Molecular Directionality of Polysaccharide Polymerization by the Pasteurella multocida Hyaluronan Synthase*

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Hyaluronan (HA), a long linear polymer composed of alternating glucuronic acid and N-acetylgalactosamine residues, is an essential polysaccharide in vertebrates and a putative virulence factor in certain microbes. All known HA synthases utilize UDP-sugar precursors. Previous reports describing the HA synthase enzymes from Streptococcus bacteria and mammals, however, did not agree on the molecular directionality of polymer elongation. We show here that a HA synthase, PmHAS, from Gram-negative P. multocida bacteria polymerizes the HA chain by the addition of sugar units to the nonreducing terminus. Recombinant PmHAS will elongate exogenous HA oligosaccharide acceptors to form long polymers in vitro; thus far no other HA synthase has displayed this capability. The directionality of synthesis was established definitively by testing the ability of PmHAS to elongate defined oligosaccharide derivatives. Analysis of the initial stages of synthesis demonstrated that PmHAS added single monosaccharide units sequentially. Apparently the fidelity of the individual sugar transfer reactions is sufficient to generate the authentic repeating structure of HA. Therefore, simultaneous addition of disaccharide block units is not required as hypothesized in some recent models of polysaccharide biosynthesis. PmHAS appears distinct from other known HA synthases based on differences in sequence, topology in the membrane, and putative reaction mechanism.

Polysaccharides are the most abundant biomaterials on earth, yet many of the molecular details of their biosynthesis and function are not clear. HA is a linear polysaccharide of the glycosaminoglycan class composed of up to thousands of β(1,3)GlcNAc-β(1,4)GlcUA repeats. In vertebrates, HA is a major structural element of the extracellular matrix and plays roles in adhesion and recognition (1). HA has a high negative charge density and numerous hydroxyl groups; therefore, the molecule assumes an extended, hydrated conformation in solution. The viscoelastic properties of cartilage and synovial fluid are in part the result of the physical properties of the HA polysaccharide. HA also interacts with proteins such as CD44, RHAMM, and fibrinogen, thereby influencing many natural processes such as angiogenesis, cancer, cell motility, wound healing, and cell adhesion (2).

HA is also made by certain microbes. Some bacterial pathogens, namely Gram-negative Pasteurella multocida Type A and Gram-positive Streptococcus Group A and C, produce extracellular HA capsules (3, 4); this coating protects the microbes from host defenses including complement and phagocytosis (5, 6). The Paramyxovirus virus (PBCV-1) directs the algal host cells to produce a HA surface coating early in infection, but the biological role of HA in the viral life cycle is not yet known (7).

The various HA synthases, the enzymes that polymerize HA, utilize UDP-GlcUA and UDP-GlcNAc sugar nucleotide precursors in the presence of a divalent Mn2+ or Mg2+ ion (7–9). The HA synthase activity from all sources is localized in the membrane fraction. The enzymes were all identified by molecular genetic means due to the innate problems of membrane protein purification. In all cases, a single species of polypeptide catalyzes the transfer of two distinct sugars; in contrast, the vast majority of other known glycosyltransferases transfer only one monosaccharide.

HasA (or SpHAS) from Group A Streptococcus pyogenes was the first HA synthase to be described at the molecular level (10). The various vertebrate homologs (Xenopus frog DG42 or XHAS1; murine and human HAS1, HAS2, and HAS3) and the viral enzyme, A98R, are quite similar at the amino acid level to certain regions of the HasA polypeptide chain (~30% identity overall). At least 7 short motifs (~5-9 residues) interspersed throughout these enzymes are identical or quite conserved. The evolutionary relationship among these HA synthases from such dissimilar sources is not clear at present. The enzymes are predicted to have a similar overall topology in the bilayer; membrane-associated regions at the amino and the carboxyl termini flank a large cytoplasmic central domain (~200 amino acids; reviewed in Ref. 8). The amino-terminal region appears to contain two transmembrane segments, whereas the carboxyl-terminal region appears to contain three to five membrane-associated or transmembrane segments depending on the species. Very little of these HAS polypeptide chains are expected to be exposed to the outside of the cell.

