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Mini review

The six hyaluronidase-like genes in the human and mouse genomes

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Abstract

The human genome contains six hyaluronidase-like genes. Three genes (*HYAL1*, *HYAL2* and *HYAL3*) are clustered on chromosome 3p21.3, and another two genes (*HYAL4* and *PH-20 / SPAM1*) and one expressed pseudogene (*HYALP1*) are similarly clustered on chromosome 7q31.3. The extensive homology between the different hyaluronidase genes suggests ancient gene duplication, followed by en masse block duplication, events that occurred before the emergence of modern mammals. Very recently we have found that the mouse genome also has six hyaluronidase-like genes that are also grouped into two clusters of three, in regions syntenic with the human genome. Surprisingly, the mouse ortholog of *HYALP1* does not contain any mutations, and unlike its human counterpart may actually encode an active enzyme. Hyal-1 is the only hyaluronidase in mammalian plasma and urine, and is also found at high levels in major organs such as liver, kidney, spleen, and heart. A model is proposed suggesting that Hyal-2 and Hyal-1 are the major mammalian hyaluronidases in somatic tissues, and that they act in concert to degrade high molecular weight hyaluronan to the tetrasaccharide. Twenty-kDa hyaluronan fragments are generated at the cell surface in unique endocytic vesicles resulting from digestion by the glycosylphosphatidylinositol-anchored Hyal-2, transported intracellularly by an unknown process, and then further digested by Hyal-1. The two β-exoglycosidases, β-glucuronidase and β-*N*-acetyl glucosaminidase, remove sugars from reducing termini of hyaluronan oligomers, and supplement the hyaluronidases in the catabolism of hyaluronan. © 2001 Elsevier Science B.V./International Society of Matrix Biology. All rights reserved.

Keywords: Hyaluronan; Lysosomal enzyme; Mucopolysacharidosis IX; Tumor suppressor gene

1. Introduction

The hyaluronidases from vertebrate somatic tissues, despite their importance, have until now defied explication. They are difficult to purify, present at exceedingly low concentrations, and have very high but unstable specific activities in the absence of detergents and protease inhibitors. The recent purification of Hyal-1 (Frost et al., 1997), the first somatic hyaluronidase to be isolated, and the explosion of information that resulted from the human genome project, facilitated rapid accumulation of new knowledge. It is now recognized that the hyaluronidases are a family of enzymes. There are six hyaluronidase-like sequences in the human genome (Csoka et al., 1999), clustered in groups of three at two chromosomal sites, on chromosomes 3p21.3 (*HYAL1*, *HYAL2* and *HYAL3*) and 7q31.3 (*HYAL4*, *PH20 / SPAM1* and *HYALP1*). *HYALP1* is an expressed pseudogene in humans. A disorder resulting from a mutation in *HYAL1* has recently been identi-

Abbreviations: CS, chondroitin sulfate; ECM, extracellular matrix; EST, expressed sequence tag; GPI-, glycosylphosphatidyl-inositol-; *HYAL1,2,3,4,* the genes for the hyaluronidases, Hyal-1,-2,-3,-4; *HYALP1*, a hyaluronidase-like sequence that is an expressed pseudogene in the human; HA, hyaluronan, hyaluronic acid; *LUCA-1*, Lung Cancer-1, a putative tumor suppressor gene site which matches the location of Hyal-1; MMPs, matrix metalloproteinases; MPS IX, mucopolysaccharidosis IX; *PH-20 / SPAM1,* the gene for the sperm-specific enzyme PH-20, or sperm adhesion molecule 1; TIMPs, tissue inhibitors of MMPs

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fied and termed Mucopolysaccharidosis IX (Natowicz et al., 1996).

2. Hyal-1, plasma hyaluronidase

The acid-active hyaluronidase in serum had been recognized for decades (De Salegui and Pigman, 1967) but has defied isolation until recently. With the development of rapid new assays (Stern and Stern, 1992; Guntenhoener et al., 1992; Frost et al., 1997), and the recognition that the activity was unstable in the absence of detergents, the serum enzyme was finally isolated. It was the first hyaluronidase to be purified to homogeneity from mammalian somatic tissues. It was then cloned, sequenced, and expressed (Frost et al., 1997). The 57-kDa protein is a single polypeptide chain of 49 kDa with approximately 8 kDa of post-translational glycosylation. It is approximately 40% identical to the enzyme found in sperm, PH-20. The highest levels of mRNA of *HYAL1* are found in the major parenchymal organs such as liver, kidney, spleen, and heart. The mouse ortholog was also cloned and expressed, (Csoka et al., 1997) and observed to be 73% identical to the human sequence.

