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Immunochemical Confirmation of the Primary Structure of Streptococcal Hyaluronan Synthase and Synthesis of High Molecular Weight Product by the Recombinant Enzyme†

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ABSTRACT: We have recently identified and cloned the gene for hyaluronan (HA) synthase, *hasA*, from group A *Streptococci* [DeAngelis, P. L., Papaconstantinou, J., & Weigel, P. H. (1993) *J. Biol. Chem. 268*, 19181–19184]. We have now generated two polyclonal monospecific antibodies against synthetic peptides corresponding to portions of the deduced protein. Both antibodies recognize a protein with an apparent molecular weight of 42 000 either from wild-type *Streptococcus pyogenes* or from *Escherichia coli* containing the cloned gene on a plasmid. Immobilized affinity-purified antibody depleted HA synthase activity from functional detergent extracts of streptococcal membranes in a specific fashion. The immobilized protein displayed HA synthase activity, and HasA was the major bound polypeptide. The recombinant HA synthase behaves identically to that from *Streptococci*, with respect to sugar nucleotide specificity and polysaccharide production. Only the authentic sugar nucleotides UDP–glucuronic acid and UDP–*N*-acetylglucosamine support HA polymerization. The recombinant enzyme elongates HA in a processive manner and rapidly produces polymers on the order of $\geq 5 \times 10^6$ Da at rates of about 10–30 monosaccharides/s at three times the apparent K_m of substrates.

The HA[1] capsule is one of the virulence factors of GAS that aids the pathogen in evading human defenses such as complement-mediated lysis and phagocytosis (Kass & Seastone, 1944; Wessels *et al.*, 1991). The bacterial HA polysaccharide, composed of alternating GlcA(β1→3) and GlcNAc(β1→4) residues, is identical to the molecule produced in higher animals (Meyer & Palmer, 1934) and, therefore, is relatively nonimmunogenic (Quinn & Singh, 1957). We recently demonstrated that two genes, *hasA* and *hasB*, can direct capsule biosynthesis in eubacteria (DeAngelis *et al.*, 1993a,b). By deletion analysis of this locus, we identified HasA as the HA synthase that polymerizes the polysaccharide from UDP–sugar nucleotides. The protein migrates at M_r = 42 000 on SDS–PAGE and has several putative transmembrane segments (DeAngelis *et al.*, 1993a,b), in agreement with the HA synthase activity residing in membranes (Markovitz *et al.*, 1959). HasA is the first glycosaminoglycan synthase to be described at the sequence level. Here we confirm the assignment of the HasA open reading frame by recognition with antibodies and characterize the substrate specificity and polymerization capacity of the recombinant enzyme.

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[1] Abbreviations: HA, hyaluronan or hyaluronic acid; GAS, group A streptococci; GlcA, glucuronic acid; GlcNAc, *N*-acetylglucosamine; UDP, uridine diphosphate; kDa, kilodalton; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; THY, Todd Hewitt with 1% yeast extract; EDTA, ethylenediaminetetraacetic acid; DTE, dithioerythritol; GlcN, glucosamine; GalNAc, *N*-acetylgalactosamine; GalA, galacturonic acid; M_r, relative molecular weight; Tris, tris(hydroxymethyl)aminomethane; TFA, trifluoroacetic acid; IgG, immunoglobulin G; TBS, Tris-buffered saline, pH 7.5; TBST, TBS plus 0.01% Tween; PBS, phosphate-buffered saline, pH 7.4; PBSD, PBS containing 0.5% dodecyl maltoside.

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EXPERIMENTAL PROCEDURES

Materials, Strains, and Plasmids. Radioimmunoassay grade BSA was from U.S. Biochemicals. Media components, unless stated otherwise, were from Difco. Fmoc peptide synthesis with Milligen reagents was performed by the laboratory of Dr. Charles Glabe (University of California, Irvine; Glabe, 1990). All other reagents were supplied by Sigma unless stated otherwise. SDS–PAGE reagents were the standard formulations (Laemmli, 1970). Gels were silver stained by the method of Blum *et al.* (1987). Protein concentration was assayed with bicinchoninic acid (Pierce; Smith *et al.*, 1985) using BSA as the standard. S43/192/4, a wild-type encapsulated GAS strain (Rockefeller Collection; Dochez *et al.*, 1919), and a Tn916 mutant with an ablated *hasA/B* locus, S43Tn7 (DeAngelis *et al.*, 1993a), were grown in THY media. The pPD41 series of plasmids (DeAngelis *et al.*, 1993a,b) contains the streptococcal HA biosynthesis locus cloned into the shuttle vector pAT19 (Trieu-Cuot *et al.*, 1991).

Membrane and Extract Preparation. Membranes from *Streptococcus pyogenes* were obtained by a modification of a protoplast method (van de Rijn & Drake, 1992). Log phase cells were harvested by centrifugation, washed with PBS, and resuspended to 1/50 volume of the original culture volume in 30% raffinose, 40 mM Tris, and 4 mM $MgCl_2$, pH 7. *N*-Acetylmuramidase SG from *Streptomyces globisporus* (Seikagaku Corp.), instead of phage lysin, was added to 0.1 mg/mL, and the mixture was incubated at 37 °C for 1 h with occasional swirling. The protoplasts were then processed as described in their method except that, after lysis in hypotonic buffer, the suspension was sonicated three times for 30 s each at about 20 W with the microtip (model W-380; Heat Systems-Ultrasonics, Inc.). The lysate was centrifuged at 10000*g* for 10 min at 4 °C, and the membranes were harvested at 100000*g* for 60 min at 4 °C. The membranes were washed twice with PBS containing 10 mM $MgCl_2$ and stored at −70 °C. Detergent extracts of the membranes were prepared using 0.5% (w/v) dodecyl maltoside (Calbiochem) as described by van de Rijn and Drake (1992), except that DTE was omitted for antibody depletion experiments and immunoaffinity purification.

