Characterization of the Lipid Linkage Region and Chain Length of the Cellubiuronic Acid Capsule of *Streptococcus pneumoniae*

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The processive reaction mechanisms of β-glycosyl-polymersases are poorly understood. The cellubiuronan synthase of *Streptococcus pneumoniae* catalyzes the synthesis of the type 3 capsular polysaccharide through the alternate additions of β-1,3-Glc and β-1,4-GlcUA. The processive multistep reaction involves the sequential binding of two nucleotide sugar donors in coordination with the extension of a polysaccharide chain associated with the carbohydrate acceptor recognition site. Degradation analysis using cellubiuronan-specific depolymerase demonstrated that the oligosaccharide-lipid and polysaccharide-lipid products synthesized in vitro with recombinant cellubiuronan synthase had a similar oligosaccharyl-lipid at their reducing termini, providing definitive evidence for a precursor-product relationship and also confirming that growth occurred at the nonreducing end following initiation on phosphatidylglycerol. The presence of a lipid marker at the reducing end allowed the quantitative determination of cellubiuronan synthase activity, corroborating the synthesis of the type 3 capsule and also confirming that growth occurred at the nonreducing end following initiation on phosphatidylglycerol. The presence of a lipid marker at the reducing end allowed the quantitative determination of cellubiuronan synthase activity, corroborating the synthesis of the type 3 capsule and also confirming that growth occurred at the nonreducing end following initiation on phosphatidylglycerol. The presence of a lipid marker at the reducing end allowed the quantitative determination of cellubiuronan synthase activity, corroborating the synthesis of the type 3 capsule and also confirming that growth occurred at the nonreducing end following initiation on phosphatidylglycerol. The presence of a lipid marker at the reducing end allowed the quantitative determination of cellubiuronan synthase activity, corroborating the synthesis of the type 3 capsule and also confirming that growth occurred at the nonreducing end following initiation on phosphatidylglycerol.

Cellubiuronic acid, the capsular polysaccharide of type 3 *Streptococcus pneumoniae*, is composed of the repeating disaccharide d-glucuronic acid (1,3)-β-D-GlcUA2-(1,4)-β-D-Glc-(1-) (1) and is synthesized by a processive mechanism similar to that for cellulose, chitin, hyaluronic acid, and other related β-glycans (2). This group of polysaccharides is synthesized by invertase GT-2A polymerases that are located in the plasma membrane with their active sites on the cytoplasmic face, and following chain initiation, the synthases are thought to be involved in the extrusion of the nascent chains to the external membrane face (3–8). The overall processive elaboration of these polysaccharides occurs poorly understood at the molecular level. In particular, there is relatively little information concerning the initiation process, the facilitation of chain extrusion, the mechanism of translocation, and the regulation of the final chain length during the assembly of these polymers. Recent investigations in this laboratory have begun to unravel some of the details of both the early and later stages of the biosynthesis of cellubiuronan.

Unlike most *S. pneumoniae* capsules, whose elaboration requires multiple glycosyltransferases, a polymerase, and an additional transport system (9), the assembly and transport of cellubiuronan in type 3 strains is carried out by the single enzyme cellubiuronan synthase (Cps3S) (3, 10, 11). Studies of the synthase in *S. pneumoniae* and recombinant Escherichia coli membranes have shown that assembly of the polysaccharide involves two distinct kinetic phases: 1) a transitory processive state wherein the chain is thought to be initiated by the formation of an oligosaccharide-lipid that is loosely associated with the synthase, and 2) a fully processive state in which the polysaccharide is tightly bound to the carbohydrate substrate recognition site, except for a brief period during the translocation stage of each catalytic cycle (5, 12). Each catalytic cycle in the extrusion mode provides for chain extension by the addition of a repeating disaccharide and requires the alternate association of the synthase with UDP-Glc and UDP-GlcUA, the formation of the glycosidic linkages of the respective sugars, and the release, translocation, and reattachment of the elongating chain at the synthase carbohydrate recognition site. Transition from the transitory mode to the fully processive extrusion mode correlates with the attainment of a threshold-length oligosaccharide of ~8 sugars (12). Nod factor chito-oligosaccharides from rhizobia are synthesized by a related group of synthases that apparently are not capable of organizing into an extrusion mode (13). Significantly, the maximum length of any reported Nod-factor oligosaccharide is 6 sugars (14).

