MINIREVIEW

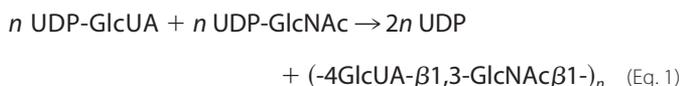# Hyaluronan Synthases: A Decade-plus of Novel Glycosyltransferases*[S]

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Hyaluronan synthases (HASs) are glycosyltransferases that catalyze polymerization of hyaluronan found in vertebrates and certain microbes. HASs transfer two distinct monosaccharides in different linkages and, in certain cases, participate in polymer transfer out of the cell. In contrast, the vast majority of glycosyltransferases form only one sugar linkage. Although our understanding of HAS biochemistry is still incomplete, very good progress has been made since the first genetic identification of a HAS in 1993. New enzymes have been discovered, and some molecular details have emerged. Important findings are the lipid dependence of Class I HASs, the function of HASs as protein monomers, and the elucidation of mechanisms of synthesis by Class II HAS. We propose three classes of HASs based on differences in protein sequences, predicted membrane topologies, potential architectures, mechanisms, and direction of polymerization.

Hyaluronan Structure and the Hyaluronan Synthase Reaction

Hyaluronan (HA)[2] is a linear heteropolysaccharide composed of $(\rightarrow 4)$-β-D-GlcUA-$(1\rightarrow 3)$-β-D-GlcNAc$(1\rightarrow)$ disaccharide repeats and is found in vertebrates and a few select microbes (1–6). HA typically resides on the cell surface or in the extracellular space, but is also found inside mammalian cells. Glycosyltransferases that polymerize HA are HA synthases (HASs; EC 2.4.1.212), earlier called HA synthetases (7–9). All HASs use UDP-sugars in the presence of Mg^{2+} or Mn^{2+} (Equation 1, overall reaction to form HA).

$$n \text{ UDP-GlcUA} + n \text{ UDP-GlcNAc} \rightarrow 2n \text{ UDP}$$

$$+ \text{(-4GlcUA-}\beta\text{1,3-GlcNAc}\beta\text{1-)}_n \quad \text{(Eq. 1)}$$

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[2] The abbreviations used are: HA, hyaluronan (hyaluronic acid); HAS, HA synthase; PmHAS, *Pasteurella multocida* HAS; SpHAS, *Streptococcus pyogenes* HAS; MmHAS, *Mus musculus* HAS; SeHAS, *Streptococcus equisimilis* HAS; aa, amino acid(s); GT2, glycosyltransferase 2; MDs, membrane domains; XlHAS, *Xenopus laevis* HAS; HsHAS, *Homo sapiens* HAS; ABC, ATP-binding cassette.

Typically, $n > 10^{3-4}$; thus, HASs make HA chains of ~ 1–10 MDa. The intrinsic fidelity of the two glycosyltransferases of HAS generates the repeating disaccharide structure. Unlike virtually all other vertebrate glycoconjugates, HA is made as a free glycan, not attached to protein or lipid. HASs do not need a primer for HA synthesis; they all initiate HA biosynthesis *de novo* with only UDP-GlcNAc, UDP-GlcUA, and Mg^{2+}. The UDP-sugars are α-linked and HASs synthesize β-linkages; thus, these are inverting (not retaining) enzymes. UDP-sugars are polymerized from cytoplasmic pools by HAS, and the growing HA chain is extruded out of the cell.

Discovery of the HASs: Dual-action Glycosyltransferases

The deduced sequences of the genes encoding the HAS proteins were identified using molecular biological techniques (*e.g.* transposon mutagenesis and expression cloning) (10–21). Classical biochemical purification methods failed with these membrane-associated enzymes. In 1993, identification of the streptococcal *hasA* gene and the biochemical demonstration that a single protein species is required to synthesize HA (13, 22) led to the identification of HAS genes in other species, including vertebrates. Only PmHAS is so different that its discovery was not aided by similarity comparisons with the prototypical *Streptococcus* HAS. The GenBank™ Data Bank lists >20 bacterial, viral, and vertebrate HAS genes or cDNAs. The nomenclature for HASs employs the first letters of the genus and species (*e.g. Streptococcus pyogenes* HAS = SpHAS). The vertebrate HAS isozymes are named in order of discovery (*e.g.* murine HAS isozymes = MmHAS1, -2, and -3).

