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Review

Hyaluronan synthases: fascinating glycosyltransferases from vertebrates, bacterial pathogens, and algal viruses

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Abstract. Hyaluronan (or hyaluronic acid or hyaluronate; HA) is a polysaccharide found in the extracellular matrix of vertebrate tissues and in the surface coating of certain *Streptococcus* and *Pasteurella* bacterial pathogens. At least one algal virus directs its host to produce HA on the cell surface early in infection. HA synthases (HASs) are the enzymes that polymerize HA using uridine diphospho-sugar precursors. In all known cases, HA is secreted out of the cell; therefore, HASs are normally found in the outer membranes of the organism. In the last 6 years, the HASs have been molecularly cloned from all the above sources. They were the first class of glycosyltransferases identified in which a single polypeptide species catalyzes the transfer of two different monosaccharides; this finding is in contrast to the usual 'single enzyme, single sugar' dogma of glycobiology. There appear to be two distinct classes of HASs based on differences in amino acid sequence, topology in the membrane, and reaction mechanism. This review discusses the current state of knowledge surrounding the molecular details of HA biosynthesis and summarizes the possible evolutionary history of the HASs.

Key words. Hyaluronan; hyaluronic acid; hyaluronate; polysaccharide; glycosyltransferase; hyaluronan synthase; enzymology; membrane proteins.

Introduction

Hyaluronan (HA) is a linear polysaccharide composed of thousands of alternating glucuronic acid (GlcUA) and N-acetylglucosamine (GlcNAc) disaccharide repeats. HA belongs to the glycosaminoglycan or the mucopolysaccharide family of carbohydrates that includes molecules such as heparin, chondroitin, dermatan, and keratan. This family of carbohydrates is essential or important for the life of vertebrates and an assortment of lower animals. Certain pathogenic bacteria cleverly utilize HA capsules as molecular camouflage. Only recently has it been shown that an algal virus directs the production of HA fibers on the host soon after infection. The HA polymer was first described in 1934 [1] but the enzymes from various microbes and vertebrates that catalyze HA biosynthesis, HA synthases (HASs) (some older reports used the term HA synthetase), were only identified in the 1990s.

HASs have the unique distinction of being the first glycosyltransferases identified at the molecular level shown to catalyze the addition of two different monosaccharides to a polymer chain. The HAS polypeptides have both selective (β-1,4)GlcNAc transferase and (β-1,3)GlcUA transferase activities. This contrasts with a pervasive central dogma of carbohydrate biosynthesis that one enzyme transfers only one sugar group.

Now that the nucleotide sequences and the recombinant forms of the HASs are available, a series of investiga-

tions have started to delve into the intricacies of these fascinating polypeptides. Some of the current issues include the mechanism of catalysis, the regulation of HA polymer size, the transport of HA polymer out of the cell, and the origin of these genes.

Biological roles of HA polysaccharide

The numerous roles of HA in the vertebrate body have been studied extensively [reviewed in refs 2–5]. Vertebrates use HA as both a structural element and as a recognition molecule. HA has been implicated in numerous phenomena including adhesion, development, cell motility, cancer, angiogenesis, and wound-healing. Due to the unique physical and biological properties of HA, this polymer is employed in eye and joint surgery and is being evaluated in other medical procedures [reviewed in ref. 6].

Certain pathogenic bacteria from the genera *Streptococcus* [7] and *Pasteurella* [8] produce nonimmunogenic HA capsules or extracellular coatings. High titers of neutralizing protective antibodies usually cannot be generated against the HA polysaccharide [9]. The mammalian body is replete with HA; therefore, any significant response against the bacterial HA capsule would cause widespread autoimmune complications. Host defenses such as phagocytosis and complement are also defeated in part by HA capsules [10–12]. HA is also suspected of playing a role in pathogen adhesion to host cells by interaction with vertebrate HA-binding proteins [13, 14]. The role of HA fiber formation in the life cycle of virus-infected *Chlorella* green algae has not yet been elucidated, but numerous distinct viral isolates collected worldwide possess the HAS gene and the ability to make HA [15, 16].

The biosynthesis of a HA chain containing thousands of sugars requires considerable energy expenditure. On the order of five ATP equivalents, two NAD cofactors, one acetylCoA group plus the glucose and the glucosamine monosaccharide components are utilized to form each individual HA disaccharide unit. Therefore, some essential purpose or a selective advantage must exist in all cases of HA production.

Enzymological studies of HASs

Before the HAS genes and polypeptides were identified, many biochemical studies characterizing the properties of HAS activity from vertebrates and bacteria were undertaken [overview in ref. 17]. Radiolabeled UDP-sugar precursors for tracing HA polymer synthesis made several types of experiments possible. In 1955, a homogenate of Rous sarcoma was shown to make HA in vitro [18]. In the late 1950s, a cell-free system from group A *Streptococcus* was used to investigate the production of HA [19, 20]. Initially, more indepth studies were possible with this bacterial system due to its ease of preparation and higher specific activity. The streptococcal enzyme activity was localized to the membrane fraction [20]. Uridine diphospho-sugar nucleotide precursors, UDP-GlcUA and UDP-GlcNAc, and divalent metal ions (Mg or Mn) were utilized by the enzyme preparations at neutral pH (fig. 1). In analogy to most other nucleotide-utilizing enzymes, the metal ions are thought to coordinate with the phosphate groups of the UDP-sugar and to form the actual substrate complex used by the enzyme; the polypeptide is not thought to be a metalloprotein. Kinetics were used to study substrate binding and the mechanism of chain growth [21]. Lipid-linked intermediates are not involved in streptococcal HA biosynthesis because the inhibitors bacitracin [21] and tunicamycin [22] had no effect on activity.