Urine, long known to contain high levels of hyaluronidase activity (Ginetzinsky, 1958), was observed to contain Hyal-1 at 100 times the specific activity of that found in plasma. An additional second activity with a molecular weight of 45 kDa is found in urine, and by amino acid sequencing was determined to be Hyal-1 with approximately 100 amino acids deleted from the carboxy region (Fig. 1), resulting in two polypeptide chains bound by disulfide linkages (Csoka et al., 1998). This does not represent a zymogen/processed enzyme relationship, since the two forms of the enzyme have similar specific activities. The two isozymes of Hyal-1 are also found in cultured cells, with the higher molecular weight isozyme predominant in the culture medium, and the shorter processed isozyme predominant in the cell layer. There are also two forms of PH-20 (Cherr et al., 1996; Meyer et al., 1997), the higher molecular weight form being a glycosylphosphatidyl-inositol- (GPI-)linked protein attached to the plasma membrane.

3. Genomic arrangement of the six hyaluronidase paralogs

The sequence of the gene for human Hyal-1 facilitated a screen of expressed sequence tag (EST) databases. This analysis revealed that the human genome contains six paralogous hyaluronidase-like se-

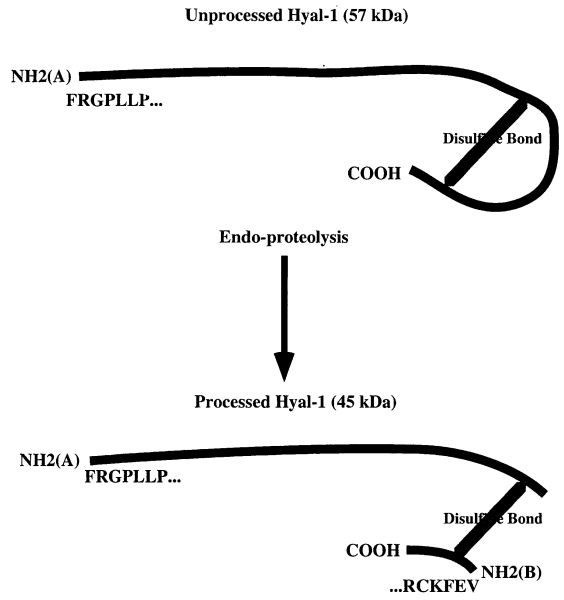

Fig. 1. Diagrammatic representation of the putative endoproteolytic processing of Hyal-1. Unprocessed Hyal-1 is shown in the upper figure. After endoproteolytic processing, two fragments are generated that produce two separate N-termini. The 22-amino acid-fragment is presumably linked to the rest of the protein by disulfide bonds (courtesy of Glycoforum's 'Hyaluronan Today,' of Seikagaku Corp., http://www.glycoforum.gr.jp/, see: http://www.glycoforum.gr.jp/science/hyaluronan/HA15/HA15E.html).

quences with approximately 40% identity to each other. They are grouped into two tightly-linked triplets on human chromosomes 3p21.3 and 7q31.3 (Fig. 2), an arrangement that probably arose from two gene duplication events followed by cluster-block duplication at some time before the emergence of modern mammals.

The three hyaluronidase sequences on chromosome 3 have similar genomic structures (Fig. 3). *HYAL1* has an additional retained intron within exon 1 not present in the other hyaluronidase sequences (Fig. 3). The three hyaluronidase genes clustered on chromosome 7 have much larger introns than those on chromosome 3, and contain an additional exon (Fig. 3).

The orthologous mouse genes are found on syntenic regions, on mouse chromosomes 9 F1–F2 (Csoka et al., 1998) and 6 A2 (unpublished data). The degree of homology between the pairs of orthologs of human and mouse is much greater than that between the six human paralogs. Therefore the divergence between the paralogs must have occurred long before the divergence of human and mouse, approximately 80 million years ago. The genome of *Caenorhabditis elegans* contains only one hyaluronidase-like sequence, but no such sequence is found in *Drosophila melanogaster*.

Another widely expressed and important human

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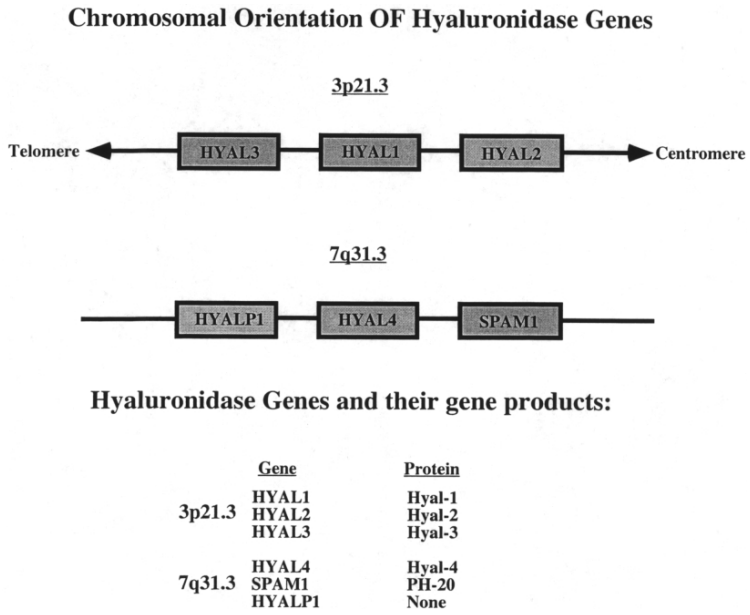

Fig. 2. Demonstration of the chromosomal orientation of the six hyaluronidase genes at their two respective chromosomal sites, and tabulation of their gene products. The relative gene order has been established for the genes on chromosome 7, but their orientation in relation to the centromere and telomere has not yet been determined. This figure is not drawn to scale. (Courtesy of Glycoforum's 'Hyaluronan Today,' of Seikagaku Corp.)

acid-active hyaluronidase, Hyal-2, is encoded by a gene at an adjacent chromosomal site to Hyal-1 (see part 4 below, and Muellegger et al. in this mini-series).