Based on β-glucosidase sensitivity of singly added [14C]Glc to the terminal end of high molecular weight cellubiuronan, it was deduced that the polysaccharide grows by repetitive β-1,3-Glc and β-1,4-GlcUA additions to the nonreducing terminus (2). Oligosaccharide-lipid assembly is thought to initiate on phosphatidylglycerol (15). To date, however, there has been no

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2 The abbreviations used are: GlcUA, glucuronic acid; PLD, phospholipase D; DOC, deoxycholate; Mes, 2-(N-morpholino)ethanesulfonic acid; UA, uronic acid; βOG, β-octyl glucoside.
Chain Length and Linkage Characterization of Cellubiuronan

Experimental Procedures

Materials

*B. circulans* cellubiuronan-specific depolymerase (2 mg of protein/ml) was prepared as previously described (16, 17, 19). *E. coli* strain JD424, containing the recombinant cellubiuronan synthase from *S. pneumoniae* strain WU2, has been described (10). Cellubiuronan di-, tetra-, and hexasaccharide standards (Glc-GlcUA), were prepared as described (12). *Streptomyces chromofuscus* phospholipase D (PLD), β-glucosidase (almond), hexokinase (yeast), phosphoglucomutase (rabbit muscle), UDP-Glc pyrophosphorylase (bovine liver), UDP-Glc dehydrogenase (bovine liver), UDP-Glc, UDP-Glc-UA, Sephacryl S-400, and S-300 were from Sigma. Sephacryl S-1000 was from Amer-...
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FIGURE 1. Separation of polysaccharide-lipid from oligosaccharide-lipid by ion exchange chromatography. Products labeled in a reaction mixture containing 2 μM UDP-[14C]GlcUA and 1 mM UDP-Glc were prepared as described under “Experimental Procedures” and applied to a 1-ml column of DEAE-cellulose. A, the column was eluted sequentially with a 0.05% Nonidet P-40 solution containing 0.25 M and then 1.0 M ammonium acetate (pH 7.5), as indicated by arrows a and b, respectively. Fractions (1 ml) were collected and monitored for radioactivity. Samples of the 0.25 M (B) and 1.0 M (C) ammonium acetate fractions were analyzed by chromatography on Sephacryl S-400, as described under “Experimental Procedures.”

centrations. The reaction was initiated with synthase, and the second round of product was collected as above. The combined pellets were suspended in 2.5 ml of a wash solution consisting of 100 mM Hepes (pH 7.5) and 10% glycerol. The washed membranes were collected by centrifugation as above, and this wash step was repeated once more. Membranes were suspended in 0.4 ml of 5 mM EDTA (pH 7.5), heated at 100 °C for 4 min, and solubilized by the addition of 0.25% Nonidet P-40.

Separation of Oligosaccharide- and Polysaccharide-Lipid

Three general purification procedures were used for the preparation of oligosaccharide-lipid, low molecular weight polysaccharide-lipid, and high molecular weight polysaccharide-lipid.

Procedure A, Preparation of Oligosaccharide-Lipid—Solubilized reaction products were applied to a 0.6 × 2.5-cm column of DEAE-cellulose (OH−), followed by sequential elution with 6 ml of 0.25 M ammonium acetate and then 10 ml of 1.0 M ammonium acetate (Fig. 1). All solutions contained 0.05% Nonidet P-40. Column fractions of 1 ml were monitored for radioactivity. Oligosaccharide-lipid (Fig. 1A) and polysaccharide-lipid (Fig. 1C) fractions with less than 1% cross-contamination were obtained, as assessed by Sephacryl S-400 chromatography (described above).

Procedure B, Preparation of Low Molecular Weight Polysaccharide-Lipid—For experiments demanding more highly purified polymer, procedure A was slightly modified such that the solubilized product was applied to DEAE-cellulose in 0.25 M ammonium acetate. The oligosaccharide-lipid passed through without binding, and virtually pure polymer-lipid was eluted with 1.0 M ammonium acetate, containing only 0.01% oligosaccharide-lipid, as determined by paper chromatography. Polymer-lipid was desalted by dialysis (three times) against 50 volumes of 0.05% Nonidet P-40 for 1 h in an ice bath and frozen at −20 °C. The oligosaccharide-lipid fraction was further purified by hydrophobic adsorption on a solid phase C-18 cartridge, as described below, and stored at −20 °C in detergent solutions.

Procedure C, Preparation of High Molecular Weight Polysaccharide-Lipid—High molecular weight chains (greater than 2000 sugars) were inefficiently eluted from DEAE-cellulose but were completely separated from oligosaccharide-lipid by gel filtration on Sephacryl S-300. Gel filtration elutions of high molecular weight polymer often included about 5% of the product that tailed into the low molecular weight elution region. This material appeared to be outside the modal distribution and was excluded. Polysaccharide in DOC solutions was dialyzed three times against 50 volumes of H2O for 1 h in an ice bath and stored frozen at −20 °C.

