Functional Molecular Mass of a Vertebrate Hyaluronan Synthase as Determined by Radiation Inactivation Analysis*

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Hyaluronan (HA), a linear polysaccharide composed of N-acetylglucosamine-glucuronic acid repeats, is found in the extracellular matrix of vertebrate tissues as well as the capsule of several pathogenic bacteria. The HA synthases (HASs) are dual-action glycosyltransferases that catalyze the addition of two different sugars from UDP-linked precursors to the growing HA chain. The prototypical vertebrate hyaluronan synthase, xl-HAS1 (or DG42) from Xenopus laevis, is a 588-residue membrane protein. Recently, the streptococcal enzyme was found to function as a monomer of protein with ~16 lipid molecules. The vertebrate enzymes are larger than the streptococcal enzymes; based on the vertebrate HAS deduced amino acid sequence, two additional membrane-associated regions at the carboxyl terminus are predicted. We have utilized radiation inactivation to measure the target size of yeast-derived recombinant xlHAS1. The target size of HAS activity was confirmed using two internal standards. First, samples were spiked with glucose-6-phosphate dehydrogenase, an enzyme of known molecular weight. Second, parallel samples of native xlHAS1 and a xlHAS1-green fluorescent protein fusion (833 residues) were compared; substantial confidence was gained by using this novel internal standard. Our test also corroborated the basic tenets of radiation inactivation theory. We found that the vertebrate HAS protein functions catalytically as a monomer.

Hyaluronan (HA),† heparin, chondroitin, dermatan, and keratan are members of the glycosaminoglycan class of carbohydrate. HA [-4]-β-D-GlcUA[1→3]-β-D-GlcNAc (1→) is a prominent glycosaminoglycan that plays roles as a structural element and a recognition molecule in vertebrates (1). The enzymes that catalyze the production of HA, the HASs, were the first examples of glycosyltransferases capable of forming the disaccharide repeat of a glycosaminoglycan that were cloned and described at the molecular level.

The initial HAS to be identified was spHAS (formerly called HasA) of the human pathogen Group A Streptococcus pyogenes. The spHAS enzyme is responsible for the polymerization of HA of the extracellular capsule (2, 3). The spHAS protein is strongly associated with the phospholipid membrane and is predicted to possess six membrane-associated segments (3, 4). The enzyme utilizes UDP-GlcUA and UDP-GlcNAc precursors found in the cytosol and extrudes the growing HA chain out of the cell during polymerization. Based on genetic and biochemical evidence, the product of a single gene, spHAS, catalyzes the transfer both GlcUA and GlcNAc residues to the nascent HA chain (3, 5). This finding is in contrast to the widely accepted “one enzyme, one sugar transferred” dogma of glycobiology (6).

A Xenopus laevis (African clawed frog) protein, xlHAS1, originally called DG42 (for differentially expressed in gastrulation), was the first vertebrate HAS to be cloned, but at the time, its function was not known (7). The amino acid sequence of xlHAS1 is quite similar to that of bacterial spHAS enzyme (3, 4). By overexpression studies utilizing mammalian hosts and expression studies utilizing a yeast host, xlHAS1 was subsequently shown to be a HAS (8–10). Some reports were made of a distinct chitooligosaccharide synthesizing activity (11–13), but whether this phenomenon is abortive catalysis or a physiological function is not clear.

Multiple HAS isoforms have been discovered in vertebrates (14). It is hypothesized that the isoforms were generated by an early gene duplication event. Xenopus contains three active HAS genes including xlHAS1 and one apparently inactive pseudogene (9). Three distinct but similar gene products possessing ~50% identity to xlHAS1, named HAS1, HAS2, and HAS3, were found in human and mouse (9). The Xenopus xlHAS1 gene is an ortholog of the mammalian HAS1 based upon conservation of exon/intron boundaries (9). xlHAS1 is expressed in the frog’s embryonic stage, whereas xlHAS2 is the main transcript in the adult (15). HA is essential for mammalian development because transgenic knockout mice lacking HAS2 fail to develop beyond midgestation (16).