Little is known about Hyal-3, the third enzyme coded for at the 3p21.3 locus. Strong hybridization expression patterns are found in mammalian testis and bone marrow. These two tissues retain a fetal and stem cell-like state for the life of the animal, suggesting that Hyal-3 may be important in stem cell regulation. We have cloned the mouse ortholog of *Hyal3* by degenerate polymerase chain reaction (PCR) performed on a P1 artificial chromosome that was previously shown to contain *Hyal1*. The mouse cDNA of *Hyal3* has been cloned by PCR and we recently found an *Hyal3* EST from a mammary tumor cDNA library in the database (GenBank accession no. BE376250). *HYAL3* ESTs from other species such as rat, pig, and cow can also be found in GenBank. Mouse Hyal-3 has approximately 80% amino acid identity to the human sequence. This degree of identity is higher than that between the human and mouse Hyal-1 orthologs (73%), which suggests that Hyal-3 may have an important function in vivo but so far it has not been conclusively shown to possess hyaluronidase activity in vitro.

The testicular hyaluronidase, PH-20/SPAM1, is important during egg fertilization by sperm. The functions and properties of this enzyme are reviewed in the article by Cherr et al. in this mini-series.

A novel hyaluronidase paralog can be identified at chromosome 7q31.3, which we termed Hyal-4. The human *HYAL4* cDNA is calculated to be 2414 nucleotides in length. Expression of *HYAL4* is restricted to placenta and skeletal muscle. Preliminary evidence indicates that Hyal-4 is a chondroitinase with no activity against HA (unpublished data). This is the first chondroitinase to be identified in vertebrate tissues. It is not surprising that a chondroitinase activity can be coded for in this gene family, considering that both Hyal-1 and PH-20 both have the ability to cleave chondroitin sulfate (CS) to a limited extent. Chondroitin sulfate is closely related to HA, the difference being that the *N*-acetyl-glucosamine of HA is replaced by *N*-acetyl-galactosamine in CS. Both polymers have exclusively β-linkages. There is sulfation of CS, but the possibility remains that the actual cleavage sites are in non-sulfated or under-sulfated regions of the randomly sulfated CS polymer. We recently identified a full-length EST of *HYAL4* in a mouse 0-day neonate cDNA library (GenBank accession no. AKO14599). Human and mouse Hyal-4 have 77% amino acid identity.

The sixth sequence, *HYALP1,* is a pseudogene in humans because two deletions exist that cause premature termination codons (Csoka et al., 1999). We previously suggested that the few mutations in the human sequence indicate that *HYALP1* may have only recently degenerated into a pseudogene on an