Studies through the 1960–1980s with cell-free preparations isolated from various mammalian tissues and cultured cell lines [a few examples include refs 23–26] found that the vertebrate and the streptococcal HAS enzymes appeared quite similar overall. As in the case of the *Streptococcus* HAS, vertebrate HAS activity is localized at the plasma membrane of the cell [24]. This subcellular location is unlike other glycosyltransferases involved in the glycosylation of glycoproteins or the elongation of the structurally related glycosaminoglycans (e.g., heparin, chondroitin) that reside in the Golgi apparatus. An interesting feature noted in these earlier

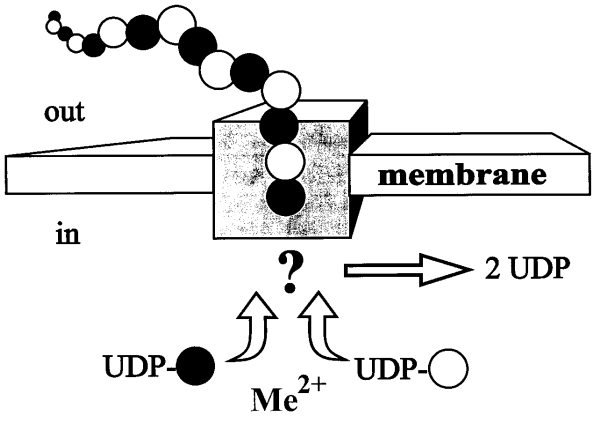

Figure 1. General model of HA biosynthesis. The HA synthase (gray cube) is found in intimate or close association with the membrane bilayer on the cell surface. Sugars (black or white circles) from UDP-sugar precursors are polymerized in the presence of divalent metal cations (Mg or Mn) by a monomeric HAS protein to form the HA chain. The HA polymer is typically 10^3–10^4 monosaccharides in length. HA is transported across the bilayer and out of the cell in an unknown fashion.

studies was that all the HAS enzymes had at least a two- to five-fold higher apparent affinity for the UDP-GlcUA substrate than for UDP-GlcNAc as measured by the Michaelis constant, K_m. Control of HA biosynthesis in vertebrates has been suggested to be in part due to the availability of UDP-GlcUA precursor. Therefore, alterations in the level and/or activity of UDP-Glc dehydrogenase (which converts UDP-Glc to UDP-GlcUA), a product of a single-copy gene in mammals [27], may be critical for controlling HA levels.

In the mid-1990s, we described two other HAS activities from *Pasteurella multocida* [28] and PBCV-1 virus [15] that were quite similar to the streptococcal and vertebrate enzymes except that the former two enzymes catalyze polymerization of HA in the presence of the Mn ion much more effectively than in the presence of Mg. HAS activity from both these sources was also localized to the membrane fraction.

After the cloning of some of the HAS genes (discussed later), experiments in the 1990s with recombinant enzyme preparations showed that the streptococcal and amphibian HASs polymerize the HA chain rapidly [29, 30]. These experiments were facilitated by the use of expression hosts that could not support HA production in vivo as they lacked the UDP-GlcUA precursor. Membrane preparations from these recombinant sources contain virgin enzymes lacking a nascent HA chain, which could be examined in vitro in a defined and synchronous fashion. In contrast, preparations of native HASs are predicted to contain a mixture of enzymes that contain nascent HA chains of various lengths. By measuring the size of the HA product produced by a recombinant HAS over time, rates of extension were calculated to be ~ 10–100 sugars/s in vitro. Therefore, native-size HA polymers of 1–10×10^6 Da could be synthesized in about 5–10 min. In the case of bacteria that divide every 20–60 min, this rapid production of HA allows the microbe to remain camouflaged from host defenses or adhered to the host tissues. For vertebrates, the potential capability to form a HA pericellular coat quickly may allow rapid modulation of cellular responses during phenomena such as development or wound-healing.

Molecular genetic identification of the HASs

The HAS proteins were identified by several means using the tools of molecular genetics. All of the known HAS glycosyltransferases appear to contain a single polypeptide species. No HAS was first identified by biochemical means alone due to their relatively low abundance in the cell and the difficulty manipulating membrane-associated proteins. In fact, some of these experiments either misidentified the protein species [31, 32] or evoked large multi-subunit complexes [33, 34].

Table 1. Nomenclature for HASs. A simplified nomenclature for the HASs uses the first letters of the genus and the species name of the source organism to identify the polypeptide. Only some examples of the vertebrate HASs are shown; homologous enzymes from other vertebrates including fish and fowl have been identified.

Enzyme	Species	Other names
spHas	*Streptococcus pyogenes* group A	HasA
seHas	*Streptococcus equisimilis* group C	–
xlHas1	*Xenopus laevis*	DG42, xhas1
hsHAS1, 2, and 3	*Homo sapiens*	HAS
mmHas1, 2, and 3	*Mus musculus*	HAS, CHASE, mHAS
cvHas	*Chlorella* virus PBCV-1	A98R
pmHas	*Pasteurella multocida* type A	PmHAS

To avoid confusion when discussing the distinct HA synthases and isozymes from various sources, a simplified nomenclature using the first letter of the genus and the species name should be utilized (table 1) [extrapolated from review, ref. 35]. The vertebrate HAS isozymes are numbered 1, 2, and 3 in order of their discovery.

Bacterial HASs

The genes for the Gram-positive group A streptococcal [36–38] and the Gram-negative *P. multocida* [39] bacterial HASs were identified by transposon mutagenesis with Tn*916*. Mutant cells in which the transposon element disrupted the HA biosynthesis locus were chosen by visual screening for loss of the capsular phenotype (small, dry colonies versus large, mucoid wild-type colonies). The transposon-tagged DNA was isolated by selective restriction enzyme digestion and gel electrophoresis. The purified DNA was directly sequenced and the data were utilized to generate DNA-based hybridization probes. Functional copies of the bacterial HAS genes, *spHas* (*hasA*) or *pmHas*, were then obtained from genomic DNA libraries in lambda phage. As described later, however, spHas and pmHas are not very similar at the protein level. In both cases, for group A streptococci and *P. multocida*, the bacterial UDP-Glc dehydrogenase gene was found directly downstream of the HAS gene. Expression of HAs in the heterologous bacterium, *Escherichia coli*, which normally does *not* make HA, was utilized to prove that the gene product from a single open reading frame conferred on the foreign host the ability to make HA polysaccharide [36, 37, 39]. Immunochemical purification of spHas utilizing an antibody generated against a synthetic peptide corre-

sponding to the deduced sequence also verified that a single polypeptide species was responsible for catalysis of HA elongation [29].