Membranes from *Escherichia coli* were isolated by a variation of a protoplast method (Ito *et al.*, 1977). All steps were at ≤4 °C. Log phase SURE cells, with various plasmids containing the HA biosynthesis locus, were harvested by centrifugation (3000*g*, 15 min) and washed once with PBS. The cell pellet was resuspended to 1% of the original culture volume in 20% sucrose and 30 mM Tris, pH 8.2, containing leupeptin and pepstatin (0.5 and 0.7 μg/mL, respectively). Lysozyme (4 mg/mL) in 0.1 M EDTA, pH 8 (0.1% culture volume), was added, and the suspension was incubated for 40 min. Phenylmethanesulfonyl fluoride was added to 46 μg/mL, and the suspension was sonicated as described for the streptococcal cells. $MgCl_2$ was added to 60 mM, and DNase and RNase (final concentration 1 μg/mL each; Promega) were added. After 10 min at 4 °C, debris was removed by centrifugation (10000*g*, 5 min, 4 °C), and the lysate was diluted 5-fold in PBS before the membranes were harvested at 100000*g* for 1 h. The membrane pellet was washed twice with PBS, containing 10 mM $MgCl_2$ and the above protease inhibitors, by repeated suspension and centrifugation. The final pellet was stored frozen at −70 °C in PBS.

HA Synthase Assay. Membrane preparations or extracts were incubated at 37 °C in 20 mM $MgCl_2$, 0.5 mM DTE, and 50 mM sodium/potassium phosphate buffer, pH 7.0, containing various amounts of UDP–sugars (0–940 μM UDP–GlcNAc and/or 0–580 μM UDP–GlcA as noted). UDP–[^{3}H]GlcNAc (30 Ci/mmol; Dupont NEN) and/or UDP–[^{14}C]GlcA (267 mCi/mmol; ICN Biomedicals) were used to measure incorporation into HA. The reactions were terminated by addition of SDS to 2% (w/v) final concentration. Incorporation into high molecular weight product was measured either by descending paper chromatography (Whatman 3MM, developed in 1 M ammonium acetate, pH 5.5/ethanol, 7:13) or by gel filtration.

Gel Filtration Analysis. Radiolabeled HA was analyzed with respect to size using Sepharose 2B (Pharmacia) in 0.15 M NaCl and 4 mM Tris, pH 8. The column (1 × 24 cm) was calibrated with high molecular weight HA (~5 × 10^6; Lifecore), blue dextran (~2 × 10^6; Pharmacia), and UDP–[^{3}H]GlcNAc. Before application to the column, samples were boiled for 1 min; the SDS concentration was reduced to 0.16% (w/v) by dilution with column buffer and then clarified by centrifugation at 15000*g* for 7 min. Portions of each fraction were mixed with scintillation cocktail and counted.

Western Blotting. After SDS–PAGE, proteins were transferred by "semidry" blotting to nitrocellulose (0.1 μm; Schleicher & Schuell) at 1 mA/cm^2 for 45 min in standard Towbin buffer with 20% methanol (Towbin *et al.*, 1979) using the Milliblot–SDE device (Millipore). The blots were then briefly air-dried, visualized with the reversible stain Ponceau S (0.5% stain in 1% acetic acid; destained with water), and blocked for 2 h in 5% BSA in TBS. TBS with 0.01% (v/v) Tween (TBST) was used routinely for washes and antibody incubations. Goat antirabbit IgG–alkaline phosphatase conjugates (ELISA grade) and *p*-nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (Bio-Rad) were used for detection on Western blots.

Monospecific Antibody Production. Synthetic peptides based on the deduced protein sequence of HasA (DeAngelis *et al.*, 1993b) were used to generate polyclonal antibodies in rabbits. Peptide A corresponds to the carboxyl terminus, CTIKNTEWGTRKKVTIFK. Peptide B is from a hydrophilic portion of the central region of the HasA molecule, ERSDADVFLTVDSDT. The latter peptide was synthesized with an additional Cys residue at the amino terminus. The cleaved peptides were dissolved in water, reduced with 10 mM dithiothreitol, and purified by reversed-phase high-performance liquid chromatography (Vydac C4 column, TFA/H_2O/acetonitrile mobile phase). The peptides were coupled by their –SH groups to the carrier protein ovalbumin, which had been activated with *N*-succinimidyl-3-(2-pyridyldithio)propionate (Pierce), a heterobifunctional cross-linker.