The amino acid sequences of streptococcal HASs, vertebrate HASs, and the viral HAS possess striking similarity. In the proposed catalytic domain (4), the vertebrate HASs share ~60% identity with each other and ~20% identity with the streptococcal and viral HASs. On the basis of the differences in primary structure and predicted topology in the membrane, at least two classes of HAS may exist. We proposed that the first three groups of enzyme should be designated Class I HASs; thus far, the Pasteurella multocida enzyme, pmHAS (17), is the only known example of a Class II HAS (18).

Class I HASs apparently perform many functions including binding UDP-GlcNAc and UDP-GlcUA precursors, catalyzing formation of glycosidic bonds, and potentially transporting the polysaccharide through the plasma membrane. Immunopurification of the spHAS (19) or the murine HAS1 (11) polypeptide...
indicates that a single polypeptide species is responsible for the catalytic transfer of both GlcNAc and GlcUA to the growing polymer chain. The Class II pmHAS protein was recently shown by molecular genetic means to have two active sites in one polypeptide (20).

Based on other known enzymes and sugar transporters, however, it would be surprising if any Class I HAS monomer, ranging in size from 418 to 588 residues (48–69 kDa), would suffice to perform all the potential HAS functions. Radiation inactivation studies of spHAS and seHAS, the homologous enzymes from Group A and C Streptococcus, respectively, have indicated that 16 molecules of lipid and a monomer of protein comprise the functional HA polymerization unit (21). In the past, it was speculated that other molecules or a complex in the mammalian cell is involved or required for HAS activity (11, 22, 23). It has recently been shown that the enriched or purified murine HAS1 polypeptide has HAS activity, but it could not be determined whether this activity was due to a single polypeptide or a homo-oligomer (24). In an effort to further characterize the vertebrate HAS, we have now employed radiation inactivation analysis to examine whether the vertebrate protein exists in a multimeric state (either homo-oligomers or hetero-oligomers). The radiation inactivation technique yields information on the functional unit size of macromolecules without the requirement for prior extraction or purification. We found that the polymerization of HA polysaccharide by xHAS1 requires a single monomer.

Furthermore, we have corroborated the basic tenets of radiation inactivation theory: irradiation of proteins at low temperature restricts radiation damage to only the direct effects (no indirect effects due to free radical propagation and diffusion or other radiation products arise), and the entire polypeptide chain is randomly damaged no matter where the initial radiation interaction occurs (25, 26). By genetic fusion, we have created a larger mass protein that still retains the original biochemical activity. Irradiation of this structure leads to a target size of the new total mass; it is the sum of the two original components. This result confirms and extends the principles already established for direct radiation damage to proteins and introduces a new parameter that can be used as an internal standard in inactivation experiments. Furthermore, it helps to clarify whether a target size corresponds to one or more polypeptides.

**EXPERIMENTAL PROCEDURES**

**Production of Recombinant Native and Fusion xHAS1 Proteins—**All reagents were from Sigma or Fisher, unless noted otherwise. The construction and use of the native size xHAS1 expression plasmid for studies in yeast have been described previously (10, 27). Basically, the xHAS1 open reading frame of 588 residues (7) was cloned into the pYES2 vector (Invitrogen) under control of the GAL1 promoter to form pYES2-DG. The insert was cloned into pYES2 with the aid of the EcoRI and NotI restriction sites within the plasmid. The ligation mixture was transformed into Escherichia coli, and the resulting colonies were screened by PCR and restriction mapping. The new plasmid, called pYES-DG-GFP, was isolated and sequenced to verify the cloning. The plasmid was transformed into Saccharomyces cerevisiae yeast by the lithium acetate/polyethylene glycol method (29). Two different yeast hosts were employed, InvSC1 (a robust diploid strain; Invitrogen) and BJ5461 (a pleiotropic protease-deficient strain; kindly supplied by Rebecca Contopoulou, Yeast Genetic Stock Center, Berkeley, CA). Western blotting with anti-DG42 antibody (7) or anti-GFP antibody (Sigma) demonstrated that the fusion protein was expressed, and HAS activity assays showed that it had similar specific activity to xHAS1 (data not shown).

