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REVIEW

# Hypotheses on the evolution of hyaluronan: A highly ironic acid

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**Hyaluronan is a high-molecular-weight glycosaminoglycan (GAG) prominent in the extracellular matrix. Emerging relatively late in evolution, it may have evolved to evade immune recognition. Chondroitin is a more ancient GAG and a possible hyaluronan precursor. Epimerization of a 4-hydroxyl in *N*-acetylgalactosamine in chondroitin to *N*-acetylglucosamine of hyaluronan is the only structural difference other than chain length between these two polymers. The axial 4-hydroxyl group extends out perpendicular from the equatorial plane of *N*-acetylgalactosamine in chondroitin. We suspect that this hydroxyl is a prime target for immune recognition. Conversion of a thumbs-up hydroxyl group into a thumbs-down position in the plane of the sugar endows hyaluronan with the ability to avoid immune recognition. Chitin is another potential precursor to hyaluronan. But regardless whether of chondroitin or of chitin origin, an ancient chondroitinase enzyme sequence seems to have been commandeered to catalyze the cleavage of the new hyaluronan substrate. The evolution of six hyaluronidase-like sequences in the human genome from a single chondroitinase as found in *Caenorhabditis elegans* can now be traced. Confirming our previous predictions, two duplication events occurred, with three hyaluronidase-like sequences occurring in the genome of *Ciona intestinalis* (sea squirt), the earliest known chordate. This was probably followed by *en masse* duplication, with six such genes present in the genome of zebra fish onwards. These events occurred, however, much earlier than predicted. It is also apparent on an evolutionary time scale that in several species, this gene family is continuing to evolve.**

*Keywords:* chondroitin / evolution / hyaluronan / hyaluronidase / immunology

## Introduction

The extracellular matrix (ECM) polymer hyaluronan (HA, hyaluronic acid) emerged relatively late in evolution. It is referred to as a "stealth molecule" (Lee and Spicer 2000), in part, because of its ability to avoid detection by the immune system. There may be a connection between its late arrival and the ability of this polymer to evade recognition, a proposal that is examined here. A mechanism for the stealth-like properties of HA is provided, as well as a scheme for the evolution of the hyaluronidase gene family from an ancestral chondroitinase. Other aspects of HA metabolism are also provided.

## HA provides a stealth-like immune shield

Hyaluronan provides a protective shield as well as a matrix scaffolding for two situations that are particularly critical to the protection and survival of vertebrate species: (1) in the cumulus mass that surrounds the vertebrate ovum (Salustri et al. 1999) and (2) in the ECM of the stem cell niche (Haylock and Nilsson 2006; Schraufstatter et al. 2010). It appears that HA may have multiple protective roles at critical points in vertebrate biology. It is important that HA be able to avoid the usual recognition systems and surveillance mechanisms that protect the organism, particularly since these are so error prone, as exemplified by the myriad of autoimmune disorders. Nature is often overly zealous in its protective mechanisms, which themselves can cause pathophysiology.

## HA emerged when metazoan organisms required internal movement of cells

Hyaluronan accompanies the progeny of stem cells as they begin to undergo programs of differentiation when, from the separate stem cell niche, they migrate through the body of the developing organism to distant sites. Hyaluronan also confers motility directly upon cells by engaging receptors that interact with the cytoskeleton (Turley 1992). HA also stimulates signal transduction pathways that facilitate motion (Turley et al. 2002). Simultaneously, the water associated with HA opens up tissue spaces through which cells are able to travel. The water of solvation for HA is actually quite small. But because of the very large negative charge associated with HA at neutral pH, there is an enormous water domain that

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accompanies the molecule. This helps to create the space through which cells then move.

As mentioned, HA seems to have appeared in evolution relatively late compared with other glycosaminoglycans (GAGs). HA may have emerged precisely when the separate stem cell niche arose. Isolated and protected from the rest of the organism, pluripotent cells in the stem cell niche proliferate and expand. Such cell populations must migrate through the body of the developing organism during embryogenesis and travel to distant sites. Not only does HA comprise a portion of the environment for stem cell development, confers the motility that enables such cells to migrate and simultaneously creates the space through which their passage occurs.

A classic example is the migration of neural crest cells derived from the primitive neuroectoderm that travel to scattered sites in the body. An HA-rich matrix lines the pathway for such migrating cells. HA becomes degraded when cells reach their destinations (Pratt et al. 1975). Perhaps cells cease their migration at that point precisely because of the disappearance of the HA-rich matrix.

## The appearance of HA may have occurred simultaneously with malignancy

The same strategy seems to have been commandeered by malignant cells. A number of studies of human cancers have demonstrated that levels of HA correlate with tumor aggressiveness (Zhang et al. 1995; Auvinen et al. 2000; Boregowda et al. 2006; Itano and Kimata 2008). The metastasizing cancer cell uses an HA-rich pavement for malignant spread, in a manner similar to that used by migrating embryonic cells. The matrix surrounding cancer stem cells is also particularly HA-rich (Al-Hajj et al. 2003). Therefore, malignancies and their attendant metastases probably arose when HA first appeared. They share together with migrating embryonic cells the need for cell movement—certainly a high price that vertebrates had to pay. In leukemias, the malignant cells home to the high endothelial venules of lymph nodes by way of a CD44 mechanism, the predominant cell surface receptor for HA (Zahalka et al. 1995; Wallach-Dayan et al. 2001). Here again, the cancer cells imitate the mechanism used by their normal counterparts.

## Chondroitin appeared prior to HA

Chondroitin appeared early in the evolution of multicellular organisms, long before HA, earlier than 540 Mya (millions of years ago) (Figure 1, polysaccharide column).

Chondroitin, but not chondroitin sulfate (CS), occurs in the nematode *Caenorhabditis elegans*. There is no evidence for the presence of HA in *C. elegans* (Yamada et al. 1999; Toyoda et al. 2000; Hwang et al. 2003). The difference between the two GAG polymers, other than chain length, is that the *N*-acetylgalactosamine of chondroitin is replaced

| Eon | Era | Period | Epoch | Model Organism | Polysaccharides | Hyal Enzymes | Mya |
|---|---|---|---|---|---|---|---|
| Phanerozoic | Cenozoic | Quarternary | Holocene | *Homo sapiens* | Chondroitin, HA | 5 (1 Pseudo) | 0.2 |
| | | | Pleistocene | | | | 1.5 |
| | | Neogene | Pliocene | *Pan troglodytes* | Chondroitin, HA | 6 | 6.0 |
| | | | Miocene | *Pongo pygmaeus* | Chondroitin, HA | 6 | 23 |
| | | Paleogene | Oligocene | | | | |
| | | | Eocene | | | | |
| | | | Paleocene | *Mus musculus* | Chondroitin, HA | 7 | 65 |
| | Mesozoic | Cretaceous | | *Gallus gallus* | | 5 | 150 |
| | | Jurassic | | | | | |
| | | Triassic | | *Anolis carolinensis* | Chondroitin, HA | 6 | 250 |
| | Paleozoic | Permian | | | | | |
| | | Carboniferous: Pennsylvanian | | | | | |
| | | Carboniferous: Mississippian | | *Xenopus laevis* | Chondroitin, HA | 4 | 300 |
| | | Devonian | | Petromyzontidae | | | 360 |
| | | Silurian | | *Danio rerio* | Chondroitin, HA | 6 | 400 |
| | | Ordovician | | *Strongylocentrotus purpuratus* | Chondroitin | 3 | 450 |
| | | Cambrian | | *Caenorhabditis elegans* | Chondroitin | 1 | 540 |
| Precambrian | | Proterozoic | | Eukaryotes | Chitin | 0 | 2500 |
| | | Archean | | Bacteria & Archea | 0 | 0 | 3800 |
| | | Hadean | | - | - | - | 4600 |

**Fig. 1.** Major subdivisions of geological times are depicted with evolutionary time-matched model organisms, their polysaccharide content and number of Hyal-like enzymes. Ages in Mya are not drawn to scale and are accredited to the International Commission on Stratigraphy.

by the *N*-acetylglucosamine present in HA. Thus, the only distinction between the two polymers is at the level of galactosamine and glucosamine with the epimerization of a single hydroxyl at carbon-4 being the only difference between the two sugars.