Female New Zealand White rabbits were immunized with conjugate (~0.4 mg of peptide/1.5 mg of ovalbumin each) in complete Freund's adjuvant subcutaneously in the back at multiple sites. Thereafter, the rabbits were boosted with 1–2 mg of free peptide in Freund's incomplete adjuvant every 3 weeks. Bleeds were taken at 2 weeks after the boost. IgG was obtained by ammonium sulfate precipitation (30–50% saturation), and the pellet was resuspended and dialyzed against PBS. Specific antibodies from the third boost were purified on columns of peptide coupled to Sulfolink agarose beads (Pierce) according to manufacturer guidelines. The total IgG was applied three times to the beads over 1 h. The beads were washed extensively with PBS, and the specific antibody was then eluted with 0.1 M glycine, pH 2.5, neutralized with 0.1 volume of 1 M Tris, pH 8, and dialyzed against PBS.

A longer version of peptide B (NKGKRHAQAWAFERS-DADVFLTVDSDT), designated peptide C, was also syn-

thesized as the acetylated amide with an extra amino-terminal cysteine. This peptide was insoluble in every solvent tried (including dimethylformamide, or neat TFA, with and without reducing agents). Therefore, the crude product was washed with PBS twice and used directly as an initial immunogen and for boosts in one rabbit as well as a supplement in boosts with peptide B for the last 2 cycles of another rabbit initially immunized with peptide B–conjugate. These rabbit sera were pooled and purified on peptide B–agarose, and the preparation was designated antipeptide B/C–IgG.

HasA Depletion and Immunoaffinity Purification. Affinity-purified monospecific antibody against peptide A was covalently coupled to hydrazide beads (1 μg of IgG/μL of resin; Avidgel F, Bioprobe Int.), *via* the IgG oligosaccharides after oxidation with periodate, according to the manufacturer guidelines. Control beads were made by omitting the IgG from the coupling buffer.

Immunoaffinity purification of streptococcal extracts was performed with the goal of identifying the polypeptide(s) involved in HA biosynthesis. The BSA blocking step above was omitted, and 100 μL of beads was loaded and washed in a scaled-up column format using 700 μg of extract protein. The beads were removed from the column and incubated for 2 h with mixing in a batchwise fashion with 125 μM peptide A in 0.5 mL of PBSD supplemented with 10% glycerol and 10 mM $MgCl_2$. A sample (10 μL) of the eluted material was assayed directly for synthase activity. The remainder of the eluate was concentrated 7-fold and dialyzed with PBS using a Centricon 10 device (10 000-Da cutoff, Amicon) before SDS–PAGE analysis.

Gram-Positive Cell Extracts. Proteins for gel analysis were released from *S. pyogenes* by first preparing protoplasts of the cells prior to solubilization with detergent. Log phase cells were treated with ovine testicular hyaluronidase (type V, 0.1 mg/mL, 37 °C for 30 min), washed in PBS, and suspended in 1/22 of the culture volume of streptococcal protoplasting buffer. After *N*-acetylmuramidase SG digestion, the cells were harvested by centrifugation at 10000*g* for 5 min, washed once in the above buffer without enzyme, suspended in 1× reducing SDS–PAGE sample buffer (Laemmli, 1970), and boiled for 5 min. The samples were clarified by centrifugation before being loaded on gels.

Metabolic Labeling and Immunoprecipitation of Bacterial Proteins. A mixture of [^{35}S]Met and [^{35}S]Cys (Tran ^{35}S-label, 1054 Ci/mmol; ICN) was used to label bacteria. *E. coli* were grown with shaking at 37 °C in minimal M9 salts (Life Technologies, Inc.) supplemented with 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.2% glucose, 10 μg/mL thiamin, and 175 μg/mL erythromycin to A_{600} = 0.4 before addition of 33 μCi/mL of radiolabel. After incubation for 15 min, a 5-min chase of unlabeled Cys and Met (33 μg/mL final concentration each) was employed before the cells were washed twice with PBS. Cells were lysed with 2% SDS in 50 mM Tris, pH 8, and boiled for 5 min. *S. pyogenes* were grown as unshaken cultures at 37 °C in complete defined media for GAS (JRH Bioscience; van de Rijn & Kessler, 1980) until A_{600} = 0.4. The cells were harvested by centrifugation and resuspended in a solution containing only the salts and glucose found in TH media. After the cells were starved for 5 min at 37 °C, 20 μCi/mL radiolabel was added 5 min before the addition of 0.5 volume of complete defined media. After an additional 30 min, the cells were washed in PBS. Protoplasts were prepared as described above before lysis with 2% SDS and boiling.

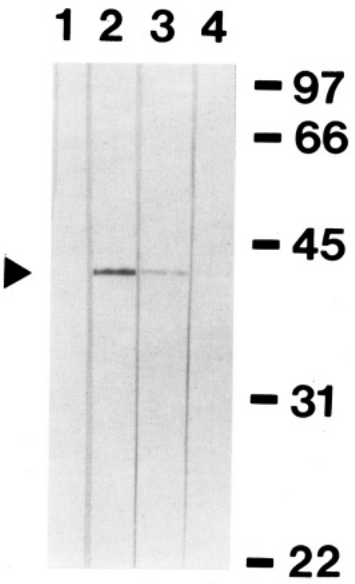

FIGURE 1: Western analysis of *S. pyogenes* membranes. A functional membrane preparation from strain S43 (18 μg of protein) was analyzed by SDS–PAGE on a 12.5% gel and transferred to nitrocellulose. The 1.3 cm wide lane was stained with Pouceau S and cut into four equivalent strips before destaining and Western analysis. Strips 1 and 2 were incubated with antipeptide A–IgG, while strips 3 and 4 were incubated with antipeptide B/C–IgG. The antibodies used in strips 1 and 4 were preincubated with 11 μM free homologous peptide for 30 min. Standards (Bio-Rad low range, marked in kDa) in an adjacent lane were visualized with Ponceau S. The identical protein at M_r = 42 000 (HasA) marked by the arrowhead is specifically recognized by both antibodies.