Vertebrate HASs

The spHas enzyme was the first HAS enzyme described at the molecular level and its sequence was therefore used as a query in a search in the protein database. Several types of protein were found to have regions of similarity to spHas: a *Xenopus laevis* (African clawed frog) protein with unknown function called DG42 (an unrecognized HAs, xlHas1), fungal chitin synthases, and *Rhizobium* bacterial nodulation factor (NodC) glycosyltransferases [37, 40]. The latter two enzymes polymerize long or short (β-1,4)GlcNAc polymers, respectively. The xlHas1 open reading frame, originally cloned as one of the most abundant message RNAs during gastrulation [41], was put into vaccinia virus [42] or *Saccharomyces cerevisiae* yeast [43] expression systems to verify that this polypeptide indeed synthesized HA.

In 1995–1996, several avenues were pursued to obtain the various mammalian HASs [reviewed in ref. 35]. Some laboratories performed direct expression cloning with mammalian host cells to identify some HAS isozymes from mouse and human. Thus far, no vertebrate HAS has been expressed in an active form in bacteria. In the first reported case, a mutant cell line defective in HA biosynthesis was corrected with a plasmid containing a HAS cDNA insert from a library [44]. In another library screening experiment, the cells containing a plasmid-borne HAS gene were able to bind to cell lines expressing CD44 (a vertebrate HA-binding protein) and were targeted for analysis [45]. Other groups capitalized on the amino acid sequence similarity between spHas and xlHas1 to obtain murine and human homolog genes via polymerase chain reaction [PCR] of cDNAs with degenerate oligonucleotides [46–48]. Cloned cDNAs were then transiently expressed in various host cell lines with either a low or no HA content. The transformed cells with the HAS plasmids formed pericellular HA coats.

Group C *Streptococcus* HAS: a correction to the literature

Degenerate PCR was utilized to obtain the authentic group C streptococcal gene, *seHas*, since hybridization with the heterologous spHas DNA probe was surprisingly ineffectual due to the widespread occurrence of numerous silent mutations and conservative substitutions in the nucleotide sequence [49]. On a protein basis, however, spHas and seHas are 72% identical. A major impact of describing the authentic seHas, a protein with an electrophoretic mobility of 42 kDa on denaturing gels, was that this report was irrefutable proof that a series of papers by the Prehm laboratory and collaborators had misidentified another streptococcal protein as HAS [31, 50]. The 56-kDa protein reported by the Prehm group was never shown to have HAS activity itself. Furthermore the 56-kDa protein did not have any sequence similarity to any streptococcal HAS or known glycosyltransferase; on the basis of homology, it is most likely a peptide transporter. Prehm and others also made a series of reports using an antiserum against the bacterial 56-kDa polypeptide to study the regulation and/or tissue distribution of an immunologically cross-reactive 50- to 52-kDa vertebrate protein erroneously identified as a HAS polypeptide [examples include refs 32, 51–53].

A viral HAS

The availability of the 330-kb *Paramecium bursaria Chlorella* virus (PBCV-1) genome sequence in the GenBank database allowed the discovery of an entirely unexpected HAS. The predicted viral open reading frame A98R was identified as a potential HAS by its similarity at the amino acid level ($\sim$30% identical) to the vertebrate and streptococcal HASs [15]. Virus-infected algal cells, but not healthy cells, produced extracellular HA fibers [15, 16]. Biochemical assay of the recombinant *E. coli*-derived A98R protein verified that it was an authentic HAS (cvHas) [15]. This report was both the first observation of a virus genome that encoded a glycosyltransferase that polymerizes a carbohydrate and the initial discovery of HA in the plant kingdom. It is also quite remarkable that the virus encodes several enzymes in the sugar precursor pathway including UDP-Glc dehydrogenase, which makes UDP-GlcUA, and glutamine:fructose-6-phosphate amidotransferase, which makes glucosamine [54].

General characteristics of the various HAS polypeptides: two classes of HAS

The amino acid sequences of streptococcal HASs, vertebrate HASs, and the viral HAS are rather distinct from the *Pasteurella* HAS. The first three enzymes are more similar at the amino acid level to the chitin synthases, Nod transferases, and certain bacterial capsule synthases [15, 37, 40]. On the other hand, pmHas is more similar to other distinct bacterial polysaccharide synthases and certain lipopolysaccharide glycosyltransferases [39]. On the basis of the differences in primary structure and predicted topology in the membrane, at least two classes of HAS may exist. We have proposed that the first three types of enzyme should be designated

class I HASs while thus far pmHas is the only known example of a class II HAS (table 2). Recent enzymological characterization of the spHas, xlHas1, and pmHas enzymes also supports the idea of two distinct classes of HAS [55].

Class I HASs

The first three types of HAS described at the molecular level, the streptococcal, the vertebrate, and the viral enzymes, are comparable in size, ranging from 417 to 588 residues (fig. 2). These enzymes appear to possess seven short sequence motifs located similarly throughout the central region of the polypeptide (table 3). A few of these putative motifs are similar to other glycosyltransferases that produce β-linked polysaccharides such as chitin, cellulose, and various bacterial capsular polysaccharides built from UDP-sugars. However, the exact role of these motifs in the structure and/or function of the polypeptide are not known. In view of the close amino acid sequence similarities among many glycosyltransferases, it is quite likely that these residues are involved in binding common determinants of UDP-sugars (e.g., uridine ring, phosphate groups) and/or catalyzing the transfer of sugar residues. Class I enzymes also have similar predicted topology in the membrane bilayer [35]. Five to seven membrane-associated regions are predicted in the various HASs, in agreement with the membrane localization of the enzyme activity. There appears to be a central cytoplasmic domain of about 260–320 residues flanked by two membrane-associated regions at the amino terminus and three to five membrane-associated regions at the carboxyl terminus. Most of the polypeptide chain is not exposed to the cell exterior. Experiments analyzing recombinant spHas with reporter enzyme fusions and with protease accessibility support these topological predictions in general [56; C. Heldermon, P. L. DeAngelis, and P. H. Weigel, unpublished data]. The exact nature of all these segments of the enzyme is not yet clear; specifically, the designation of a transmembrane spanning helix, reentrant helix, or a peripherally membrane-associated domain is difficult to assess for some regions.