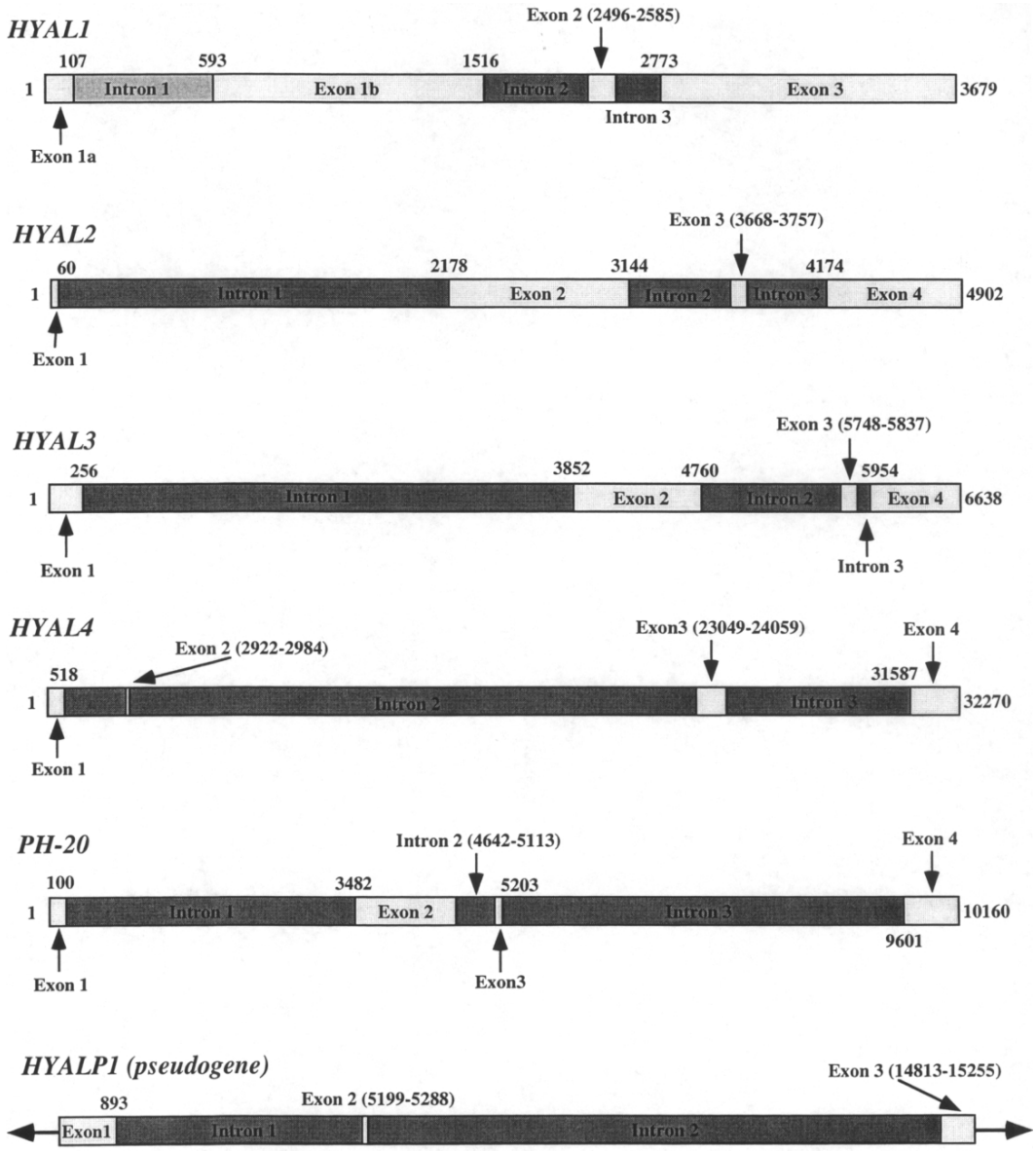

Fig. 3. Genomic structure of the hyaluronidase genes. Exons are indicated by yellow rectangles and introns are indicated by dark green rectangles. Numbers indicate beginning and end of exonic nucleotides unless otherwise shown. In the *HYAL1* mRNA isoform that is not translated into protein (see text), intron 1 is retained within exon 1 (indicated by the light green rectangle). Exons and introns are drawn to scale for each individual gene, but relative gene sizes are not drawn to scale because of the almost 10-fold spread in size. All of the genes in the hyaluronidase cluster on chromosome 3 have similar exon and intron structures, but this structure is not preserved on chromosome 7. (Courtesy of Glycoforum's 'Hyaluronan Today,' of Seikagaku Corp).

evolutionary time-scale, and may be functional in other species. Indeed, we recently identified a full-length EST of *HYALP1* from a mouse testis cDNA library (GenBank accession no. AK016575) that does not contain any mutations. *HYALP1* may therefore encode an active hyaluronidase enzyme in the mouse and possibly other mammals. Human and mouse Hyalp-1 have approximately 68% amino acid identity (with forced virtual translation of the human ortholog).

Alignment of the conceptual translation of the cDNAs of the six hyaluronidase-like genes in human and mouse, using the PIMA (Smith and Smith, 1992) multiple sequence alignment program, is shown in Fig. 4. Conserved blocks may represent regions critical to enzymatic activity. An unrooted phylogenetic tree of all 12 of the human and mouse hyaluronidase paralogs can be seen in Fig. 5. The tree was generated using ClustalW and Phylip (Protpars) (Felsenstein, 1989), two of the suite of bioinformatics programs at BioNavigator by Entigen Corporation (http://www.entigen.com).