Protein A–agarose beads (preincubated for 30 min in 5% BSA in TBS) were used to immobilize affinity-purified antibodies for immunoprecipitation of the radiolabeled extracts. The beads and specific IgG against peptides B/C were incubated for 1 h with gentle mixing, and the beads were washed with TBST twice by repeated centrifugation (1000*g*, 1 min) and suspension. The total cell extracts (~2 × 10^6 cpm from 1 mL of culture) were diluted 20-fold in TBST and clarified by centrifugation at 10000*g* for 5 min before addition of the antibody beads. After 1 h, the beads were washed six times with TBST. Bound proteins were eluted by boiling the beads for 2 min in 2 volumes of 1× reducing SDS–PAGE buffer. Half of the sample was electrophoresed, and the gels were dried directly for autoradiography using Kodak XAR-5 film.

RESULTS

Antibody Analysis of HasA. Antibodies to two different synthetic peptides corresponding to the deduced primary sequence of HasA recognize a protein of M_r = 42 000 from the wild-type *S. pyogenes* strain S43 as seen by Western blotting (Figure 1). The binding of each monospecific antibody was blocked by preincubation with the homologous peptide. Neither antibody detected a similar protein in extracts from the S43Tn7 mutant which is missing the *hasA* gene (not shown), thus confirming the antibody specificity for HasA. Immobilized specific IgG against peptide A removed HA synthase activity from solubilized streptococcal membranes (Figure 2A). Homologous, but *not* heterologous, peptide blocked this effect. Furthermore, at least 18% of the starting HA synthase activity was then recovered associated with IgG beads after the extract was washed away. The activity was specifically bound by the IgG; beads preincubated with the homologous peptide A had only 2% of the starting HA synthase activity, but beads treated with the heterologous peptide B

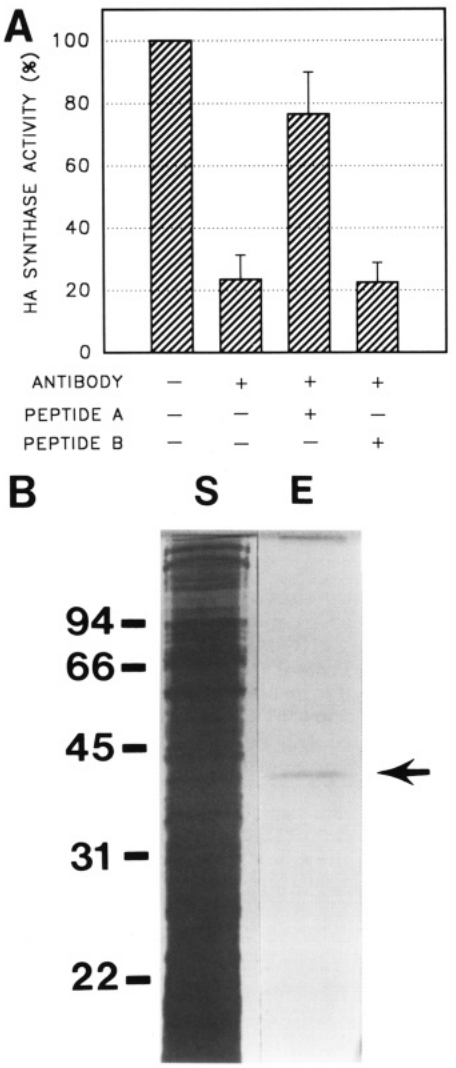

FIGURE 2: Immunodepletion of HA synthase activity and purification of HasA. Panel A: Control Avidgel beads or antipeptide A–IgG beads were blocked with 2.5% BSA in TBS for 30 min on ice and washed with PBSD, and 10 μL of beads was resuspended in 4 volumes of PBSD. Some samples of antibody beads were preincubated with 28–40 μM peptide A or B (as indicated) for 30 min at 23 °C with gentle mixing by rolling before addition of 15 μL of a dodecyl maltoside extract of S43 membranes (24 μg protein). After an additional 60-min incubation with mixing at 23 °C, the beads were removed by centrifugation (2000*g* for 10 s). Portions of the supernatant were assayed for HA synthase activity using 300 μM UDP–GlcNAc and 120 μM UDP–GlcA (2×10^4 cpm per assay; 100 μL) for 1 h at 37 °C. Although the data are not shown in this figure, the beads were also washed with 10 volumes of PBSD and likewise assayed for synthase activity, except that the tubes were mixed during the incubation and SDS termination steps and the beads were removed prior to paper chromatographic analysis (see text). The histogram shows the average HA synthase activity in the supernatants from two separate experiments (±SD) using extracts prepared on different days. The total enzyme activity from the supernatants of samples incubated with control beads with no antibody was set at 100% and was the equivalent of 10 nmol of GlcA transferred/h. The monospecific antibody to HasA substantially depletes HA synthase activity from functional extracts in a specific fashion. Panel B: Membrane extract (700 μg of protein) was applied to a column of antipeptide A–IgG beads and eluted with free peptide A as described in Experimental Procedures. After concentration, one-quarter of the eluted material was analyzed by SDS–PAGE on a 12.5% gel. These silver-stained lanes show the starting material (S; 3.5 μg of protein, 1.5 nmol of GlcA/h) and the eluted material (E; 2.8 nmol of GlcA/h). Standards are marked as in Figure 1. The eluted material contains HasA (arrow) and retains HA synthase activity.

