both have a promoting effect on the heparanase activity (magenta colored). The function of the 3-O-sulfate group in blue remains controversial. In principle it is thought to inhibit the enzyme, but it may also have a promoting effect due to its negative charge.

Although the mice appeared normal, fertile, and exhibited a normal life span, pregnant transgenic mice had a significant increase in the number of implanted embryos compared with control mice. There also was a higher miscarriage and embryonic lethality confirming the necessity of normal HS structures during embrionic growth and morphogenesis (Lin et al, 2000; Zcharia et al, 2004). Because of less intact HS, thereby also affecting the basement membrane structure, the kidney function was insufficient. Elevated levels of proteins were found in the urine, indicating that heparanase is able to disrupt the filtration barrier as well as to have an impact on the reabsorption function of the kidneys (Weinstein et al, 1992; Levidiotis et al, 2001; Katz et al, 2002; Zcharia et al, 2004). The mammary glands of virgin transgenic mice showed similar development and maturation to the ones of normal mice at day 12 of pregnancy. An even more pronounced development was observed when those transgenic mice became pregnant, most probably due to heparanase-induced overbranching, hyperplasia and widening of ducts (Zcharia et al, 2004). Furthermore, the heparanase overexpressing mice showed accelerated hair growth by enhancing the vascularization and maturation of the hair follicle (Yano et al, 2001; Zcharia et al, 2004).

Upon aging the expression levels of the heparanase become negligibly low. In an adult organism the enzyme only appears during wound repair (Vlodavsky and Friedmann, 2001), fracture repair (Saijo et al, 2003), tissue regeneration (Dempsey et al, 2000), and immune surveillance (Zcharia et al, 2001). In addition, following vascular injury, it is believed that heparanase, probably secreted from infiltrating and extravasating leukocytes, degrades heparan sulfate to induce smooth muscle cell proliferation, which is normally inhibited by the intact HS chains (Campbell et al, 1992; Francis et al 2003).

B. Pathophysiology

With regard to the specific structural interaction of HSPGs with various extracellular matrix and basement membrane macromolecules, they play a key role in self-assembly, modeling and insolubility of ECM components, as well as cell adhesion, harvesting and locomotion (Kjellen and Lindahl, 1991; Iozzo, 1998; Vlodavsky et al 1999). Cleavage of the HS sidechains by HS-degrading enzymes, such as heparanase, therefore results in disassembly of the extracellular matrix and in the permeability of the underlying basement membranes, occurring as a crucial triggering process in the extravasation of blood borne cells (Parish et al, 1987; Vlodavsky et al, 1992, 1994; Nakajima et al, 1988; Parish et al, 1999). Immunohistochemical investigations revealed that the heparanase enzyme appeared primarily in neutrophils, macrophages, platelets, keratinocytes, capillary endothelium and neurons, but very seldom in normal epithelia. While the expression levels of the enzyme in normal tissues are very low and its incidence is restricted to the placenta and to lymphoid organs, elevated levels of heparanase were observed in tumour bearing animals and cancer patients suffering for example from bladder cancer (Gohji et al, 2001), colon cancer (Friedmann et al, 2000), gastric cancer (Tang et al, 2002), breast cancer (Maxhimer et al, 2002), oral cancer, oesophageal cancer (Ikuta et al, 2001), pancreatic cancer (Koliopanos et al, 2001; Kim et al, 2002; Rohloff et al, 2002), brain cancer (Marchetti et al, 2001), endometrial cancer (Watanabe et al, 2003) and acute myeloid leukemia (Bitan et al, 2002; Vlodavsky et al, 2002; Sanderson et al, 2004). Moreover, the expression of the human heparanase correlates with the metastatic potential of human tumour cells. Since this is also the case for other extracellular matrix degrading enzymes, i.e. for the group of matrix metalloproteinases (MMPs) - the feature of inducing angiogenesis in addition to the initiation of cell invasion becomes more obvious (Hulett et al, 1999; Parish et al, 2001; Goldshmidt et al, 2002; Takaoka et al, 2003; Watanabe et al, 2003). Together with the degradation of ECM components (collagens, laminins, fibronectin, vitronectin etc) a continuous cleavage of heparan sulfate proteoglycans by the human heparanase is fulfilled. This process increases the bioavailability and activity of growth factors and other bioactive molecules, which have been tethered and inactivated by binding to heparan sulfate structures, and promotes the migration and proliferation of endothelial cells (ECs) to form vascular sprouts. Heparanase has also been implicated in the degradation of subendothelial basement membrane by leucocytes (Naparstek et al, 1984; Parish et al, 2001). During chronic inflammation leukocytes enter and accumulate in inflammatory areas by the continous extravasation through the blood vessel wall. Several in vitro studies have confirmed this assumption (Vlodavsky et al, 1992; Bartlett et al, 1995; Parish et al, 1998; Hulett et al, 1999). Both tumour masses and inflammatory sites provide the acidic environment which human heparanase requires for degradation of heparan sulfate structures. The enzyme has its maximal endoglycosidase activity between pH 5,0 and 6,0 (more precisely from pH 5,6 to 5,8) and is inactivated at pH greater than 8,0. Under physiological conditions (pH 7,4) heparanase binds heparan sulfate but does not degrade its substrate. This is in accordance with the findings that inactive recombinant heparanase enzyme still binds to HS molecules without subsequent degradation and therefore enables the adhesion of cells (Goldshmidt et al, 2003).