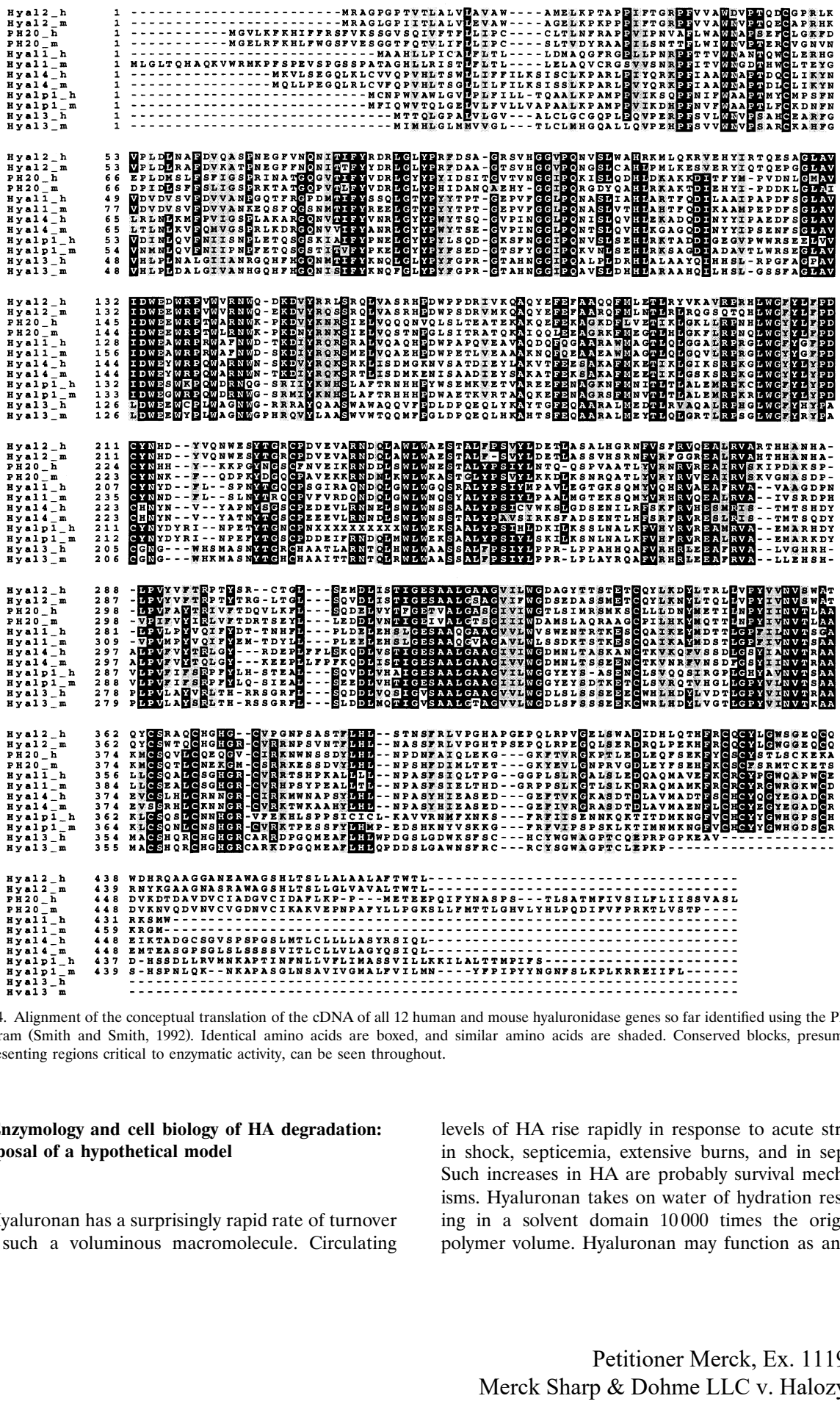

Fig. 4. Alignment of the conceptual translation of the cDNA of all 12 human and mouse hyaluronidase genes so far identified using the PIMA program (Smith and Smith, 1992). Identical amino acids are boxed, and similar amino acids are shaded. Conserved blocks, presumably representing regions critical to enzymatic activity, can be seen throughout.

4. Enzymology and cell biology of HA degradation: proposal of a hypothetical model

Hyaluronan has a surprisingly rapid rate of turnover for such a voluminous macromolecule. Circulating levels of HA rise rapidly in response to acute stress, in shock, septicemia, extensive burns, and in sepsis. Such increases in HA are probably survival mechanisms. Hyaluronan takes on water of hydration resulting in a solvent domain 10 000 times the original polymer volume. Hyaluronan may function as an in-

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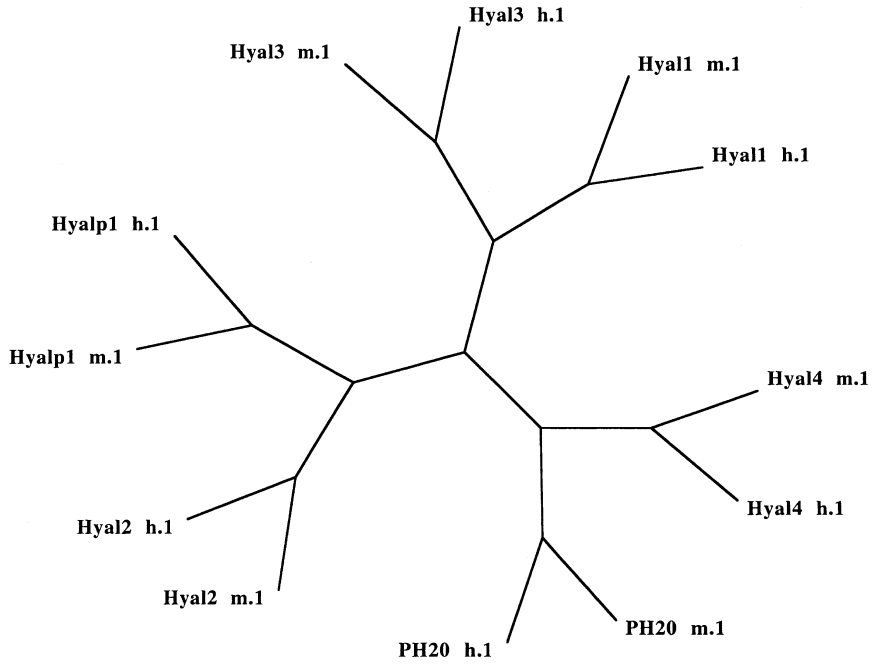

Fig. 5. Phylogenetic analysis of the 12 human and mouse hyaluronidases. h = human, m = mouse. The dendrogram was generated on the Bionavigator web site at http://www.bionavigator.com. Programs used to generate the dendrogram were ClustalW and Phylip (see text for references).

travascular volume expander preventing circulatory collapse.

