We identified two conserved polar amino acids within different membrane domains (MD) of Streptococcus equisimilis hyaluronan synthase (seHAS), Lys48 in MD2 and Glu327 in MD4. In eukaryotic HASs, the position of the Glu is very similar and the Lys is replaced by a conserved polar Gln. To assess whether Lys48 and Glu327 interact or influence seHAS activity, we investigated the effects of changing Lys48 to Arg or Glu and Glu327 to Lys, Asp, or Gln. Mutants, including a double switch variant with Lys48 and Glu327 exchanged, were expressed and assayed in Escherichia coli membranes. SeHAS(E327Q) and seHAS(E327K) were expressed at low levels, whereas seHAS(E327D) and the Lys48 mutants were expressed well. The specific enzyme activities (relative to wild type) were 17 and 7% for the K48R and K48E mutants and 26 and 38% for the E327Q and E327D mutants, respectively. In contrast, seHAS(E327K) showed only 0.16% of wild-type activity but was rescued over 46-fold by changing Lys48 to Glu. Expression of the seHAS(E327K,K48E) protein was also rescued to near wild-type levels. Based on size exclusion chromatography coupled to multidangle laser light scattering analysis, all the variants synthesized hyaluronan (HA) of smaller weight-average molar mass than wild-type enzyme (3.6 MDa); the analysis, all the variants synthesized HYALURONAN (HA) of smaller size exclusion chromatography coupled to multiangle laser light scattering. In the present study, the growing recognition that certain biological responses may be dependent on HA of a particular size meshes nicely with the discovery that, although they catalyze the same reaction, different HASs can differ in the size distribution of HA they produce (20–22). For example, membranes from cells expressing HAS1 or HAS3 synthesized HA of 0.2–2.0 MDa, whereas HA of >2 MDa was produced by membranes from cells expressing HAS2 (20, 21). If similar results are applicable to human cells in vivo, then the actual HA size distribution made by HAS1, HAS2, or HAS3 could be different for specific biological functions. The expression patterns for the three human HAS genes in different adult and embryonic tissues are quite distinct, as are their temporal patterns of expression during embryogenesis (23, 24). HA of different size made by the three HAS isozymes in specific tissues at different times may, therefore, be involved in different physiological functions.

After its discovery in 1934 by Meyer and Palmer (1), hyaluronan (HA) was found as a ubiquitous, often abundant extracellular matrix component in vertebrate tissues, particularly in cartilage, skin, and vitreous humor (2–5). HA is also made by some pathogenic bacteria (6). HA is a linear unbranched alternating polymer composed of β (1, 4)-N-acetyl-D-glucosamine and β (1, 3)-D-glucuronic acid. Native HA is very polydisperse; typically the smallest molecules are ≤100 kDa and the largest molecules approach 10 MDa. HA is made by the enzyme HA synthase (HAS); since the discovery in 1993 of the first HAS gene, from Group A Streptococcus (7–9), similar HAS genes (or cDNAs) have been identified in other bacteria, frogs, humans, many other mammals, and even in a virus that infects algae (10–14). These >20 HAS enzymes comprise a protein family with many common features and regions of amino acid sequence identity and similarity. The Pasteurella multocida enzyme is the only HAS not in this large family, because it is different in its structure, topology, and mechanism of action (12).

Though simple in structure, HA is necessary for normal development in vertebrates and plays important functions both in normal health and in various diseases. Many studies have supported the growing consensus that HA is not just a structural molecule but is also a cell signaling molecule capable of modulating complex cell behaviors, including cardiac cushion formation (15), angiogenesis (16, 17), and multidrug resistance (18). Surprisingly, many of these cell signaling effects are mediated by smaller, but not larger, HA. For example, angiogenesis is stimulated by small, but not large, HA (16, 17) and activated macrophages are induced to express a large number of genes in response only to HA that is small (19). Small HA fragments stimulate signal cascades in target cells through specific cell surface receptors, in particular CD44 (18). Thus, large and small HA molecules can have different physiological functions.