Depolymerase Hydrolysis

Digestion with the cellubiuronan-specific B. circulans depolymerase was carried out with detergent, as indicated, in 50 mM Mes (pH 6.0) with 2 μg/ml of depolymerase at 35 °C for the indicated time. Samples containing DOC formed a precipitate with Mes (pH 6), which was diminished by the addition of either Nonidet P-40 or β-octyl glucoside (βOG) and did not appear to interfere with hydrolysis. The extent of hydrolysis was monitored by chromatography on 3MM paper in an ascending solvent of butanol/acetic acid/H2O (44:16:40) for 5–14 h. The chromatogram was cut into 1-cm sections, and radioactivity was determined by liquid scintillation counting.

Molecular Weight Determination by Lipid-linked Limit Glycan Analysis

Depolymerase hydrolysates were separated by gel filtration on Biogel P-4 or fractionated on C-18 solid phase cartridges as described below. The limit glycan attached to the lipid eluted in the detergent micelle region in the former and in the hydrophobic region in the latter. The proportions of radioisotope in hydrophilic and hydrophobic oligosaccharide fractions were used to calculate the polymer/lipid-linked limit glycan ratio. This number, in conjunction with the average size of the lipid-linked limit glycan, which was determined by DEAE-cellulose chromatography, was used to calculate the number of GlcUA residues in the polysaccharide chain.

Biogel P-4 chromatography of hydrolysates was carried out on 1 × 37-cm columns irrigated at a flow rate of 0.15 ml/min with a solution of 0.2 M ammonium acetate (pH 7.4) and 0.05% Nonidet P-40. Samples were concentrated to 0.5 ml under a
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Direction of Polysaccharide Chain Growth

\( E. coli \) membranes (576 \( \mu \)g of protein) were incubated for 7 min at 35 °C in a 5-ml reaction mixture consisting of 10 \( \mu \)M \( ^{3}H \)Glc (3.4 \( \times \) 10\(^6\)) cpm), 10 \( \mu \)M UDP-GlcUA, 100 mM Heps (pH 7.5), and 10 mM MgCl\(_2\). The reaction was terminated in an ice-water bath, and the membranes were collected by sedimentation at 100,000 \( \times \) g for 30 min. The membranes were suspended with a Teflon pestle in 0.5 ml of cold unlabeled reaction mixture as above. The enzyme suspension was then added to a 5-ml reaction mixture at 35 °C, which was similar to the above except that it contained 2.5 \( \times \) 10\(^6\) cpm of 10 \( \mu \)M UDP-\(^{14}C\)GlcUA and unlabeled 10 \( \mu \)M UDP-Glc. The incubation was repeated for another 7 min at 35 °C. The reaction was terminated in an ice-water bath, and the membranes were collected by sedimentation and washed as described under "Preparation of Oligosaccharide- and Polysaccharide-Lipid." The polysaccharide-lipid was further purified by procedure B above under "Separation of Oligo- and Polysaccharide-Lipid." The polysaccharide-lipid was hydrolyzed for 25 min in 0.05% Nonidet P-40 with 6 \( \mu \)g of depolymerase in a final volume of 5 ml as described above. The lipid-linked limit glycan was isolated by hydrophobic C-18 fractionation as described above, and the oligosaccharides were released by PLD and fractionated by ionic exchange chromatography on DEAE-cellulose as described above.

RESULTS

At any fixed concentration of UDP-GlcUA, the cellubiuronan polymerization reaction rapidly comes into a steady state in which the synthase is distributed between two modes, synthesizing either oligosaccharide-lipid or polysaccharide-lipid (12). Upon transition into the fully processive state of polysaccharide-lipid synthesis, the size of the growing polymer is thought to be determined by the UDP-sugar concentrations. As shown in Fig. 2, discrete populations of distinctive size chains were observed under different UDP-GlcUA concentrations, when the cellubiuronan products were synthesized in vitro using \( E. coli \) membranes containing the recombinant synthase. The relative elution positions of the polysaccharide products indicate a very large increase in chain size as the concentration of UDP-GlcUA was increased from 1 to 11.5 \( \mu \)M. To definitively establish the presence of a lipid constituent at the reducing end of the cellubiuronic acid polymer and in turn to use the lipid...
presence as a marker to develop a set of polysaccharide standards of defined size, we used the cellubiuronan-specific depolymerase, an endoglycosidase from *B. circulans*, to analyze the above products.