High levels of HA are present normally in the joint capsule, in the vitreous of the eye, in Wharton's jelly of the umbilical cord, in amniotic fluid and fetal tissues, and in all tissues undergoing rapid proliferation or repair. Enhanced levels of HA occur in inflammation, edema, the swelling following organ transplantation, stroke, or myocardial infarction, in sepsis, wound repair, and in carcinogenesis. Hyaluronidases and their attendant control mechanisms are apparently of critical importance in normal and abnormal biology. It is the modulation of catabolic turnover, the modulation of hyaluronidase activities, that provides rapid response mechanisms for changing levels of HA, rather than the synthetic reactions.

Hyal-2, which can be linked by a GPI-anchor to the outer cell membrane (Rai et al., 2001), has an unusual substrate specificity, cleaving high-molecular-weight HA polymers to intermediate size fragments of approximately 20 kDa. Lung fibroblasts possess an acid-active hyaluronidase in plasma membrane extracts with a similar size specificity for HA as Hyal-2 (Sampson et al., 1992). A model is proposed suggesting that Hyal-2 and Hyal-1 are the major mammalian hyaluronidases in somatic tissues, and that they act in concert to degrade high molecular weight HA to the tetrasaccharide. Large (20 kDa) HA fragments are generated at the cell surface in unique endocytic vesicles (Tammi et al., 2001) resulting from digestion by the GPI-anchored Hyal-2 in an acidic environment, transported intracellularly by an unknown process, and then further digested by Hyal-1. The two β-exoglycosidases remove sugars from reducing termini of HA oligomers, and supplement hyaluronidases in their catabolism of HA, as shown in Fig. 6a.

One problem with this hypothesis is that early endosomes are not as acidic as pH 4.0, the pH optimum of Hyal-2. A consequence of this is that either Hyal-2 has a higher pH optimum when in the plasma membrane, or HA is digested in unusually acidic endosomes. Hyal-1 is not able to bind HA at neutral pH. However, if Hyal-2 is able to bind extracellular HA at neutral pH, perhaps it is actually a co-receptor for internalization of HA, as well as a catabolic enzyme.

In vertebrate tissues the degradation of HA occurs by the concerted action of four enzymes: the two hyaluronidases, Hyal-1 and Hyal-2; and the two exoglycosidases that remove sugars sequentially from the non-reducing termini of HA oligomers, β-glucuronidase and β-*N*-acetyl glucosaminidase. The endolytic cleavage generates increasing numbers of non-reducing termini that are substrates for the exoglycosidases. The relative contribution of the endo- and exo-cleavage reactions may vary from tissue-to-tissue, but their individual contributions to overall

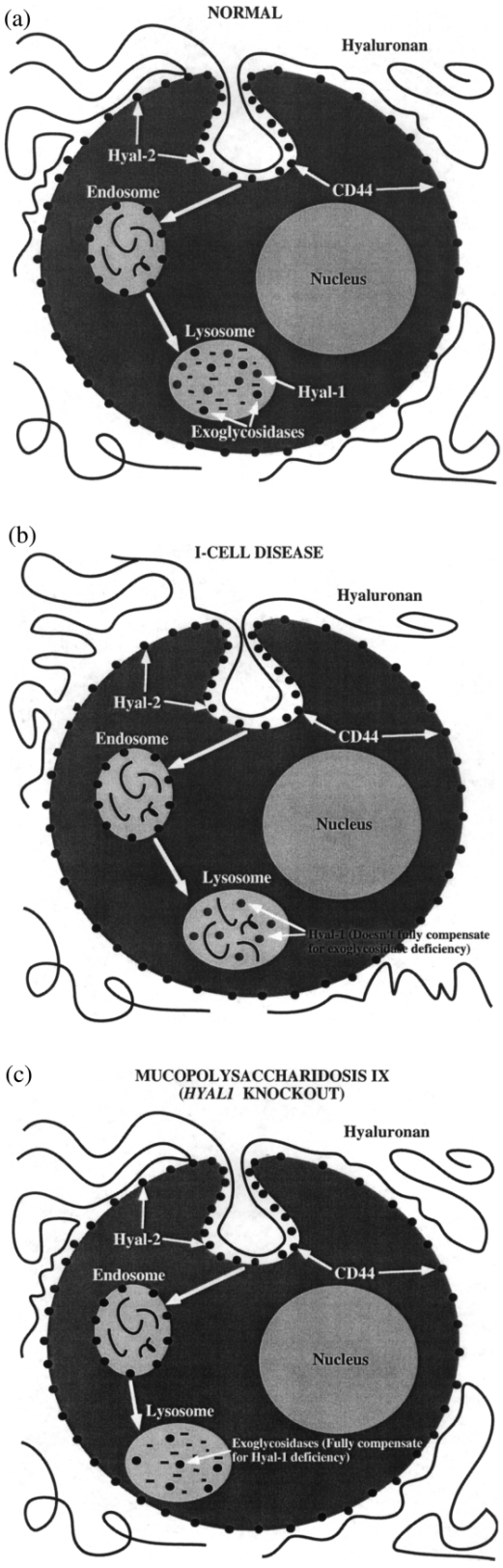

Fig. 6.

