Characterization of the Linkage between the Type III Capsular Polysaccharide and the Bacterial Cell Wall of Group B Streptococcus

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The capsular polysaccharide of group B Streptococcus is a key virulence factor and an important target for protective immune responses. Until now, the nature of the attachment between the capsular polysaccharide and the bacterial cell has been poorly defined. We isolated insoluble cell wall fragments from lysates of type III group B Streptococcus and showed that the complex contained both capsular polysaccharide and group B carbohydrate covalently bound to peptidoglycan. Treatment with the endo-N-acetylmuramidase mutanolysin released soluble complexes of capsular polysaccharide linked to group B carbohydrate by peptidoglycan fragments. Capsular polysaccharide could be enzymatically cleaved from group B carbohydrate by treatment of the soluble complexes with β-N-acetylmuramidase, which catalyzes hydrolysis of the β-d-GlcNAc(1→4)β-d-MurNAc subunit produced by mutanolysin digestion of peptidoglycan. Evidence from gas chromatography/mass spectrometry and 31P NMR analysis of the separated polysaccharides supports a model of the group B Streptococcus cell surface in which the group B carbohydrate and the capsular polysaccharide are independently linked to the glycocalyx backbone of cell wall peptidoglycan; group B carbohydrate is linked to N-acetylmuramic acid, and capsular polysaccharide is linked via a phosphodiester bond and an oligosaccharide linker to N-acetylglycosamine.

Group B Streptococcus (GBS) is a common cause of serious infections in neonates, including bacteremia, pneumonia, and meningitis. Almost all GBS strains isolated from neonates with invasive infection are encapsulated with one of several capsular polysaccharides (1–3). Studies in experimental animals have provided evidence that the capsular polysaccharide is linked via a phosphodiester bond and an oligosaccharide linker to N-acetylglycosamine.

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† The abbreviations used are: GBS, group B Streptococcus; GC/MS, gas chromatography/mass spectrometry; MurNAc, N-acetylmuramic acid, ELISA, enzyme-linked immunosorbent assay; FPLC, fast protein liquid chromatography.

tagenesis are attenuated in their ability to cause lethal infection in neonatal rats (4, 5). Studies of opsonophagocytic killing of type III GBS in vitro demonstrated a direct correlation between the amount of capsule produced by a strain and its level of resistance to complement-mediated phagocytic killing by human blood leukocytes (6). Other studies suggest not only that the capsule is important in virulence but also that the amount of capsule produced under different circumstances may vary, perhaps as a means to enhance adaptation of the organism to various ecological niches within the human host (7). Despite the importance of the capsular polysaccharide in the pathogenesis of GBS infection, relatively little is known about the biochemistry of capsule biosynthesis or the nature of the linkage of the capsular polysaccharide to the bacterial cell surface.

Nine GBS capsular types have been identified serologically, and the repeating unit structure of each has been defined (8–15). The type III capsular polysaccharide is one of the three major capsular types associated with invasive neonatal infection and the most common serotype in GBS meningitis (2, 16). The repeating unit structure of GBS type III polysaccharide is illustrated in Fig. 1. Although previous investigations have suggested the capsular polysaccharide is linked to peptidoglycan in the cell wall (17, 18), the nature of the attachment of the capsular polysaccharide to the GBS cell wall has not been clearly defined. In this study, we present evidence to show that the type III GBS capsular polysaccharide is covalently linked via a phosphodiester bond and a linker oligosaccharide to N-acetylmuramylglucosamine residues of the disaccharide repeating unit of cell wall peptidoglycan, while the group B antigen is linked to N-acetylmuramic acid.

EXPERIMENTAL PROCEDURES

GBS Strains and Growth of Bacteria—GBS strains included two clinical isolates of type III GBS, strains M781 and COH1, and an unencapsulated mutant of strain COH1, strain COH1–13 (5, 19, 20). GBS were grown to exponential phase in Columbia broth at 37 °C with shaking at 200 rpm. Unless otherwise specified, all studies were performed with strain M781.