With respect to the reaction pathway utilized by this group of enzymes, mixed findings have been reported from indirect experiments. The Group A streptococcal enzyme was reported to add sugars to the nonreducing terminus of the growing chain as determined by selective labeling and degradation studies (11). Using a similar approach, however, two laboratories working with the enzyme preparations from mammalian cells concluded that the new sugars were added to the reducing end of the nascent chain (12, 13). In comparing these various studies, the analysis of the enzymatically released sugars from the streptococcal system added more rigorous support for their
interpretation (11). In another type of experiment, HA made in mammalian cells was reported to have a covarly attached UDP group as measured by an incorporation of low amounts of radioactivity derived from $^{32}$P-labeled UDP-sugar into an anion polymer (14). These data implied that the last sugar was transferred to the reducing end of the polymer. Thus it remains unclear if these rather similar HAS polypeptides from vertebrates and streptococci actually utilize different reaction pathways.

We recently reported the identification and molecular cloning of a unique HA synthase, PmHAS, from the fowl cholera pathogen, Type A P. multocida (15). Expression of this single 972-residue protein allowed the Escherichia coli host cells to produce HA capsules in vivo; normally, E. coli does not make HA. Overall, the deduced PmHAS sequence is very different from the other known HA synthases. There appear to be only two short potential sequence motifs ((D/N)DGS(S/T); DSD(D/T/Y)) in common between PmHAS and Group A HasA. Instead, a portion of the central region of the new enzyme is more homologous to the amino termini of other bacterial glycosyltransferases that produce different capsular polysaccharides or lipopolysaccharides. Furthermore, even though PmHAS is about twice as long as any other HAS enzyme, it only has two predicted transmembrane-spanning helices separated by ~320 residues. Thus at least a third of the polypeptide is predicted not to be in the cytoplasm.

In this report, definitive proof is presented that PmHAS adds sugars to the nonreducing end of the growing polymer chain, in contrast to the reports with mammalian enzymes. Furthermore, it is shown that the correct monosaccharides are added sequentially in a stepwise fashion to the nascent chain. This is the first direct demonstration of HA polyascharide polymerization in such a fashion.

**EXPERIMENTAL PROCEDURES**

All reagents were from Sigma or Fisher unless noted otherwise.

**HA Synthase Isolation and Assays—**Membrane preparations containing recombinant PmHAS (rPmHAS) (GenBank™ accession number AP036904) were isolated from E. coli DH5α:pPmHAS as described (15). Membrane preparations containing native PmHAS were obtained from the P. multocida strain P-1059 (ATCC #15742) as described (9), except that 1 mM β-mercaptoethanol was substituted for thioglycollate throughout the procedure. PmHAS was assayed in 50 mM Tris, pH 7.2, 20 mM MnCl$_2$, and UDP-sugars (UDP-[14C]GlcUA, 0.3 Ci/mmol; NEN Life Science Products) and UDP-GlcNAc at 30 °C. The reaction products were analyzed by various chromatographic methods as described below. Membrane preparations containing other recombinant HAS enzymes, Group A streptococcal HasA or Xenopus DG42 produced in the yeast Saccharomyces cerevisiae, were prepared as described previously (16).

**Acceptor Oligosaccharides—**Uronic acid was quantitated by the carbazole method (17). Even-numbered HA oligosaccharides ([GlcNAc-GlcUA]$_n$) were generated by degradation of HA (from Group C Streptococcus) with either ovine testicular hyaluronidase Type V ($n = 2–5$) or Streptomyces hyaluronidicus HA lyase ($n = 2$ or $3$) in 30 mM sodium acetate, pH 5.2, at 30 °C overnight. The latter enzyme employs an elimination mechanism to cleave the chain, resulting in an unsaturated ΔGlcUA residue at the nonreducing terminus of each fragment (18). For further purification and desialylation, some preparations were subjected to gel filtration with P-2 resin (Bio-Rad) in 0.2 M ammonium formate and lyophilization. Odd-numbered HA oligosaccharides ([GlcNac-GlcUA-GlcNAc]$_n$) ending in a GlcNAc residue were prepared by mercuric acetate treatment of partial HA digests generated by HA lyase ($n = 2–7$; gift of Dr. G. Sugumaran; Ref. 19). The masses of the HA oligosaccharides were verified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Sugars in water were mixed with an equal volume of 5 mg/ml 6-azo-2-thiothymine (Azo) and 0.2 M sodium acetate and quickly dried on the target plate. The negative ions produced by pulsed nitrogen laser irradiation were analyzed in linear mode (20-kV acceleration; Perceptive Voyager™).

