Hyaluronan (HA) is a nonsulfated glycosaminoglycan that has long been known to play structural roles in vertebrates. Recently, it has become increasingly obvious that this linear polysaccharide has many more uses than simply scaffolding or space filler. HA has been found to be involved in development, cell signaling, cell motility, and metastasis. These roles are often dictated by the length of the HA polymer, which can vary from a few to about 10,000 sugar residues in length. Three distinct isoforms of HA synthase exist in mammals. It has been shown previously by others that each isoform produces HA that differs in size distribution, but the regulatory mechanism is not yet known. Mutations have been described that alter the size distribution of the HA produced by the streptococcal HA synthases. We show that by mutating on particular amino acid residue of a vertebrate HA synthase, depending on the introduced side chain, the size of HA produced can be either reduced or increased. We postulate that several cysteine residues and a serine residue may be involved in binding directly or indirectly to the nascent HA chain. These data support the theory that the relative strength of the interaction between the catalyst and the polymer may be a major factor in HA size control.

HA² is a glycosaminoglycan composed of repeats of the alternating disaccharide (−4)-β-D-GlcUA(1−3)-β-D-GlcNAc(1−→). The enzymes that catalyze the formation of HA, the HA synthases, are dual action glycosyltransferases that catalyze the transfer of both GlcUA and GlcNAc (1, 2). Birds and mammals, including humans, each contain three HAS isoforms called HAS1, 2, and 3 (1, 2). These enzymes share considerable homology with each other as well as with HASs from group A and C Streptococcus bacteria and a virus (3). The membrane-associated enzymes utilize UDP-linked sugar precursors in the cytosol and extrude the growing HA chain through the plasma membrane (1, 4).

The roles that HA plays in the vitreous humor of the eye (5) and in the formation of cartilage (6) have been known for many years. Considering the high concentration of HA (>100 mg/liter) in these areas (7, 8), it is not surprising that structural functions were the first biological uses discovered. Only recently have a wider variety of roles for HA been discovered. In vertebrates, HA is also involved in development, cell migration, and signaling (9). Many hyaluronan-binding proteins (10) are implicated strongly in signal transduction processes (11); thus, HA is much more than just a space-filling molecule. The sizes of the HA molecules involved appear to dictate their biological activities (12, 13). For example, HA with an average size of >10⁶ Da appears to be involved in maintaining structure and viscosity (14), mediating cell-matrix adhesion (15), and reducing contact inhibition and promoting migration and metastasis (16–20). However, HA with an average size of <10⁶ Da has been implicated in angiogenesis (21–28), cellular proliferation and migration (22, 23, 26, 28–32), and inflammation (33–47). The various vertebrate HAS isozymes produce HA of different sizes (48). Therefore, it is likely that cells can manipulate the properties of HA produced by controlling the expression of the different isozymes depending on the particular developmental stage, tissue type, and external stimuli (2, 49–52).

The basis for size control of HA product has recently begun to be investigated (53). It is likely that the differences in the HAS sequences of the different isoforms are responsible at least in part for the variation in HA size distribution. One or more residues that are conserved between species but vary between isoforms may be responsible for the differences in HA size distribution. This hypothesis is based on the fact that when the different recombinant enzymes are expressed in the same system, the HA product size differs. In most experiments, a recombinant vector was used to express a HAS gene either in a foreign host, yeast, or in a HA-deficient mammalian cell line. In a direct comparison utilizing membrane preparations from yeast, spHAS (from Streptococcus pyogenes) made larger HA polymers than xHAs1 (from Xenopus laevis, originally called DG42) in vitro (54). Marine HAS1, 2, and 3 were also directly compared in vitro and in vivo (48). First, membrane preparations derived from mammalian cells transfected with the various marine HAS isozymes demonstrated that the relative product size was HAS2 > HAS1 > HAS3. Second, HA in the culture medium from mammalian cells transfected with HAS1, 2, or 3 also showed differences; HAS1 and 3 appeared to produce HA with more equivalent size distributions in vivo but still smaller than HAS2. Another group showed that the size distribution of HA products formed by xHAS1 and xHAS2 differed substantially when examined in vitro (51).