Preparation of GBS Cell Wall Complex—GBS cells in the midexponential phase of growth were harvested by centrifugation, ground extensively in liquid nitrogen with a prechilled mortar and pestle, and homogenized 1:2 (w/v) with ice-cold 70 mM HEPES buffer, pH 8.2, containing 5% glycerol, 4 mM EDTA, 2 mM 2-mercaptoethanol, 1 mM diithiothreitol, and 0.4 mM phenylmethylsulfonyl fluoride (21). After removal of cell debris and unbroken cells by centrifugation (1000 × g), the cell lysate was partitioned by 10 volumes of chloroform/methanol to make a final ratio of chloroform/methanol/water of 8:4:3 (v/v/v). Particles were disaggregated by sonication in an ultrasonic bath for 2 min, and the mixture was separated into three phases by centrifugation at 1000 × g for 15 min: an upper aqueous phase, a lower organic phase, and an interface between the aqueous and organic phases containing insoluble material. This insoluble material was washed several times.
and dialyzed against distilled water; it is referred to below as the cell wall complex.

**Enzymatic Treatment of Cell Wall Complex—**Duplicate samples of cell wall complex (2 mg) were incubated either with mutanolysin (155 units in 50 μM sodium acetate buffer, pH 5.5, containing 10 mM calcium chloride at 37 °C overnight) or with endo-β-galactosidase (22) in 50 mM sodium acetate buffer, pH 7.4, loaded onto a 3×3×70 mm) in the absence of SDS. The gels were purchased from Jule Inc. (New Haven, CT) and contained 0.09 M Tris, 0.08 M boric acid, and 2.6 mM pyrophosphate. The gels were run for 1 h at 200 V constant voltage.

**Purification of Peptidoglycan from the Cell Wall Complex—**A sample of cell wall complex (11.8 mg) remaining after endo-β-galactosidase treatment was further purified by successive treatment with DNase (0.1 mg/ml) and RNase (0.1 mg/ml) in 100 mM potassium phosphate buffer, pH 7.1, at 37 °C overnight; repeated washing with 2% Triton X-100 in 10 mM Tris buffer, pH 7.0, containing 1 mM EDTA; and incubation in the same buffer and 0.5 mM sodium hydroxide at 50 °C for 24 h. After being washed twice with distilled water, the insoluble material remaining (0.6 mg) was hydrolyzed with 6 N hydrochloric acid for amino acid analysis (model 6300 amino acid analyzer; Beckman, Palo Alto, CA).

**Purification of Type III Capsular Polysaccharide and Group B Carbohydrate from GBS Cells—**Base-treated GBS type III capsular polysaccharide was purified as described (12). Group B carbohydrate was prepared as described (23).

**ELISA for Type III Capsular Polysaccharide—**Immunoreactive type III polysaccharide in samples of cell wall complex was detected by an ELISA inhibition assay, essentially as described previously (24). Antiserum used in ELISA was from rabbits immunized with type III GBS polysaccharide-tetanus toxoid conjugate vaccine. Cell wall complex (0.2 μg) was added to ELISA wells coated with type III capsular polysaccharide before the addition of rabbit antiserum at a final dilution of 1:100. The remainder of the ELISA procedure was performed as described (4).

**Component Sugar Analysis—**Samples were analyzed by high performance anion exchange chromatography using a gradient system (Dionex, Sunnyvale, CA) equipped with a pulsed amperometric detector (model PAD2) and a pellicular anion exchange column (PA-1; 4 × 250 mm). The detector sensitivity was set at 500 nA with a 0.5 mA applied pulse potential. The instrument was connected to Dionex AI-450 chromatography automation software version 3.32 for analysis and data processing. Samples were hydrolyzed with 2 M trifluoroacetic acid at 100 °C for 6 h for analysis of neutral sugars and muramic acid. Hydrolyzed samples were applied through a microinjection valve with a 50-μl loop. Neutral sugars were eluted with 20 mM sodium hydroxide at a flow rate of 1 ml/min. Muramic acid was eluted with 100 mM sodium hydroxide containing 75 mM sodium acetate. For detection of sialic acid, samples were treated with 6% acetic acid at 100 °C for 1 h, applied to the column as above, and eluted with 60 mM sodium hydroxide.