C. Inhibition

The knowledge of heparanase biological function collected so far, is sufficient for its consideration as a promising target for cancer therapy. Great efforts are being made in order to develop a potent inhibitor, and sulfated polysaccharides, like heparin, dextran sulfate, xylan sulfate, fucoidan, carageenan-gamma and laminaran sulfate, which have primarily anticoagulant activity, are already known to be effective inhibitors of tumour metastasis. Their inhibitory effect in experimental metastasis is more related to their ability to inhibit the heparanase enzyme than to their anticoagulant properties (Parish et al, 1987; Nakajima et al, 1988; Vlodavsky et al, 1994; Miao et al, 1999; Parish et al, 1999).

In addition, other polyanionic molecules, such as phosphomannopentaose sulfate (PI-88) and maltohexaose sulfate, have proven to be as potent as heparin concerning the inhibition of heparanase activity (IC_{50} ~ 1 – 2 μg/ml),

confirming the assumption that the oligosaccharide chain length and the degree of sulfation are more important than sugar composition and type of linkage (N- or O-sulfated) (Vlodavsky et al, 1994, 2001; Lapierre et al, 1996; Parish et al, 1999). PI-88 has successfully passed through the preclinical studies as its application confirmed the inhibition of tumour growth and tumour angiogenesis. It is currently being tested on cancer patients in a Phase II clinical trial (Parish et al 1999).

Furthermore, in a parallel study, by screening 10000 culture broths of microorganisms (actinomycetes, fungi and bacteria), a specific actinomycete-strain (RK99-A234) emerged for the reason of compensating heparanase enzyme activity. The responsible neutralizing interaction partner was identified as RK-682 (IC_{50} ~ 17μM), already known to inhibit protein tyrosine phosphatases (Hamaguchi et al, 1995; Ishida et al, 2004). Using RK-682 as a lead compound structure a more selective heparanase inhibitor was designed, namely 4-Benzyl-RK-682 (Ishida et al, 2004).

Finally, Suramin (IC_{50} < 10μM in vitro), a polysulfonated naphthylurea, and Trachyspic acid (IC_{50} ~ 50μM), isolated from Talaromyces trachyspermus, have to be mentioned in order to complete the short enumeration of potent heparanase inhibitors known so far. The inhibitory mechanisms of both are as yet unknown, although the structure of Trachyspic acid is similar to 4-Benzyl-RK-682 concluding that both of the substances bind and block as mimic substrates the heparanase's cleavage site (Nakajima et al, 1991; Shiozawa et al, 1995; Hirai et al, 2002).

IV. Biophysical remarks and conclusions

A. Development of heparanase activity assays described in the literature

Although the human heparanase and its role in regulating proteoglycan function in angiogenesis have been known for many years, the enzyme as a potential pharmaceutical target has not yet received as much attention as other angiogenetic factors, like for example FGF and VEGF. The main starting point for interfering in non physiological angiogenic processes has so far been the synthesis of specific heparan sulfate glycosaminoglycan sequences with high affinity for growth factor binding, thereby silencing downstream signalling in angiogenesis.