Functional testing of recombinant proteins revealed HASs to be the first glycosyltransferases in which a single protein catalyzes the transfer of two different monosaccharides. Studies with purified recombinant SpHAS, SeHAS (23–25), MmHAS1 (26), and PmHAS (27) confirmed that no other proteins are needed to initiate or catalyze HA synthesis. This finding of dual-action, or bifunctional, enzymes is in contrast to the usual "one enzyme, one sugar linkage" dogma of glycobiology. It was also unexpected that such small proteins (417–588 aa and one of 972 aa) would perform multiple functions, including (*a*) binding of two distinct UDP-sugars, (*b*) binding of two distinct HA acceptor or donor species, (*c*) transfer of two different sugars in two different linkages, (*d*) repetitive sugar polymerization, and (*e*) HA transfer across the membrane.

Two Distinct Types of Bacterial HASs

Many bacteria and Archaea produce polysaccharides, but only certain animal and human pathogens produce extracellular HA capsules (28). HA is found in some Gram-positive streptococci (*e.g. S. pyogenes*, *S. equisimilis*, *Streptococcus uberis*, and *Streptococcus zooepidemicus*) and in Gram-negative Type A *P. multocida*. Mutant microbes without HA capsules are less virulent than parental strains because of increased susceptibility to host defenses, *e.g.* complement and phagocytosis (29, 30). The streptococcal HASs are $\sim 70\%$ identical to each other and

TABLE 1

Proposed HAS classification system

Two classes of HASs were initially posited in 1999 (7). On the basis of differences in predicted topology and architecture and additional biochemical data on reaction mechanisms, we now propose three distinct HAS classes: I-R, I-N, and II (R, reducing; N, nonreducing). Assignments in parentheses require further confirmatory studies. Su, *S. uberis*; Cv, *Chlorella* virus.

| | Class I | | Class II |
	R	N	
Members	SpHAS, SeHAS, SuHAS (MmHAS, HsHAS)	XlHAS1 (CvHAS)	PmHAS
No. of GT2 modules	One	One	Two
Predicted topology	Integral membrane	Integral membrane	Peripheral (or soluble)
HA chain growth	Reducing	Nonreducing	Nonreducing
Intrinsic polymerization	Processive	Processive	Nonprocessive
No. of UDP-sugar sites	?	?	Two
No. of HA polymer sites	?	?	Two (probably)

~25% identical to the vertebrate HASs. PmHAS is totally different from all other HASs.

The Animal HAS Family

HA is not found in all animals and arose ~500 million years ago in some chordates (31). Thus far, primitive extant animals, including hydra, sponges, insects (*e.g. Drosophila*), nematodes (*e.g. Caenorhabditis*), echinoderms, and tunicates (Urochordata, a branch of Chordata), do not contain HA or HAS genes (31, 32). In contrast, candidate genes encoding enzymes that make chondroitin/chondroitin sulfate and heparan sulfate, structurally related glycosaminoglycans, are in all animal genomes down to primordial hydra. This distribution is not simply because HAS cannot function in cells of lower organisms because MmHAS2 expression in fly results in high HA production *in vivo* (33).

Multiple HAS isozymes encoded by three to four genes are present in chordates (34). No known animal possesses only one HAS gene that might suggest an early ancestor/founder, but there is evidence for two rounds of gene duplication (34). *Xenopus* has three active HAS genes and one pseudogene, whereas mice and humans lack a nonfunctional gene. The mammalian HAS1, -2, and -3 isozymes are ~55–70% identical and >90% identical within an isozyme family. Knock-out mouse developmental studies show that HAS2 is essential (35), but HAS1 and HAS3 have lesser roles.