**Purification of Type III Capsular Polysaccharide from Cell Wall Complex—**A 50-g sample of type III GBS cell lysate, prepared as described above, was heated in an autoclave to 120 °C at 20 p.s.i. The lysate was clarified by centrifugation at 1000 × g for 15 min. The insoluble material at the aqueous/organic interface was washed three times with water, dialyzed against water, and treated sequentially with 0.1 mg/ml DNase, 0.1 mg/ml RNase, 0.1% trypsin, and 0.1% pepstatin in 100 mM potassium phosphate buffer, pH 7.1, with washing between steps. The residual insoluble material was treated with mutanolysin (10 units/mg of insoluble material) in 50 mM sodium acetate buffer, pH 5.5, containing 10 mM calcium chloride at 37 °C overnight, and then with 0.1% trypsin or pepstatin at 37 °C for 4 h to inactivate the mutanolysin. The resultant solution containing crude soluble type III polysaccharide was clarified by centrifugation at 1500 × g and then dialyzed against distilled water and lyophilized. A sample of 100 mg of the lyophilized material was dissolved in 5 ml of 50 mM Tris buffer, pH 7.8, and loaded onto a Resource Q column (Amersham Pharmacia Biotech). The sample was eluted with a 0–0.5 M sodium chloride gradient in the same buffer using a gradient FPLC system (Amersham Pharmacia Biotech) at a flow rate of 2 ml/min. Fractions were assayed by ELISA inhibition for immunoreactive type III polysaccharide or group B carbohydrate as described previously (24, 25). Fractions containing type III polysaccharide were pooled, dialyzed, and lyophilized. The lyophilized material was dissolved in 10 ml Tris buffer, pH 7.4, loaded onto a 3 × 90-cm column of Sepharose S300 (Amersham Pharmacia Biotech) and eluted with the same buffer at a flow rate of 4 ml/min. Fractions were analyzed as above for type III polysaccharide and group B carbohydrate. Fractions containing type III polysaccharide were pooled, dialyzed, and lyophilized.

**Polycrylamide Gel Electrophoresis of GBS Polysaccharides—**GBS polysaccharide samples were analyzed by electrophoresis on nondenaturing 4–15% polyacrylamide gradient slab minigels (90 × 70 × 0.75 mm) in the absence of SDS. The gels were purchased from Jule Inc. (New Haven, CT) and contained 0.09 M Tris, 0.08 M boric acid, and 2.6 mM pyrophosphate. The gels were run for 1 h at 200 V constant voltage in 0.5× sample buffer. Polysaccharides were visualized by staining with Alcian blue and silver (26). The molecular mass range of the GBS polysaccharides was estimated by comparison of the mobility of the polysaccharides with protein molecular mass standards (Sigma). β-N-Acetylgalactosaminidase Treatment of the Purified Cell Wall-associated Type III Polysaccharide and Subsequent Purification—Soluble complexes of cell wall-associated type III polysaccharide were treated with β-N-acetylgalactosaminidase from Streptococcus pneumoniae (Sigma) in 50 mM sodium acetate buffer, pH 5.5, containing 10 mM magnesium chloride at 22 °C for 4 days and at 37 °C overnight. The sample was then dialyzed against water and lyophilized. The lyophilized material was dissolved in 50 mM Tris-HCl, pH 7.8, loaded onto a Resource Q FPLC column, and eluted as described above. Fractions were assayed by ELISA inhibition for immunoreactive type III polysaccharide or group B carbohydrate as described (24, 25). Fractions containing type III polysaccharide were pooled, dialyzed, and lyophilized, and used for chemical analysis of sugars and amino acids.

**GC/MS Analysis—**GC/MS was performed on a DB-17 (J & W Scientific, Folsom, CA) 30-m capillary column containing (50% phenyl) methylpolysiloxane on an HP 6890 Series gas chromatograph/HP5973 MSD (Hewlett Packard, Wilmington, DE). The flow rate was constant (1 ml/min). For trimethylsilyl derivatives, the temperature program...
started at 100 °C for 2 min, increased at 5 °C/min from 100 to 275 °C over 35 min, and then remained at 275 °C for 3 min for a total run time of 40 min. The temperature program for alditol acetate derivatives started at 150 °C for 2 min, followed by a 4 °C/min rise to 200 °C, a 1 °C/min rise to 225 °C, a 4 °C/min rise to 280 °C, and finally a constant temperature of 280 °C for 6 min. The injector temperature was 230 °C.