An intriguing observation suggests that chondroitin indeed proceeded HA in evolution and carried out functions that were later provided by HA. Primitive mesenchymal cell migration in the sea urchin embryo requires a chondroitin-containing proteoglycan (Lane and Solursh 1991).

## When did HA first appear?

There continues to be a major question as to when HA first appeared in vertebrate evolution. The first step in the development of chordates may have occurred in the sea squirt, a urochordate in the phylum Chordata that evolved ~450 Mya. These tunicates are sessile creatures in their adult forms, but the free-swimming larvae have a primitive notochord. The genome of one of these creatures, *Ciona intestinalis*, contains no evidence of HA anabolism or synthesis (Satoh et al. 2006). There is no HA synthase-like sequence and chondroitin appears to be the predominant GAG. The beginnings of a primitive immune system are attributed to these organisms (Nonaka and Satake 2010).

The zebra fish has six hyaluronidase-like sequences, as well as several HA synthase genes, indicating that HA is expressed in such vertebrates. The first evidence for HA must have occurred earlier in vertebrate evolution. HA is observed in amphioxus (Spicer and McDonald 1998a), in the subphylum cephalochordata. This may be the first organism to express HA, having evolved ~400 Mya. There is controversy, however, whether this organism is actually an ancestor of vertebrates. Amphioxus may be more closely related to echinoderms (Shu et al. 2004). Therefore, it remains unclear when HA first appeared in vertebrate evolution.

## Evolution of the hyaluronidase-like sequences

There are six hyaluronidase-like sequences in the Human Genome. These were discovered following the isolation and characterization of the first somatic hyaluronidase that was purified from human plasma (Afify et al. 1993; Frost et al. 1997; Csóka et al. 1998). The remaining sequences were detected using the Expressed Sequence Tag (EST) data bank (Csóka et al. 1999, 2001; Stern and Jedrzejas 2006).

Three sequences were found clustered at one chromosomal location (3p21), and a similar tight cluster of three at another site (7q31). From this, it was surmised that an original sequence emerged at some point in evolution, followed by two duplication events to yield three such sequences. It was then suspected that *en masse* duplication occurred, bringing the total from three to six, as observed in the genome of a vertebrate, the zebra fish, *Danio rerio*. The genome of *C. elegans* has only one such sequence. Three sequences occur in the genome of the sea squirt, *C. intestinalis*, thus confirming this prediction (Figure 1, Hyal-like Enzymes column). Figure 1, in the Hyal-like enzymes column, illustrates the evolution of the Hyal-like sequences: From one in *C. elegans*, to three in the sea squirt, to six such sequences in the zebra fish.

The two duplication events must have occurred at time points between the evolution of *C. elegans* and that of the sea squirt, between 540 and 450 Mya, perhaps during the Cambrian explosion of 500 Mya (Zhuravlev and Riding 2001). It would be interesting to identify an organism with only two hyaluronidase-like sequences, if such exists.

In the past decade, a large number of eukaryotic genomes have been sequenced. It was predicted that the *en masse* duplication event, from three to six hyaluronidase-like sequences, occurred when placental mammals separated from nonplacental mammals. However, the duck-billed platypus (*Ornithorhynchus anatinus*), as egg-laying mammal, has six hyaluronidase-like sequences, as does the zebra fish, *D. rerio*. Thus, it appears that the duplication event, from three to six, occurred very much earlier than predicted. The common ancestor of fish and mammals lived 400 Mya, indicating that the duplication event occurred before that time, possibly between 450 and 400 Mya. It has been suggested that such duplication events occurred before the origin of jawed fishes, perhaps at the divergence of ray-finned and lobe-finned fishes (Postlethwait et al. 1998).

It is curious that the domestic chicken, *Gallus gallus*, and the African clawed frog, *Xenopus laevus*, have discarded one and two hyaluronidase-like sequences, respectively, while the mouse, *Mus musculus*, has added a seventh gene (Kim et al. 2005).

The chromosomal locations of the six hyaluronidase-like sequences in the human genome are depicted in Figure 2, three clustered on chromosome 3p21.3 and a similar cluster on 7q31.3. The orientation of the 3p cluster is established. HYAL1, the serum enzyme, is straddled by *HYAL3*, which is telomeric, and *HYAL2*, centromeric. Orientation of the 7q cluster is also shown. The chondroitinase-like *HYAL4* is

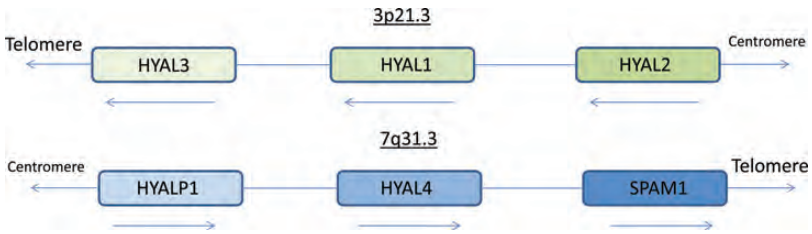


**Fig. 2.** Schematic presentation of the chromosomal locations of the six hyaluronidase genes at their two respective chromosomal sites. The relative gene order has been established in relation to their telomeric and contromeric orientations. The figure is not drawn to scale.

flanked by the centromeric pseudogene (in the human) *HYALP1*, while the sperm-associated gene *SPAM1*, also known as *PH20*, is telomeric. Though *C. intestinalis* contains no HA synthase-like genes, it does have three hyaluronidase-like sequences. These genes may code for chondroitinase-like enzymes, as discussed below.

## Which HYAL cluster came first?

The somatic hyaluronidases lay neglected for a long period (Kreil 1995). This is attributed to their mechanism of action. Prokaryotic hyaluronidases had become well described because their mechanism of action is an elimination reaction that can easily be followed using a spectrophotometer. In contrast, eukaryotic hyaluronidases use a hydrolytic mechanism; this reaction is not accompanied by a spectrophotometric change. Special assays had to be formulated before serious analyses could be conducted (Guntenhöener et al. 1992; Frost and Stern 1997). Information then accumulated rapidly describing the two key somatic hyaluronidases, HYAL1 (Frost et al. 1997; Csóka et al. 1998) and HYAL2 (Lepperdinger et al. 1998, 2001; Andre et al. 2011). Attention to this gene cluster was augmented with observations of gene deletions of this region in human lung cancers, suggestive of a tumor suppressor gene (Lerman and Minna 2000). Further promotion of the importance of this gene cluster occurred when HYAL2 was identified as a cell entry receptor for certain oncogenic retroviruses (Miller 2003). This flurry of activity brought much attention to the enzymes on chromosome 3p.21, promoting the assumption that they reflect the original gene cluster. However, HYAL4 on chromosome 7q21 is associated with the putative primordial chondroitinase activity. An argument can be made, therefore, that this reflects the original gene cluster.