In addition to regulation via the innate kinetics or enzymological properties of the HASs, control of the precursor levels has been hypothesized as being important for size control. Certain treatments (e.g. proinflammatory cytokines) can cause cells to increase the expression of UDP-Glc dehydrogenase (55). The actual levels of UDP-GlcUA were not measured, but it was...
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postulated that HA production could be regulated by such a mechanism. The predominant HA size control mechanism, whether intrinsic factors (e.g. enzymological properties of the synthase), extrinsic factors (e.g. UDP-sugar substrate concentration or substrate/enzyme ratio), or a combination of both, has not been reported.

Recently, it was shown that mutation of certain single cysteine residues in sHAS and hHAS (from Streptococcus equisimilis) cause the enzyme preparations to either produce HA of smaller or slightly larger average size distribution than that of wild type enzyme in vitro (53, 56). We have used site-directed mutagenesis to examine the difference in the size distribution of HA produced by various mutants of a vertebrate enzyme, xHAS1. We have not only discovered a variety of single mutations that cause either a smaller or a larger HA product but also one particular amino acid that can be mutated to give rise to either a smaller or a larger HA product depending on the substituting residue. We have also examined these mutants to assess whether there is a relationship between enzyme kinetics and chain length.

EXPERIMENTAL PROCEDURES

Production of Recombinant xHAS1 Wild Type, Cysteine Mutants, and Ser77 Mutant Enzymes—All of the reagents were from Sigma or Fisher Scientific. The construction and the use of the xHAS1 expression plasmid for studies in yeast were previously described (54, 57). Basically, the xHAS1 polypeptide was cloned into the pYES2 vector (Invitrogen) under control of the GAL1 promoter to form pYES/ΔG+. Site-directed mutagenesis was performed on pYES/ΔG+ using a QuickChange™ kit (Stratagene). Based on the preliminary results from photoaffinity labeling experiments probing the active site of xHAS1,4 the Ser77 and Tyr107 codons were altered using pairs of synthetic oligonucleotides containing partially degenerate codons. For Ser77, the codon NNY (where N = any base and Y = C or T) was used to obtain a variety of mutants. For Tyr107, the codon YT (7) was used to obtain the Y107F mutant. Y107S was also generated but not investigated. Plasmids derived from independent transformants were sequenced to determine the identity of the substitution at residues 77 or 107 as well as to verify the entire open reading frame. The following Ser77 mutants were generated: alanine (S77A), aspartate (S77D), phenylalanine (S77F), isoleucine (S77I), threonine (S77T), valine (S77V), and tyrosine (S77Y/R271G; a secondary mutation was found upon sequencing). The cysteine mutants (C117S, C210S, C239S, C298S, C304S, C307S, and C337S) were obtained in a manner similar to that previously described (58). The plasmids were transformed into Saccharomyces cerevisiae BJ5461 yeast (a pleiotrophic protease-deficient strain; Yeast Genetic Stock Center, Berkeley, CA) by the lithium acetate (58) (or PCR) method (59).

Yeast with recombinant plasmids were routinely grown to a suitable biomass (A600 reached 0.3) in uracil-deficient synthetic medium with 0.1% glucose and 5% glycerol. Upon induction with galactose (final concentration, 1% w/v), xHAS1 wild type or mutant enzyme accumulated on a 1-kb DNA ladder standard (Stratagene) was visualized with the primary reagent composed of serum (1:1,000) from rabbits (gift of I. Dawid; 50). Protein A-alkaline phosphatase detection with the primary reagent composed of serum (1:1,000) from rabbits (gift of I. Dawid; 50) was used to visualize the immunoreactive bands.