For detection of sugars and amino sugars, samples were hydrolyzed with 2 M trifluoroacetic acid at 100 °C for 5 h, dried with nitrogen gas, derivatized with Trisil (Pierce) at 50 °C for 30 min, dried, and then resuspended in 2 ml of hexane and filtered through a clean cotton column. For detection of reducing terminal sugar, the samples were reduced with sodium borohydride and then hydrolyzed with trifluoroacetic acid. The hydrolyzed sample was treated with acetic anhydride at 100 °C for 1 h, washed with toluene, and partitioned with methylchloro-ride and water. The organic phase was dried for GC/MS analysis (27). The alditol acetate ions were monitored by GC/MS and expected to
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GBS Capsular Polysaccharide Is Linked to Cell Wall Pepti-doglycan—In experiments to characterize glycosyltransf-erase(s) involved in capsular polysaccharide biosynthesis, we observed the incorporation of galactose from UDP-galactose into insoluble material that partitioned to the aqueous/organic interface of a chloroform/methanol/water mixture (5). To study the interface material more closely, the insoluble product was collected and washed with both aqueous and organic solvents. After treatment with protease and alkali, amino acid analysis was performed on the residual insoluble material. It was found to contain lysine, alanine, and glutamine/glutamic acid in a molar ratio of 1:2.8:1.4, with minor amounts of other amino acids. This analysis is consistent with the previously reported composition of GBS peptidoglycan and provided evidence that the insoluble material contained bacterial cell wall components (17).

To investigate whether this cell wall complex also contained type III capsular polysaccharide, a lysate from a preparative scale culture of type III GBS was fractionated in chloroform/methanol/water, and the insoluble material containing the cell wall complex was collected from the interface between aqueous and organic phases. The complex was subjected to acid hydroly-

ysis and analyzed for component sugars by Dionex high per-
formance anion exchange chromatography. With an elution program to detect neutral sugars, glucose, galactose, glucosa-
mine, and rhamnose were identified; with hydrolysis conditions and elution programs for acidic sugars, muramic acid and sialic acid were detected (Fig. 2). This mixture of sugars suggested that the cell wall complex included not only peptidoglycan, which contains N-acetylmuramic acid and N-acetylglycosamine but also capsular polysaccharide, which contains galactose, glucose, N-acetylmuramic acid, and sialic acid (10), and group B carbohydrate, which contains rhamnose, N-acetylglycosamine, galactose, and glucitol (28).

Analysis of cell wall complex isolated from an acapsular mutant strain COH1–13 revealed a similar sugar composition except for the absence of sialic acid and glucose, the two sugars found exclusively in the capsular polysaccharide and not in group B antigen or peptidoglycan.

As a more specific assay for the presence of capsular polysaccharide in the cell wall complex of the wild type strain, the complex was treated with endo-β-galactosidase, an enzyme used previously to depolymerize the type III polysaccharide (22). Endo-β-galactosidase is highly specific for a β-1-Gal(1→4)-β-1-Glc linkage that occurs once per backbone repeating unit in the type III capsular polysaccharide. Component sugar analysis by high performance anion exchange chromatography of the material released by the enzyme detected galactose, glucose, and glucosamine in the expected ratio of 2:1:1 as exclusive sugars, using a neutral sugar program. This sugar analysis, together with the highly specific action of endo-β-galactosidase, provided further evidence that the cell wall complex contained type III capsular polysaccharide.

Immunoreactive type III capsular polysaccharide was detected by the capacity of cell wall complex to inhibit the reaction of type III polysaccharide–specific rabbit antiseraum with purified type III GBS polysaccharide in ELISA. Cell wall complex from type III strains M781 and COH1 produced more than 90% inhibition in this immunoassay, while cell wall complex from the acapsular mutant strain COH1–13 inhibited less than 20%. Immunoreactive type III polysaccharide could be released into solution from the GBS cell wall complex of strain M781 by treatment with 0.5 M ammonium hydroxide for 2 h at 37 °C; because the glycosidic bonds in the repeating unit of the type III polysaccharide are resistant to cleavage by base, this result suggested that a base-sensitive linkage was involved in the

GBS Propionyl Polysaccharide Is Linked to Cell Wall Pepti-doglycan—To study the composition of the cell wall complex, a lysate from a preparative scale culture of type III was fractionated in chloroform/methanol/water, and the insoluble material containing the cell wall complex was collected from the interface between aqueous and organic phases. The complex was subjected to acid hydroly-

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attachment of the capsular polysaccharide to the cell wall.