contained enzyme activity quantitatively indistinguishable from the untreated IgG beads. When the material bound to

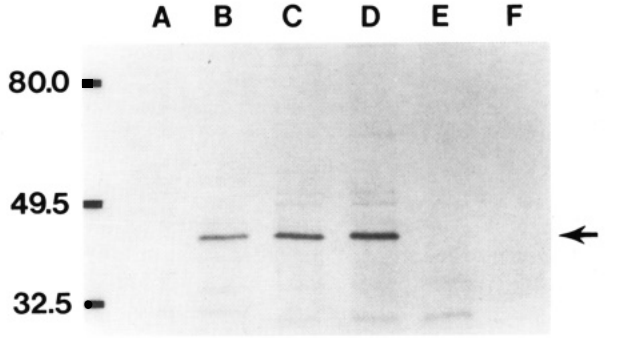

FIGURE 3: Immunoprecipitation of bacterial extracts. Immunoprecipitates prepared from ^{35}S-radiolabeled cell extracts using the antipeptide B/C–IgG were analyzed by SDS–PAGE. The autoradiogram (13-day exposure) reveals that the same protein (arrow) at 42 kDa is made in both wild-type *S. pyogenes* S43 and some *E. coli* transformants. The mixture lane (C) was flanked by lanes of the two individual samples. Lanes: A, S43Tn7 mutant; B, S43; C, one-half S43 plus one-half *E. coli* SURE (pPD41Δ5); D, SURE (pPD41Δ5); E, SURE (pPD41Δ*Eco*RV); F, SURE cells. Standards (Bio-Rad prestained low range) are marked in kDa on this 12.5% gel.

IgG beads was eluted with either 8 M urea (not shown) or free peptide A (Figure 2B) and analyzed by SDS–PAGE, the HasA protein was the major band consistently observed by silver staining. The peptide-eluted material, although obtained in low yield (~3% based on activity), retained HA synthase activity. The urea preparation was inactive even after substantial dilution into assay buffer. The above results suggest that HasA is the sole protein needed for HA synthase activity.

Comparison of Recombinant and Native HasA. The *E. coli* SURE membrane preparations from cells containing intact *hasA* produced HA. Specific activities of 500 and 2000 pmol of GlcA transferred h^{-1} (μg of protein)$^{-1}$ were observed for membranes from cells containing pPD41Δ5 and pPD41Δ*Pst*I, respectively. Membranes from cells with the truncated version of *hasA* found on pPD41Δ*Eco*RV did not possess active HA synthase and had a background equivalent to host cells alone (no more than 11 and 12 pmol h^{-1} mg^{-1}, respectively).

In contrast to streptococcal samples, a recombinant version of the 42-kDa protein in extracts of *E. coli* SURE cells containing pPD41Δ5 or pPD41Δ*Pst*I could not be detected by Western blots utilizing a secondary IgG/alkaline phosphatase conjugate. Therefore, we employed immunoprecipitation of radiolabeled extracts to compare the proteins from different sources. The plasmid constructs pPD41Δ5 or pPD41Δ*Pst*I can direct production of the HA polysaccharide, but pPD41Δ*Eco*RV cannot; an intact *hasA* gene is in the first two plasmids but not the latter (DeAngelis *et al.*, 1993b). IgG raised against the B/C peptides immunoprecipitated a protein migrating at M_r = 42 000 from *S. pyogenes* S43 or *E. coli* SURE (pPD41Δ5) cell extracts but not from the S43Tn7 mutant or SURE (pPD41Δ*Eco*RV) extracts (Figure 3). The same size protein was also immunoprecipitated from SURE (pPD41Δ*Pst*I) cells (not shown). To examine more closely if the *E. coli* version of HasA was identical in size to the wild-type S43 version, we mixed equal amounts (by radioactivity) of both immunoprecipitates and analyzed them by SDS–PAGE (Figure 3; lane C). There was no detectable difference in the migration or appearance of the HA synthase from either source. Therefore, HasA is synthesized and processed to give a functional enzyme in a similar fashion in Gram-positive *S. pyogenes* and Gram-negative *E. coli*.