An Algal Virus HAS

A phycodnavirus (*Chlorella* virus) that infects freshwater green algae also possesses a HAS, CvHAS (12), with a sequence more similar to vertebrate than streptococcal HASs, but the evolutionary pathway of this gene is unclear. Early in viral infection, algal cells are coated with HA fibers (36), but their function is unknown. This is the first and only report of HA naturally occurring in plants and the first report of a viral carbohydrate-producing enzyme.

Differences in Structural and Biochemical Properties of HASs

The various types of HASs have biochemical and protein sequence differences (Table 1); apparently, multiple classes of HASs evolved independently to perform the same overall synthesis reaction by two distinct modes. New sugars may be added to the reducing (Equation 2, reducing end elongation reaction)[3]

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[3] These glycosyl-UDPs are shown with the reducing end on the right and highlighted differentially (italics or boldface) to illustrate the fate of the various UDP groups in this reaction.

or nonreducing (Equation 3, nonreducing end elongation reaction) terminus of the HA polymer (supplemental Fig. 1).

$$\textbf{HA-GlcNAc-UDP} + \textbf{GlcUA-}\textit{UDP} + \textbf{GlcNAc-}\text{UDP} \rightarrow \textbf{UDP}$$

$$+ \textit{UDP} + \textbf{HA-GlcNAc}(\beta 1,4)\textbf{GlcUA}(\beta 1,3)\textbf{GlcNAc-}\text{UDP} \quad \text{(Eq. 2)}$$

$$\textit{UDP}\text{-}\textbf{GlcUA} + \textbf{UDP-GlcNAc} + \textbf{HA} \rightarrow \textit{UDP} + \textbf{UDP}$$

$$+ \textbf{GlcUA}(\beta 1,3)\textbf{GlcNAc}(\beta 1,4)\text{-}\textbf{HA} \quad \text{(Eq. 3)}$$

HASs were previously divided into two groups (7): Class II containing PmHAS and Class I containing all other HASs. Further analysis is required to determine catalytic mechanisms, but note that the two UDP-sugars are acceptors and the HA-UDP chain is the donor during synthesis at the reducing end, whereas the converse occurs for nonreducing end growth (8).

The Class I streptococcal, vertebrate, and viral HASs (Fig. 1) (7) share aa sequence similarities, especially in their central regions. These enzymes have a single glycosyltransferase 2 (GT2) family (see www.cazy.org/) module with similarity to chitin synthases. Class I HASs are integral membrane proteins with four to six predicted trans-MDs and one to two membrane-associated MDs (8). The overall topology for SpHAS was determined by a reporter protein fusion approach (37). The active sites are intracellular (37), as are the UDP-sugars. All Class I HAS topologies are predicted to be comparable because the first two-thirds of these proteins are similar to SpHAS.

Class II PmHAS (Fig. 1) (7) has two GT2 family modules. PmHAS is a peripheral membrane protein with neither intrinsic affinity for bilayers nor lipid requirement for activity. Truncation analysis suggests that a C-terminal region (aa 703–972) docks peripherally with integral membrane transport proteins common to many encapsulated Gram-negative bacteria (38).

Class I HASs May All Be Lipid-dependent

Specific phospholipids (*e.g.* cardiolipin) are required for activity of membrane-bound or purified SeHAS or SpHAS (23, 25, 39, 40) and MmHAS2. The functional catalytic unit of SeHAS or SpHAS, as assessed by radiation inactivation analysis, is a HAS monomer with ~16 cardiolipins (41), as illustrated in supplemental Fig. 2. XlHAS1 is also active as a monomer with an additional mass of ~23 kDa that was not identified (42). Kimata *et al.*[4] found that dodecyl maltoside-solubilized recombinant insect cell-derived MmHAS2 requires subsequent addi-