**Capsular Polysaccharide Linked to the GBS Cell Wall Also Is Covalently Bound to Group B Carbohydrate**—To study directly the physical association between the capsular polysaccharide linked to the cell wall complex and the cell wall-associated group B carbohydrate, we analyzed type III capsular polysaccharide purified from the cell wall complex. The complex was treated with RNase, DNase, and Pronase, washed extensively, and then treated with mutanolysin, a muramidase that cleaves the glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine in the glycan strands of peptidoglycan (29). Capsular polysaccharide released by mutanolysin was purified by anion exchange FPLC, followed by gel filtration chromatography. Fractions from each column were assayed for N-acetylglucosaminidase activity and for group B carbohydrate by ELISA inhibition. On both columns, group B carbohydrate co-eluted with the capsular polysaccharide.

In the native gradient polyacrylamide gel electrophoresis shown in Fig. 3, the type III polysaccharide purified from cell wall complex appeared as a diffuse band spanning a broad $M_r$ range of 62,000–320,000. By comparison, type III polysaccharide purified after base treatment to remove group B carbohydrate migrated as a diffuse band of 37,000–320,000, while purified group B carbohydrate (free of peptidoglycan or capsular polysaccharide) appeared as a diffuse band corresponding to an estimated $M_r$ range of 25,000–50,000 (Fig. 3). A Western immunoblot of the same sample of cell wall-associated type III polysaccharide reacted with the antisera specific for type III polysaccharide and group B carbohydrate, indicating that the two polysaccharides co-migrated in the electrophoresis (not shown). This result together with the larger apparent molecular mass of the cell wall-associated compared with base-treated type III polysaccharide provided evidence that the type III polysaccharide and the group B carbohydrate are covalently linked.

**Type III Capsular Polysaccharide Can Be Separated from Group B Carbohydrate by Cleavage of Peptidoglycan Fragments with $\beta$-N-Acetylglucosaminidase**—Results discussed above indicated that capsular polysaccharide released from the cell wall complex by mutanolysin remained covalently linked to group B carbohydrate, either because the capsular polysaccharide was attached directly to group B carbohydrate or because both polysaccharides were attached to small subunits of peptidoglycan that survived mutanolysin digestion. Complete digestion of peptidoglycan by mutanolysin is expected to yield individual disaccharide subunits of $\beta$-D-GlcNAc(1→4)$\beta$-D-MurNAc, with some degree of peptide cross-linking to other disaccharide units. To test whether the capsular polysaccharide and the group B carbohydrate were independently linked to these peptidoglycan fragments, the capsular polysaccharide-group B carbohydrate complex released from the GBS cell wall complex by mutanolysin was further digested by treatment with $\beta$-N-acetylglucosaminidase, a treatment shown in pilot experiments to cleave the disaccharide fragments of $\beta$-D-GlcNAc(1→4)$\beta$-D-MurNAc produced by mutanolysin digestion of peptidoglycan. $\beta$-N-Acetylglucosaminidase treatment resulted in a shift of the polysaccharide to a lower apparent $M_r$ distribution on polyacrylamide gel electrophoresis analysis (Fig. 4). The $\beta$-N-acetylglucosaminidase-treated material was fractionated by ion exchange FPLC. In contrast to the elution profile obtained before enzyme treatment, the treated material resolved into distinct peaks of type III capsular polysaccharide and group B carbohydrate (Fig. 5). Separation of the two polysaccharides by $\beta$-N-acetylglucosaminidase treatment implies that they are attached independently to peptidoglycan.

**Characterization of the Linkage between the Type III Capsular Polysaccharide and the GBS Cell Wall**—Following the release of the capsular polysaccharide from group B carbohydrate by $\beta$-N-acetylglucosaminidase treatment and separation of the two polysaccharides by ion exchange FPLC, each polysaccharide was analyzed separately by GC/MS of trimethylsilyl derivatives of the component monosaccharides. The group B carbohydrate fraction contained rhamnose, glucosamine, galactose, glucose, muramic acid, and a trace amount of glucitol (Fig. 6). Amino acid analysis of the same fraction revealed the amino acids lysine, alanine, and glutamic/glutamine acid in a molar ratio of 1:3:1.2, which is in agreement with the amino acid composition reported previously for GBS peptidoglycan (17). The presence of muramic acid and amino acids from the cross-linking peptide bound to group B antigen indicated that, after $\beta$-N-acetylglucosaminidase digestion, group B antigen remains covalently linked to these constituents of the cell wall peptidoglycan.