Other oligosaccharides that are structurally similar to HA were also tested in HAS assays. The structure of heparan pentamer derived from E. coli K5 capsular polysaccharide is (β1,4GlcNACα1,4)-
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FIG. 2. Time course of HA polymerization; effect of HA oligosaccharides. Two parallel reactions containing rPmHAS with even-numbered HA oligosaccharides (105 μg of membrane protein/point with a mixture of HA hexamer, octamer, and decamer, 4.4 μg total (solid circles)) or 6-fold more rPmHAS without oligosaccharide acceptor (630 μg of protein/point (open circles)) were compared. The enzyme preparations were added to prewarmed reaction mixtures containing UDP-[3H]GlcUA (240 μM, 6 × 10^4 dpm/point) and UDP-GlcNAc (600 μM) in assay buffer. At various times, 50-μl aliquots were withdrawn, terminated, and analyzed by paper chromatography. The exogenously supplied acceptor accelerated the bulk incorporation of sugar precursor into polymer product by PmHAS, but the acceptor was not absolutely required.

FIG. 3. HA tetramer elongation into larger polymers by rPmHAS. Gel filtration analysis on Sephacryl S-200 (20-ml column, 0.7-ml fractions) shows that rPmHAS makes HA polysaccharide using HA tetramer acceptor and UDP-sugars. Dextrans of ≥80 KDa (≥ 400 monosaccharides) elute in the void volume (Vo, arrow). The starting tetramer elutes in the included volume (Vi, arrow). Membranes (190 μg of total protein), UDP-GlcUA (200 μM), UDP-GlcNAc (600 μM), and radiolabeled [3H]HA tetramer (1.1 × 10^4 dpm) were incubated for 3 h before gel filtration (solid squares). As a negative control, a parallel reaction containing all the components except for UDP-GlcNAc was analyzed (open squares). The small primer was elongated into higher molecular weight product if both precursors were supplied.

unsaturated sugar is missing the C4 hydroxyl of GlcUA (18), which would normally be extended by the HA synthase. The lack of subsequent polymerization onto this terminal unsaturated sugar is analogous to the case of dideoxynucleotides causing chain termination if present during DNA synthesis. A closed pyranose ring at the reducing terminus was not required by PmHAS, since reduction with borohydride did not effect the ability of the HA tetramer to serve as an acceptor; this finding also allowed the use of borotritide labeling to monitor the fate of oligosaccharides.

Other Recombinant HASs Do Not Utilize HA Oligosaccharide Acceptors—Neither recombinant Group A HasA nor recombinant DG42 produced in yeast elongated HA-derived oligosaccharides into larger polymers. First, the addition of HA tetramer or a series of longer oligosaccharides neither stimulated nor inhibited the incorporation of radiolabeled UDP-sugar precursors into HA by these enzymes significantly (≥ ±5% control value). In parallel experiments, the HAS activity of HasA or DG42 were not affected by the addition of chitin-derived oligosaccharides (data not shown). Second, the recombinant enzymes did not elongate radiolabeled HA tetramer in the presence of UDP-sugars (Table 1). These same preparations of enzymes, however, were highly active in the conventional HAS assay in which radiolabeled UDP-sugars were polymerized into HA.