One reason for the lack of interest in designing a heparanase inhibitor is mainly determined by initial major difficulties in the establishment of an assay that could easily monitor enzyme activity by following the degradation of its HS substrate, unlike bacterial lyases which cleave HS- or heparin chains by an elimination mechanism, a reaction that can be detected spectroscopically as unsaturated products are generated (Linhardt et al, 1986). The human as well as the mammalian heparanases in general constitute hydrolases cleaving without double bond formation. Thus more sophisticated assays for activity determination had to be developed in order to facilitate heparanase purification from tissues for subsequent protein characterisation. Besides, the definition of the heparanase specific substrate turned out to be difficult, because most of the HS molecules purified from diverse tissues have already been cleaved and the unprocessed oligosaccharide chain could not be reconstructed. In some cases heparin has been used, but because it is highly modified, it does not fullfill the domain structure of heparan sulfate (Oosta et al, 1982; Lyon and Gallagher, 1998). Despite difficulties in the beginning, major progress in the development of different heparanase activity assays could be reported in the last few years.

Radioactive- (^{35}S) and fluorescence- (FITC) labeled HS substrates were used to detect the size-shift of the degraded glycosaminoglycan to smaller fragments upon incubation with active heparanase. The thus obtained cleavage products were finally analysed by gel filtration chromatography (Toyoshima and Nakajima, 1999; Vlodavsky et al, 1999). These assays are highly sensitive but not suitable for screening large amounts of substrate samples. In addition the handling of the single separated steps of the reaction procedure as well as the detection were quite laborious but nevertheless were accepted. When it came to attempts to searching for an inhibitor, however, a simple and efficient heparanase assay method became absolutely indispensable in order to guarantee a promising high throughput analysis. This approach became possible by forming polyacrylamide tablets containing defined amounts of HS stained with Alcian blue. The colour density of the tablets correlates with the HS concentration and is quantified. After adding active heparanase, degraded fragments are excluded out of the tablets which results in a decrease of the colour density, a process which can easily be visually detected (Ishida et al, 2004). Another assay method is described on the principle of ultrafiltration. Based on limited cleavage sites of HS chains, the degradation of radioactively labeled glycosaminoglycans with approximately 30 kDa molecular size results in products ranging from 7 to 10 kDa is exploited. A subsequent separation of the cleaved fragments is performed by using a special molecular weight cutoff, which exhibits minimal permeability to the undigested HS, while permitting maximum permeability to the digested products which can then be detected by radioactive scintillation counting (Nakajima et al, 1988; Tsuchida et al 2004).

Several reasons retarded the research progress for the human heparanase, among which the already well characterised growth factors with a great potential for interference in angiogenesis and the lack of a powerful enzymatic assay played a decisive role. In addition, the protein is not very abundant in vivo and therefore getting enough material to purify adequate amounts of enzyme for characterisation is still a challenge. Beside the very small amounts of protein, another difficulty is related to the unstable nature of the heparanase. Several attempts to purify human heparanase or heparanase subunits from diverse tissues to homogeneity resulted in the loss of enzymatic activity. The cloning of the full length cDNA and of the two subunits in diverse expression systems (mammalian cells or insect cells) is reported by various groups (Hulett et al, 1999; Vlodavsky et al, 1999; Elkin et

al, 2001; Nardella et al, 2004) and delivers sufficient amounts of active heparanase but exact details concerning the nature of the active complex, formed by the respective subunits after posttranslational processing are so far unknown.

B. Novel aspects in the molecular characterisation of the human heparanase

The design of specific inhibitors seems to make only slow progress. With the exception of PI-88 which is currently being tested in phase II clinical trials in Australia, there are no other heparanase inhibitors in clinical studies to our knowledge. Furthermore, a multitude of existing inhibiting molecules, like suramin, are not suitable for in vivo experiments because of severe side effects and toxicity (Parish et al, 2001). Added to this, the mechanisms of inhibition are yet unknown or cannot precisely be correlated with heparanase action, as for example PI-88 also shows high binding affinities to growth factors, like FGF-1, FGF-2 and VEGF (Cochran et al, 2003).

In the literature, no paper describes a structure based design of heparanase inhibitors and the protein itself remains a structurally uncharacterised enzyme. In addition nearly no published article investigates the structure of human heparanase or its biophysical properties. Encouraged by this gap in heparanase characterisation and in order to provide information for structure based drug design, a method which has been successfully applied for other proteins, we decided to produce this enzyme in our labs using a different cloning strategy to that reported so far. We cloned the respective subunits, 8 kDa and the 50 kDa, known to form the active heterodimer, separately. Rather laborious efforts were made to purify the two fragments after several purification attempts had failed. But finally, the activity of the enzyme could be tested, as mentioned above, by analysing degraded FITC labeled heparan sulfate. Neither the 8 kDa fragment nor the 50 kDa polypeptide on their own showed cleavage activity when being incubated with HS. But the reconstitution of both subunits in crude lysates led to an active recombinant human heparanase, which can now be exactly characterised with regard to its biophysical properties.