Similar analysis of the type III capsular polysaccharide fraction showed galactose, glucose, and glucosamine, which are the expected component neutral sugars for GBS type III capsular...
polysaccharide. The absence of rhamnose in this fraction is consistent with complete separation of the capsular polysaccharide from group B antigen. In addition, selective ion monitoring for the characteristic muramic acid ion at 185 m/z did not detect muramic acid in the capsular polysaccharide fraction (Fig. 7). In contrast to the group B carbohydrate fraction, only trace amounts of amino acids were found in the capsular polysaccharide fraction. These results suggested that capsular polysaccharide is not covalently linked to N-acetylmuramic acid of peptidoglycan.

To define the nature of the attachment between the capsular polysaccharide and peptidoglycan, the capsular polysaccharide fraction was subjected to reduction, followed by acid hydrolysis and acetylation. GC/MS analysis of the acetylated derivatives revealed peaks corresponding to sugar acetates of galactose, glucose, and glucosamine and a peak at 35.7 min whose electron intact mass spectrum included characteristic ions of m/z 144, 156, and 318, representing the amino sugar alditol, 1,3,4,5,6-penta-O-acetyl-2-acetamido-2-deoxy-D-glucitol. These results are consistent with the presence of N-acetylglucosamine at the reducing terminus of the capsular polysaccharide.

31P NMR analysis of the capsular polysaccharide fraction showed a signal with a chemical shift of (δ ~ 0.5), characteristic of a phosphodiester (Fig. 8) (30). As expected, no phosphorus signal was detected in the spectrum of capsular polysaccharide purified after base treatment, suggesting that the β-elimination site in type III capsular polysaccharide is a phosphodiester. In order to better characterize the nature of the link between the capsular polysaccharide and the cell wall peptidoglycan, a sample of capsular polysaccharide was first reduced with sodium borohydride and then subjected to base treatment to cleave the phosphodiester linkage. The products of this β-elimination reaction were separated into polysacchar-
ride (>3-kDa) and oligosaccharide (<3-kDa) fractions. To identify the reducing terminus of the polysaccharide exposed by the β-elimination reaction, the polysaccharide fraction was reduced with sodium borodeuteride. After acid hydrolysis and acetylation, GC/MS analysis revealed strong peaks representing acetates of the component sugars of the capsular polysaccharide with characteristic mass ions of \( m/z \) 157, 200, 245, and 331. As expected, the signal for the deuterium-labeled alditol acetate representing the reducing terminus of the polysaccharide was a very small one at 26.3 min (Fig. 9A) with the characteristic mass ions of \( m/z \) 217, 218, 259, 260, 289, 290, 361, and 362, representing deuterium-labeled \( \alpha \)-glucitol hexaacetate (Fig. 9B). This result indicated that the reducing terminus of the capsular polysaccharide linked to the phosphate group is a glucose residue.

The oligosaccharide (<3-kDa) fraction was subjected to acid hydrolysis, reduction with sodium borodeuteride, acetylation, and GC/MS analysis of the resultant alditol acetates. Peaks were detected at 26.16 min (glucitol hexaacetate), 26.55 min (galactitol hexaacetate), 17.31 min (arabinofuranose hexaacetate), and 36.6 min (1,3,4,5,6-penta-O-acetyl-2-acetamido-2-deoxy-\( \alpha \)-glucitol), consistent with the presence in the oligosaccharide of glucose, galactose, arabinose, and \((N\text{-acetyl})\)glucosamine. Partial hydrolysis yielded disaccharide and trisaccharide fragments; however, the extremely small amounts of material in the oligosaccharide fraction did not permit a complete determination of the structure of this linker oligosaccharide. The mass spectra revealed deuterium labeling of alditol acetates corresponding to all of the component sugars except glucosamine, indicating that \((N\text{-acetyl})\)glucosamine was the reducing terminal residue. This sugar is proposed to represent the \( N\text{-acetyl} \)glucosamine of the disaccharide repeating unit of peptidoglycan to which the capsular polysaccharide-phosphate-oligosaccharide is linked. Thus, results of the linker analysis are in agreement with those described earlier that showed \( N\text{-acetyl} \)glucosamine at the reducing end of the polysaccharide released from the cell wall complex by \( \beta\text{-N-acetyl} \)glucosaminidase. GC/MS analysis of trimethylsilyl derivatives of the hydrolyzed oligosaccharide fraction confirmed the presence of the component sugars identified above as well as a trimethylsilyl-phosphate peak detected at 7.0 min with characteristic mass ions of \( m/z \) 299 and 314.