HA degradation have not been evaluated. However, they appear to make a major contribution to HA degradation in fibroblasts. In I-cell disease, in which trafficking of lysosomal enzymes is defective and a deficiency of these β-exoglycosidases exists, an accumulation of cytoplasmic HA occurs (unpublished observations), a situation shown in Fig. 6b.

5. Mucopolysaccharidosis IX (MPS IX): Hyal-1 deficiency

Genetic deficiencies of the different hyaluronidase enzymes could result in clinically distinct syndromes. Deficiency of the human plasma hyaluronidase, Hyal-1, has been reported, and termed Mucopolysaccharidosis IX (Natowicz et al., 1996; Triggs-Raine et al., 1999). The patient has a remarkably normal phenotype. There is some growth retardation (5th percentile), bilateral peri- and intra-articular soft tissue masses, transient painful swelling of these masses with occasional effusions, and generalized cutaneous swelling. She has mildly dysmorphic facial features. Ultrastructural studies reveal histiocytes filled with numerous large membrane-bound vacuoles suggestive of a lysosomal storage disease. Fibroblasts contain lesser numbers of such vacuoles. The patient's circulating HA level is 40 times normal.

A Hyal-1 deficient mouse has been produced using targeted mutagenesis (unpublished), the mouse equivalent of the patient with MPS IX. The homozygous Hyal-1 deficient mouse is viable, but the Hyal-2 deficient mouse is probably not (Kreil et al., personal communication). As shown in Fig. 6c, the β-exoglycosidases may be able to compensate for the defi-

Fig. 6 (a) Diagrammatic representation of a newly-proposed model of HA degradation in cells. Hyal-2, in cooperation with CD44 or another HA receptor, cleaves high-molecular-weight HA polymers to intermediate size fragments of approximately 20 kDa in unique acidic endocytic vesicles. The products of Hyal-2 digestion are then further degraded to low-molecular-weight oligosaccharides by the action of Hyal-1 together with the two β-exoglycosidases, β-glucuronidase and β-*N*-acetyl glucosaminidase. (b) In I-cell disease, in which trafficking of lysosomal enzymes is defective and a deficiency of these β-exoglycosidases exists, an accumulation of HA occurs (unpublished observations). Therefore Hyal-1 cannot fully compensate for the missing β-exoglycosidases. (c) Representation of the postulated situation in Mucopolysaccharidosis IX and in the Hyal-1 knock-out mouse. Hyal-2 cuts high molecular weight HA chains, as in the normal situation. The oligomers are delivered to lysosomes, where continued degradation by the β-glycosidases occurs. Their activities may partially or totally compensate for the absence of Hyal-1 activity. This explains why the elimination of Hyal-1 is not a lethal event during embryonic development, while the elimination of Hyal-2 is lethal.

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ciency of Hyal-1, but there is no compensatory mechanism available for the loss of Hyal-2 activity. It is quite probable that Hyal-2 is the rate limiting step in the cellular degradation of HA, because it is on the plasma membrane and therefore acts as a gatekeeper for HA internalization. It is also conceivable that Hyal-2 may interact with HA receptors such as CD44 (Aruffo et al., 1990) and LYVE-1 (Banerji et al., 1999) to regulate HA internalization (see the review by Knudson et al., in this mini-series).

6. The role of hyaluronidases in cancer

Hyaluronan has been invoked as mechanisms for tumor invasion and metastatic spread. The levels of HA surrounding tumor cells often correlate with tumor aggressiveness and poor outcome (Zhang et al., 1995). Overproduction of HA enhances anchorage-independent tumor cell growth (Kosaki et al., 1999; Liu et al., 2001). Loss of hyaluronidase activity, permitting accumulation of HA, may be one of the several steps required by cells in the multi-step process of carcinogenesis. Hyaluronidases would then qualify as candidate tumor suppressor gene products. The *HYAL1* gene (also known as *LUCA-1,* or LUng CAncer-1) indeed maps within a candidate tumor suppressor gene locus, defined by homozygous deletions and by functional tumor suppressor activity (Wei et al., 1996). Hemizygosity, or loss of one of two alleles, occurs in this region in many oral, head and neck, and lung carcinomas. Mutations in the remaining allele would be required to have homozygous loss (loss of function in both alleles). However, only two missense mutations in *HYAL1* have been reported in carcinomas despite extensive searches (Lerman and Minna, 2000). We recently identified a lesion that does produce functional silencing of the *HYAL1* gene product in some oral cancers (Frost et al., 2000). The unusually large 5′ UTR, found by PCR, indicated the presence of a retained intron (see Fig. 3). Neither hyaluronidase enzyme activity nor Hyal-1 protein could be detected in a number of cancer cell lines, though the mRNA for Hyal-1 was present. The retained intron prevented translation, possibly because of the large number of start and stop codons it contains. It is not known how widespread such a mode of gene silencing is in carcinomas. In other malignancies, hypermethylated CpG nucleotides in the 5′ HYAL1 promoter region have been identified by metabisulfite sequencing, that correlates with loss of gene expression. Treatment of such cell lines with demethylating agents and histone deacetylase inhibitors results in derepression of gene expression to normal levels (Angelborg et al., manuscript in preparation). These findings emphasize the importance of the loss of hyaluronidase in the process of cancer development, and the multiple pathways of gene silencing. Loss of hyaluronidase might provide the cancer cell with the HA-rich environment that stimulates growth, movement, and metastatic spread, or may be altogether unrelated to glycosaminoglycan catabolism. Cancer cells have adapted a wide range of mechanisms for silencing unwanted gene products. Apparently, silencing of tumor suppressor genes can occur not only at the level of DNA, but also at the level of mRNA.