rPmHAS Elongates HA via Stepwise Addition of Single Sugars—TLC was utilized to monitor the PmHAS-catalyzed elongation reactions containing 3H-labeled oligosaccharides and various combinations of UDP-sugar nucleotides. Fig. 4A clearly shows that rPmHAS elongated HA-derived tetramer by a single sugar unit if the next appropriate UDP-sugar precursor was available in the reaction mixture. GlcNAc derived from UDP-GlcNAc was added onto the GlcUA residue at the nonreducing terminus of the tetramer acceptor to form a pentamer. On the other hand, inclusion of only UDP-GlcUA did not alter the mobility of the oligosaccharide. If both HA precursors were supplied, then various longer products were made. In parallel reactions, control membranes prepared from host cells with vector plasmid did not alter the mobility of the radiolabeled HA tetramer under any circumstances (not shown). In similar analyses monitored by TLC, rPmHAS did not utilize labeled chitopentaose as an acceptor (Fig. 4B).

HA-derived oligosaccharides with either GlcUA or GlcNAc at
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The various recombinant enzymes were tested for their ability to convert HA tetramer into higher molecular weight products. The reactions contained radiolabeled HA tetramer (5–8 × 10^5 dpm), 750 μM UDP-GlcNac, 360 μM UDP-GlcUA, 20 mM XCL1, 50 mM Tris, pH 7–7.6 (the respective X cation and pH values for used for each enzyme were: PmHAS, Mn^2+; 7.2; Xenopus DG42, Mg2^+/7.6; Group A streptococcal HasA, Mg2^+;7.0), and enzyme (units/reaction listed). As a control, parallel reactions in which the metal ion was chelated (22 mM EDTA final; EDTA column, rows with +1 were tested; without free metal ion, the HAS enzymes do not catalyze polymerization. After a 1-h incubation, the reactions were terminated and subjected to descending paper chromatography. Only rPmHAS could elongate HA tetramer even though all three membrane preparations were very active in the conventional HAS assay (incorporation of 1[^14C]GlcUA from UDP-GlcUA into polymer when supplied UDP-GlcNac). r-, recombinant.

| Enzyme       | Units | EDTA | Incorporation of HA4 into polymer (pmol) |
|--------------|-------|------|----------------------------------------|
| rPmHAS       | 6b    | –    | 240                                    |
| rHasA        | 9,800 | +    | ≤0.2                                   |
| rDG42        | 11,500| +    | ≤0.1                                   |

^a pmol of GlcUA transfer/h in the conventional HAS assay.

Secondly, the nascent polymer chain is still bound to the nascent chain. This feature is particularly relevant for HA elongation at the active site of the enzyme. Possible mechanisms for maintaining the growing polymer chain at the active site of the enzyme are immediately obvious. First, the enzyme possesses a carbohydrate polymer binding pocket or cleft. Second, the nascent chain is covalently attached to the enzyme during its synthesis. Third, the enzyme binds to the nucleotide base or the lipid moiety of the precursor while the nascent polymer chain is still covalently attached. Thus far, the molecular details of the vast majority of polysaccharide synthases are lacking.

**PmHAS and Acceptor Oligosaccharides**—The HAS activity of the native PmHAS enzyme found in *P. multocida* membrane preparations was not stimulated by addition of HA oligosaccharides; theoretically, the endogenous nascent HA chain initiated in vivo renders the exogenously supplied acceptor unnecessary. However, recombinant PmHAS produced in an E. coli strain that lacks the UDP-GlcUA precursor and, thus, lacks a nascent HA chain, is able to bind to and to elongate exogenous HA oligosaccharides. As mentioned above, there are three likely means for a nascent HA chain to be held at or near the active site. In the case of PmHAS, it appears that a HA-binding site exists near or at the active site of the putative HA polymer-binding pocket of PmHAS will bind and elongate at least an intact HA trisaccharide (reduced tetramer). The monosaccharides GlcUA or GlcNAc, however, even in combination at high concentration, are not effective acceptors. Oligosaccharide binding to PmHAS appears to be quite selective, because the heparosan pentamer, which only differs in the glycosidic linkages from HA-derived oligosaccharides, does not serve as an acceptor. Future studies will further examine the structural requirements for the acceptor molecule as well as the identity of the oligosaccharide-binding site on the PmHAS polypeptide.