Yeasts with recombinant plasmids were routinely grown to a suitable biomass in uracil-deficient synthetic media with 0.1% glucose and 5% glycerol until A\textsubscript{600} was 0.3. Upon induction with 1% galactose, active xHAS1 or xHAS1-GFP fusion accumulated in the plasma membrane fraction. Crude membranes were prepared by disruption with silica/zirconia beads (0.5 mm) in a MiniBead-Beater-8 (Biospec) and harvested by ultracentrifugation (27). The membrane pellet was suspended in 50 mM Tris, pH 7.5, 10% glycerol, 1 mM dithiothreitol, 0.1 mM EDTA, 1 mM benzamidine, 0.2 mM 4-(2-aminophenoxy)-benzenesulfonfluoride, and 5 μg/ml pepstatin. Protein was quantitated by the Coomassie Blue dye binding assay (30) (Pierce) using a bovine serum albumin standard. The total protein concentration of samples destined for radiation inactivation was determined, and samples destined for radiation inactivation (one polypeptide) were stored at −80 °C until assay.

**Radiation Procedure—**Samples were irradiated at −135 °C in the gas phase of evaporating liquid nitrogen with a beam of 13-MeV electrons produced by a linear accelerator (Armed Forces Radiobiology Research Institute, Bethesda, MD). Two to four aliquots were not irradiated to serve as the zero dose control. Radiation dose was determined by the thermoluminescent dosimeters. Typical exposure rates were 10–30 megarads/h. Upon opening the vials, but before thawing, nitrogen gas was used to purge any remaining oxygen generated by the intense radiation (35). The volume in each vial was then divided into several 0.5 ml aliquots that were stored at −80 °C until assay.

**HAS Assays—**The incorporation of sugars into high molecular weight HA polysaccharide was monitored by using UDP-[14C]GlcUA (∼290 mCi/mmol; PerkinElmer Life Sciences) precursor as described previously (10, 27). The membrane preparations were incubated at 30 °C for 30 min to 2 h in a buffer containing 50 mM Tris, pH 7.5, 20 mM MgCl\textsubscript{2}, 1 mM diithiothreitol, 120 or 300 μM UDP-GlcUA, and 600 or 1200 μM UDP-GlcNAc. Reactions were terminated by the addition of 50 mM EDTA (pH 8) and 60% formamide (w/v). Descending paper chromatography (65:35, ethanol:1 M ammonium acetate, pH 5.5) was utilized to separate products from substrates; the radioactive polymers at the origin of the paper chromatogram were detected by liquid scintillation counting. To assess nonspecific incorporation, parallel control reactions without UDP-GlcNAc were used (<5% of the specific binding at all doses). Assays for quantitation of xHAS1 or xHAS1-GFP synthase activity were set so that <5% of the radiolabeled substrate was consumed, and the enzyme concentration was in the linear range. Both enzymes lost <5% of their original HAS activity after three freeze-thaw cycles.

**GFP-DH Assays—**The GFP-DH standard enzyme activity was measured at 25 °C in 1-ml assays containing 56 mM Tris, pH 7.8, 3.4 mM MgCl\textsubscript{2}, 0.2 mM NADP, 5.3 mM glucose-6-phosphate, and 0.5–50 μl of the irradiated membrane preparations (34). Reaction rates were obtained by monitoring the change in A\textsubscript{485} over time. Reactions with the same amount of membranes but lacking the glucose-6-phosphate were used to obtain baseline reaction rates.