**Depolymerase Hydrolysis of Cellubiuronan Polysaccharide Products**—Cellubiuronic acid polysaccharide products labeled with $[^{14}C]$GlcUA were subjected to hydrolysis with the cellubiuronan-specific depolymerase. The random endoglycosidic cleavage of the polysaccharide by the depolymerase (17), as opposed to removal of tetrasaccharide units from the end of the polymer, was confirmed by the gradual reduction in the size of a single polysaccharide peak when assessed by gel filtration following hydrolysis with low levels of depolymerase (data not shown). Preferential cleavage in the interior region of the chain was demonstrated by the rapid accumulation of hexasaccharide, along with tetrasaccharide, in the early stage of depolymerase digestion (Fig. 3). As the reaction continued, the hexasaccharide product gradually disappeared concomitant with an increase of disaccharide and tetrasaccharide. The cleavage of hexasaccharide, yielding di- and tetrasaccharide, occurred at a much slower rate than the initial hydrolysis of chain segments that were octasaccharides or longer. The size and anionic charge of the di-, tetra-, and hexasaccharide products were confirmed as previously described (12).

**Limit Glycans Obtained by Depolymerase Digestion of Oligosaccharide-Lipid Products**—Cellubiuronan oligosaccharide-lipid products labeled with $[^{14}C]$GlcUA were synthesized in vitro using *E. coli* membranes containing the recombinant synthase, as described under “Experimental Procedures.” Prior to depolymerase hydrolysis, most of the oligosaccharide-lipid eluted in the detergent micelle fraction when analyzed by gel filtration (Fig. 4A). The oligosaccharides were released with PLD, and shown by ion exchange chromatography on DEAE-cellulose to consist of a major triuronosyl (UA) component, along with lesser amounts of mono-, di-, and tetrauronosyl oligomers (Fig. 4B). Depolymerase hydrolysis of the oligosaccharide-lipid fraction occurred at a much slower rate than with long polysaccharide chains (Fig. 3) and was strongly affected by the presence of detergents. When the oligosaccharide-lipid fraction was reconstituted in Nonidet P-40 micelles, 26% of the remaining after depolymerase hydrolysis was isolated from the lipid moiety by PLD and analyzed by DEAE-cellulose chromatography (right) as described under “Experimental Procedures.” A and B, oligosaccharide-lipid products prior to depolymerase digestion; C and D, following depolymerase hydrolysis in 0.1% Nonidet P-40; and E and F, following depolymerase hydrolysis in 25 mM βOG. The elution position of Glc on Biogel P-4 is indicated by the arrow at G. The gel filtration elution positions of di-, tetra-, and hexasaccharide, indicated by arrows at 1, 2, and 3, respectively, were also used as mono-, di-, and tri-UA markers, respectively, on DEAE-cellulose. The product eluting from DEAE-cellulose in fraction 41 was previously shown (12) by gel filtration to correspond to a tetra-UA oligomer (Glc-GlcUA$_2$-Glc-glycerol) and is indicated by the arrow at 4.

**Limit Glycans Obtained by Depolymerase Digestion of Oligosaccharide-Lipid Products**—Cellubiuronan oligosaccharide-lipid products labeled with $[^{14}C]$GlcUA were synthesized in vitro using *E. coli* membranes containing the recombinant synthase, as described under “Experimental Procedures.” Prior to depolymerase hydrolysis, most of the oligosaccharide-lipid eluted in the detergent micelle fraction when analyzed by gel filtration (Fig. 4A). The oligosaccharides were released with PLD, and shown by ion exchange chromatography on DEAE-cellulose to consist of a major triuronosyl (UA) component, along with lesser amounts of mono-, di-, and tetrauronosyl oligomers (Fig. 4B). Depolymerase hydrolysis of the oligosaccharide-lipid fraction occurred at a much slower rate than with long polysaccharide chains (Fig. 3) and was strongly affected by the presence of detergents. When the oligosaccharide-lipid fraction was reconstituted in Nonidet P-40 micelles, 26% of the labeled GlcUA was released after 1 h of digestion, primarily as tetrasaccharide with minor amounts of di- and hexasaccharide, as shown by gel filtration (Fig. 4C). The lipid-linked limit glycan remaining after depolymerase hydrolysis was isolated from the
detergent micelle fraction following Biogel P-4 chromatography, and the residual oligosaccharides were released by PLD and analyzed by ion exchange chromatography on DEAE-cellulose. Depolymerase hydrolysis in Nonidet P-40 (Fig. 4, compare B and D) resulted in the complete elimination of the tetra-UA component, a reduction in the relative amount of tri-UA oligosaccharides, and increases in the relative amounts of di- and mono-UA oligosaccharides.

The relatively low proportion of disaccharide in Fig. 4C suggests a slower rate of release of disaccharide from oligosaccharide-lipid than from polysaccharide, as shown in Fig. 3. This pattern indicates that the depolymerase is restricted in the removal of disaccharide fragments from the nonreducing termini and leads to the suggestion that most of the disaccharide comes from the reducing end of the hexasaccharide product subsequent to its cleavage from the parent substrate. In conjunction with a comparison of the oligomer patterns in Fig. 4, C and D, the data indicate that primarily tetrasaccharide was cleaved from the nonreducing termini of the oligosaccharide-lipid. A small amount of hexasaccharide probably came from the tetrauronosyl oligomer and was subsequently partially cleaved into di- and tetrasaccharide.