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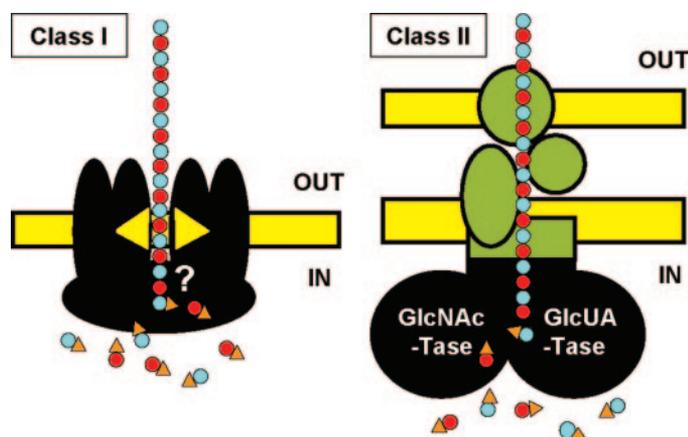[4] K. Kimata and co-workers, personal communication.

FIGURE 1. **Schematic models of Class I and II HASs.** GlcNAc and GlcUA (*red* and *blue spheres*, respectively) from the UDP-sugars (UDP, *orange triangles*) inside the cell are polymerized by the HASs (dual-action or bifunctional glycosyltransferases) into long polymer chains. HA is transported out of the cell to form extracellular coats or matrices in vertebrates and infected algae or capsules in bacteria. The Class I enzymes (*left*, *black*) are lipid-dependent integral membrane proteins with multiple MDs embedded in the lipid bilayer that catalyze sugar addition and may transport the hydrophilic HA polymer across the cell membrane of eukaryotes or Gram-positive bacteria. Associated lipid molecules (*triangles*) may facilitate pore formation for direct HA translocation, and/or an ABC transporter system (not shown) may translocate HA indirectly. For Class I-R enzymes, the growing reducing end of the HA chain is inside the cell and attached to UDP (as shown). For Class I-N enzymes, the growing nonreducing end of the HA chain is inside (not shown). Class II PmHAS (*right*, *black*) catalyzes the sugar addition reactions and probably docks with a polysaccharide transport apparatus composed of multiple proteins (*green shapes*) to translocate the HA across the two membranes of Gram-negative bacteria. The enzyme is a fusion of two glycosyltransferases (*Tase*) that add GlcNAc (Domain A1) and GlcUA (Domain A2) at the nonreducing end of the HA chain.

tion of one particular phospholipid for activity. Thus, it seems likely that all Class I HAS family members are lipid-dependent, and the historic difficulties in purifying active detergent-solubilized Class I HASs can be attributed to the loss of tightly bound phospholipid (40).

Some Class I HASs Polymerize HA by Addition to the Reducing End

New sugars can be added to a linear saccharide chain at either the nonreducing (Equation 3) or reducing (Equation 2) terminus during biosynthesis depending on the nature of the enzyme (supplemental Fig. 1). SpHAS was first reported to add sugars to the nonreducing end (43), but later studies using crude membranes (44, 45) or purified enzymes (46) demonstrated that SeHAS and SpHAS elongate HA at the reducing end. In each cycle of sugar addition at the reducing end, the released UDP is derived from the previously added monosaccharide (*i.e.* HA-UDP). The hyaluronyl-UDP is activated by its attachment to UDP and is the donor in each transfer reaction, analogous to protein and fatty acid synthesis (47, 48). The direction of synthesis for eukaryotic HASs is less clear; either some reports are in error, or both types of elongation occur, although the HAS sequences are all similar. Recombinant amphibian XlHAS1 adds sugars at the nonreducing end (45), but native mouse (49) and human (50) HASs extend HA at the reducing end. Nonreducing end addition was reported for recombinant truncated *Escherichia coli*-derived HsHAS2 (51), although only very small oligosaccharides were made.

Class II HAS Adds to the Nonreducing End

Recombinant *E. coli*-derived PmHAS elongates exogenous HA oligosaccharide acceptors (*e.g.* tetrasaccharides) *in vitro* by stepwise addition of individual monosaccharides to the nonreducing terminus (supplemental Fig. 1) (52). These acceptors are not absolutely required for HA formation by PmHAS, but stimulate polymerization by bypassing the relatively slow initiation step. A biotechnological spin-off (Hyalose, LLC) capitalizing on this kinetic feature is the production of size-defined HA polymers; *in vitro* reactions employing stoichiometrically controlled purified PmHAS, acceptors, and UDP-sugars are synchronized to yield essentially monodisperse HA products in the range of ~20 to ~2000 kDa (27).