The growing recognition that certain biological responses may be dependent on HA of a particular size meshes nicely with the discovery that, although they catalyze the same reaction, different HASs can differ in the size distribution of HA they produce (20–22). For example, membranes from cells expressing HAS1 or HAS3 synthesized HA of 0.2–2.0 MDa, whereas HA of >2 MDa was produced by membranes from cells expressing HAS2 (20, 21). If similar results are applicable to human cells in vivo, then the actual HA size distribution made by HAS1, HAS2, or HAS3 could be different for specific biological functions. The expression patterns for the three human HAS genes in different adult and embryonic tissues are quite distinct, as are their temporal patterns of expression during embryogenesis (23, 24). HA of different size made by the three HAS isozymes in specific tissues at different times may, therefore, be involved in different physiological functions.

There is currently great interest in understanding the mechanisms by which cells regulate HAS activity and the factors that influence the size of HA made by HAS. This is the first report in which the molar masses of HA products made by membranes containing wild-type or mutant HAS have been analyzed by SEC-MALLS (size exclusion chromatography coupled to multidangle laser light scattering). In the present study, we examined whether two conserved amino acids, each of which is localized within a different membrane domain (MD) of HAS, are important for HAS function. The results show their involvement in the ability of HAS to synthesize high molar mass HA and support the possibility that MD2 and MD4 are close enough for these two residues to interact directly, and possibly with HA.
Polar Residue Mutations in HAS MDs Alter HA Size

EXPERIMENTAL PROCEDURES

Vectors, Primers, and Reagents—The expression vector pKK223 was from Amersham Biosciences. Escherichia coli SURE cells and QuikChange™ site-directed mutagenesis kits were from Stratagene. Mutagenic oligonucleotides were synthesized by Genosys Biotechnologies, Inc. (Spring, TX) and were purified by reverse-phase chromatography. Cy-5 fluorescent sequencing primers were synthesized by the Great American Gene Co. (Ransom Hill Bioscience, Inc.). UDP-GlcNAc and UDP-GlcUA were from Fluka and Sigma, respectively. UDP-[14C]GlcUA (300 mCi/mmol) was from PerkinElmer Life Sciences. All other reagents were the highest grade available from Sigma unless otherwise noted.

Site-directed Mutagenesis—The seHAS gene with a fusion at the 3′-end encoding a His6 tail (seHAS-His6) was cloned into pKK223 (25). Mutagenic sense primers (altered codons are italic and boldface) were designed to change Glu327 to Gln (5′-CTATGGACATTACCTTCAAGG-TGTCTATGTTTATG), Asp (5′-CTATGGACATTACCTTCAAGGGTG-TCTATGTTTATG), or Lys (5′-CTATGGACATTACCTTCAAGGTTG-CTATGTTTATG) and to change Lys48 to Arg (5′-GCTTACCTATTAGTCAGAATGTCCCCTATCCTTT) or Glu (5′-GCTTACCTATTAGTCGAAATGTCCCCTATCCTTT). Two complementary oligonucleotide primers encoding the desired mutation were used to create each single residue mutation. The seHAS(H8E,E327K) double mutant was made using a single mutant plasmid DNA as the template. Mutagenesis was carried out using the QuikChange method according to the manufacturer’s instructions. The pKK223 plasmid containing the seHAS-His6 gene was grown in SURE cells, purified using a Spin Mini Prep kit (Qiagen), and used as the template for the primer extension reaction with a pair of mutagenic primers. PCR amplification conditions using pfu DNA polymerase were: 16 cycles of 95 °C for 1 min, 58 °C for 1 min, and 68 °C for 18 min after which mutated plasmids were treated with DpnI to digest the methylated and hemimethylated parental DNA. The digested pDNA was transformed into SURE cells, and colonies were screened for the desired mutations by sequencing the isolated pDNA using fluorescently labeled terminators (ABI Prism 377 MODEL 11756 JOURNAL OF BIOLOGICAL CHEMISTRY)