Table 2. Two classes of HASs. Based on the differences in the predicted nature of the deduced polypeptide sequences as well as the potential reaction pathways, it appears that at least two distinct classes of HAS exist (N and C, amino and carboxyl termini, respectively; cyto, cytoplasmic domain; M, membrane-associated region; sol, non-membrane-associated region).

	Class I	Class II
Members	spHas, seHas, cvHas vertebrate Has1, 2 and 3	pmHas
Polypeptide size	417–588 residues	972 residues
Predicted topology	N-M-M-cyto-$(M)_{3-5}$-C	soluble *or* N-sol-M-sol-M-C
Molecular directionality of polymer growth	unclear	nonreducing terminus extension
Acceptor utilization	not likely	yes

Class II HAS

The *P. multocida* enzyme, pmHas, differs quite significantly from the other enzymes and is the only known member of class II (table 2). The 972-residue pmHas protein is roughly twice as large as the class I enzymes. The central portion of the pmHas polypeptide is quite similar to certain bacterial glycosyltransferases that synthesize capsules or lipopolysaccharides (fig. 3); this observation may indicate a catalytic domain within pmHas. Perhaps only two amino acid sequence motifs (no. 1 and 3 of table 3) are similar among the proposed class I and II groups. The pmHas enzyme is predicted to have two transmembrane helices by some computer analysis programs (e.g., TMpred), but other programs classify the enzyme as a soluble protein (e.g., SOSUI). In any case, most of the polypeptide chain is predicted *not* to be associated with the membrane on the basis of the primary structure. pmHas enzyme activity is found associated with the membrane fraction after lysis of the native [28] or recombinant [39] bacterial cells; therefore, it may be that pmHas associates with the membrane via an as yet unknown partner or a polymer transport apparatus.

Observations on the molecular mechanism of HA biosynthesis

Monomeric HASs catalyze polymer elongation

In comparison to the polypeptide size of many other glycosyltransferases and carbohydrate transport proteins, the HASs seem rather small to encode two distinct activities let alone possibly transport the hydrophilic and charged HA polymer out of the cell. The functional sizes of the glycosyltransferase catalytic activity of some of the HASs have been determined by radiation inactivation studies. This technique utilizes the general principle that a large-molecular-mass target is destroyed by a low dose of radiation, while smaller targets require higher doses to be inactivated [57]. No problematic detergent extraction or laborious purification is required with this methodology to determine the size of membrane proteins. The spHas and seHas functional units contain one monomer of the polypeptide [58]. As discussed later, the actual active enzyme is also hypothesized to contain $\sim$16 lipid molecules. Data from experiments with xlHas1 and pmHas