**Molecular Mass Determination—**The fraction of the surviving HAS activity (A\textsubscript{S}) or the amount of xHAS1 polypeptide (P\textsubscript{S}) was plotted on a semilog scale as a function of radiation dosage. The data from each series of irradiated samples was fit by least squares analysis to a line that passed through 1.0 at zero dosage. The slope of the line (k) = (D\textsubscript{S})\textsuperscript{-1}, where D\textsubscript{S} is the dose in megarads that reduces the activity or the amount of a protein species to 37% of the irradiated sample. The empirical and theoretical relationship is molecular mass = (6.4 × 10\textsuperscript{8} × S\textsubscript{D})\textsuperscript{1/5}, where S\textsubscript{D} = 2.8 for irradiation at −135 °C (34). Thus, the molecular mass.
lecular mass (in kDa) = 1792 k (35). In the case of measuring enzyme activity, the target size corresponds to the mass of the enzyme’s functional unit (strictly defined as the sum of the masses of all the structures required to express activity in the assay).

RESULTS AND DISCUSSION

Many of the molecular details of HASs and of the majority of other glycosyltransferases in general are not known, except for their deduced amino acid sequence. xlHAS1 contains predicted membrane-associated segments or transmembrane helices clustered at both the amino and carboxyl termini. The predicted positioning of the membrane-associated regions in xlHAS1 and mammalian HASs is similar to that found in spHAS (4), except that there appear to be two more membrane-associated regions at the carboxyl terminus in the vertebrate enzyme. The function or potential role of these additional segments is not known.

By gene fusion techniques, a new protein can be created that is an extended linear polypeptide. The fusion of the HAS gene to the GFP gene leads to the synthesis of a larger protein. It had previously been shown (25) that radiation damage occurred throughout a natural polypeptide, no matter where the initial primary ionization had occurred; thus, it was anticipated that the same processes would occur in a fusion protein. A single primary ionization anywhere in the polypeptide would lead to structural damage randomly throughout that polymer. Biochemical functions associated with each of the protein domains in the fusion would be lost, as they were in the arom complex (26), and target analysis of surviving activity will yield a mass equal to that of the entire polypeptide. A polypeptide of mass \( i \) that is genetically fused to a different polypeptide will yield a polypeptide of mass \( i/j \), where \( j \) is the mass of the fused protein together with the mass of any linker sequence. If a single polypeptide is required for expression of activity, the radiation target size will change from \( i \) in the native state to

![FIG. 1. Schematic of xlHAS1 and xlHAS1-GFP proteins. The xlHAS1-GFP DNA construct (HAS-GFP) was prepared using the PCR-ligation-PCR method. The xlHAS1 and xlHAS1-GFP proteins in membrane preparations from recombinant yeast were irradiated and assayed for synthase activity.](http://www.jbc.org/)

### TABLE I

| Run no. | Enzyme | Dosimetry target size | G6P-DH adjusted target size | Predicted size |
|---------|--------|-----------------------|-----------------------------|----------------|
| 1       | L.m. G6P-DH | 105 kDa | 82 kDa | 109 kDa |
|         | xlHAS1 | 85 kDa | 112 kDa | 107 kDa |
|         | xlHAS1-GFP | 85 kDa | 72 kDa | 25 kDa |
| 2       | L.m. G6P-DH | 85 kDa | 85 kDa | 109 kDa |
|         | xlHAS1 | 70 kDa | 98 kDa | 128 kDa |
|         | xlHAS1-GFP | 70 kDa | 28 kDa | 37 kDa |
| 3       | S.c. G6P-DH | 99 kDa | 99 kDa | 115 kDa |
|         | xlHAS1 | 104 kDa | 133 kDa | 130 kDa |
|         | xlHAS1-GFP | 104 kDa | 24 kDa | 26 kDa |
| Average ± S.D. | xlHAS1 | 85 ± 15 kDa | 92 ± 11 kDa | 69 kDa |
|         | xlHAS1-GFP | 111 ± 13 kDa | 122 ± 13 kDa | 96 kDa |
|         | Δ Mass | 26 ± 2 kDa | 29 ± 7 kDa | 27 kDa |