RESULTS AND DISCUSSION

We have reported previously that xHAS1 synthesizes HA at a rate of ~80 monosaccharides/s and produces chains of between 3 × 106 and 2 × 107 Da (54). These sizes were based on results obtained on a Sephacryl S-500HR gel filtration column using dextrans (a1–6 glucans) as standards. In this study, we

P. E. Pummill, R. R. Drake, and P. L. DeAngelis, unpublished observation.

The size of HA polymers was analyzed by gel filtration chromatography on a Phenomenex PolySep-GPC-P 5000 or 6000 column (300 × 7.8 mm) eluted with 0.2 mM sodium nitrate at 0.6 ml/min on a Waters 600E system. All of the samples were clarified by centrifugation at 16,000 × g for 5 min prior to injection. Radioactive components were detected with a LKB 12502 RadioFlow Detector (EG & G Berthold) and the Zinsser mixture (1.8 ml/min). The columns were standardized with various size dextrans (580, 145, 50, and 20 kDa) or, more appropriately, MANT-labeled HA with average molecular masses of 1300, 600, and 80 kDa (determined by MALDI). Each radiolabeled sample was spiked with an internal fluorescent dextran standard; elution times were then adjusted to within ±0.1 min of that standard. Because of discrepancies in the elution times of dextrans compared with HA, HA standards were made by substoichiometric labeling (~1 MANT/50 monosaccharides) of hydroxyl groups of the streptococcal HA polysaccharide (1300 kDa by MALDI) with N-methylisatoic anhydride (61). The 600-kDa standard was obtained by subfractionation of bulk HA using preparative high pressure liquid chromatography. Expected ultraviolet absorbance in water (2-nm intervals for 30 min total on ice) of the bulk HA with a Heat Systems Ultrasonic W-380 sonicator with a Micropip™ (power setting 4) was used to produce the 80-kDa standard. The size determination of HA by gel filtration was performed by MALDI. The HA polymers (100 µg) were loaded on two tandem Tosoh BioSep TSK-GEK columns (6000PWXL following by 4000PWXL; each 7.5 mm × 30 cm) and eluted in 50 mM sodium phosphate, 150 mM NaCl, pH 7, at 0.5 ml/min. The eluant flowed through an Optilab DSP interferometric refractometer and then a Dawn DSF laser photometer (632.8 nm; Wyatt Technology, Santa Barbara, CA) in the multi-angle mode. The software package of the manufacturer was used to determine the absolute average molecular mass by using a dn/dc coefficient of 0.153.

Some of the reactions tested with Pronase/Microen were run on a 1.35% agarose gel in 1× TAE (40 mM Tris acetate, 2 mM EDTA, pH 8.5) at 30 V for several hours. The gels were dried, and the labeled HA was visualized using a PhosphorImager™ and ImageQuant™ software from Molecular Dynamics. The identity of the radiolabeled material as authentic HA was assessed by Streptomyces HA lyase digestion. Prior to gel drying, the 1-kb DNA ladder standard (Stratagene) was visualized by photography of ethidium bromide fluorescence upon exposure to UV light. HA sizes were estimated by comparison with the DNA standard according to a previous study (62).

Immunochromatographic Detection of Polypeptides—The xHAS1 and mutant proteins were quantitated by Western blot analysis of relative size of specific HAS activity. After SDS-PAGE separation, the proteins in the gel were transferred to nitrocellulose by semi-dry transfer. The blot was blocked with bovine serum albumin and incubated with the primary reagent composed of serum (1:1,000) from rabbits (gift of I. Dawid; 50). Protein A-alkaline phosphatase detection with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium was used to visualize the immunoreactive bands.