Substrate Specificity of the Recombinant Enzyme. HA synthase utilizes both UDP–GlcA and UDP–GlcNAc to form

Table 1: Sugar Nucleotide Specificity of Hyaluronan Synthase Expressed in *E. coli* (PD41Δ5) Membranes[a]

second sugar nucleotide present (μM)	[^{14}C]GlcA cpm (%)	[^{3}H]GlcNAc cpm (%)
none	474 (1.8)	0 (0)
UDP–GlcA (128)		33000 (100)
UDP–GlcNAc (320)	26000 (100)	
UDP–Glc (160)	309 (1.1)	0 (0)
UDP–GalA (150)		0 (0)
UDP–GalNAc (300)	375 (1.4)	

[a] Crude membranes (22 μg of protein) from early log cells were incubated at 37 °C for 1 h with either 128 μM UDP-[^{14}C]GlcA (3.4 × 10^4 cpm) or 160 μM UDP-[^{3}H]GlcNAc (1 × 10^5 cpm). The radiolabeled sugar nucleotide was used in the presence of the indicated second nonlabeled sugar nucleotide. Precursors and product were separated by paper chromatography, and radioactivity remaining at the origin was determined. Hyaluronidase treatment for 30 min decreased incorporation into the product formed with authentic substrates by ~87%. No enzyme activity is seen in the absence of free Mg^{2+} (excess EDTA).

the alternating structure of the HA polysaccharide (Markovitz *et al.*, 1959). We assessed the sugar nucleotide specificity of recombinant HasA by measuring the incorporation of radiolabeled normal sugar precursors. The galactose–nucleotide analogs, the C4 epimers of glucose, did not substitute for the natural precursors of HA. UDP–GalA could not support incorporation of UDP–[^{3}H]GlcNAc into polysaccharide, and likewise UDP–GalNAc failed to produce polymer when used with UDP–[^{14}C]GlcA (Table 1). Also UDP–Glc was not incorporated with either radiolabeled precursor (Table 1). Therefore, the observed sugar nucleotide specificity of the recombinant HasA is that expected for an authentic HA synthase.

Kinetics of HA Elongation by Recombinant HA Synthase. We used pulse–chase analysis to follow the kinetics of polymerization of HA polysaccharide by the *E. coli* recombinant synthase. The pPD41Δ*Pst*I construct possesses an intact *hasA* gene and a nonfunctional, truncated streptococcal UDP–Glc dehydrogenase gene, *hasB*. *E. coli* SURE cells containing this plasmid make the HasA protein, HA synthase, but they make almost undetectable amounts of HA *in vivo*, since this strain contains very low levels, if any, of UDP–Glc dehydrogenase needed to make the precursor UDP–GlcA (DeAngelis *et al.*, 1993b). Membranes from this recombinant strain were incubated with sugar nucleotides using a pulse–chase protocol. The products of these reactions were fractionated on Sepharose 2B. We observed the rapid, simultaneous and extensive polymerization of both radiolabeled precursors (Figure 4). The calculated polymerization rate is on the order of ~10–30 monosaccharides/s at 3 times the reported apparent K_m concentration of substrates (Dougherty & van de Rijin, 1992). Membranes from *S. pyogenes* S43 or *E. coli* SURE (pPD41Δ5) cells yielded similar profiles of HA elongation (not shown). In all cases the specific *Streptomyces* hyaluronidase completely degraded the high molecular weight double-labeled product found in the void-volume fractions. After digestion, both ^{14}C and ^{3}H radioactivity migrated in the totally included fractions (not shown). Membranes from *E. coli* SURE (pPD41Δ*Eco*RV) cells did not produce labeled polymers detectable by Sepharose 2B or by paper chromatography.

The growth of HA chains was also analyzed by using UDP–GlcNAc and undiluted UDP–[^{14}C]GlcA (Figure 5). The use of one sugar nucleotide in limiting concentration allowed the observation of intermediate size polymers, before and during the chase, that could not otherwise be detected due to the very rapid saccharide polymerization by the HA synthase. Relatively discrete, rather than very broad size distributions were observed, indicating that the enzyme is processive. The enzyme in membranes required the presence of both sugar nucleotide precursors for HA production (data not shown). If only UDP–[^{14}C]GlcA is incubated with the membranes from *S. pyogenes* or *E. coli* that can produce HA, the radioactivity appears in the totally included volume of Sepharose 2B (not shown).