## The introns within the 7q cluster are much larger than those on 3p

There are major difference between gene sizes of the chromosome 7 and the chromosome 3 clusters. Those on chromosome 7 range from 10 to 32 K nucleotides, compared with 3.6–6.6 K on chromosome 3 (Figure 3). Most of these differences are contributed to by the sizes of the introns. In fact, the entire *HYAL1* gene could fit into intron 2 of *HYAL4*. The exon sizes, however, have remained relatively constant, as have exon patterns. A curiosity of *HYAL1* is a retained intron, which when translated, contains multiple stop codons, with premature termination (Frost et al. 2000). This phenomenon of alternative splicing is another level for regulation of enzyme expression. The basis of this regulation or the factors involved are unknown.

## The appearance of HA required commandeering a chondroitinase

There are six hyaluronidase-like sequences in the human genome (Csóka et al. 2001) and in the chimpanzee, but seven in murine animals (Kim et al. 2005). *C. elegans* has only one hyaluronidase-like sequence, and that gene codes for a chondroitinase enzyme and not for a protein with hyaluronidase activity (Kaneiwa et al. 2008). One of the hyaluronidase-like sequences in the vertebrate, HYAL4, has now been demonstrated to code for an enzyme with exclusively chondroitinase activity (Chatel et al. 2010; Kaneiwa et al. 2010). Working retrospectively, the following scenario can be postulated. The first hyaluronidase-like sequence was exclusively a chondroitinase, chondroitin being the only GAG present in *C. elegans*, and from this chondroitinase, hyaluronidases evolved. There are striking sequence homologies between the following proteins: Human HYAL1, murine Hyal1, HYAL4 and the chondroitinase sequence from *C. elegans*, as shown in Figure 4.

An important proviso must be added, however. These proteins coded for by hyaluronidase-like sequences may have functions other than degradation of GAG polymers. Hyal2 functions additionally as a control for glycocalyx deposition and also interacts with ezrin-radixin-moesin (ERM), major cytoskeletal elements through cluster of determination 44 (CD44) binding (Duterme et al. 2009). Hyaluronidases may have functions other than and perhaps even more important than their enzymatic activities. They may act as adhesion or as anti-adhesion proteins. Enzymes are blithely unaware and totally indifferent to what we call them. Many proteins have functions in addition to the activities that we measure or that their "names" imply. Caution is indicated, therefore, in hypothetical formulations such as those described here.

At some later time, HA evolved and duplications of the original chondroitinase sequence occurred, in order for HA catabolism to occur. To this day, other hyaluronidases, such as HYAL1, 2 and PH-20, retain residual chondroitinase activity, a reference possibly to the substrate of their ancestral enzyme.

The *HYAL4* gene should be the one that resembles most closely the ancestral gene. The current authors attempted to show, using homology search engines and dendrogram analyses, that the *C. elegans* chondroitinase gene and human *HYAL4* bear an ancestral relationship and are more closely related to each other than to any of the other sequences (A.C. and R.S., unpublished observations). However, this exercise proved unsuccessful. Subsequent evolutionary drift may be the basis of this failure.

Following the appearance of six hyaluronidase-like sequences in the zebra fish, a seventh sequence appeared in the mouse genome, with the unfortunate appellation of *Hyal5* (Kim et al. 2005). Subsequent evolutionary progress proceeded to discard this seventh sequence, since it is no longer present in the genome of the great apes or in that of the human (Figure 1). This event must have occurred at some point between 65 and 6 Mya.

One of the hyaluronidase-like sequences in the human genome, *HYALP1*, is transcribed but not translated. It is a pseudogene because there are at least two stop codons in the first three exons (Csóka et al. 1999; Zhang et al. 2010). However, it is translated in most mammals, including primates and has been termed Hyal6. It may be concluded that this is a sequence perhaps on its way to being deleted altogether in humans.

In summary, the mouse has seven hyaluronidase-like sequences and primates have six, while humans have six, with one silent sequence. The mouse sequences are provided

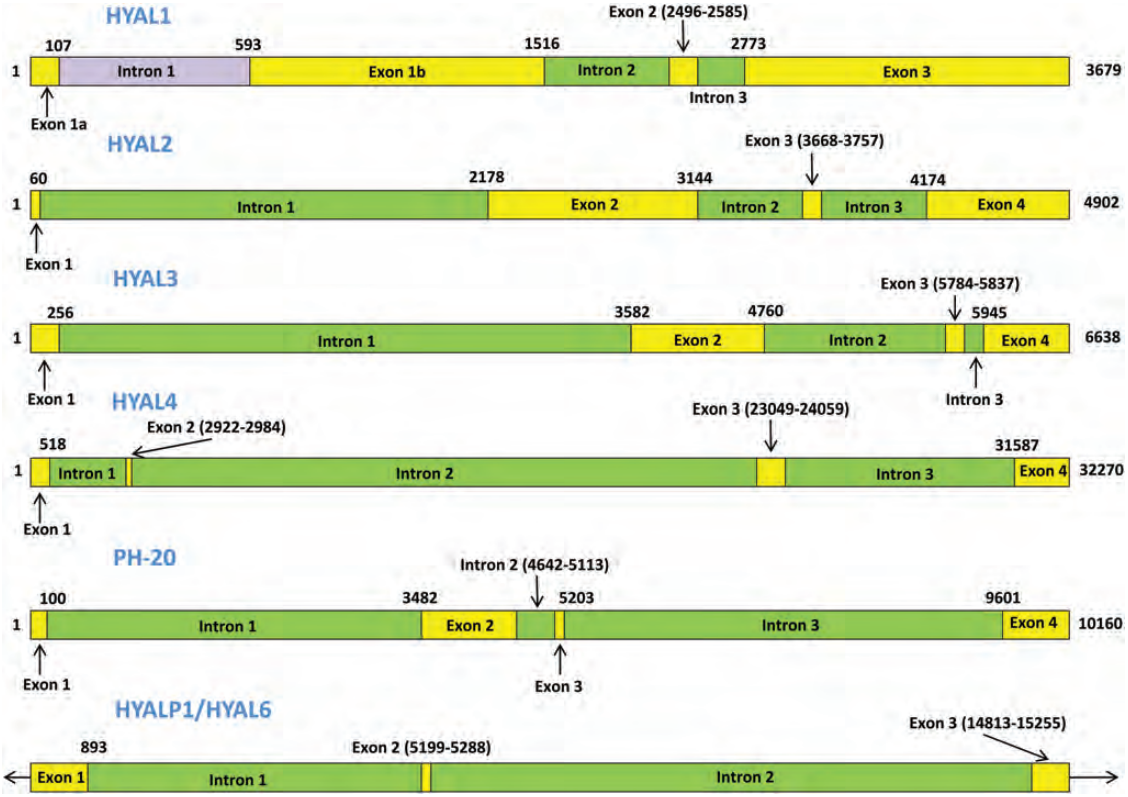


**Fig. 3.** Genomic structure of the hyaluronidase genes. Exons are indicated by yellow rectangles and introns are indicated by dark green rectangles. Numbers indicate the beginning and the end of exonic nucleotides unless otherwise shown. In the *HYAL1* mRNA isoform that is not translated into protein, 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 the genes in the hyaluronidase cluster on chromosome 3 have similar exon and intron structures, but this structure is not preserved on chromosome 7.

in Figure 5. Alignment of the conceptual translation of the cDNA of all seven mouse hyaluronidase genes was obtained using the CLUSTAL W program (Higgins et al. 1996; Chenna et al. 2003). Identical amino acids are boxed, and similar amino acids are shaded. Conserved blocks, representing the regions most critical to enzymatic activity, can be seen throughout. Alignment was performed using the CLUSTAL W sequence alignment program on GenomeNet, a Japanese network of data bases (http:www.genome.jp/tools/clustalw/).