Enzymatic Activity of seHAS Mutants—E. coli SURE cells transformed with plasmids containing various seHAS mutants were grown in Luria Bertani medium at 32 °C to A600 ~0.8 and induced with 1 mM isopropyl-β-thiogalactoside for 3 h. Cells were harvested and membranes were prepared as described previously (26). The specific activities of seHAS variants were determined at 37 °C in 100 μl of 50 mM sodium and potassium phosphate, pH 7.0, with 20 mM MgCl2, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 1.0 μM pepstatin, and 2 μM leupeptin, 240 μM UDP-GlcUA, 0.7 μM UDP-[14C]GlcUA, and 0.6 mM UDP-GlcNAc. Membranes (0.5–40 μg of protein) were added to initiate the enzyme reaction. Reactions were terminated by the addition of SDS to a final concentration of 2% (w/v), and incorporation of [14C]GlcUA into HA was determined by descending paper chromatography (7). All samples were assayed in duplicate or triplicate using two or three independent membrane preparations. Results are presented as the mean ± S.E. Assays were performed under conditions that were linear with respect to time and protein concentration. Km and Vmax values were determined as described by Tlapak-Simmons et al. (26).

Determination of seHAS Protein Concentration in Membranes and Normalization of seHAS Activity—E. coli membranes containing wild-type or mutant seHASs were solubilized and subjected to SDS-PAGE in 10% (w/v) gels (27), and seHAS protein in each membrane preparation was quantified (25) by image analysis of stained gels using a Fluorchem™ 8000 (Alpha Innotech Corp). Integrated values for seHAS bands in membranes were compared with standard curves using pure seHAS to estimate seHAS protein/mg of membrane protein (28). These data were then used to normalize variant HAS activity in membranes with wild type.

Determination of HA Size Produced by seHAS Variants—The absolute masses of the HA synthesized by wild-type or mutant seHASs were determined by SEC-MALLS analysis of unlabeled HA products. Documentation of the SEC-MALLS procedures to study HA synthesized by membranes containing HAS is described in more detail elsewhere (29). Membranes were suspended by brief sonication at 0 °C and added to 0.5–1.0 ml of assay buffer (in microcentrifuge tubes) prepared as above but with 2% (v/v) glycerol, 0.1 mM EDTA and without MgCl2 or substrates. UDP-GlcUA and UDP-GlcNAc were added to 1 mM final concentrations, the mixture incubated at 30 °C for 10 min, and calf intestinal alkaline phosphatase was added to a final concentration of 0.02 unit/μl. MgCl2 was then added to a final concentration of 20 mM, and the samples were incubated for 4 h at 30 °C in a vibrating Taitec mixer (San Jose, CA). Synthesis was terminated by adding EDTA and UDP to final concentrations of 40 and 10 mM, respectively, chilling on ice for ~20 min, and then heating at 100 °C (1 min/0.1 ml) to inactivate the phosphatase.

Chromatographic separation of samples was performed using two PL aquagel-OH60 (Polymer Laboratories) columns in series at a flow rate of 0.5 ml/min in 50 mM sodium phosphate, pH 7.0, 150 mM NaCl at 22 °C. MALLS analysis was performed continuously on the eluate using a DAWN DSP Laser Photometer in series with an OPTILAB DSP Interferometric Refractometer (both from Wyatt Technologies, Inc.). Samples (200 μl) in microcentrifuge tubes were incubated in a 100 °C bath for 2 min just prior to injection. Data were analyzed using Astra v4.73, a dn/dc value of 0.153, an A2 value of 0.0023, and first order Zimm or second order Berry fits, respectively, for HA of <2 MDa or >2 MDa.

General—Agarose gel electrophoresis was performed as described by Lee and Cowman (30). Protein was determined by the method of Bradford (31) using bovine serum albumin as the standard. Western analysis to detect seHAS was performed according to the procedure of Burnette (32) using a polyclonal rabbit anti-peptide IgG (33) with modifications as described previously (25).

RESULTS AND DISCUSSION

Amino acid alignment of HAS family members revealed several well conserved charged or polar residues present within verified MDs. Charged or polar amino acids are not typically found within MDs, although this occurs in many proteins, because they create an unfavorable energetic situation when present within the hydrophobic bilayer. One mechanism to alleviate this is to have a second polar or oppositely charged residue near enough to allow formation of an ion pair or H-bonded pair. For example, Arg144 in the lactose permease in MD-VIII forms an H-bond with Glu269 in MD-VIII, and this interaction helps align and stabilize the formation of two H-bonds between Arg144 and the substrate lactose (37).