Together, these results provide evidence that the type III GBS capsular polysaccharide is linked through a reducing terminal glucose residue, via a phosphodiester bond and an oligosaccharide linker molecule, to \( \beta\text{-N-acetyl} \)glucosamine on the disaccharide repeating unit of peptidoglycan. Fig. 10 shows a schematic representation of the proposed relationships between the capsular polysaccharide, group B carbohydrate, and cell wall peptidoglycan.

**DISCUSSION**

The capsular polysaccharide is a major surface structure of GBS isolates associated with human infection. However, as with other Gram-positive bacterial species, the nature of the attachment between the GBS capsular polysaccharide and the bacterial cell is incompletely understood. In Gram-negative bacteria, the capsular polysaccharide is linked via a lipid moiety to the bacterial outer membrane (31, 32). By contrast, evidence from studies of *S. pneumoniae*, *Staphylococcus aureus*, and GBS suggest that the capsular polysaccharide of each of these Gram-positive pathogens is covalently attached to the bacterial cell wall (17, 18, 33, 34). Treatment of the bacterial cells with enzymes that cleave peptidoglycan releases the capsular polysaccharide from the cell, suggesting a direct or indirect linkage of capsule to cell wall peptidoglycan. deCueninck et al. (17) found that mutanolysin treatment of type III GBS cell walls yielded soluble complexes that contained constituents of peptidoglycan, group B carbohydrate, and capsular polysaccharide. The complexes could be cleaved by base treatment to release capsular polysaccharide free of peptidoglycan. In similar experiments, Yeung and Mattingly (18) analyzed capsular
Linkage of Group B Streptococcus Capsule to Cell Wall

was linked via a phosphodiester bond and a linking oligosaccharide to N-acetylmuramic acid.

In general, \(\beta\)-N-acetylglucosaminidase cleaves the glycosidic bond of a terminal residue of N-acetylmuramic acid. However, in the present study, enzyme treatment of the capsular polysaccharide-group B carbohydrate complex produced products of \(M_r \sim 10,000\) to \(>100,000\), a result that implies cleavage of internal linkages within the complex. Several pieces of evidence suggest the site of action of the enzyme is the \(\beta\)-D-GlcNAc(1\(\rightarrow\)4)\(\beta\)-D-MurNac bond in polysaccharide-substituted disaccharide fragments of peptidoglycan produced by mutanolysin digestion. 1) Sequential treatment of purified GBS peptidoglycan with mutanolysin followed by \(\beta\)-N-acetylglucosaminidase resulted in the release of free N-acetylmuramic acid (data not shown), a result that confirms susceptibility of the \(\beta\)-D-GlcNAc(1\(\rightarrow\)4)\(\beta\)-D-MurNac bond to \(\beta\)-N-acetylglucosaminidase (the released free N-acetylmuramic acid presumably represents residues that were unsubstituted in the native state or residues from which the capsular polysaccharide was cleaved during purification of the peptidoglycan). 2) The capsular polysaccharide was released from insoluble cell wall complexes by \(\beta\)-N-acetylglucosaminidase only after digestion of the peptidoglycan with mutanolysin, which exposes N-acetylmuramic acid residues. 3) \(\beta\)-N-Acetylglucosaminidase digestion of the complexes separated capsular polysaccharide from group B carbohydrate, an observation that implies the enzyme acts on a moiety that links the two polysaccharides. 4) Muramic acid remained with the group B carbohydrate after \(\beta\)-N-acetylglucosaminidase treatment, while reducing terminal N-acetylmuramic acid residues were detected in the capsular polysaccharide fraction. Thus, evidence from several different experimental approaches indicates that \(\beta\)-N-acetylglucosaminidase cleaves the glycosidic linkage to muramic acid of (polysaccharide-substituted) N-acetylmuramic acid residues exposed by mutanolysin digestion of peptidoglycan.