The data suggest that the hyaluronidase-like sequences are continuing to evolve. This appears to be occurring at a rapid rate on an evolutionary time scale. It would be interesting to establish what forces drive such evolution. How are evolution, survival, gene silencing, genetic adaptation and efficiency related to each other on a mechanistic level?

A phylogenetic tree with branch lengths for 34 hyaluronidase-like sequences from nine species (*C. elegans*, hydra, sea squirt, sea urchin, xenopus, zebra, fish, chicken, mouse and human) is shown in Figure 6. The *C. elegans* chondroitinase is at the base of the tree.

## The HA mechanism for avoiding detection

Galactose, synthesized from glucose, is a major constituent of multicellular organisms, important in many processes, particularly those involved in immunological recognition. Galactose is commonly located at the nonreducing termini of structurally complex N-glycans. The most straightforward example is the ABO blood groups, the complex sugars critically important for blood transfusions. They must be identified in Blood Banking in order to avoid immune-based red cell hemolysis. The entire issue rests on a terminal galactosamine of blood group B that must be distinguished from an *N*-acetylgalactosamine of blood group A. The orientation of the hydroxyl group is the only difference between the terminal glucose and galactose of the two blood group antigens. Yet this simple hydroxyl orientation appears to be sufficient to cause massive immune-based red cell hemolysis in a transfusion blood type miss-match. That is the critical and only difference between the two A and B blood groups and an example of how important galactose residues may be in immune recognition. In a multitude of glycoconjugates, it is the last sugar to be added. Many lectins and antibodies appear to be able to recognize this galactose moiety. In fact, this family of lectins are now termed galactins (Barondes et al. 1994).

The galactose component of *N*-acetygalactosamine has a prominent advantage over other hexose sugars in recognition phenomena. It has an axial 4-OH group at a distinct position away from the core of the sugar, extending out perpendicular from the equatorial plane of the molecule. That axial 4-OH

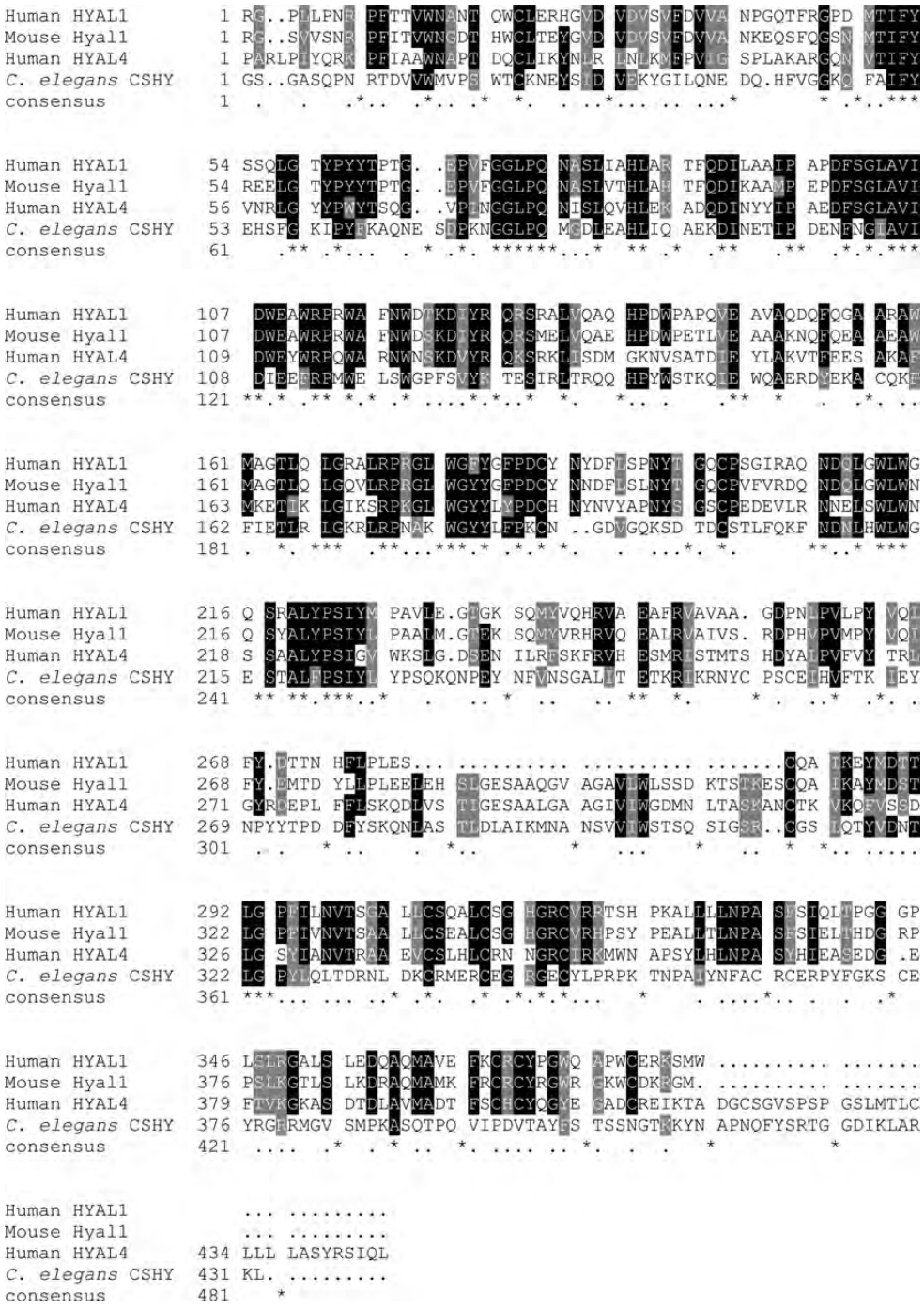


**Fig. 4.** Alignment of the conceptual translation of the cDNAs of human HYAL1, mouse Hyal1, the human chondroitinase, HYAL4 and the presumed primordial chondroitinase as reflected in the genome of *C. elegans*. The remarkable conservation of these sequences is apparent, despite the change in substrate in the course of evolution from chondroitin to HA.

group is epimerized to the equivalent 4-OH in glucose that occurs in the plane of the molecule, the energetically more stable position.

The advantage of glucose over galactose or of *N*-acetylglucosamine over *N*-acetylgalactosamine in avoiding recognition thus becomes obvious. The relatively late emergence of HA in evolution as a survival mechanism may be precisely to avoid the evolving primitive immune and lectin recognition systems that galactose provides. The stealth-like HA has the intrinsic ability to avoid the recognition mechanisms utilized by animal tissues, a recognition that occurs predominantly by detection of the thumbs-up axial 4-hydroxy group of galactose.