In seHAS, Lys48 and Glu327 are within MD2 and MD4, respectively, and these residues are absolutely conserved among the streptococcal enzymes (Fig. 1). Although Lys48 in seHAS is not conserved in the other HAS family members, an absolutely conserved polar Glu residue occurs...
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FIGURE 1. An intramembrane polar pair corresponding to seHAS Lys48 and Glu327 is conserved within the large HAS family. The partial alignment shows the amino acid sequences around the regions corresponding to residues Lys48 (A) and Glu327 (B) in seHAS. These two residues are completely conserved among the three streptococcal HASs from *ubereis*, *equisimilis*, and *pyogenes*. Although not identical, these two residues are generally conserved within the larger family of eukaryotic HASs, e.g. from chicken (gHAS), mouse (mmHAS), frog (xHAs), human (hHAS), or an algal virus (vHAs). In the case of seHAS(Lys48), all other HAS family members contain a similar polar residue, glutamine, at the same relative position. The seHAS(Glu327) residue is positionally conserved; all eukaryotic family members contain a highly conserved Glu within three residues of the seHAS position.

at the same position (Fig. 1A). Among eukaryotic HAS family members, the Glu327 residue of seHAS is highly conserved positionally; all HAS family members have a Glu within 3 amino acids of the position in seHAS (Fig. 1B). Lys48 could be charged or uncharged and participate in H-bond pairs within the streptococcal HASs. Like Lys, the polar side chain of Gin can engage in multiple H-bonds as an acceptor or donor. We have suggested that the MDs of HASs form an internal pore-like structure through which a growing HA chain can move and that HASs have the ability to translocate HA through the membrane as it is elongated (34). Because the streptococcal and probably also the eukaryotic HASs are phospholipid dependent (34–36), such a pore is likely to be formed by specific interactions within a HAS-lipid complex. The pore would also likely require specific interactions between MDs within HAS.

To examine the possibility that Lys48 and Glu327 interact or are otherwise important for HAS function, we created mutants in which Lys48 was changed to Arg or Gin and Glu327 was changed to Asp, Gin, or Gin. All of these seHAS mutants were expressed, based on Western analysis (Fig. 2A), although the protein levels varied greatly (Fig. 2B). The E327D, K48R, and K48E variants showed near-wild-type expression levels (Fig. 2B). In contrast, the E327Q and E327K variants were expressed poorly. Interestingly, the E327K and E327Q variants were less disruptive than changes to a similar polar residue. The UDP-sugar Kmax values, relative to wild type or the indicated variant seHAS, were altered very little; they differed from wild type by up to ~2-fold (not shown). Although seHAS variants with Glu327 changed to Asp or Gin had good activity, the E327K variant had dramatically less activity (Table 1). Note that the relative migrations of seHAS(E327D) and seHAS(K48E) were both significantly slower than wild-type seHAS (indicated by the dashed line).

FIGURE 2. Expression and quantitation of recombinant seHAS variants at Lys48 and Glu327 in *E. coli* membranes. Membranes were prepared from cells expressing wild-type or the indicated variant seHAS, subjected to SDS-PAGE, and the gels either stained with Coomassie Brilliant Blue for quantitation of HAS protein expression (B) or electroblotted to nitrocellulose for detection of HAS protein by Western analysis (A). Note that the relative migration of seHAS(E327D) and seHAS(K48E) were both significantly slower than wild-type seHAS (indicated by the dashed line).

### TABLE 1

| seHAS variant | Vmax (% of wild type) | Molar mass of HA products (MDa) |
|---------------|-----------------------|-------------------------------|
| Wild type     | 100                   | 3.64 ± 0.07                   |
| E327D         | 38 ± 2.0              | 3.20 ± 0.01                   |
| E327Q         | 27 ± 4.1              | 1.52 ± 0.38                   |
| E327K         | 0.16 ± 0.2            | Not detected                  |
| K48E          | 6.7 ± 0.2             | 0.68 ± 0.02                   |
| K48R          | 17.2 ± 2.6            | 1.89 ± 0.10                   |
| E327K & K48E  | 7.5 ± 0.3             | 0.61 ± 0.09                   |

The ~42-kDa protein. This anomalous behavior indicates that, despite boiling in SDS, these proteins retain substantial secondary or tertiary structure and do not completely unfold. Interestingly, the SDS-PAGE migration rates of seHAS(E327D) and seHAS(K48E) were both significantly slower than wild-type or the other variants, indicating that these two proteins were more unfolded and more sensitive to denaturation by SDS. Normal migration was restored by adding the E327K change to the seHAS(K48E) protein (Fig. 2A).

