JOURNAL OF BACTERIOLOGY, June 1988, p. 2618–2624
0021-9193/88/062618-07$02.00/0

Vol. 170, No. 6

Isolation and Characterization of the Cell-Associated Region of Group A Streptococcal M6 Protein

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Received 11 December 1987/Accepted 29 February 1988

DNA sequence analysis of the complete M6 protein gene revealed 19 hydrophobic amino acids at the C terminus which could act as a membrane anchor and an adjacent proline- and glycine-rich region likely to be located in the cell wall. To define this region within the cell wall and its role in attaching the molecule to the cell, we isolated the cell-associated fragment of the M protein. Assuming that the cell-associated region of the M protein would be embedded within the wall and thus protected from trypsin digestion, cells were digested with this enzyme, and the wall-associated M protein fragment was released by phage lysin digestion of the peptidoglycan. With antibody probes prepared to synthetic peptides of C-terminal sequences, a cell wall-associated M protein fragment (molecular weight, 16,000) was identified and purified. Amino acid sequence analysis placed the N terminus of the 16,000-molecular-weight fragment at residue 298 within the M sequence. Amino acid composition of this peptide was consistent with a C-terminal sequence lacking the membrane anchor. Antibody studies of nitrous acid-extracted whole bacteria suggested that, in addition to the peptidoglycan-associated region, a 65-residue helical segment of the C-terminal domain of the M protein is embedded within the carbohydrate moiety of the cell wall. Since no detectable amino sugars were associated with the wall-associated fragment, the C-terminal region of the M6 molecule is likely to be intercalated within the cross-linked peptidoglycan and not covalently linked to it. Because the C-terminal region of the M molecule is highly homologous to the C-terminal end of protein A from staphylococci and protein G from streptococci, it is likely that the mechanism of attachment of these proteins to the cell wall is conserved.

M protein is an antiphagocytic molecule located on the surface of the group A streptococcal cell wall (21, 31). From physicochemical and amino acid sequence analyses of the M5 (22), M6 (12, 16), and M24 proteins (2), it is clear that M protein is composed of two alpha-helical protein chains wound around each other to form a coiled-coil structure (12, 27). Although the majority of structural and immunochemical data on the M protein is derived from an amino-terminal fragment released from the streptococcus by limited pepsin digestion (2, 3, 22–24, 27), information regarding the C-terminal portion of this molecule is beginning to emerge (12, 16, 17, 19, 20, 28).

DNA sequence analysis of the M6 gene revealed the complete amino acid sequence of this M molecule, which identified three different repeat regions (16). In addition, it revealed a region at the C-terminal end which is likely to be responsible for attaching the M molecule to the cell. This segment of the M protein contains a potential membrane anchor region composed of 19 hydrophobic amino acids with a charged tail probably extending into the cytoplasm. Adjacent to the membrane anchor is a proline- and glycine-rich region which is likely to be located within the cell wall. The nearly regular placement of proline and glycine within the sequence suggests that this region may form a regular structure (12, 16). This C-terminal region was found to have strong sequence homology with the so-called cell anchor region of staphylococcal protein A (5, 16) and the C-terminal end of protein G, an immunoglobulin-binding protein from group G streptococci (5). Among these cell wall-associated proteins from gram-positive organisms, only protein A from Cowan I *Staphylococcus aureus* has been partially characterized (15). An attempt to isolate the cell wall-associated portion of this protein by trypsin digestion followed by lysostaphin extraction of the cell wall has been achieved with limited success (29). The partial characterization of this region is based on subtractive amino acid sequence analysis (29) of the protein A from the Cowan I strain and nucleotide sequence of the protein A gene from staphylococcal strain 8325-4 (15). Although the available data regarding the nature of the association of the C-terminal region of protein A with the cell wall peptidoglycan are not conclusive (15, 29, 30), there is no direct evidence to indicate that the so-called cell wall-associated region of proteins from gram-positive organisms is actually found within the cell wall.

From earlier studies, it is clear that the M protein is associated with the cell wall fraction of the streptococcal cell (9). In this study we isolated the cell wall-associated region of the M6 molecule directly from the streptococcus to determine both the region of the molecule located within the wall and the mechanism by which the molecule is attached. Since the M6 protein contains 61 lysines and 16 arginines, 7 and 1 of which, respectively, are located in the presumed wall region (16), a method was devised to isolate this cell wall-associated segment of the M molecule on the assumption that its location would protect it from digestion with trypsin. Group C streptococcal phage-associated lysin, an *N*-acetylmuramyl L-alanine amidase which cleaves the peptidoglycan (8, 13), was then used to release the protected fragment from the cell wall. By the use of monoclonal antibodies to the native M6 molecule (19, 20), polyclonal sera against synthetic peptides from sequences derived from the so-called cell wall region and adjacent regions, along with N-terminal sequence and amino acid analysis of the purified wall-associated peptides, the region of the M6 molecule embedded within the cell wall was deduced.