If HA indeed emerged from chondroitin, then the change of the hydroxyl position between glucose and galactose must

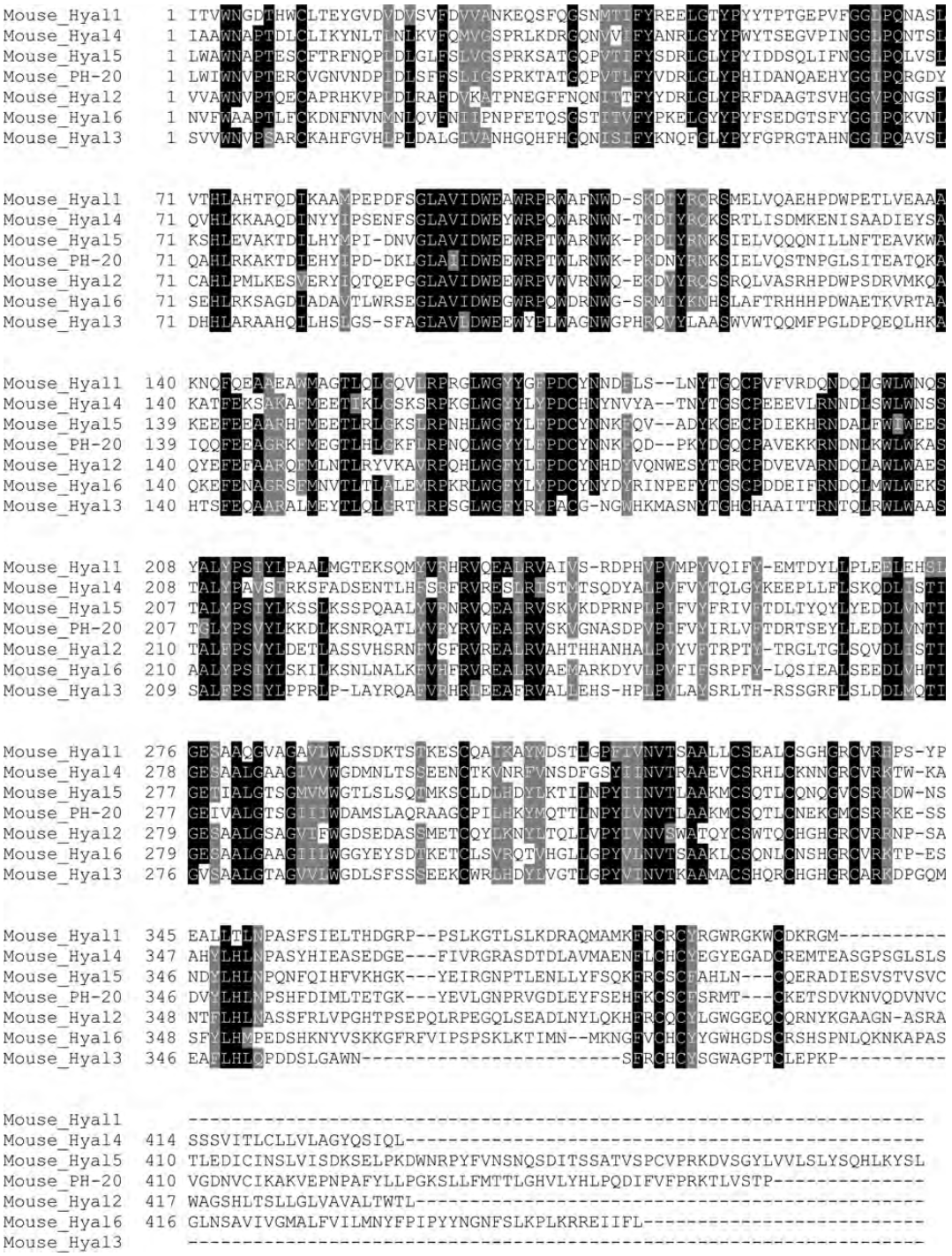


**Fig. 5.** Alignment of the conceptual translation of the cDNA of all the seven mouse hyaluronidase genes using the CLUSTAL W program (Higgins et al. 1996). Identical amino acids are boxed, and similar amino acids are shaded. Conserved blocks, representing the regions most critical to enzymatic activity, can be seen throughout. Alignment was performed using the CLUSTALW sequence alignment program on GenomeNet (http://www.genome.jp/tools/clustalw/). GenomeNet is a Japanese network of database and computational services for genome research operated by the Kyoto University Bioinformatics Center.

be evaluated. The *N*-acetylgalactosamine reverts to a more primordial or primitive *N*-acetyglucosamine. This change in orientation of the hydroxyl group seems to be a step backwards, but provides an evolutionary survival mechanism for more complex organisms.

## Evolution of galactose from glucose: The Leloir pathway

In the evolution of the hexose sugars, galactose may have evolved from the glucose molecule. The emergence of HA, if it arose from chondroitin, requires the ability of the orientation