The enzymes were analyzed by either high performance gel filtration chromatography or agarose gel electrophoresis. For gel filtration analyses, the membranes (40–1400 µg of total protein) were incubated with 0.3–0.6 mM UDP-[14C]GlcUA (0.1–0.2 µCi) and 1.2–2.4 mM unlabeled UDP-GlcNAc in 50 mM Tris, pH 7.5, and 20 mM MgCl2 at 30 °C for various times. Control experiments validated that the HA product size was unaffected by the variations in UDP-sugar and enzyme concentrations. Membranes and the other reaction components were prewarmed separately to 30 °C prior to mixing to start the HAS reaction. At the end of the reaction time, the samples were either stopped by the addition of SDS (final concentration, w/v) or by addition of SDS to 0.5% followed by Pronase® treatment (final concentration, 1 mg/ml; overnight at 37 °C; Roche Applied Sciences). After Pronase digestion, the unincorporated precursors and small molecules were removed by ultrafiltration with a Microcon® 3 unit (three buffer changes; 3000 Da molecular mass cut-off, Amicon).

The assays were set so that the UDP-sugar precursors and small molecules were removed by ultrafiltration with a Microcon® 3 unit (three buffer changes; 3000 Da molecular mass cut-off, Amicon). The results obtained on a Sephacryl S-500HR gel filtration column using dextrans (a1–6 glucans) as standards. In this study, we

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have determined that these dextran polymers elute after HA polymers of similar size (dextrans are probably more compact than HA polymer of the same molecular mass) on the Phenomenex PolySep columns. Based on the MANT-labeled authentic HA standards, we have revised the HAS-catalyzed elongation rate for xlHAS1 to \( \frac{3 \text{ monosaccharides/s}}{11011} \) in vitro and the final product chain length to between \( 100000 \) and \( 1000000 \) Da in vitro. This rate is similar to that of mmHAS3 (from mouse) expressed in fibroblasts (48), and the size range is similar to or slightly larger than that obtained for xlHAS1 expressed in COS-1 cells (2, 51), as determined by agarose gel analysis.

We discovered that certain xlHAS1 mutants with substitutions for the serine at position 77 synthesize HA products with different size distribution (Table I). Of the Ser77 mutants created, S77F and S77I created a larger HA product and S77T created a smaller HA product. On the other hand, S77A, S77D, S77V, S77Y/R271G, and Y107F all made HA with an average size similar to that of wild type xlHAS1 (data not shown). As can be seen in Fig. 1, the average size of HA produced by S77T starts to fall behind that of wild type after \( 5 \text{ min} \). The average

### Table I

| xlHAS1 enzyme | Protein expression | HAS activity | \( K_m \) (UDP-GlcNAc) | Size of HA compared with WT |
|---------------|--------------------|--------------|------------------------|---------------------------|
| WT | + + + | 400 ± 100; 260° | 190 ± 40 | equivalent |
| S77A | + + + | 130° | 90 | larger |
| S77D | + | ND | 70° | 90 ± 50 | larger |
| S77F | + + + | 160° | 90° | 50 | larger |
| S77I | + | ND | 240° | 210 ± 60 | smaller |
| S77T | + + + | 180° | ND | 90 ± 40 | equivalent |
| S77Y/R271G | + | 160° | 90° | 90 | equivalent |
| Y107F | + + + | 220° | 90° | 40 | equivalent |
| C117S | + + + | 120 | 340 | larger |
| C239S | + + + | 110 | 320 | larger |
| C337S | + + + | 700 ± 170 | 400 ± 100 | smaller |

* 0.6 mM UDP-GlcUA.

* ND, not determined.
size of HA produced by S77T is smaller than that of wild type even after incubation for 60 min. S77F and S77I make HA with a larger average product size than wild type for all time points after 5 min.

We also tested several cysteine to serine mutants from our previous study (58) for changes in the size of the HA product. As shown in Fig. 2, C117S and C239S consistently made larger HA than that of wild type, whereas C337S made HA that was consistently smaller. No difference was observed with any of the other cysteine to serine mutants (data not shown). The results obtained from the gel filtration experiments shown in Figs. 1 and 2 were confirmed by electrophoretic analysis of the HA produced by wild type and mutant enzymes. The average HA product size was larger than that of wild type for C117S, C239S, S77F, and S77I but smaller for C337S and S77T (Fig. 3).