This pattern of hydrolysis is even more apparent with oligosaccharide-lipid in small micelle-forming detergents like βOG and DOC that allow more extensive depolymerase hydrolysis. Digestion of oligosaccharide-lipid in βOG released 65% of the radioactivity, primarily as tetrasaccharide (Fig. 4E; compare with 26% release during digestion with Nonidet P-40 in Fig. 4C). Presumably, the smaller hydrophilic headgroup or the greater micelle curvature allowed more accessibility of the depolymerase catalytic site. Following PLD release of the depolymerase-resistant micellar fraction, the predominant residual glycan appeared to contain a single uronic acid (Fig. 4F). These data suggest that all of the original tetra-UA-lipid was reduced in size to di- or mono-UA-lipid and that almost all of the tri-UA-lipid was reduced to mono-UA-lipid. There was no apparent cleavage of the di- or mono-UA-lipid. Glycosidic cleavage of the oligosaccharide-lipid by the highly specific depolymerase was a further indication of the homology of the sugar linkages in the cellubiuronan oligosaccharide- and polysaccharide-lipid products.

Limit Glycans Obtained by Depolymerase Digestion of Polysaccharide-Lipid Products—To accurately determine the length of the lipid-linked cellubiuronan polysaccharide chains, the polymer products were first completely separated from the oligosaccharide-lipid fraction, as described under “Experimen
tal Procedures.” Polysaccharide-lipid synthesized in a reaction mixture containing 6 μM UDP-GlcUA, separated from oligosaccharide-lipid, and hydrolyzed with depolymerase for 10 min in 0.05% Nonidet P-40, as described under “Experimental Procedures.” The hydrolysate was fractionated by gel filtration on a column of Biogel P-4 (A), and the oligosaccharides were liberated from the micelle (mic) fraction with PLD and analyzed by DEAE-cellulose chromatography (B), as described under “Experimental Procedures.” Standards, indicated by the arrows, were the same as in Fig. 4.

Lar region of the Biogel P4 column, was analyzed by ion exchange chromatography and found to consist of a mixture of relatively large oligosaccharides (Fig. 5B). The predominant residual glycan appeared to be a pentauronosyl glycan, based on the anion exchange elution position. The pattern of large oligomers attached to the lipid anchor clearly demonstrated the removal of contaminating oligosaccharide-lipid during the purification of polysaccharide-lipid prior to depolymerase hydrolysis. There appeared to be a slight loss of the largest lipid-linked members by the C-18 fractionation method (data not shown). Both gel filtration and C-18 hydrophobic adsorption were equally effective in fractionating more exhaustive digests. Lipid-linked limit glycans of minimal size were obtained by longer digestions of polysaccharide-lipid with depolymerase in βOG and DOC. Examples of hydrolysates of polysaccharide produced in polymerization reactions in the presence of 1 μM UDP-GlcUA are shown in Fig. 6. Digestion of intermediate duration (30 min) in βOG liberated hexa- and disaccharide at a

![Figure 5. Limit glycan analysis of polysaccharide-lipid following depolymerase hydrolysis in Nonidet P-40.](image-url)

Cellubiuronan polysaccharide was polymerized in a reaction mixture containing 6 μM UDP-GlcUA, separated from oligosaccharide-lipid, and hydrolyzed with depolymerase for 10 min in 0.05% Nonidet P-40, as described under “Experimental Procedures.” The hydrolysate was fractionated by gel filtration on a column of Biogel P-4 (A), and the oligosaccharides were liberated from the micelle (mic) fraction with PLD and analyzed by DEAE-cellulose chromatography (B), as described under “Experimental Procedures.” Standards, indicated by the arrows, were the same as in Fig. 4.
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FIGURE 6. Limit glycan analysis of polysaccharide-lipid hydrolyzed with depolymerase in β-octyl glycoside and DOC. Cellubiuronan was polymerized in a reaction mixture containing 1 μM UDP-GlcUA, and the purified polysaccharide was subjected to depolymerase hydrolysis in 25 mM βOG for 30 min (top) or in 8 mM DOC and 10 mM βOG for 2 h (bottom), as described under “Experimental Procedures.” A, the former hydrolysate was fractionated on Biogel P-4; B, the micellar oligosaccharide components were separated on DEAE-cellulose as in Fig. 5. C, the latter hydrolysate was passed through a hydrophobic matrix (fraction 1), and the matrix was sequentially washed with H2O, (fractions 2–4) and eluted in fractions 5 and 6 with chloroform/methanol/H2O (1:2:1), as described under “Experimental Procedures.” D, the organic soluble material was dried, and oligosaccharides were released by PLD and analyzed by DEAE-cellulose chromatography as described under “Experimental Procedures.” Fractions in B and D were normalized based on conductivity. Standards indicated by the arrows were the same as in Fig. 4.