All the seHAS variants had synthase activity, although seHAS(E327K) was barely detectable (Table 1). Vmax values, relative to wild type, ranged from 0.16% for seHAS(E327K) to 38% for seHAS(E327D). Amino acid changes to similar residues at positions Lys48 or Glu327 were generally less disruptive than changes to a dissimilar polar residue. The UDP-sugar Kmax values for the seHAS mutants were not altered drastically; they differed from wild type by up to ~2-fold (not shown). Although seHAS variants with Glu327 changed to Asp or Gin had good activity, the E327K variant had dramatically less activity (Table 1). However, both the activity and expression of seHAS(E327K) were greatly increased by combining this mutation with the reciprocal change at position 48 to create the seHAS(K48E,E327K)
mutant. This double switch variant was expressed as well as wild type (Fig. 2B) and exhibited 7.4% of wild-type activity. The conversion of Lys48 to Glu essentially rescued the seHAS (E327K) enzyme, increasing activity of this variant by ~46-fold.

If the Lys48 and Glu327 residues in seHAS mediate an interaction between MD2 and MD4, then their disruption could destabilize a putative intramolecular pore through which the enzyme translocates HA. Perturbing this pore might alter the rate at which HAS could translocate a growing HA chain (resulting in a decreased synthesis rate) and also affect the ability of HAS to retain the growing HA chain, resulting in smaller HA products.

There are no reports using SEC-MALLS to characterize HA made by membranes containing HAS, despite the fact that MALLS is one of the best techniques to assess the size distributions and absolute masses of polydisperse HA. We recently developed a procedure to perform such analyses (29) and used this new approach to quantify possible differences in HA size made by these seHAS variants (Figs. 3 and 4). The weight-average molar mass values are summarized in Table 1. The gel filtration profiles show that the distributions of HA products remained normal (i.e. approximately symmetric) even though the mutants made smaller HA (Fig. 3). The seHAS(K48E) and seHAS(K48E,E327K) variants made the smallest HA, with essentially identical molar masses. The weight-average masses of HA products made by the wild-type enzyme and seHAS(K48E,E327K) were 3.64 and 0.61 MDa, respectively (Fig. 4, Table 1). Fig. 4 illustrates the high quality light scattering data that can be obtained for HA made by membranes expressing seHAS.

HA synthesis mediated by the streptococcal HASs occurs at the reducing end (38–40), and growing HA-UDP chains are assembled in a processive manner (25, 33, 39). These enzymes are unable to rebind and extend HA chains once they are released. Although good progress has been made in understanding HAS topology (28) and the mechanism of HA chain elongation, several key questions about HAS function remain unanswered. One question is how HAS controls HA product size. A second concerns the mechanism by which the HA products are transferred to the cell exterior, since the active sites of the enzyme are intracellular (28). Others have suggested that HAS makes HA inside the cell and that HA-specific ABC transporters then transport HA to the outside of the cell (41).

We have suggested that membrane-bound HASs are inherently able to translocate HA chains across the bilayer during biosynthesis (10, 13, 34). This hypothesis is based on a variety of findings, including that the UDP-sugar binding sites are located at or very near the membrane-HAS interface (42) and, unlike virtually all other glycosyltransferases, the HASs are phospholipid dependent (34, 35) and have multiple transmembrane domains (28). In addition, the genetic and biochemical data show that if substrates are present, HAS is the only protein required for HA biosynthesis. When bacteria that do not normally make HA are transformed with the hasA gene and genes for the synthesis of UDP-sugar precursors, the cells make and, importantly, secrete HA into the medium. For example, no other exogenous genes except hasA are needed for Enterococcus faecalis (7) or Bacillus subtilis (43) cells to accumulate HA in the culture medium.