There are several reasons why mutation of a single serine or cysteine in the synthase might cause a change in HA product size distribution. For the Ser77 mutations, it is possible that a phosphorylation site is being altered, but this explanation is unlikely because the amino acid sequence around Ser77 is not a consensus sequence for phosphorylation (Prosite program). In addition, not all mutations of Ser77 caused a change in HA size. For the cysteine mutations, it is possible that a disulfide bond is being eliminated. This explanation is less likely because of the facts that the majority of the peptide sequence for Class I HA synthases is predicted to be in the cytoplasm (63) and that no disulfide bonds were found in the streptococcal HASs (56, 64).

Mutations in the synthase may cause a change in the HA product size distribution for several other reasons. As shown in Table II, an alteration in the HAS enzyme (a) stability, (b) UDP-sugar substrate binding, (c) relative elongation rate, or (d) HA product interaction might result in a change in the size distribution of the HA produced by the enzyme. To test the first possibility (a), time course experiments were performed on wild type and mutant enzymes to confirm that the various enzymes were stable over the duration of the HAS reaction time. All of

**Fig. 2.** Gel filtration of HA product for xlHAS1 wild type and cysteine mutants. The reactions were carried out as described under “Experimental Procedures” and analyzed with a PolySep-GFC-P 5600 (A and B) or 6000 (C–E) column. Incubation times were as follows: 2.5 min (A), 5 min (B), 15 min (C), 30 min (D), and 60 min (E). A, B, and D were stopped with 2% SDS. C and E were stopped with 0.5% SDS and treatment with Pronase followed by ultrafiltration. The data points are shown for xlHAS1 wild type (black dots), whereas moving average trend lines represent the data for the mutants.

**Fig. 3.** Agarose gel analysis of HA product of xlHAS1 wild type and mutant enzymes. Duplicate reactions from C of Figs. 1 and 2 (30 min) were also run on a 1.35% agarose gel and visualized as described under “Experimental Procedures.” Lane 1, wild type xlHAS1; lane 2, C117S; lane 3, C239S; lane 4, C337S; lane 5, S77F; lane 6, S77I; lane 7, S77T. The positions of the 1-kb DNA ladder standards are indicated with bars (corresponding to HA of ∼200, 250, 300, 400, 500, 700, and 900 kDa, from bottom to top, in size) (62). The arrow marks the position of the sample well. Wild type xlHAS1 produced HA (WT marked with a bracket) with a size of 2–9 × 10^5 Da in this experiment. The agarose gel data qualitatively corroborates the gel filtration analyses.
Modification of a synthase via mutagenesis may change its enzymological properties. The predicted effects of an enzymological property on HA product size with respect to reaction time are listed. We assume that as long as the enzyme was stable, even a slow enzyme could achieve a large size product (e.g. the ultimate achievable size) given a sufficiently long incubation. WT, wild type.

| Mutant enzyme property | Reduced stability | Decreased substrate affinity | Increased substrate affinity | Decreased elongation rate | Increased elongation rate | Decreased product affinity | Increased product affinity |
|------------------------|------------------|-----------------------------|-----------------------------|--------------------------|--------------------------|---------------------------|---------------------------|
| Compared with WT       | none or slightly smaller | smaller | larger | smaller | larger | smaller | larger |
| Relative effect on HA product size |
| Short incubation | larger | none | none | none | none | none | none |
| Long incubation | smaller | none | none | none | none | none | none |

The enzymes showed a similar linear increase in HA production with time over a 60-min incubation period (Fig. 4). Therefore, in these cases, relative stability does not cause differences in HA size distribution.