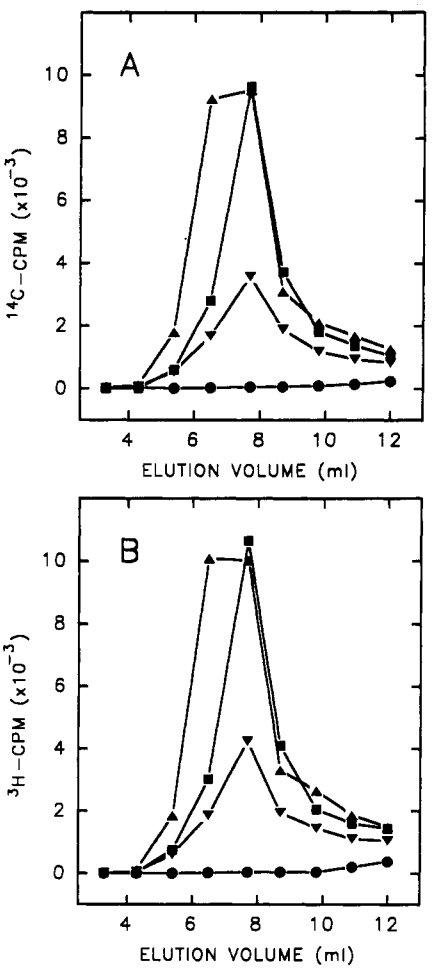

FIGURE 4: Double-labeled, pulse–chase analysis of HA synthesized by the recombinant HasA. Membranes (108 μg of protein) from SURE (pPD41Δ*Pst*) cells were incubated with undiluted ^{3}H- and ^{14}C-labeled sugar nucleotide precursors (12 or 3.9 × 10^5 cpm, respectively) for 40 s at 37 °C before addition of both unlabeled precursors (425 μM UDP–GlcNAc, 170 μM UDP–GlcA final concentration; 460-μL final volume). Samples (80 μL) were then withdrawn at 2 (●), 11 (▼), 32 (■), and 67 (▲) min, and the size profile of the products was analyzed on Sepharose 2B. Only the fractions containing high molecular weight HA in the vicinity of the void volume (6.3 mL) are displayed. Panels: A, ^{14}C channel; B, ^{3}H channel. The membranes incorporate both radioisotopes quickly and in parallel into high molecular weight HA.

DISCUSSION

Both monospecific IgGs described here recognize the M_r ~ 42 000 protein from the GAS strain S43 but do not detect a similar species in the acapsular S43Tn7 mutant with the ablated *has* locus. The recombinant HasA derived from cloned DNA is identical to the wild-type *S. pyogenes* HasA. These immunochemical results confirm the deduced primary sequence of HasA. Although the identity of the amino terminus of the active enzyme has not been determined directly, based on ongoing studies it is likely that the protein starts at the alternate initiation codon GTG (Val), which is 24 residues upstream from the first Met, as noted previously (DeAngelis

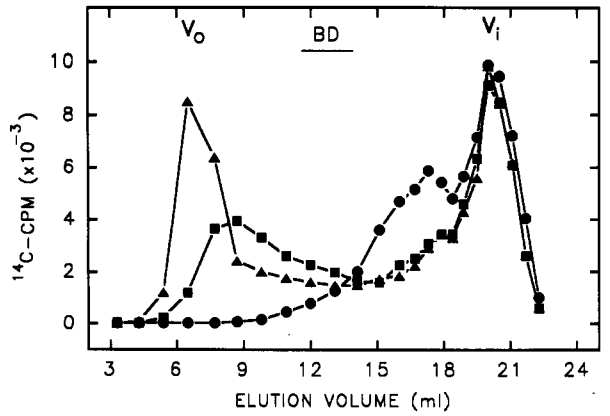

FIGURE 5: Pulse–chase analysis of HA elongation. Membranes (70 μg of protein) from *E. coli* SURE (pPD41Δ*Pst*) cells were incubated with undiluted UDP-[^{14}C]GlcA (2.5×10^5 cpm) and 940 μM UDP–GlcNAc in 256 μL for 5 min at 37 °C, and a portion (80 μL) of this pulse-labeled reaction mix (●) was taken. Unlabeled UDP–GlcA was then added to the remaining reaction mix to a final concentration of 580 μM, and samples (80 μL) were taken at 5 (■) and 22 min (▲) for gel filtration analysis over Sepharose 2B. The void (V_o) and totally included volumes (V_i) as well as the peak of blue dextran (BD) are marked. The progressive increase in predominantly high molecular weight products during the chase period shows that HA is made in a processive fashion.

et al., 1993b). A previous report (Lansing *et al.*, 1993) that the HA synthase gene from group C *Streptococcus equisimilis* strain D181 had been cloned was in error. Their cloned gene, which was completely different from *hasA*, was not shown to direct HA biosynthesis. Subsequently, PCR sequence analysis indicated that group C strain D181 does, in fact, contain the same internal coding region found in the authentic group A *hasA* (DeAngelis *et al.*, 1994).

Two lines of evidence support our conclusion that the *hasA* gene encodes the authentic HA synthase. First, as we initially demonstrated, the *hasA* gene is necessary and sufficient to direct HA biosynthesis in heterologous bacteria (DeAngelis *et al.*, 1993a,b). If the microbe produces UDP–GlcA in addition to the omnipresent UDP–GlcNAc, which is essential for cell wall synthesis, then expression of the intact *hasA* gene on a plasmid allows the cell to make HA. However, in a subsequent report that confirmed our published sequence for *hasA* (except for one nucleotide difference), Dougherty and van de Rijn (1994) speculated that HasA may catalyze transfer of only one of the two sugars, GlcNAc, to the nascent HA chain. This hypothesis was based on the similarity (30% identity) of the HA synthase with NodC, an enzyme from *Rhizobium* that produces oligosaccharides of β-1,4-linked GlcNAc. Another as yet uncharacterized protein, detected by photoaffinity labeling with azido-UDP–GlcA as a 33-kDa band (van de Rijn & Drake, 1992), was invoked as the possible GlcA transferase (Doughtery & van de Rijn, 1994).

Our results strongly argue against this hypothesis. In particular, an additional streptococcal factor was *not* required for HA synthesis in either *Enterococcus faecalis* or *E. coli* (DeAngelis *et al.*, 1993a); the DNA insert of pPD41Δ5 does not contain a gene encoding a 33-kDa protein or any other extraneous gene. The sole difference between our two constructs, pPD41Δ5 and pPD41Δ*Eco*RV, is the status of the *hasA* gene. SURE cells with the latter plasmid, which possesses a truncated gene, do not produce intact HasA as assessed by immunoprecipitation, as well as our earlier minicell experiments (DeAngelis *et al.*, 1993b), and do not synthesize HA. Membranes from SURE cells with the pPD41Δ5 construct, on the other hand, contain intact HasA and can transfer GlcA to the HA polysaccharide upon addition of the sugar nucleotide precursor to membranes. This correlation in a defined genetic system is very strong evidence that *hasA* is indeed the HA synthase and possesses both glycosyl transferase activities required to polymerize HA.