Our results do not support the earlier suggestion that the capsular polysaccharide is attached to the peptide cross-bridges of peptidoglycan (18). Rather, the data indicate that the capsule is linked to N-acetylmuramic acid residues of the glycan backbone. Potentially available sites on N-acetylmuramic acid residues of the GBS peptidoglycan are at C-3 and C-6. Substitution at C-6 may be more compatible with the observed sensitivity in our studies of the GBS capsular polysaccharide-peptidoglycan fragment complex to digestion with \(\beta\)-N-acetylglucosaminidase. We speculate that substitution at C-6 is more likely to permit \(\beta\)-N-acetylglucosaminidase to act on a (substituted) \(\beta\)-linked N-acetylmuramic acid residue, since a substituent at the 6-position is separated from the pyranose ring by an additional C-C bond compared with a substituent at the 3- or 4-position. The additional C-C bond separating C-6 from the pyranose ring not only places the substituent group at the 3- or 4-position. The additional C-C bond separating C-6 from the pyranose ring not only places the substituent group at the 3- or 4-position, but also confers greater flexibility to the portion of the molecule bearing the substituent. These effects may permit more efficient utilization by \(\beta\)-N-acetylglucosaminidase of a \(\beta\)-linked N-acetylmuramic acid substituted at C-6 than one substituted at C-3. In a separate experiment, we found that the \(\beta\)-N-acetylglucosaminidase preparation used in our studies cleaved 4-methylumbelliferyl-\(\beta\)-D-glucopyranoside, albeit more slowly than the standard fluorogenic substrate 4-methylumbelliferyl-N-acetyl-\(\beta\)-D-glucopyranoside (data not shown), indicating that the enzyme can cleave \(\beta\)-linked N-acetylmuramic acid residues bearing a substituent group at C-6.

Others have reported evidence of attachment of accessory polysaccharides to the glycan portion of peptidoglycan in several Gram-positive organisms. Examples include teichoic acid

![Schematic representation of a proposed model for the linkage of capsular polysaccharide and group B carbohydrate to peptidoglycan of GBS. The arrows denote cleavage sites of mutanolysin (1) and \(\beta\)-N-acetylglucosaminidase (2).](http://www.jbc.org/)
of Bacillus subtilis, teichuronic acid of Micrococcus luteus, and arabinoxylan of mycobacteria (35–37). Where it has been examined, linkage of these polymers appears to be to C-6 of the N-acetylmuramic acid residues in contrast to the linkage of the GBS capsular polysaccharide to N-acetylgalacosamine residues. In the case of GBS, N-acetylmuramic acid residues may be unavailable or energetically less favorable for attachment of the capsular polysaccharide because the group B carbohydrate is linked at this site. These results indicate that the capsular polysaccharide is distinctive among GBS accessory polysaccharides not only in its central role in virulence but also in its mode of attachment to the bacterial cell. It remains to be determined whether linkage of the capsular polysaccharide to N-acetylgalacosamine residues of peptidoglycan is a feature unique to GBS or a general property of encapsulated Gram-positive bacteria.

Results of these studies provide direct evidence that both the capsular polysaccharide and group B carbohydrate are covalently bound to peptidoglycan of the GBS cell wall. They indicate further that the two polysaccharides are attached independently and at separate sites. The general features of this model of the GBS cell surface are likely to apply generally to other encapsulated Gram-positive bacteria. These data represent strong evidence that the mechanism and site of attachment of capsular polysaccharides to the Gram-positive cell surface is fundamentally different from that in Gram-negative bacteria and from the linkage of other accessory cell wall polysaccharides in Gram-positives. Characterization of the site of attachment and of the nature of the linkage between the capsular polysaccharide and the GBS cell wall provides new insight into the basic structure of this important pathogen and may suggest potential targets for novel antimicrobial drug design for this and other encapsulated Gram-positive bacteria.

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