To investigate the second possibility (b), that altered kinetics of substrate binding might be playing a role in changing the size of the HA product, the apparent \( K_m \) values (which correspond to the apparent affinity of the enzyme for substrate) of the Ser77 mutated enzymes were determined. A potential caveat of this evaluation is that the \( K_m \) values measured here are only approximations of the affinity of the enzyme for substrate; the values obtained are also affected by the ability of the enzyme to transfer sugars to the growing HA chain. Compared with wild type, S77F and S77I consistently had slightly lower \( K_m \) values for both substrates (Table I). It is possible that this higher apparent substrate affinity might explain their higher HA product sizes at 15–60-min time points. However, the differences in the \( K_m \) values are rather small (e.g. 2-fold), and there is little difference in the size of HA produced during short incubations where the effect of altered substrate affinity would likely be most apparent. Also, in these elongation time courses, substrate concentrations were about 3-fold higher than the \( K_m \) value, a condition where all of the various enzymes should be saturated with UDP-sugars.

The apparent \( K_m \) values for the cysteine to serine mutants have been reported previously (58). Of the mutants showing changes in HA product size, only C337S had altered substrate affinity. The mutation of Cys337 to serine caused a 3-fold increase in the \( K_m \) value of xHAS1 for UDP-GlcUA (Table I), which might explain the production of HA with lower average size by C337S. However, because C337S failed to make larger HA even after long incubation times, altered kinetics is probably only part of the explanation. We assume that even a “slow” enzyme would eventually make a long product given sufficient reaction time. Therefore, the third possibility (c), that the differences in HA product size might be due to altered relative elongation rates, also does not appear to be the major size determining factor.

The fourth possibility (d), that the larger size product seen in longer incubations is due to an alteration in the affinity of the

**TABLE II**

| Mutant enzyme property | Reduced stability | Decreased substrate affinity | Increased substrate affinity | Decreased elongation rate | Increased elongation rate | Decreased product affinity | Increased product affinity |
|------------------------|------------------|-----------------------------|-----------------------------|--------------------------|--------------------------|---------------------------|---------------------------|
| Compared with WT       | none or slightly smaller | smaller | larger | smaller | larger | smaller | larger |
| Relative effect on HA product size |
| Short incubation | larger | none | none | none | none | none | none |
| Long incubation | smaller | none | none | none | none | none | none |

**Fig. 4.** Time course of HA production by xHAS1 wild type and mutants. HAS reactions (total volume, 300 µl) containing 200–600 µg of total membrane protein were incubated at 30 °C. The aliquots (50 µl) were removed at various time points. The HAS reactions were stopped, and the amount of HA produced was determined as described under “Experimental Procedures.” Enzyme designation is as follows: xHAS1 (closed circles, solid line), C117S (open circles, dashed line), C239S (open squares, dashed line), C337S (open diamonds, dashed line), S77F (closed squares, solid line), and S77T (closed diamonds, solid line). The enzymes have similar stability.

**Fig. 5.** Effect of NaCl on HA product size for xHAS1. The reactions were carried out as described under “Experimental Procedures” with varying amounts of NaCl. The incubations were for 30 min, and the reactions were stopped with 2% SDS and analyzed with a PolySep-GFC-P 6000 column. The moving average trend lines are shown for all experiments. The lines for the reactions containing 0 and 1 M NaCl are labeled; between these minimal and maximal salt concentrations are the lines for 0.1, 0.2, 0.4, and 0.7 M NaCl. DPS, disintegration/s. Increasing ionic strength increases the HA chain size.

**Fig. 6.** Comparison of vertebrate HAS protein sequences. This multiple sequence alignment (Multalin program) (71) compares the protein sequence of xHAS1 (residues 61–156) with the sequences of the three different human HAS isozymes. The HAS protein sequences are presented in single-letter code with residues that are conserved among all vertebrate HASs in bold type and residues that are conserved among the members of more than one isozyme as capital letters. Ser77 and Tyr107 (marked with *asterisks*) are conserved among all vertebrate HASs.
enzyme for the HA polysaccharide product, seems to be the most likely in view of our data. S777 did not seem to have altered kinetics because it had similar $K_m$ values, protein expression, and HAS activity to that of wild type (Table I), and yet it made HA with a smaller average size than all other enzymes tested. An alteration in the affinity for product is also the most likely explanation for the larger HA product size in C117S and C239S because their kinetic values are similar to enzymes tested. An alteration in the affinity for product is also yet it made HA with a smaller average size than all other enzymes. It is obvious that the HAS enzymes have an intrinsic ability to regulate the size of HA produced because the HA size distribution reaches a maximum after ~30 min, and the size of HA produced does not increase even if the reaction is allowed to proceed for 2 h (data not shown). This apparent regulation may be based on the ability of the enzyme to retain the nascent HA product. The tighter the enzyme can hang on to the chain, the more sugars can be added to the product before it is torn away from the enzyme by external forces; this theory was proposed by Weigel and co-workers (53), but no direct experimental evidence was provided at that time. To date, no one has directly measured the affinity of the enzyme for HA product. It can be envisioned that force measurements using atomic force microscopy could be made to directly determine the strength of the HA chain/enzyme interaction, but this experiment has not been reported.