**DISCUSSION**

Potential Polymer Retention Mechanisms—An intrinsic and essential feature of polysaccharide synthesis is the repetitive addition of sugar monomer units to the growing polymer. The glycosyltransferase is expected to remain in association with the nascent chain. This feature is particularly relevant for HA biosynthesis, as the HA polysaccharide product in all known cases is transported out of the cell; if the polymer was released, then the HAS would not have another chance to elongate that particular molecule. Three possible mechanisms for maintaining the growing polymer chain at the active site of the enzyme are immediately obvious. First, the enzyme possesses a carbohydrate polymer binding pocket or cleft. Second, the nascent chain is covalently attached to the enzyme during its synthesis. Third, the enzyme binds to the nucleotide base or the lipid moiety of the precursor while the nascent polymer chain is still covalently attached. Thus far, the molecular details of the vast majority of polysaccharide synthases are lacking.

**Acceptor use of various recombinant HA synthases**

The various recombinant enzymes were tested for their ability to convert HA tetramer into higher molecular weight products. The reactions contained radiolabeled HA tetramer (5–8 × 10^5 dpm), 750 μM UDP-GlcNac, 360 μM UDP-GlcUA, 20 mM XCL1, 50 mM Tris, pH 7–7.6 (the respective X cation and pH values for used for each enzyme were: PmHAS, Mn^2+; 7.2; Xenopus DG42, Mg2^+/7.6; Group A streptococcal HasA, Mg2^+;7.0), and enzyme (units/reaction listed). As a control, parallel reactions in which the metal ion was chelated (22 mM EDTA final; EDTA column, rows with +1 were tested; without free metal ion, the HAS enzymes do not catalyze polymerization. After a 1-h incubation, the reactions were terminated and subjected to descending paper chromatography. Only rPmHAS could elongate HA tetramer even though all three membrane preparations were very active in the conventional HAS assay (incorporation of 1[^14C]GlcUA from UDP-GlcUA into polymer when supplied UDP-GlcNac). r-, recombinant.

**FIG. 4. TLC analysis of PmHAS extension of HA tetramer.** Panel A, radiolabeled HA tetramer (HA4, 8 × 10^5 dpm 3H) with a GlcUA at the nonreducing terminus was incubated with various combinations of UDP-sugars (360 μM UDP-GlcUA (A); 750 μM UDP-GlcNac (N), no UDP-sugar (O)), and rPmHAS (55 μg of membrane protein) in assay buffer for 60 min. The reactions (7 μl total) were terminated by heating at 95 °C for 1 min and clarified by centrifugation. Portions (2.5 μl) of the supernatant were spotted onto the application zone of a silica TLC plate and developed with solvent (1.25:1:1). The beginning of the analytical layer is marked with an arrow. The positions of odd-numbered HA oligosaccharides (S lane) are marked as number of monosaccharide units. This autoradiogram (4-day exposure) shows the single addition of a GlcNAc sugar onto the HA tetramer acceptor to form a pentamer when only the subsequent precursor is supplied (N). The mobility of the labeled tetramer is unchanged if only the inappropriate precursor, UDP-GlcUA (A) or no UDP-sugar (O) is present. If both UDP-sugars are supplied, then a ladder of products with sizes of 5, 7, 9, 11, and 13 sugars is formed (+AN). Panel B, in a parallel experiment, chitosanose (8 × 10^4 dpm 3H (Chito5)) was tested as an acceptor substrate. Under no condition was this structurally related molecule extended by rPmHAS.
sugars are supplied (1) of the supernatant were spotted onto the TLC plate and developed in 1.5:1:1 solvent. This autoradiogram (1-month exposure) shows the single addition of a sugar onto an acceptor when only the appropriate precursor is supplied (HA4, N lane and HA5, A lane). If both UDP-sugars are supplied (+AN lanes), then a ladder of products with final sizes of 6, 8, and 10 sugars is formed from either HA4 or HA5 in 2 min. After 20 min, a range of odd- and even-numbered product sugars are observed in reactions with HA4 and both UDP-sugars. In the 20-min reaction with HA5 and both UDP-sugars, the HA products are so large that they do not migrate from the application zone.