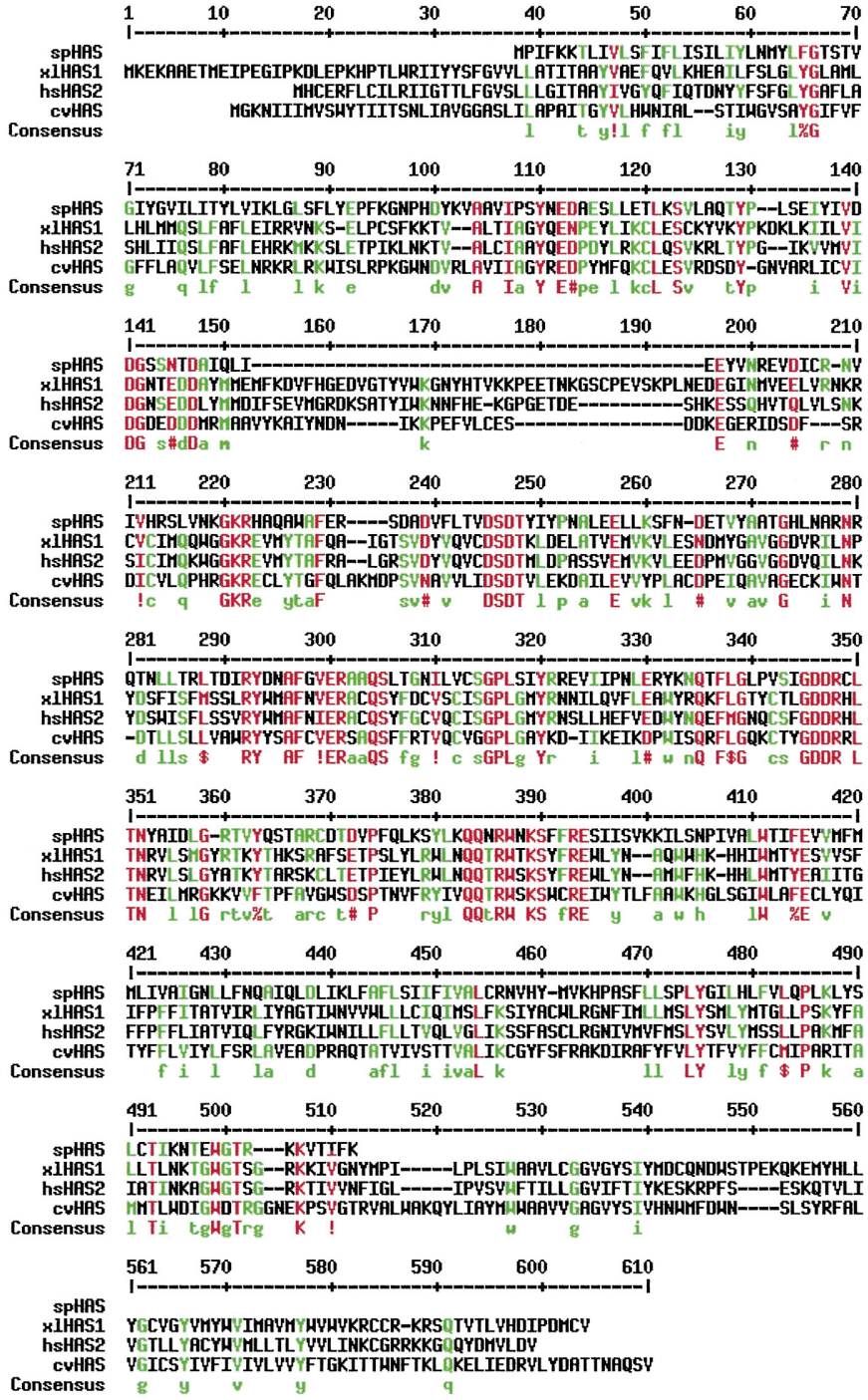

Figure 2. Comparison of class I HAS protein sequences. This multiple sequence alignment [Multalin program; 71] compares some representative members of the class I HAS family. The HAS protein sequences (in single-letter code) are remarkable similar in some regions (red letters signify identical or 90% consensus; green letters signify 50% consensus; consensus sequence symbols are: !, any one of I or V; $, any one of L or M; %, any one of Y or F; #, any one of N, D, E, or Q).

[P. E. Pummill, E. S. Kempner, and P. L. DeAngelis, unpublished data] also appear to be compatible with the single-polypeptide model. The role of lipids is not clear for the vertebrate enzymes, but pmHas does not appear to have a lipid or a membrane requirement [W. Jing and P. L. De Angelis, unpublished data].

Directionality of HA biosynthesis and utilization of acceptor oligosaccharides

The direction of HA biosynthesis catalyzed by any particular HAS polypeptide has two mutually exclusive possibilities: addition of the new sugar to the nonreducing terminus or to the reducing terminus. In the field of glycobiology, examples of both types of chain growth have been found for other oligosaccharides and polysaccharides. Another issue intertwined with directionality is the use and/or requirement for a primer molecule in HA biosynthesis. The HAS polypeptide could potentially add onto a distinct primer or acceptor in a repetitive fashion, but the enzyme might not be sufficient for initiating the HA molecule. As there appear to be two classes of HAS, there may be two answers for the directionality and primer questions. These issues have certainly posed a puzzling enigma over the last few decades [overview in ref. 17].

The first theoretical model of HA biosynthesis based on direct logic proposed that three active sites (on one or two proteins) were involved in transferring the sugars from precursors to the nonreducing terminus of the nascent chain in an alternating fashion [20]. Subsequent work by that laboratory on enzyme preparations containing native spHas utilizing selective labeling and degradation experiments gave supporting data with regard to the directionality of synthesis [21]. On the other hand, two other groups working with native mammalian HAS preparations using similar means concluded that HA was extended by addition of sugars to the reducing end [25, 59]. In another type of experiment, HA made in mammalian cells was reported to have a covalently attached UDP group as measured by incorporation of radioactivity derived from ^{32}P-labeled UDP-sugar into an anionic polymer, implying that the last sugar was transferred to the reducing end of the polymer [60]. Therefore, it remains unclear if these structurally similar HAS polypeptides from vertebrates and streptococci actually utilize different reaction pathways. It is obvious that direct experiments with defined systems utilizing purified enzyme will be necessary to address these issues.

Recently, it has been postulated that certain vertebrate HAS enzymes, *Xenopus* xlHas1 and a *Brachydanio* zebrafish homolog in particular, can produce chitin oligosaccharides under certain conditions [61, 62]. Another possibility forwarded was that chitin oligosaccharide primers are used to initiate HA chains and that polymerization would occur at the nonreducing terminus of the primer [62]. These theories were devised in part to explain some unexpected experimental results. The reports describe the synthesis of chitin oligosaccharides in vitro by extracts containing recombinant xlHas1 (produced in *E. coli* or murine systems) or by extracts of early vertebrate embryo [61, 62]. The addition of exogenous chitinase also reduced HA production catalyzed by embryos extracts in vitro [62]. Well-defined enzyme systems will be needed to address this difficult issue in the vertebrate system.

However, due to some unique enzymological attributes of pmHas, we could show that this enzyme adds monosaccharides to the nonreducing termini of the nascent HA chain [55]. This work was facilitated by the observation that recombinant pmHas could extend exogeneously supplied oligosaccharides derived from testicular hyaluronidase digests of HA (e.g., GlcNAc → GlcUA → GlcNAc → GlcUA) in vitro. An intact nonreducing end acceptor was required because the unsaturated GlcUA residue (which is missing the hydroxyl group to be extended) of *Streptomyces* HA-lyase-generated oligosaccharides was not elongated by pmHas. On the other hand, the reducing end pyranose residue could be opened by treatment with borohydride with no effect on the ability of the oligosaccharide to serve as an acceptor. Recombinant pmHas exhibits considerable selectivity for the acceptor oligosaccharide. The monosaccharides, GlcUA or GlcNAc, either separately or in combination at high concentrations, did not serve as acceptors. Heparosan oligosaccharides, which have the same sugar composition as HA but different glycosidic linkages, also did not serve as acceptors.

Table 3. Potential common sequence motifs of HASs. Seven short stretches of amino acid sequence (single-letter code) appear to be very similar among the class I HASs. These motifs appear with the same relative order and approximate spacing in all of the polypeptide chains. pmHas, the class II enzyme, may possess slight variants of motif nos. 1 and 3. Other glycosyltransferases (e.g., chitin and cellulose synthases) may possess motifs similar to nos. 1, 3, 6 and 7. No functional or structural roles have yet been assigned for these motifs. (Start residue refers to the consensus sequence numbering system in fig. 2; bold residues are identical; x designates a more variable residue).