The attractive forces between the synthase and nascent HA chain could be mediated in several ways including (a) a covalent intermediate generated during catalysis, (b) hydrogen bonding of enzyme with hydroxyls or amides of HA, (c) hydrophobic interaction of the enzyme with the apolar faces of the HA pyranose rings, and (d) ionic bond interaction between positively charged residues and the negative groups of HA. Also, if the reducing end theory of chain elongation is correct (65–67), then (e) the enzyme may interact with a UDP moiety at the end of the growing chain. On the other hand, the effect of repulsive forces could also serve to sever the HA/HAS connection; the obvious example is that a negative patch of amino acid residues on the enzyme could repel the negatively charged HA molecule. This repulsive model may be less likely than some of the attractive model scenarios because the HA polymer sizes are quite large for all mutants; the relative charge repulsion generated by a ~500- or ~100-kDa polymer is probably not that different.

We performed a simple experiment in an attempt to rule out several of the hypotheses described above. Increasing concentrations of NaCl, up to 1 M, caused xHAS1 to produce HA with a larger average size (Fig. 5). Higher ionic strength would probably not enhance attraction in scenarios (a), (b), or (c) above. However, high salt concentrations increase hydrophobic interactions (68); therefore this result suggests that this weak force is utilized to maintain the HA product in the proper position to receive the next sugar monomer. Again, it is less likely that the higher salt is shielding the HA chain from an electrostatic repulsion by potential proximal negative groups on the enzyme. Precedent for a hydrophobic interaction between the HA chain and an enzyme was set recently in the case of HA lyase (69, 70). The HA lyase from Streptococcus pneumoniae utilizes hydrophobic interactions between its own tryptophan and phenylalanine residues and the carbohydrate rings of the HA substrate to properly position the HA chain for degradation. At this time, we cannot distinguish whether the enzyme is binding the apolar face of the sugars (c) or the uridine group (e).

Because Ser$^{77}$ is conserved among all vertebrate HASs (Fig. 6), there are obviously other residues that are responsible for the variation observed in the size of the HA produced by these different enzymes. If there is a direct interaction between Ser$^{77}$ and the HA product chain, the fact that changing Ser$^{77}$ to an amino acid with a more hydrophobic side chain causes an increase in the HA size distribution would support the hypothesis of a hydrophobic interaction. However, because changing Ser$^{77}$ to aspartate (a negatively charged amino acid) did not alter the HA size distribution, a direct interaction between Ser$^{77}$ and the HA chain is unlikely. It is also possible that the hydrogen bond concentration is causing a conformational change resulting in alterations in the hydrophobic or electrostatic interactions elsewhere in the protein (or the membrane) and thereby affecting the affinity of the enzyme for the growing HA chain. The identity and the microenvironment of the active site are not yet known. Obtaining the three-dimensional structure of a HAS with a nascent HA chain should help identify the correct hypothesis.

Overall, these results may be an indication that the length of the HA chain is dictated by how tightly the enzyme retains the HA product. The mutations we have investigated probably either cause an increase or a decrease in the ability of the enzyme to bind the HA product, thus making it larger or smaller, respectively.

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