For the further development of structure and substrate specific inhibitors it is absolutely essential to resolve the 3 dimensional structure of the active heterodimer with and without ligands. As this project may turn out to be time consuming regarding the size and the difficult handling of the protein we will further support this aim of structure determination by performing biophysical techniques to study the molecular properties of the human heparanase.

In addition we have performed a more theoretical approach to mimic the possible structure of the human heparanase in vivo, namely the molecular modeling. BLAST (NCBI tools) searches with the full length amino acid sequence of the human protein resulted in a few significant sequences. The mouse-, rat- and chicken-heparanase show high similarity to the human sequence. Less similar to the query are two putative proteins from Arabidopsis thaliana, with as yet unknown functions. Some further proteins of glycosyl hydrolases from bacteria have been found, but these similarities seem to be rather unreliable.

Although secondary predictions (Hulett et al, 2000; Nardella et al, 2004; and our findings) result in an alternating α/β series, similar to the (α/β) TIM barrel protein fold, “swiss-model”, or “SDSC1” - programmes on the expasy homepage for protein structure homology modeling - and some others could not design a three dimensional structure of the human protein (“sorry, no suitable template for modeling could be found”).

With the knowledge of the identified active residues (E^{225} and E^{343}) and putative basic amino acids near to these sites we started to calculate a possible structure for the human heparanase **(Figure 4)**.

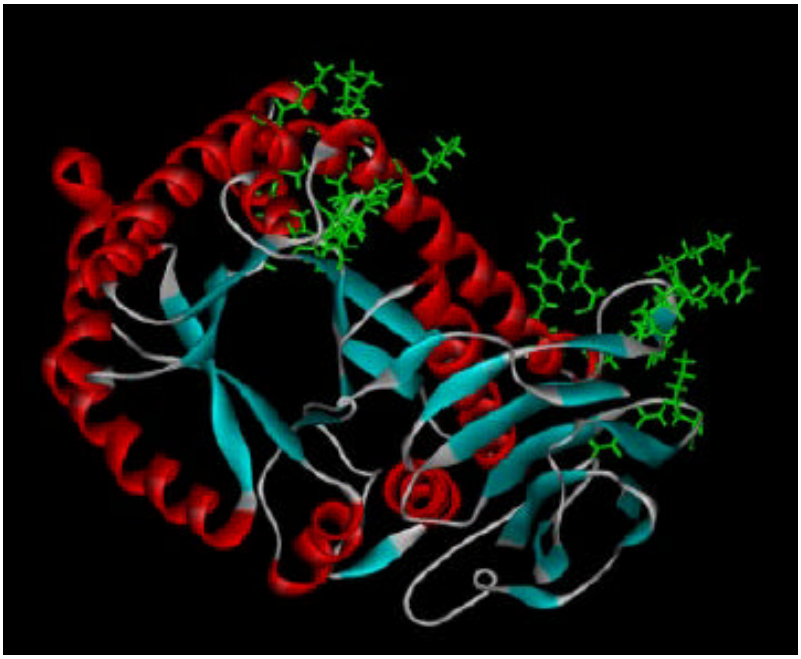


Figure 4. Molecular Model of the human heparanase
Molecular modeling of the 50 kDa subunit of the human heparanase (amino acid residue 158 to 514) without its putative transmembrane domain. Functionally important amino acids are indicated in green: the two acitve sites Glu^{225} and Glu^{343}, positive loaded amino acids near to the proton donor and acceptor, that can bind heparan sulfate, Lys^{231}, Lys^{232} and Lys^{337}, Lys^{338}. Sectors of basic amino acid residues from Gln^{157} to Asn^{162} and Pro^{271} to Met^{278} may also interact in the substrate binding towards the enzymatic active site.

The model should facilitate and illustrate - together with the biophysical characterisation - investigations on intramolecular and molecular effects and interactions of the human heparanase with its surrounding environment.

C. Future perspectives

Even if the three dimensional structure of the protein heterodimer is established in the near future by X-ray diffraction or NMR-methods, the biophysical techniques particularly allow studying exactly the dynamics and conformational effects of the human heparanase in context with ligands. With the resulting findings it would be possible to understand better the affinities and activities of this enzyme in its immediate environment, to search for natural compounds which inhibit more efficiently and to design a specific, competitive heparanase-action-inhibitor to establish a new promising cancer therapy.

Acknowledgments

This work was supported by OENB Jubiläumsfondsprojekt No 10855 and by the Austrian Science Fund project No P15969.

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