*Monomer size predicted from the deduced DNA sequence.

xlHAS1 (588 aa; 69 kDa)

HAS-GFP (833 aa; 96 kDa)

![FIG. 2. Target size analysis of xlHAS1 and xlHAS1-GFP. Residual HAS activity was determined for each radiation dose a minimum of four times. These results were then graphed logarithmically versus radiation dose, and the slope of the least squares line (k) was determined. The plot for Run 1 (Table I), a representative of the plots obtained for each irradiation run, is depicted. xlHAS1, ; xlHAS1-GFP, ■. Error bars denote ± S. D.](http://www.jbc.org/)
i + j in the fused form. Similarly, a requirement for multiple polypeptides will yield target sizes of n(i) or n(i + j) for the two forms.

In our radiation inactivation studies, we employed two types of internal standards to calibrate and refine our target size of xlHAS1. As in many previous studies (36, 37), we included an enzyme of known size, G6P-DH, to compare with our experimental protein. The calculated standard target size for each enzyme of known size, G6P-DH, to compare with our experimental protein, xlHAS1, by fusing it to GFP. We then made a direct comparison between the native and the fusion proteins. The fusion polypeptide, xlHAS1-GFP (Fig. 1), consisting of xlHAS1 and the Aequorea GFP is a functional HAS. xlHAS1-GFP (predicted mass, 96 kDa) is 27 kDa larger than xlHAS1 (predicted mass, 69 kDa), with similar specific activity. The results of a representative radiation inactivation experiment are shown in Fig. 2. The sizes obtained for xlHAS1 and xlHAS1-GFP from three different irradiation runs are shown in Table I. Results are given based on radiation doses determined both by dosimetry and by calculation using the G6P-DH standard. The measurement of radiation doses using an internal biological monitor, like G6P-DH, is more reliable because it identifies the dose actually delivered to the sample (33). The difference in experimental target size between xlHAS1 and the fusion protein, 25–37 kDa, corresponds to the GFP monomer. In this construct, each enzyme molecule was fused to a single GFP monomer, so that an increase in target size equivalent to one GFP structure shows that only one HAS polypeptide is required for synthase activity.

Experimental error can sometimes cloud association of a radiation target size with the mass of either a monomer or dimer. Application of the fusion technique permits comparison of the target sizes with the two expected masses, as well as the difference of the two target sizes with the mass of the fused polypeptide. This general principle can be applied to any protein whose gene can be manipulated. These modifications enhance and reinforce the analyses possible by radiation target theory.

The predicted xlHAS1 mass (69 kDa) and the experimental target size (85 ± 15 as determined by dosimetry or 92 ± 11 as determined by G6P-DH adjustment), however, are not identical. It appears there is some additional mass (~20 kDa) in the target size that is not accounted for by the xlHAS1 polypeptide. In the case of the streptococcal HASs, this excess mass is believed to be lipid (21). This interpretation was based on the fact that the size obtained from HAS assays performed on intact, irradiated membranes was larger than the size obtained by solubilizing the irradiated membranes, adding cardiolipin (a stimulatory phospholipid), and then performing the HAS assay. Unlike the streptococcal HASs, we have not been able to purify an active form of xlHAS1 either with or without the addition of cardiolipin or total vertebrate lipid extracts. Also, addition of lipids to membrane preparations did not alter the HAS activity (data not shown). The HAS activity of detergent-solubilized, enriched murine HAS1 was reported to be unaffected by added phospholipid (11). If the additional mass observed in the xlHAS1 radiation inactivation target size were due to lipid, the mass observed could correspond to ~14 molecules of cardiolipin or ~25 molecules of phospholipid. Work is in progress to determine whether lipid is involved in the HAS catalytic or transport mechanism.

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