Based on radiation inactivation analysis of two streptococcal HASs (35) and Xenopus HAS1 (36), the active membrane-bound enzymes contain only a single HAS protein (rather than an oligomer), but the protein is associated with an additional mass of ~23 kDa. This extra mass could not be identified for the active HAS1 enzyme but was iden-
tified as cardiolipin for the streptococcal enzymes (35). Thus, an active streptococcal HAS is one protein in complex with about 16 molecules of cardiolipin. When purified in the absence of cardiolipin, the streptococcal HASs show very low activity, but when this phospholipid is restored enzyme activity increases 10-fold (34). Therefore, all HAS family members might require lipid to synthesize HA, but different HASs may require different types of lipids. We proposed that cardiolipin molecules help the enzymes to create a pore-like passage within the HAS-lipid complex through which a growing HA chain passes (34).

If the MDs of HAS form a pore through which HA passes during biosynthesis, then there are likely to be very specific interactions between MDs that stabilize this structure and mediate its cycle of making and breaking interactions with the HA chain as the HA-UDP is alternately translocated or extended. The results show that changing either Lys48 or Glu327 can inhibit enzyme activity and alter HA product size and support the possibility that Lys48 and Glu327 are binding partners that mediate interaction between MD2 and MD4. This latter conclusion is supported strongly by the rescue of both protein expression and enzyme activity in the double switch mutant compared with the E327K variant. Although other explanations can be proposed for rescue of the E327K variant by introducing the K48E change, the simplest is that the Glu and Lys residues at these two positions directly interact. Based on the topology of seHAS (28), Lys48 and Glu327 are within the same region of the bilayer (Fig. 5A) and could interact physically.

Because of the very short extracellular loops of HAS, MD1-MD2 and MD4-MD5 are likely to be adjacent MD pairs (e.g. Fig. 5B).

It is also possible that the two polar residues within MD2 and MD4 interact with polar groups in HA and are involved in the HA alignment.
or translocation process during synthesis. When the enzyme is not engaged in HA synthesis, then the two side chains might directly interact as suggested above. An interesting hybrid of these two ideas is that Lys48 and Glu327 could switch between these two states in an alternating way (i.e. interacting with each other versus with HA) during chain elongation and translocation. Translocation of HA through the enzyme would likely require the coordinated movement of one or more MDs bound to the chain to achieve HA movement. This would have to be a cyclic process in which the interactions between residues in HAS and specific groups within the HA chain were made and then broken as the chain was translocated in a ratchet-like manner through the membrane to the cell exterior.

Although changing either residue influences HA product size, Glu327 appears to be more critical for seHAS activity than Lys48. Changing Glu327 to the very similar Asp had minimal effect on protein expression and reduced HA product size by only ~12%, whereas changing Glu327 to Lys destabilized the protein, reduced expression, and drastically inhibited activity. The K48R and K48E mutants were both well expressed, indicating that these changes did not destabilize the protein. The slower migration of seHAS(K48E) in SDS-PAGE and its smaller HA product size (~19% of wild type) are consistent with the mutant protein being less compact and hindered in either HA bond formation, HA translocation, or both. We interpret all of these results to indicate that Glu327 participates in H-bonding or ionic interactions that are structurally important. The relative tolerance to changes at Lys48 suggests that if this residue interacts with Glu327, then Glu327 likely also has important interactions with other residues. The two side chains could interact either directly or indirectly through water, phospholipid head groups, or other side chains.

The results in the present study indicate that there is not a simple correlation between the effects of a mutation on enzyme activity compared with enzyme stability as assessed by migration in SDS-PAGE. A possible explanation for this is that both Lys48 and Glu327 also likely interact with other residues in HAS and possibly with groups in HA as noted above. If these two residues interact in a complex, alternating cyclic manner with side chains in the protein and in HA, then correlations among enzyme activity, protein stability, and domain flexibility may not be apparent for individual mutants or may be too complex to sort out without substantially more structural information.

A pore region within HAS would have multiple contacts with the growing HA–UDP chain. Differences in the energetics of these intermolecular contacts among the three eukaryotic HAS isoforms could alter the balance between HA retention and HA release forces and result in inherent differences in HA size distributions made by HAS variants or native HAS isoforms (44). For example, a mutation that decreased HA retention by HAS could result in decreased average HA product size. Further studies are needed to test this model and to elucidate the roles of Lys48, Glu327, and other conserved amino acids in the ability of HAS to make HA of a particular size.

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