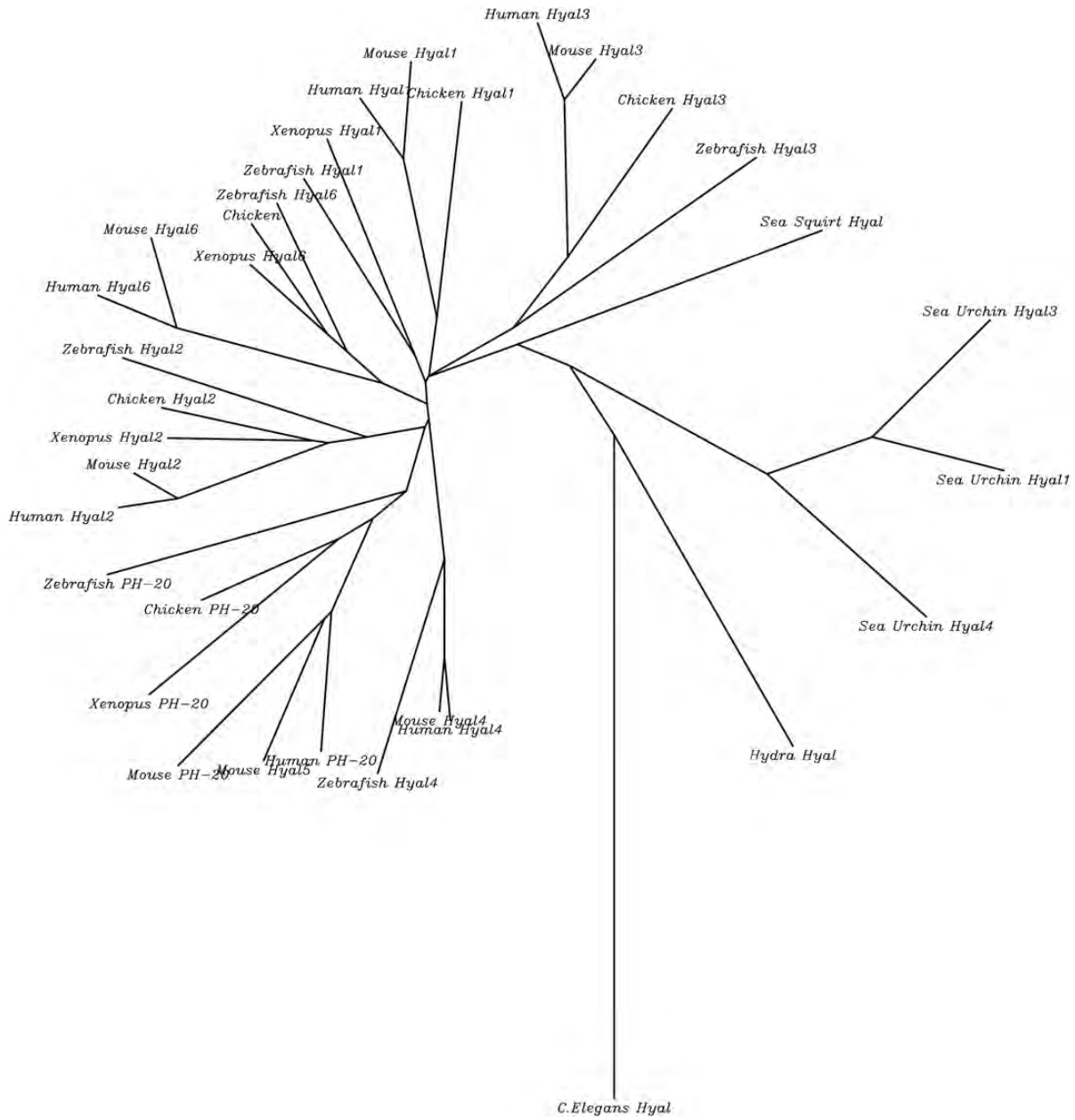


**Fig. 6.** Unrooted phylogenetic tree with branch lengths for 34 Hyal sequences from nine species (*C. elegans*, hydra, sea squirt, sea urchin, xenopus, zebrafish, chicken, mouse and human). Alignment was first performed using CLUSTALW on the GenomeNet server (see above), and a dendrogram was subsequently generated using the CLUSTALW data.

of the hydroxyl in galactose to return to that of the glucose molecule—such a reversible reaction mechanism does exist. The biochemistry of such a series of reversible enzymatic steps between glucose and galactose was established in the laboratory of Argentinian Nobel biochemist Luis Leloir (Holden et al. 2003) and is known as the Leloir pathway. This pathway is entirely reversible and permits interconversion of the two sugars. Three separate enzymes are required for the interconversion of glucose and galactose, for changing the stereochemical configuration at carbon-4 (Frey 1996). The enormous effort involved in the evolution of three separate enzymes required for accomplishing this single epimerization is a reflection of its biological importance. There are no parallels in any other biochemical system.

Though galactose is a more evolved sugar compared with glucose, a return to glucose, in the case of the HA polymer would appear to be a regression. However, this "strategic retreat" can provide progress. The evolution of chondroitin to become HA is another example in which backwards can be forwards. On the other hand, if HA arose from chitin, it would reflect the evolutionary pressure for organisms to generate this stealth-like polymer, while simultaneously providing the pressure to commandeer an anabolic enzyme for HA from the chondroitinase gene family. Hyaluronan has a very rapid

rate of turnover in vertebrate organisms, most of which is dependent on hyaluronidase activity. The half-life of circulating HA is 3–5 min (Reed et al. 1990). In a 70 Kg individual, there are 15 g of HA, 5 g of which turns over daily (McCourt et al. 1999). There may have been great evolutionary pressure to generate a catabolic enzyme for a molecule as dynamic as HA.

## The HA synthases

The three members of the HA-synthase family, HAS-1, -2 and -3, are now recognized as able to synthesize the HA polymer, with strict alternating additions of the two sugars. The two sugar substrates are uridine diphosphate (UDP)-glucuronic acid and UDP-*N*-acetylglucosamine, added sequentially at the reducing end of the growing polymer (Prehm 1983). The enzymes have a high degree of sequence homology; they are embedded membrane proteins with seven passes on the inner surface of the plasma membrane. The HA product is extruded through the plasma membrane into the extracellular space as it is being synthesized. This permits unconstrained polymer growth, which could not occur with synthesis in the Golgi apparatus or the endoplasmic reticulum (ER) where most other sugar polymers are synthesized without destruction of the cell. HA also occurs within cells, but it is not clear whether such HA is synthesized directly or transported back into the cell following synthesis.

The three HAS isozymes produce different size polymers and are differentially regulated (Itano and Kimata 1996; Weigel et al. 1997), by transcriptional, translational and post-translational levels, including alternative splicing, sub-cellular localization and epigenetic processes. The three genes are located on three different chromosomes, even though they have 50–71% identity. They occur at 19q13.4, 8q24.12 and 16q22.1, respectively. HAS2 is considered the important enzyme, as it synthesizes a long polymer with a MW of $\sim 2 \times 10^6$ Da. Mice with *HAS2* gene deletion die at mid-gestation (Camenisch et al. 2000), while gene deletion of *HAS1* or *3* has no apparent effect on fetal development.

## The HA receptors

CD44 is considered the predominant receptor for HA, though it is not its only ligand. CD44 is a membrane glycoprotein consisting of ten constant exons in humans, with ten variant exons inserted in various combinations at a single extra-membrane site. The constant isoform comprising exons 1–5 and 16–20 is designated CD44S. An enormous number of splice variants, designated CD44v, containing the variant exons can be generated (Screaton et al. 1992; Lesley and Hyman 1998), some of which have highly specific functions in embryogenesis, matrix assembly, malignant transformation and metastatic spread (Knudson 2003; Naor et al. 2008). CD44E, a variant containing v8-10 is expressed on epithelial cells. CD44 interacts with a number of other ligands including osteopontin, collagens and matrix metalloproteinases (MMPs).

The CD44 receptor for HA may be one of the most complex and most intriguing proteins in all of vertebrate biology. The following reviews recent progress in a CD44 variant. This sialofucosylation variant is a selectin-binding form of CD44 termed hemtopoietic cell E-selectin/L-selectin ligand (HCELL). It is found on cancer cells, hematopoietic stem cells and leukemic blast cells. It is a bone-homing receptor that directs stem cells to the bone marrow (Sackstein et al. 2008). Clearly, CD44 is involved in a myriad of critical functions and underscores the enormous number of biological reactions in which HA participates.

The HA receptor, receptor for HA-mediated motility (RHAMM) preceded CD44 in evolution, and is far more ancient in origin. A microbial ortholog for RHAMM occurs which is a chromosome segregation protein with 45% homology and 25% identity. RHAMM homology exists in *C. elegans*, with no evidence for CD44. Also. the sea urchin has a RHAMM-like protein, again without evidence for CD44, according to a basic local alignment search tool (BLAST) search. These observations are reflected in the position of the RHAMM and CD44 bars in Figure 7. This makes sense because the RHAMM receptor has many functions in addition to being an HA-binding protein. For example, RHAMM associates with microtubules and plays a role in the regulation of mitosis (Maxwell et al. 2003; Tolg et al. 2010; Telmer et al. 2011).

Two other HA receptors have come into prominence recently, reflecting the ever-increasing role HA is documented to play in biology: lymphatic vessel endothelial hyaluronan receptor (LYVE-1) and hyaluronic acid receptor for endocytosis (HARE), also known as Stabilin-2.

HARE-like proteins are evident in the sea-urchin genome, indicating a very ancient origin, similar to RHAMM (Figure 7). HARE mediates systemic clearance of HA, CSs and heparin from both the vascular and the lymphatic circulations. It exists in two isoforms of 190- and 315-kDa. It can be identified in sinusoidal endothelial cells of lymph nodes and liver (Zhou et al. 2000; Harris et al. 2008).