ratio of 2.5 and yielded a released/bound isotope ratio of 63 (Fig. 6A). PLD digestion of the residual lipid glycan produced di- and triuronosyl components in approximately equal quantity, with very minor amounts of the mono- and tetra-UA oligomers (Fig. 6B). In order to obtain the monouronosyl component in sufficient quantity for further characterization, the synthase reaction in the presence of 1 μM UDP-GlcUA was scaled up to yield 900,000 cpm of polymer along with 1.5 × 10^6 cpm of oligosaccharide-lipid. Following a 2-h depolymerase digest of purified polysaccharide in mixed micelles of βOG and DOC, the level of disappearance exceeded hexasaccharide by 2:1 (data not shown), the radioisotope ratio of the released/bound products was 1:113 (Fig. 6C), and the lipid-bound residual glycan consisted mostly of mono- and di-UA oligomers (Fig. 6D). The persistence of the di-UA oligomer after extensive digestion with the depolymerase affirms the above observation that removal of a disaccharide unit from the nonreducing saccharide terminus does not occur.

Identity of Oligo- and Polymer-Lipid Limit Glycans—The mono-UA oligosaccharides obtained by depolymerase digestion of the oligosaccharide-lipid (Fig. 4F) and polysaccharide-lipid (Fig. 6D) were further characterized by chromatography on an analytical Biogel P-4 column before and after β-glucosidase digestion. The predominant component in both products appeared to have identical properties, being slightly smaller than the cellubiuronan tetrasaccharide standard (Glc-GlcUA)4 before β-glucosidase digestion and slightly larger than the disaccharide standard (Glc-GlcUA)2 after digestion (Fig. 7, A and B), consistent with the expected monourononic oligosaccharide structure of Glc-GlcUA-Glc-glycerol. The β-glucosidase preparation contains a low level of β-glucuronidase, which may explain the production of a small amount of radioactivity co-migrating with free GlcUA following a digestion of 24 h (Fig. 7A). The digestion in Fig. 7B was for 3.5 h and went only to about 75% completion, and no free GlcUA was observed. These data strongly support a reaction process whereby cellubiuronan polysaccharide formation occurs by the continued glycosidic addition of Glc and GlcUA to the nonreducing terminus of the oligosaccharide-lipid following its transition into a tightly binding complex with the synthase.

The polysaccharide-derived mono-UA oligomer in Fig. 7B was not uniform, as is evident by a leading shoulder of the major peak of material both before and after β-glucosidase digestion. The elution position prior to digestion corresponds to that of Glc-GlcUA-glycerol, and a similar product has been observed in oligosaccharide-lipid fractions synthesized under some reaction conditions (12). In a reaction containing only 1 μM UDP-GlcUA, the synthase was estimated to have cycled (see “Experimental Procedures”) on the order of 10^5 times for every successful transition to the fully processive state. Thus, even a slight increase in the transition coupling efficiency of a GlcUA-glycerol moiety as compared with Glc-glycerol would have magnified the level of the former in the polysaccharide-lipid product. In sum, these data suggest that oligosaccharide-lipid
of sufficient acceptor recognition size and consisting of either a GlcUA- or Glc-glycerol linkage will bind to the synthase carbohydrate acceptor engagement site.

**Modulation of Polysaccharide Chain Length by UDP-GlcUA**—Polysaccharide was synthesized in reactions containing 1 mM UDP-Glc and UDP-GlcUA ranging from 1 to 11.5 μM. The length of incubation at each concentration was sufficient to achieve a steady state of chain size production of maximum size (38). The chain length at each UDP-GlcUA concentration was determined by lipid-linked limit glycan analysis following depolymerase digestion. At any fixed concentration of UDP-GlcUA, the ratio of depolymerase-releasable material increased and the size of the lipid-linked limit glycan decreased when depolymerase digestion was more extensive and carried out in smaller micelle-forming detergents (shown for 1 μM UDP-GlcUA in Table 1). As shown in Table 1, a 21-fold increase in polysaccharide chain length resulted from an 11.5-fold increase in the UDP-GlcUA concentration. These data imply that in response to the increasing nucleotide sugar activity at the donor binding domain, the growing chain remains bound at the acceptor domain for a continuously increasing time period, and they are in agreement with previous data (12, 19), indicative of an interaction between the donor and acceptor domains.