7. Unanswered questions and concluding remarks

Details of the catabolic mechanisms involved in HA catabolism remain elusive. It is not known why there is an acid-active hyaluronidase in the circulation and in urine. Moreover, the absence of a circulating catalytically active hyaluronidase in some species poses clear questions as to its function (Fiszer-Szafarz, 1984). In cultured cells, whether stromal or epithelial, most hyaluronidase activity is quickly secreted into the culture medium and is not retained by the cell layer. Does this reflect the in vivo situation? It is unlikely that hyaluronidase activity is retained in vivo in an active form within the extracellular matrix (ECM) where it would cause great havoc. If it is found within the ECM, it may be in an inactive or suppressed form, perhaps bound to an inhibitor. Such a situation would parallel the relationship between the metalloproteinases (MMPs) and the tissue inhibitors of MMPs, (TIMPs) that exert exquisite control over MMP activity (see the article by Mio and Stern in this mini-series).

Are hyaluronidases secreted and then taken up secondarily by cells for delivery to lysosomes? Such a situation is less likely to be recapitulated in vitro by cultured cells, because of the rapidity with which enzymes become diluted in the large volume of medium. Is receptor-mediated endocytosis involved in hyaluronidase uptake by cells? If so, what is the nature of these receptors? Evidence from patients with Mucolipidoses II and III indicates that Hyal-1 is unusual, and different from most lysosomal enzymes (Natowicz and Wang, 1996). Its activity is not elevated in the plasma of patients with these genetic disorders, indicating either that Hyal-1 is not targeted to lysosomes, or that it is targeted by a mechanism other than the usual phospho-mannosyl recognition pathway.

CD44 has been implicated in HA uptake and degradation (Aruffo et al., 1990). How do CD44 and its variant exons participate in the overall process of HA uptake and degradation by hyaluronidases? Since

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Hyal-2 has now been shown to exist on the apical side of the plasma membrane, do Hyal-2 and CD44 interact and thus cooperatively regulate the catabolic internalization of HA?

The controls for modulating levels of hyaluronidase expression are just beginning to be understood. In cultured cells, whether stromal or epithelial, an inverse pattern of HA and hyaluronidase activity occurs. As sparsely plated cells grow and begin to fill the dish, HA levels are high, and acid-active hyaluronidase activity is barely detectable. When cells become contact inhibited and cease proliferating, hyaluronidase activity rises sharply and levels of HA deposition fall rapidly, a scenario that is not observed in transformed cells (unpublished observations).

Keratinocytes obtained from human skin can be made to differentiate in culture when the calcium levels are increased from 0.05 to 1.30 mM. The medium containing high calcium causes cells to stratify and to begin synthesizing keratins. With differentiation, levels of HA synthesis and deposition decrease (Lamberg et al., 1986) and Hyal-1 hyaluronidase production increases 25-fold (Frost and Stern, 1997). The emerging picture is that undifferentiated, rapidly proliferating cells have high levels of HA, while cells lose their HA-rich matrix in order for proliferation to slow and for a program of differentiation to commence. Hyaluronidase activities may be a key to mediating such transitions.

Two isoforms of *HYAL1* mRNA are found even in normal tissues. The larger transcript contains the retained intron shown in Fig. 3. An obvious question is whether the ratio between the two isoforms regulates translation and thus expression of Hyal-1 activity.

Recently, hyaluronidase levels in dermal fibroblasts (Stair-Nawy et al., 1999) were examined during the process of their differentiation into chondrocytes in a micrcomass culture system (Nicoll et al., 1999). We found dramatic increases in Hyal-1 expression with differentiation, as well as modest increases in Hyal-2 and Hyal-3. The process of differentiation in various isolated organ and tissue systems can provide many more opportunities for studying the multiple mechanisms for the modulation of hyaluronidase, as first suggested by Bryan Toole in his classic studies of the role of HA in embryology (Toole, 1991).

Acknowledgements

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