Updated HAS Classification System: Three Classes

In light of new data, the original system of two HAS classes (7) needs to be revised (Table 1). We propose to maintain all integral membrane HAS proteins with a single GT2 module in Class I, but further subdivide these into Class I-R (reducing end polymerization; *e.g. Streptococcus*, mouse, and human HASs) and Class I-N (nonreducing end polymerization; *e.g.* XlHAS1). Another reason to keep the original Class I is that these HASs appear to both catalyze sugar transfer and facilitate HA transfer across the membrane. We anticipate that initial assignments of some members to the Class I-N or Class I-R group will require more experimental confirmation and may change with future studies. The very distinct PmHAS remains the only known Class II HAS.

Models of HAS Architecture

No three-dimensional structure is available for any HAS, but models for HAS architecture, function, and mechanisms of catalysis have been discussed (supplemental Fig. 1 and 2) (7–9, 28, 48, 53). Two types of putative functional domains, termed Domains A and B, were proposed to be present in many β-glycosyltransferases that use nucleoside diphosphosugars as donors (54). Processive enzymes, which add many sugars without releasing their polymer chain, possess both Domains A and B, whereas enzymes that add a single sugar usually have only Domain A. Class I-R and Class I-N HASs do not release their growing chains during synthesis and possess both putative Domains A and B. Class II PmHAS possesses two tandem copies of Domain A and no Domain B (38).

Studies of SeHAS have provided insight into the molecular organization of Class I HASs (55, 56). Four cysteine residues, conserved in the Class I family, are clustered very close together at the inner surface of the membrane and are close to or within UDP-sugar-binding sites (56). These results indicate that HAS active site(s) are at the membrane-protein junction and support the "Pore Hypothesis" for HA synthesis (25). supplemental Fig. 2 shows the first experimentally based model for the organization and nearest neighbors of the six MDs of SeHAS (55) that create this putative pore.

Kinetic analyses of Class II PmHAS and its mutants show that two relatively independent active sites with separate UDP-sugar-binding sites (one each in Domains A1 and A2) exist in one protein (38, 57). A pair of PmHAS variants, one disrupted at Domain A1 and the other at Domain A2, acted together to

polymerize HA at rates similar to the wild-type enzyme (38). HA synthesis is thus nonprocessive because HA chains must be released by one mutant PmHAS enzyme to be acted on by the other mutant enzyme. Cross-competition between GlcUA- and GlcNAc-terminated oligosaccharides for PmHAS-mediated elongation was not observed (53), indicating at least two acceptor-binding sites. Studies of optimal HA oligosaccharide acceptor length suggest that ~3 or 4 monosaccharide units of the nascent HA chain are bound by the enzyme (53).

The number and the nature of UDP-sugar- and HA-binding site(s) for Class I HASs are still as unclear as in 1959, when the first schematic models were presented (58). Mutations at conserved aa thought to be in UDP-sugar-binding or catalytic sites often inactivate HA polymerization completely; thus, key aa for various HAS functions have been difficult to map. However, mutations in MmHAS1 motifs that are conserved in other Domain A/B glycosyltransferases resulted in formation of short chitin oligosaccharides instead of HA (26).

Transfer of Newly Made HA to the Cell Surface: Pore or Transporters?

Class II PmHAS *in vivo* is probably docked with the capsular polysaccharide transport machinery, which helps retain the HA chain in the microenvironment of the two active sites and mediates HA export. Transporters typically use ~12 transMDs to create a pore for substrate transfer (*e.g.* lactose permease (59)). Class I HAS proteins have six to eight MDs, but these enzymes might use multiple interactions with the phospholipids essential for activity to create a larger pore-like passage through which HA could move (supplemental Fig. 2) (25, 41). The paradox is as follows: how can HAS bind and retain HA tightly enough to be processive, yet the HA chain move easily within the enzyme after every one to two sugar additions? Forces generated by Brownian motion might separate HA from HAS. One answer is that if HA is within an intraprotein pore, HAS can more readily release and rebind the HA without complete dissociation.