**Direction of Polysaccharide Chain Growth**—In a dual isotope reaction, cellulobionan polysaccharide-lipid was pulse-labeled for 7 min in a polymerization mixture that contained UDP-[3H]Glc. The membranes were collected by sedimentation and then chased for 7 min in a reaction mixture containing UDP-[14C]GlcUA. The purified polysaccharide had a 3H/14C ratio of 1.6 (Fig. 8A). Likewise, the isotope ratio of the aqueous oligosaccharides released by depolymerase hydrolysis was 1.6 (data not shown). The limited depolymerase hydrolysis resulted in a pool of lipid-linked limit glycan composed of oligomers ranging in size from 2 to 8 uronosyl units, having a 3H/14C ratio of ~11 (Fig. 8B). The 3H-enriched content of the lipid linkage segment of the polysaccharide demonstrated that this region of the chain was synthesized during the early stage of the polymerization reaction and is strong evidence for an elongation mechanism of sequential addition at the nonreducing end of the growing chain. The small quantity of 14C in this segment of the polysaccharide pool was probably due to a low level reverision of synthase from the fully processive to the transitory processive state as a consequence of a low rate of chain ejection. In addition, the polymerization reaction concentrations of a 10 μM concentration of each of the UDP-sugar substrates may not
have been sufficient to drive 100% of the synthase into the fully processive state during the first 7 min of the reaction.

**DISCUSSION**

Hydrolysis of cellubiuronan polysaccharide chains with cellubiuronan depolymerase yielded significant amounts of low molecular weight lipid-linked oligomers, providing the first definitive evidence that the polysaccharide is assembled on a lipid primer. In previous investigations, significant quantities of preformed chains in isolated *S. pneumoniae* membranes were releasable, indicating the absence of a hydrophobic aglycone constituent (19), whereas newly formed chains tightly adhered to these membranes (5). The latter data, in conjunction with the accumulation of glucosylphosphatidylglycerol, was suggestive of chain initiation on a phosphatidylglycerol anchor. The chemical structural evidence provided by the present studies is crucial in the irrefutable establishment of the presence of a reducing terminal cellubiuronan lipid component, eliminating the possibility of more than one initiation process. The identity of the initial chain constituent is particularly important given the lack of donor and acceptor specificity of the cellubiuronan synthase in the initial step when it lacks the co-factor guidance of a substrate in the acceptor binding site (see below).

Mechanisms that would specifically modulate the chain length of polysaccharides are an area of longstanding interest (22–25). Control of polymer size by substrate concentration has been suggested for several polysaccharide systems, but data supporting this hypothesis are limited (18, 23). The fully processive reaction catalyzed by cellubiuronan synthase appears to be dependent on a tight association of the growing chain with the carbohydrate recognition site, and disruption of this association by an abortive translocation mechanism as a function of the UDP-sugar substrate concentrations would appear to be a feasible chain length regulation mechanism (12, 19). To develop a thorough quantitative relationship between the substrate concentration and the chain length synthesized in vitro with *E. coli* membranes, we have used the lipid anchor as a marker following depolymerase hydrolysis to determine the length of cellubiuronan chains. The employment of various detergents during depolymerase hydrolysis to obtain limit glycans of different lengths has enhanced the reliability of this method, particularly for analysis of very long chain polysaccharides. Using this methodology, this investigation has established that an increase in the concentration of UDP-GlcUA from 1 to 11.5 μM resulted in an increase in polysaccharide chain length from 320 to 7400 sugar residues. The demonstration of the relationship between the concentration of UDP-GlcUA and cellubiuronan chain length was essential in the development of a kinetic model of chain length modulation by substrate concentration (38).

Although a large body of evidence has been amassed regarding donor catalytic aspects of inverting and retaining single glycosidic transfers (26), the elucidation of the role of the acceptor in the complex catalysis that provides for the simultaneous glycosidic extension and vectorial extrusion carried out by processive GT-2A glycosyltransferases remains in its infancy. With the goal of developing a more detailed picture of the transition of the synthase into the extrusion mode, we have sought to more fully establish a definitive precursor-product relationship between the transitory and fully processive biosynthetic states. Initial attempts to chase oligosaccharide-lipid into polysaccharide-lipid products were unsuccessful. We have therefore used structural analysis in this study to demonstrate continuity between these products. Similar limit glycan structures were obtained following depolymerase hydrolysis of both oligosaccharide- and polysaccharide-lipid fractions, indicating that both of these structures were initiated by a similar process. The generation of relatively large lipid-linked limit glycans containing up to 10 uronosyl residues by depolymerase hydrolysis of the polysaccharide-lipid (Fig. 5) is conclusive evidence of the presence of a lipid constituent linked to the polysaccharide, thus clearly demonstrating catalytic continuity of chain extension as the cellubiuronan synthase made the transition between the two synthetic modes.