The second line of evidence for assigning the HasA protein as the authentic HA synthase is that a monospecific IgG against the deduced carboxyl terminus specifically removes HA synthase activity from extracts. This immunopurified HA synthase, when supplied with sugar nucleotides, can still function when bound by antipeptide A–IgG. Furthermore, HasA is the major protein bound to, or specifically eluted from, this affinity matrix as seen by silver staining of SDS–PAGE gels; no other proteins of similar stoichiometry are observed at any molecular weight, including 33 kDa.

The recombinant HasA enzyme from *E. coli* membranes mimics the streptococcal version with respect to both sugar nucleotide precursor specificity and size of the polysaccharide product. This finding is fortunate, since GAS are difficult to manipulate genetically and also possess an endogenous hyaluronidase activity that degrades their own capsular polysaccharide in late stages of growth (van de Rijn, 1983). Using radiolabeled substrates, <1% misincorporation could be detected if one authentic precursor and one closely related, unnatural analog were incubated with functional membrane preparations. Each of the sugar nucleotide analogs tested differs from the natural substrates used by the HA synthase at only one substituent of the pyranose ring. Although an extensive survey of functional substrates has not been reported for streptococcal membranes containing the HA synthase, this is the expected outcome for the authentic enzyme. Apparently several factors are important for substrate recognition: (i) the equatorial orientation of the C4 hydroxyl of both precursors as well as the presence of (ii) the C6 carboxylate of UDP–GlcA and (iii) the deoxy, acetamido group at C2 of UDP–GlcNAc.

Membranes containing HasA, from either *E. coli* (pPD41Δ5) or *E. coli* (pPD41Δ*Pst*I) or *S. pyogenes*, rapidly polymerize the precursor sugars to form HA polysaccharide. The final product from the *E. coli in vitro* system possesses a M_r on the order of $\sim 5 \times 10^6$, the observed size for the native HA polysaccharide. The results of the pulse–chase experiments suggest that the recombinant HA synthase is highly processive. The vast majority of radioactivity incorporated during the pulse is rapidly chased into high molecular weight products. It is not likely, therefore, that a growing HA chain dissociates or is released from the enzyme until it has achieved a size of (7–20) $\times 10^3$ monosaccharides.

In summary, our results show that the HasA protein is probably the only component of the authentic, functional HA synthase. Furthermore, due to the ease of manipulation of *E. coli*, studies with the recombinant version of HasA should be of substantial utility in elucidating the structure/function relationship of the HA synthase, as well as the catalytic mechanism of glycosaminoglycan biosynthesis.

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REFERENCES

Blum, H., Beier, H., & Goss, H. J. (1987) *Electrophoresis 8*, 93–99.

DeAngelis, P. L., Papaconstantinou, J., & Weigel, P. H. (1993a) *J. Biol. Chem. 268*, 14568–14571.

DeAngelis, P. L., Papaconstantinou, J., & Weigel, P. H. (1993b) *J. Biol. Chem. 268*, 19181–19184.
DeAngelis, P. L., Yang, N., & Weigel, P. H. (1994) *Biochem. Biophys. Res. Commun. 199*, 1–10.
Dochez, A. R., Avery, O. T., & Lancefield, R. C. (1919) *J. Exp. Med. 30*, 179–213.
Dougherty, B. A., & van de Rijn, I. (1994) *J. Biol. Chem. 269*, 169–175.
Glabe, C. G. (1990) *Technique 2*, 138–146.
Ito, K., Sato, T., & Yura, T. (1977) *Cell 11*, 551–559.
Kass, E. H., & Seastone, C. V. (1944) *J. Exp. Med. 79*, 319–330.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Lansing, M., Lellig, S., Mausolf, A., Martini, I., Crescenzi, F., O'Regan, M., & Prehm, P. (1993) *Biochem. J. 289*, 179–184.
Markovitz, A., Cifonelli, J. A., & Dorfman, A. (1959) *234*, 2343–2350.
Meyer, K., & Palmer, J. W. (1934) *J. Biol. Chem. 107*, 629–634.
Quinn, A. W., & Sing, K. P. (1957) *Proc. Soc. Exp. Biol. Med. 95*, 290–294.
Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., & Klenk, D. C. (1985) *Anal. Biochem. 150*, 76–85.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad Sci. U.S.A. 76*, 4350–4354.
Trieu-Cuot, P., Carlier, C., Poyart-Salmeron, C., & Courvalin, P. (1991) *Gene 102*, 99–104.
van de Rijn, I. (1983) *J. Bacteriol. 156*, 1059–1065.
van de Rijn, I., & Kessler, R. E. (1980) *Infect. Immun. 27*, 444–448.
van de Rijn, I., & Drake, R. R. (1992) *J. Biol. Chem. 267*, 24302–24306.
Wessels, M. R., Moses, A. E., Goldberg, J. B., & DiCesare, T. J. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 8317–8321.