An alternative explanation is that HA is transported by an ABC system (60, 61), which can export many different bacterial polysaccharides (62). In this model, HAS synthesizes intracellular HA, which is recognized and transported by a membrane-bound ABC transport system. An ABC transport system seems unlikely to be involved in HA externalization for the Class I HASs because this model requires that *Enterococcus faecalis*, *Bacillus subtilis*, and *Drosophila* express ABC transport systems that lack specificity and can transport a foreign polysaccharide, HA (13, 33, 63). Perhaps HA can be brought to the cell surface by a combination of both HAS pore and ABC transporter mechanisms.

HA Size Matters: Is HAS Control Involved?

HA size (*i.e.* mass) is now recognized to be an important factor governing the ability of HA to alter cell behavior (4, 5). In particular, very small HA oligosaccharides (*e.g.* ~10–20 sugars) have potent effects in many *in vitro* or *ex vivo* systems, including angiogenesis models. It is generally assumed that small HA oligosaccharides would be derived from large extracellular matrix HA, which is degraded inside cells via exo-β-glycosidases and

hyaluronidases (64) during local or systemic turnover (6). However, it is also possible that HASs might be regulated to synthesize very small HA, thus producing small HA oligosaccharides directly. Tissue HA levels vary during embryonic and oocyte development and wound healing (3, 6). The levels of the three HAS mRNAs or isozymes also vary from amphibians to mammals, but data required for a cohesive view of HAS regulation are still being generated. Many connections have been reported between the proliferation, survival, and metastasis of cancer cells and the relative expression of HAS isoforms or HA levels (65).

The relative specific activities of the three HsHAS isozymes and the size distributions of their *in vitro* HA products are different (66). Changing a single aa in XlHAS1 alters the HA size distribution *in vitro* to either a bigger or smaller size depending on the substitution (67), and many mutations of SpHAS or SeHAS result in smaller HA (55, 68). In all cases, the biochemical explanation for how HASs control HA size is unknown. One hypothesis is that the more time a chain is held and acted on by HAS, the larger the HA; conversely, if the chains are readily released by HAS, then the HA will be smaller. Another possibility is that HA is released when HA retention forces within a HA·HAS complex are exceeded by the HA release forces (*e.g.* movement or collisions of the growing HA chain) (48). Increasing the ionic strength of an *in vitro* XlHAS1 reaction yields longer HA (67), suggesting that hydrophobic forces (*e.g.* between hydrophobic aa side chains and the hydrophobic faces of sugars in HA) are involved in nascent HA retention. The demonstration for HsHAS3 that HASs can be phosphorylated (69) and the finding that mammalian HAS trafficking and catalytic activity are dynamic and controlled (70) also add new levels of complexity for how HA production and HAS location are regulated.

Areas for Future Research

Several areas stand out to us as focal points for future progress.

Biochemistry—The identity, number, and nature of substrate-binding sites for any Class I HAS are unknown. Whether the initiating or terminating sugars in released HA chains are unique is unclear. Knowing these details could aid in the understanding of HAS regulation and development of useful drug targets.

Cell Biology—The field needs better HAS isozyme-specific antibodies that allow immunodetection of native nondenatured HAS1, -2, or -3 so that the cellular localization and distribution of each isoform can be elucidated in different cell types or tissues. Understanding HA homeostasis requires knowing how both the HAS and hyaluronidase genes and proteins are regulated.

HA Biology—Regulation of HA quantity and size has potentially great significance for altering cell behavior in health and disease (*e.g.* inflammation and cancer). Determining whether small HA oligomers are present *in vivo* outside cells and have effects similar to those found *in vitro* and in model systems is a priority. Are there also other hitherto unidentified regulatory proteins that alter HAS activity or localization? We look for-

ward to the next decade and many more answers to these and other questions.

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