Motif No.	Start residue	Sequence
1	139	**V**(D/I)**DG**xx(N/E/D)(T/D)**D**
2	219	(G/K/R)**GKR**(E/H)
3	245	**DSDT**x(L/I)
4	292	**RY**xx**AF**x(I/V)**ER**(A/S)(C/A)**QS**
5	312	(C/V)(**I** *or* V/C)(S/G)**GPL** (G/S)x**Y**(R/K)
6	345	**GDDRxLTN**
7	383	**QQ**(T/N)**RW**(N/T/S)**KS**(F/Y/W) (F/C)**RE**

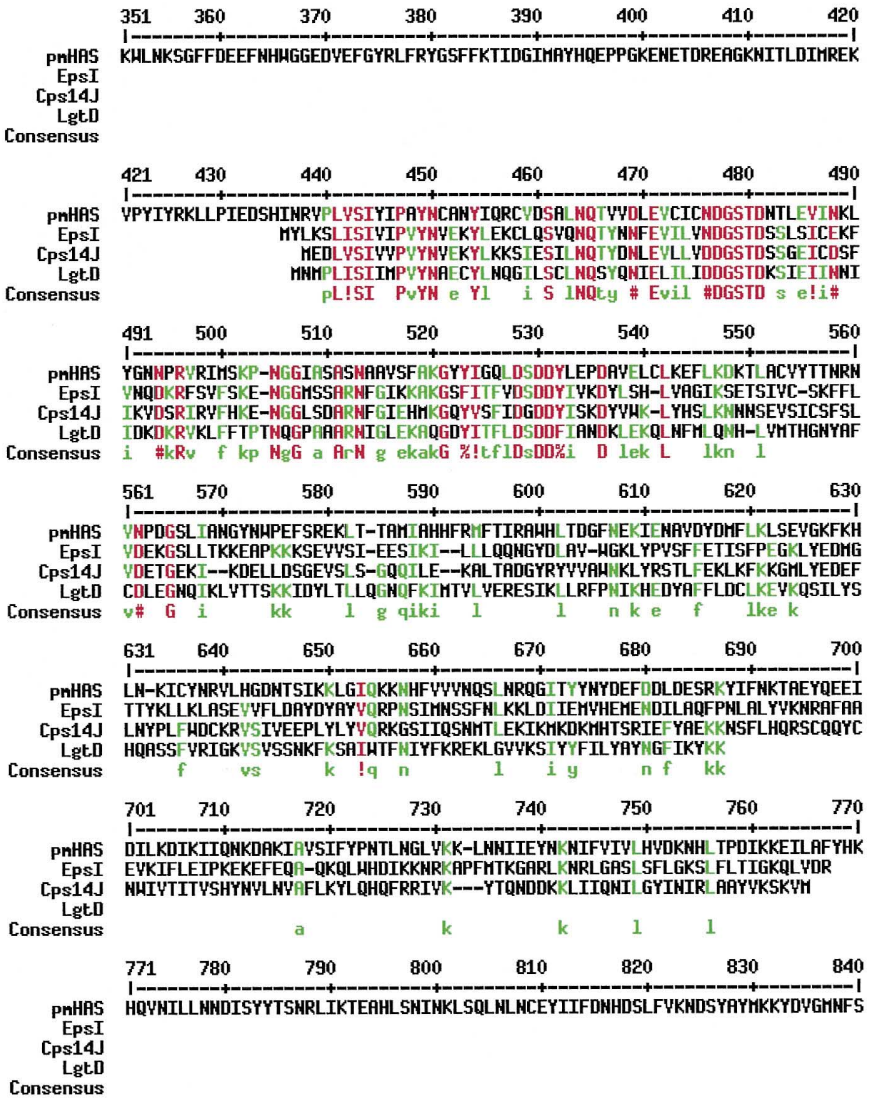

Figure 3. Comparison of pmHas, a class II HAS, to other glycosyltransferases. This multiple sequence alignment (as described in fig. 2) illustrates that the central region of the pmHas protein (residues 351–836) is similar to other bacterial glycosyltransferases involved in the biosynthesis of capsular polysaccharide (*Streptococcus thermophilus* EpsI, *S. pneumoniae* Cps14J) or lipopolysaccharide (*Haemophilus influenzae* LgtD homolog). The grouping of these other enzymes in the alignment may signify a domain structure within pmHas.

Chitooligosaccharides neither stimulated pmHas-catalyzed HA production nor served as an acceptor.

However, the HA-derived acceptor was not absolutely required; reactions containing recombinant pmHas enzyme (that lacks a nascent HA chain) without acceptor oligosaccharide still produced HA, albeit at lower rates. This observation suggests that polymerization of the first few sugars of the HA chain is the rate-limiting kinetic step in vitro which can be circumvented by addition of HA oligosaccharides.

Therefore, pmHas may use a carbohydrate binding site to retain the nascent HA at the active site; the exogeneously supplied HA-oligosaccharide can simply occupy this site of recombinant pmHas and be elongated into a polymer in vitro. Interestingly, we observed that neither recombinant spHas nor recombinant xlHas1 utilized the same oligosaccharides in an analogous fashion [55]. Likewise, earlier studies with native spHas reported that various HA-derived fragments neither stimulated nor inhibited HAS activity in vitro [21].

Overall, these findings further support the class I/class II distinction of HASs.

Formation of the disaccharide repeat unit

Recent work and supposition on the *Acetobacter* bacterial glycosyltransferase that produces cellulose, a β(1,4)-linked glucose homopolysaccharide, have led to theories that both glycosidic bonds of the disaccharide unit are formed simultaneously [63, 64]. The completed disaccharide would then move out of the active site, and the next two precursors would enter and be coupled. As the UDP-sugar precursors are α-linked, performing both transfers at the same time by an inversion step would remove the need for either the enzyme or the substrate to rotate during catalysis. This strategy was in part invoked to remove potential topological hindrances in producing the insoluble cellulose fibrils. As many glycosyltransferases that make β-linked polysaccharides appear to share some sequence motifs, this simultaneous disaccharide formation theory was extended to other enzymes including the class I HASs [63, 64].