In view of the conflicting history for the direction of growth of hyaluronan (27–32), we sought to more firmly establish this feature of cellubiuronan assembly. The pulse-chase feature of the direction of growth experiment, in conjunction with the dual isotope labeling pattern, demonstrated both kinetically and structurally that cellubiuronan polysaccharide chains are first initiated as oligosaccharide-lipids and that the transition into the fully processive state occurs by successive nonreducing terminal additions of Glc and GlcUA, in agreement with the previous cellubiuronan biosynthetic model (12). Cellulose is also thought to elongate by addition at the nonreducing termini (33), and there is evidence of initiation on a lipid primer (34); however, there are minimal data on the overall kinetic processes carried out by the cellulose catalytic complex. Chito-oligosaccharide synthases from rhizobia also catalyze nonreducing end sugar additions (35), but this class of enzymes does not appear to organize into an extrusion mode. Rather, the Nod factors are released internally and then transported to the outside by a separate protein system (13).

To obtain comparative structural information regarding the initial glycosidic linkage of the oligosaccharide- and polysaccharide-lipid products, limit glycan structures were obtained from each of these fractions following cellubiuronan depolymerase hydrolysis in DOC/βO, mixed detergent micelles. These detergents, forming small micelles with a maximum degree of curvature, allowed the greatest penetration of cellubiuronan depolymerase into the micelle interior, releasing primarily lipid-linked mono-UA oligomers. Analysis of the PLD-hydrolyzed mono-UA products obtained from both the original oligosaccharide- and polysaccharide-lipid products indicated that the predominant lead sugar was Glc. Significantly, a small amount of GlcUA also appeared to occur as the polysaccharide linkage sugar to the lipid anchor.

Whereas a large number of glycosyltransferases are specific for one donor and one acceptor in catalyzing a single glycosidic linkage, cellubiuronan synthase demonstrates a puzzling lack of specificity, in certain situations, both for the appropriate donor and acceptor. In the fully processive state, the enzyme is highly specific in catalyzing the formation of β1,3-glucosyl additions

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3 W. T. Forsee, R. T. Cartee, and J. Yother, unpublished results.
to GlcUA and β1,4-glucuronosyl) additions to Glc. At the outset of the transitory state, however, the synthase demonstrates an unusual lack of specificity in that it is able to utilize the glycerol moiety of phosphatidylglycerol as an acceptor for Glc (5) and in some cases for GlcUA (12). These data suggest that the oligosaccharide segment bound to the substrate acceptor site apparently acts as a co-factor in determining the affinity of nucleotide sugar donor substrates and that in the absence of bound carbohydrate, the enzyme demonstrates deceased fidelity in recognizing the appropriate acceptor substrate (12). The demonstration of polysaccharide-lipid linkages with either Glc or GlcUA as the leading sugar attached to glycerol indicates that the formation of the oligosaccharide-enzyme catalytic complex and the fully processive polymerization mechanism is unaffected by the initial sugar in the chain. The only requirement appears to be a repeating disaccharide chain segment of sufficient length to lock the peptide into the fully processive state. The initial formation of a threshold-size oligosaccharide lipid and its recognition and binding at the carbohydrate acceptor site appear to be pivotal events in providing the spatial peptide arrangement for the fully processive assembly of cellubiuronan capsular polysaccharide.

The role of the acceptor site in processive assemblies is still largely hypothetical (6, 25, 26, 32, 33), and deciphering the architectural features that can provide for initiation, extrusion, and extension of the polysaccharide chain is a major challenge in this field. Bustamante et al. (36) have emphasized the importance of taking into account a variety of mechanical aspects that are involved in complex enzyme catalysis. Based on kinetic and structural studies, we have dissected out several mechanical processes in the catalysis of cellubiuronan assembly. (a) Previous kinetic determination of the ejection $K_m$ and $K_i$ values (19) is consistent with a mechanical process, as is the translocation step that presumably occurs after every other glycosidic addition. (b) Donor and acceptor specificities discussed above in conjunction with the presence of only a single UDP-sugar substrate binding site (37) may necessitate a repetitive allosteric process to provide for its sequential interaction with UDP-Glc and UDP-GlcUA. (c) Structural studies of the oligosaccharide- and polysaccharide-lipid products reinforce previous speculation of a reorientation of the carbohydrate binding site within the catalytic center to allow the extrusion of the elongating polysaccharide to the external side of the membrane during the fully processive polymerization biosynthetic phase (12). (d) Finally, the results obtained in this study are consistent with a steadily diminished capacity of ejection of the growing chain by UDP-Glc from the carbohydrate-substrate binding site as the concentration of UDP-GlcUA increases (19) and have provided the basic foundation for a kinetic model of cellubiuronan polysaccharide chain modulation (38).

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