LYVE-1 is also an integral membrane glycoprotein involved in lymphatic HA transport, with a role in lymphatic spread of tumor metastases (Jackson 2003). Similar to CD44, evidence for LYVE cannot be found earlier than the zebra fish (Figure 7). Thus, both CD44 and LYVE-1 are HA receptors that have evolved more recently, corresponding to the emergence of vertebrates (Figure 7).

## HA is immunosuppressive

It has long been established that polymers of HA are themselves intrinsically immunosuppressive (McBride and Bard 1979; Delmage et al. 1986) and anti-inflammatory, particularly in their higher molecular weight forms. The HA contained in the ECM of adherent cells protects them from lymphocyte-mediated cytolysis (McBride and Bard 1979). Hyaluronan suppresses septic responses to lipopolysaccharides (Muto et al. 2009) and acts to maintain immune tolerance (Bollyky et al. 2009). In a more circuitous fashion, HA can induce production of immunosuppressive macrophages (Kuang et al. 2007). Apparently, HA evolved not only to evade immunosuppression, but also developed intrinsic immunosuppressive properties, both direct and indirect. High-molecular-weight HA is not only immunosuppressive, but also anti-inflammatory and anti-angiogenic. All of those properties reflect an organism

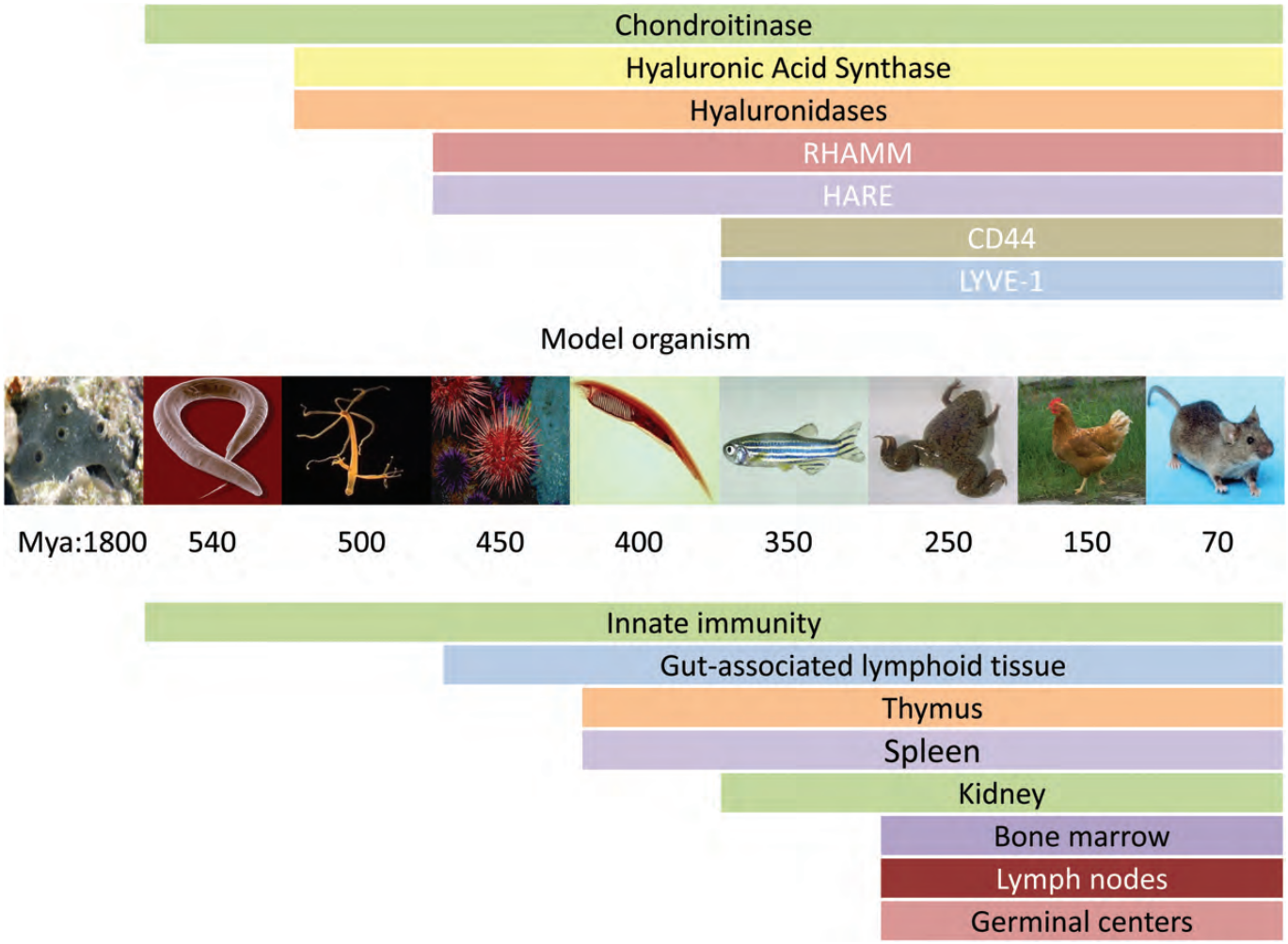


**Fig. 7.** Evolutionary sequence of immunity (lower horizontal bars) shown alongside model organisms (middle evolutionary sequence) and components of the GAG metabolizing system (upper horizontal bars). The organisms illustrated, from left to right are: A sponge, the nematode *C. elegans*, hydra, sea urchin, lancelet, also known as amphioxus, zebra fish, frog, chicken and mouse. Chondroitinases gave rise to hyaluronidases. Evidence is provided in the text that the HA receptors RHAMM and HARE preceded CD44 and LYVE-1. Innate immunity existed before adaptive immunity. The lymphoid tissue of the gastro-intestinal system, the largest of all lymphoid organs preceded appearance of the thymus and spleen. Bone marrow, lymph nodes and their germinal centers were a later evolutionary development.

in an intact healthy state, while fragmented HA has the opposite properties, a signal of tissues under stress. In fact, various sizes of HA fragments have evolved to become major immune regulators (Jiang et al. 2011). Hyaluronan size appears to be an information-rich system (Stern et al. 2006).

### Various human pathogens utilize HA to avoid host recognition

HA is in the capsule of several human and animal bacterial pathogens. These HA-containing bacterial structures are virulence factors for such organisms, having evolved in order to disguise themselves, to evade recognition by the mammalian immune system (Kendall et al. 1937; Carter and Annau 1953; MacLennan 1956). Such molecular mimicry could be accomplished by lateral gene transfer, though there is no direct evidence for this for the HA synthases. Evidence for lateral gene transfer does come from studies of hyaluronidase. The venom of bees, wasps and hornets contain a hyaluronidase that is 30% identical to the human sperm hyaluronidase, PH20 (Marković-Housley et al. 2000). Their structures, as determined by X-ray crystallography are superimposable (Stern and Jedrzejas 2006), suggestive of a lateral gene transfer. However, some of these bacterial synthases that produce HA may represent convergent evolution, that HA synthesis evolved and that HA sysnthases were created independently more than once in microbial evolution (DeAngelis 1999, 2002a, b). Indirect evidence for such a conclusion also comes from the observation that the direction of HA synthesis occurs in two different directions in different organisms, either at the reducing end or at the nonreducing end of the growing HA polymer, (DeAngelis 1999), unlike the metazoan HA synthases, with addition strictly at the reducing end.