We showed experimentally that recombinant pmHas, a class II enzyme, adds single monosaccharides in a sequential fashion to the nonreducing termini of the nascent HA chain [55]. This general mechanism is also utilized to form the disaccharide repeat structure of the other major glycosaminoglycans, heparin and chondroitan [reviewed in ref. 65]. pmHas elongates HA-derived acceptors ($\geq$ tetramer) into polymers containing hundreds of sugars in vitro if both UDP-sugar precursors are provided simultaneously. However, if only a single, appropriate UDP-sugar precursor was supplied, then the acceptor oligosaccharide was lengthened by one sugar at the nonreducing terminus. Individual transfer of either GlcUA or GlcNAc was demonstrated utilizing acceptors that terminated at the nonreducing end with GlcNAc or GlcUA, respectively. Therefore, HA biosynthesis by pmHas does not require the simultaneous formation of the disaccharide repeat unit to generate the alternating structure of the HA molecule. The intrinsic specificity and fidelity of each half-reaction (e.g., GlcNAc added to a GlcUA residue or vice versa) is apparently sufficient to synthesize authentic HA chains. For the class I enzymes, no rigorous proof has been reported for the reaction pathway of polymer growth.

Modulation of transferase activity by substrates

In early studies of cell-free streptococcal extracts, it was noted that the Michaelis-Menten plots for the dependence of HAS activity on UDP-sugar concentration had a sigmoidal curve for UDP-GlcNAc but a rectangular hyperbolic curve for UDP-GlcUA [21]. Recently, kinetic characterization of crude membrane-bound and purified, lipid-reconstituted streptococcal HASs was undertaken [66]. In both cases, the apparent K_m values increased four- to tenfold upon purification but the calculated V_{max} remained at least 50% that of the crude membranes. Cosubstrate inhibition was detected in which high levels of one sugar nucleotide (e.g., UDP-GlcUA) altered the apparent kinetic values for the other precursor (e.g., UDP-GlcNAc). These observations led to a 'cross-talk' hypothesis: the actions and forces at one transferase site affect the activity of the other site.

Control of HA chain length

The differences in size distribution of the HA products from the reactions catalyzed by various HA synthases and isozymes in vitro suggest that HA polymer size is controlled in part by the nature of the polypeptide. Yeast-derived recombinant forms of spHas and xlHas1 assayed under virtually identical conditions resulted in HA with different size profiles [30]. The size profiles of the HA polymers produced by the recombinant murine Has1 and Has2 isozymes were larger than Has3 expressed in the identical mammalian cell line [48, 67].

The mechanism for controlling the polysaccharide size distribution is not yet defined, but several alternatives are obvious. First, a region or a site on the HAS polypeptide might sense polymer length directly or indirectly, and then modulate activity of the catalytic site. This feat may be unusual as the HA chain can be extremely long ($\sim$0.1–2 μm) and somehow the length information must reach the active site over a distance. One possibility is that HAS possesses a force sensor that causes HA chain release or halts the chain polymerization when a certain amount of dynamic force (produced by shear or Brownian motion of the solvent) pulls on the chain. This mechanism may be as simple as each particular HAS active site retaining the nascent HA chain with only so much strength. For example, a stronger interaction between protein and carbohydrate would yield a longer polymer on average before the HA chain was ripped way from the enzyme. In another perhaps less likely scenario, some sort of 'molecular ruler' (possibly mediated by a distinct protein) may communicate when a specific length is spanned by the nascent HA chain and a signal may be transmitted to the active site to shut down transferase activity. Second, subtle differences in the kinetics (e.g., substrate binding affinity, transfer rate) between the HAS polypeptides may result in size distribution differences. However, some comparisons among the bacterial and vertebrate HASs show that the range of K_m values and transfer rates measured in vitro are not very dissimilar.

Role of lipids in HAS function

HASs are membrane associated, and the class I enzymes are predicted to be integral membrane proteins, raising the question of the role of lipids in catalytic activity and/or polymer transport. Earlier work focused on purifying native spHas found that detergent extraction of streptococcal membrane preparations resulted in the loss of most HAS activity; reconstitution of the extract with the lipid cardiolipin, however, restored the activity of the preparation [68]. Subsequent detailed analysis of purified recombinant spHas and seHas showed that the enzyme had a preference for the lipid cardiolipin, but phosphatidylserine could also stimulate HAS activity [69]. Some differences in lipid preference were observed for this pair of streptococcal enzymes. For example, phosphatidic acid and phosphatidylethanolamine stimulated seHas but not spHas. Certain other lipids, such as phosphatidylcholine, sphingomyelin, and sulfatides, did not stimulate or even inhibited HAS activity of both streptococcal enzymes. Therefore, the streptococcal enzymes were interpreted as having a specific binding site for a lipid that was a cardiolipin or structurally related molecule [69]. The putative endogenous native lipid has not yet been identified.

The most active lipid in vitro, cardiolipin, changed the apparent K_m or relative affinity for its UDP-sugar substrates of the enzyme [69]. The K_m of UDP-GlcUA increased and the value for UDP-GlcNAc decreased. Overall, however, the maximal velocity increased. Using radiation inactivation to determine the target size of the functional unit of spHas and seHas, it was concluded that some extra mass was present in addition to the contribution of the mass of the HAS polypeptide (calculated from the deduced sequence and measured experimentally by mass spectroscopy) [58]. Experiments involving solubilization and reconstitution with cardiolipin suggested that a tightly bound lipid component was essential for activity. About 16 lipid molecules and a spHas or a seHas monomer were hypothesized to be the functional unit of streptococcal HAS [58]. These lipids were proposed to participate in the formation of a pore through the membrane to facilitate transport of the polar HA polymer across the hydrophobic core of the membrane bilayer [69].