HA-derived acceptors is likely to be due to the circumvention of the initial kinetic obstacle.

**Acceptors and Other HA and Glycosaminoglycan Synthases**—Previously no HA synthase had been shown to utilize an exogenous acceptor or primer sugar. In an early study of a cell-free HA synthesis system, preparations of native Group A streptococcal HAS were neither inhibited nor stimulated by the addition of various HA oligosaccharides, including the HA tetramer derived from testicular hyaluronidase digests (11). These membrane preparations were isolated from cultures that were producing copious amounts of HA polysaccharide. The cells were hyaluronidase-treated to facilitate handling. Therefore, it is quite likely that the native streptococcal enzyme was isolated with a small nascent HA chain attached to or bound to the protein much as suspected in the case of native PmHAS. Theoretically, the existing nascent chain formed in vivo would block the entry and subsequent utilization of an exogenous acceptor by the isolated enzyme in vitro. With the advent of molecularly cloned HAS genes, it is possible to prepare virgin enzymes lacking a nascent HA chain if the proper host is utilized for expression. The yeast *S. cerevisiae*, an organism whose genome has been totally sequenced, does not possess the UDP-glucose dehydrogenase that is required for UDP-GlcUA precursor synthesis. Nonetheless, the virgin yeast-derived recombinant streptococcal or vertebrate enzymes did not utilize HA acceptor oligosaccharides in our experiments in vitro. Possible explanations for this finding are that the enzymes lack an accessible binding site for the HA-derived acceptor chains tested, or the enzymes utilize a different polymer retention mechanism.

Recently, it has been postulated that certain vertebrate HAS enzymes, *Xenopus* DG42 and the *Brachydanio* zebrafish homolog in particular, can produce chitin oligosaccharides under certain conditions (23, 24). Another possibility forward was that chitin oligosaccharide primers are used to initiate HA chains, and polymerization would occur at the nonreducing terminus (24). More defined enzyme systems will be needed to address this difficult issue in the vertebrate system. With respect to PmHAS, however, chitotetraose and chitopentaose neither stimulated HA production nor served as acceptors in our experiments.

In the case of the biosynthesis of the other glycosaminogly-
PmHAS—We have found that recombinant PmHAS adds single monosaccharides in a sequential fashion to the nonreducing termini of the nascent HA chain. Elongation of HA polymers containing hundreds of sugars was demonstrated in vitro. The simultaneous formation of the disaccharide repeat unit is not necessary for generating the alternating structure of the HA molecule. The intrinsic specificity and fidelity of each half-reaction (e.g. GlcUA added to a GlcNAC residue or vice versa) apparently is sufficient to synthesize authentic HA chains.

A great technical benefit resulting from the alternating disaccharide structure of HA is that the reaction may be dissected by controlling the availability of UDP-sugar nucleotides. By omitting or supplying precursors in a reaction mixture, the glycosyltransferase may be stopped and started at different stages of synthesis of the heteropolysaccharide. In contrast, there is no facile way to control in a stepwise fashion the saccharide structure of HA is that the reaction may be dissected by controlling the availability of UDP-sugar nucleotides. In contrast, there is no facile way to control in a stepwise fashion the glycosyltransferase enzymes that produce important homopolysaccharides such as chitin, cellulose, starch, and glycogen. The lessons learned with PmHAS in the future, however, may be applicable to the study of other enzyme systems.

Two Classes of HA Synthases—It has been established that one polypeptide species transfers both GlcUA and GlcNAC during HA biosynthesis in all known cases (7, 8, 10, 15, 16, 22), but at least two evolutionary paths may have led to the creation of HA synthases. I propose that two distinct classes of enzyme exist based on differences in the amino acid sequences, the predicted polypeptide topology in the membrane bilayer, and the putative reaction pathway. Class I HASs would include the previously described streptococcal, viral, and vertebrate enzymes. At present, P. multocida PmHAS is the only known member of the Class II HA synthase. In the near future, it will be interesting to examine and to compare the reaction mechanisms of the glycosaminoglycan synthases and other β-glycosyltransferases in more detail.

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