Nonbacterial pathogens also use this strategy. Such a mechanisms has evolved in the human pathogenic yeast

*Cryptococcus neoformans* (Jong et al. 2007). Other major vertebrate parasites have cleverly evolved HA-binding proteins that enable them to sneak under the tent and to obtain a free ride on the HA molecule. These include ascaris, the giant intestinal roundworm nematode (Ponce-Leon et al. 2009) and the leishmania organism responsible for the disorder leishmaniasis (Rao et al. 1999).

## An alternative scenario: Chitin as the evolutionary origin of HA

Despite the fact that chondroitinase sequence was utilized for HA catabolism, the synthetic origins of the polymer may be from an entirely different source.

An alternative scenario posits that HA came originally not from chondroitin, but rather from chitin. All vertebrate GAG's are synthesized by way of the Golgi apparatus and are made together with an attached core protein. The exception is HA, which is synthesized on the cytoplasmic surface of the plasma membrane and is made without a covalently bound protein. Chitin is a ubiquitous β-linked polymer, with a repeating monosaccharide sequence of *N*-acetylglucosamine. Like HA, it is made without a core protein. The chitin synthase uses a single UDP-sugar, the HA synthases having evolved a bifunctional alternating UDP-sugar addition mechanism. A structural mechanism for the evolution of a bifunctional glycosyltransferase from a monofunctional precursor through the addition of a specific peptide has recently been described (Sobhany et al. 2008, 2012).

The three major β-chain sugars on the Earth are cellulose, chitin and HA. There is evidence that all the three polymers descended from a proto-β-chained sugar polymer, as their respective synthase genes bear homologies (Somerville and Colwell 1993; Spicer and McDonald 1998b; Richmond and Somerville 2000).

Chitin constitutes the cell wall of ancient eukaryotic organisms such as yeast, from the kingdom of fungi. The cell wall appears to be the ancient site of synthesis of polymers such as chitin, not only in yeast but also in arthropods such as crustaceans, spiders and insects (Merzendorfer 2011). Chitin is also a monotonous polymer without post-synthetic modifications. All other GAGs are more complex. They undergo modifications such as sulfation, insertion of alpha linkages and other reactions in a highly ordered sequence-specific manner. The utilization of 3′-phosphoadenosine 5′-phosphosulfate (PAPs) and its synthase is used for various biochemical transformations involving sulfation (Venkatachalam 2003). Sulfation within the Golgi apparatus has advantages over the inner surface of the plasma membrane exposed only to cytoplasmic concentrations of such a highly specialized molecule. Such enzymatic modifications can occur more accurately on a two-dimensional grid, as is present in the Golgi. That sugar sequence precision would not be possible using a linear one-dimensional mechanism of synthesis. We posit, therefore, that the plasma membrane, the ancient location for chitin synthesis, became subsequently the site for HA synthesis. This is the major argument for chitin rather than chondroitin being the evolutionary precursor for HA.

A singular curiosity is the occurrence of several chitinases in vertebrates, though none of their tissues contains chitin. There is a chitinase in humans on chromosome 1 expressed in monocytes and macrophages (Boot et al. 1995; Escott and Adams 1995). The popular sentiment states that this enzyme provides evolutionary advantage as a protection against parasite and yeast infections, though there is no evidence to support this. A BLAST search shows ~25% amino acid identity and 45% homology with yeast (*Saccharomyces cerevisiae* S288c) chitinase (NCBI Reference Sequence: NP_010659.1) (A.C., unpublished observation).

Synthesis of sugar polymers may have occurred first in evolution on the inner surface of plasma membranes. However, the ER and Golgi are also a very ancient sites of complex sugar synthesis and transport (Klute et al. 2011). This is counter-intuitive, but the trafficking of complex sugars to and from the Golgi was already in place by the time of the divergence of the major eukaryotic lineages, nearly two billion years ago. It is abundantly evident that the synthesis of complex sugars was a requirement for the evolution of organisms from the very beginning (Varki 2011). It cannot be easily established, however, whether the Golgi or cell wall synthesis came first, or perhaps even simultaneously.

## Overall scheme for the evolution of HA and the immune system

A highly theoretical scheme for the evolution of HA, its enzymes and receptors and their relationship to the development of the immune system is provided in Figure 7. The evolutionary sequence of immunity is shown alongside the model organisms: The sponge, the nematode *C. elegans*, hydra, sea urchin, lancelet, also known as amphioxus, zebra fish, frog, chicken and mouse. Chondroitinases gave rise to hyaluronidases. HA synthases occurred at about that time from some ancestral primordial β-chain synthesizing enzyme.

Chondroitin, CS and even heparins occur in the hydrozoan *Hydra maagnipapillata* (Yamada et al. 2007, 2011), with no evidence for HA. In *C. elegans*, only chondroitin occurs, with no CS. In Figure 7, hydra follows the nematode *C. elegans*. However, it cannot be readily established which is the most ancient organism. The hydra has only a single orifice, while *C. elegans* has two orifices that simulate a gastrointestinal tract. It is on this basis that the hydra might be placed in an evolutionary sequence preceding *C. elegans*. But the GAG content of hydra suggests that it is the more developed organism. A more careful comparison of their genomes may resolve this issue of which is the more ancient on the evolutionary scale.

Innate immunity is the most primitive component of immune recognition, while lymph nodes with germinal centers reflect the most recent evolutionary development (Rast et al. 2006; Smith et al. 2010). Innate immunity existed before adaptive immunity. The lymphoid tissue of the gastrointestinal system, the largest of all lymphoid organs preceded appearance the thymus, and spleen. Bone marrow, lymph nodes and their germinal centers were a later evolutionary development.

## Conclusion

HA is truly a stealth molecule, as was first described over a decade ago (Lee and Spicer 2000). The mechanism for endowing HA with such stealth has been there all along.

By examining the hyaluronidase gene family, it appears that human evolution is continuing. We have not yet reached wherever we are going. The mouse has seven hyaluronidase-like sequences. The chimpanzee, one of our closest relatives, has shed one of these genes over the course of the 60 millions of years that separate chimpanzees from mice. The human genome also contains six such sequences, but one has become a pseudogene, apparently on its way to also becoming discarded over the course of the 6 Mya since humans and chimpanzees parted company. The primate genome is becoming more efficient, perhaps because many of the micro RNA's are taking on regulatory functions, making it possible to do more with less. It is unfortunate that none of us will be here to observe what happens next.

## Note

The notations for the human proteins are in upper case letters, while their equivalent coding nucleic acid sequences, whether DNA or RNA, are given in italics, e.g. HYAL1, HYAL2 and *HYAL1*, *HYAL2*, respectively. The general convention for nonhuman proteins or their equivalent coding nucleic acid sequences are only the first letter in upper case, e.g. Hyal1, Hyal2 and *Hyal1*, *Hyal2*, respectively.

## Conflict of interest

None declared.

## Abbreviations

BLAST, basic local alignment search tool; CD44, cluster of determination 44; CS, chondroitin sulfate; ECM, extracellular matrix; ERM, ezrin-radixin-moesin; ER, endoplasmic reticulum; EST, Expressed Sequence Tag; GAGs, glycosaminoglycans; HA, hyaluronan, hyaluronic acid; HYAL, hyaluronidase (human); Hyal, hyaluronidase (murine); HARE, hyaluronic acid receptor for endocytosis; HCELL, hemtopoietic cell E-selectin/L-selectin ligand; LYVE-1, lymphatic vessel endothelial hyaluronan receptor; MMP, matrix metalloproteinase; Mya, Millions of years ago; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; RHAMM, receptor for HA-mediated motility; UDP, uridine diphosphate.

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