Similar experiments to assess the role of lipids in the vertebrate HASs have not been successful. In most cases, the enzyme loses most or all of its ability to polymerize HA in vitro upon detergent treatment. As the enzyme is found in very small amounts in the cell, no purification has been effected in high enough yield to test rigorously the role of lipids. However, it is likely that all class I enzymes utilize the same general mechanisms for HA biosynthesis.

Preliminary experiments with recombinant pmHas suggest that a membrane bilayer and/or a bound lipid component are not required for catalysis of HA polymerization [W. Jing and P. L. DeAngelis, unpublished data]. In encapsulated Gram-negative bacteria such as *E. coli* and *Haemophilus*, several proteins encoded in the capsule biosynthesis loci are essential to transport the polysaccharide out of the cell. The mechanism of polymer translocation through two membrane bilayers is still the target of much investigation. It may be that the pmHas polypeptide is responsible for HA catalysis, and that other proteins either perform or assist in polymer translocation across the membrane.

Evolutionary relationships of HAS

Even with the availability of the deduced amino acid sequences of many polysaccharide glycosyltransferases, their evolutionary relationships are not clear. Are these enzymes part of a single ancestral superfamily or did multiple families arise independently? Has the same catalytic mechanism been developed several times over the eons? It is quite remarkable that the class I HASs from distinct organisms, ranging from an algal virus to certain Gram-positive bacteria to human, possess similar motifs. The origin of the streptococcal enzyme has been postulated to be a case of recent horizontal transfer of a HAS gene from the mammalian host to a pathogenic bacterium [35]. However, it may be more likely that the enzyme was the result of functional convergent evolution. spHas and seHas have closer sequence similarity to the *Streptococcus pneumoniae* type III enzyme Cps3S, which makes a Glc-GlcUA polymer, than to members of the vertebrate HAS family. Furthermore, the bacterial UDP-Glc dehydrogenase gene is tightly linked with the *spHas* or *pmHas* genes. This close association between the precursor-forming enzymes and the polysaccharide glycosyltransferases is typical for other encapsulated bacterial pathogens. In contrast, the human and the murine UDP-Glc dehydrogenase genes reside on a separate chromosome [27] from any of the three HAS genes [70].

pmHas may also be a case of a clever pathogenic bacterium evolving a HAS among all the possibilities of anionic carbohydrate polymers. The selective advantage of producing a capsule of relatively nonimmunogenic material added to their virulence and these microbes flourished. pmHas and the streptococcal HASs were probably founded by distinct lineages of polysaccharide glycosyltransferases because the polypeptides differ so greatly in sequence and in predicted topology.

The evolution of the vertebrate HAS enzyme family appears to involve the typical scenario of gene duplication and divergence [67]. The position and nature of the exon/intron junctions as well as sequence analysis of *HAS* genes of frog, mouse, and human imply that the

initial ancestral *HAS* in animals was duplicated to form a HAS1 lineage and a HAS2 lineage. A subsequent pair of duplication events early in the vertebrate phylogenetic tree resulted in (i) the formation of *HAS1* and a HAS-related homolog, and (ii) the formation of *HAS2* and *HAS3*. In *Xenopus*, the HAS-related gene (called *xHas-rs*) may be a pseudogene, as the recombinant protein was found to be inactive in all assays in vitro [67]. In mammals, no such related counterpart has been detected, and it was suggested that it was probably lost from the chromosome [67]. Preliminary work with amphioxus (*Branchiostoma floridae*), a primitive invertebrate chordate, suggests that the *HAS* gene family evolved well before the emergence of the vertebrate lineage [A. P. Spicer, personal communication].

A very intriguing, but as yet unclear, tale is the acquisition of a HAS gene by the *Paramecium bursaria Chlorella* virus. This viral protein is only slightly more similar to the vertebrate enzymes than to the bacterial HASs [30 vs. 26% identity, respectively; 15]. Thus far no other algal or plant glycosyltransferase has strong similarity to cvHas. No introns are seen in the viral HAS sequence (this interesting virus actually has *two* types of introns in a few other genes). The bacterial HA biosynthesis loci cluster the required HAS and UDP-Glc dehydrogenase genes in an operon. In contrast, the viral UDP-Glc dehydrogenase gene (*A609L*) is found ~ 240 kilobases away from the *cvHas* gene. A609L is more similar to the bacterial UDP-Glc dehydrogenases than the eukaryotic enzymes [51–56 vs. 25% identity, respectively; 54]. The glutamine:fructose-6-phosphate amidotransferase (GFAT) or GlcNAc synthase gene (*A100R*) is, instead, adjacent to the *cvHas* gene. The viral GFAT enzyme is of about equal similarity to bacterial and eukaryotic proteins [40–47 vs. 32–34% identity, respectively; 54]. The virus must have acquired or engineered a HAS gene quite some time ago because distinct isolates from freshwater worldwide (e.g., U.S.A., Argentina, China) possess the ability to make HA [16].

Summary of potential future research areas

The HASs are rather small polypeptides that are fully capable of catalyzing the polymerization of the heteropolysaccharide HA. The initial steps of HA chain formation may prove to be unique and distinct from the repetitive mechanism of HA chain elongation. The identity, the number, and the nature of the UDP-sugar substrate binding sites are as yet unknown. The mechanism of catalysis for other glycosyltransferases has been posited as the reverse of the degradative hydrolytic mechanism, but no rigorous chemical, kinetic, or genetic tests have been reported for any biosynthetic enzyme. The potential role of the HAS enzyme and bound lipid in transporting or translocating the nascent HA polymer across the membrane is still under investigation. Regulation of the size of the HA polymer has potentially great significance for altering cell behavior or adhesion, but the mechanism is not known. The potential similarities and differences of the cell surface HASs to the other Golgi-bound glycosaminoglycan synthases should also prove interesting. The evolutionary relationships of the HASs themselves and to the myriad glycosyltransferases should prove captivating to both biochemists and bioinformaticians.

Answers to these issues should aid our overall pursuit of good health and beneficial biotechnology as well as enhance our appreciation of Nature's enzymatic solutions for biosynthesis.

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