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MATERIALS AND METHODS

Streptococcal strain. Type 6 streptococcal strain D471 was from the Rockefeller University collection.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot (immunoblot) analysis. Proteins were separated on 14% sodium dodecyl sulfate–polyacrylamide gel slabs and electrotransferred onto nitrocellulose membrane as described previously (10). Specific proteins bound to the nitrocellulose membrane were visualized with monoclonal (1:1,000) and polyclonal (1:500) antibodies by the method of Blake et al. (4).

To detect any contamination of cell wall material in the purified peptide, the nitrocellulose paper bearing the immobilized peptide was first blocked in 0.1 M Tris hydrochloride buffer (pH 8.0)–0.4 N NaCl–0.5% Tween 20 for 2 h with shaking. The blot was reacted with *N*-acetylglucosamine-specific lectin, 1 μg of wheat germ agglutinin (E-Y Laboratories Inc., San Mateo, Calif.) per ml for 3 h, followed by rabbit anti-wheat germ agglutinin antibody (1:500; E-Y Laboratories) for 3 h and developed as described previously (4). Anti-*N*-acetylglucosamine specific antibody (1:500) was also used to detect the presence of *N*-acetylglucosamine in the wall-associated peptide (11).

Immunoreagents. Specific immunoreagents were used to identify the various regions of the M molecule. Monoclonal antibody 10B6, prepared against the native M6 molecule of strain D471, is reactive with an epitope at residues 275 through 289 on the M6 molecule (19, 20). Polyclonal rabbit sera against M6 synthetic peptides were prepared as described below. Antisera with the following specificities were included in the present study (numbers within parentheses indicate residue numbers): anti-SM6(308–327); anti-SM6 (339–352), whose epitope is located immediately N terminal to the proline and glycine region; and anti-SM6(381–398), whose epitope is within the proline- and glycine-rich region.

Tryptic digestion and lysin extraction. Streptococci were grown in 40 liters of chemically defined medium (32) overnight at 37°C. The bacteria were harvested, washed twice in 200 mM ammonium bicarbonate, and suspended in 500 ml of this buffer. The M protein exposed on the surface of the streptococcal cell wall was digested with trypsin (Worthington Diagnostics, Freehold, N.J.) at a concentration of 100 μg/ml for 30 min at 37°C under constant rotation. The digestion was terminated by the addition of soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, Mo.) at a concentration of 150 μg/ml, followed by centrifugation at 8,000 × *g* for 30 min to sediment the organisms. To remove any residual enzymatic activity, the digested bacteria were washed three times with 0.05 M phosphate buffer (pH 6.1) containing trypsin inhibitor (100 μg/ml). The pellet was then suspended in 500 ml of 30% raffinose (Sigma) in 0.05 M phosphate buffer (pH 6.1) containing 50 μg of trypsin inhibitor per ml, 0.005 M dithiothreitol, and 10 μg of chloramphenicol (Parke-Davis) per ml. Phage-associated lysin, prepared as described previously (8), was added (120 U/ml), and the mixture was incubated for 90 min at 37°C under constant rotation to digest the cell wall and release the cell wall-associated peptide (11). The protoplasts were then separated from the wall extract by centrifugation at 10,000 × *g* for 30 min. The supernatant was brought to 45% saturation with ammonium sulfate and placed at 4°C for 18 h, and the resulting fine precipitate was sedimented at 8,000 × *g* for 30 min. The supernatant was then adjusted to 75% saturation and again placed at 4°C for 18 h. The precipitate was again sedimented at 8,000 × *g* and frozen at −70°C until used for the purification.

Purification of the cell wall-associated peptide from the trypsin-lysin digest. The 75% precipitate (crude extract) was suspended in 40 ml of 0.005 M sodium acetate (pH 5.5) and dialyzed extensively against this buffer. The extract was then applied to a column (13.5 by 1.5 cm) of carboxymethyl (CM-) cellulose (CM-52; Whatman Chemical Separation Ltd., England) equilibrated in 0.005 M sodium acetate (pH 5.5). The column was washed with this buffer until the absorbance at 220 nm reached baseline. The adsorbed peptides were eluted with a linear gradient from 0.1 to 0.5 M in a total of 140 ml. The peptides in each fraction were monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis as described above. Fractions reactive with anti-SM6(339–352) and anti-SM6(381–398) polyclonal sera were pooled, dialyzed against 0.05 M ammonium bicarbonate, and lyophilized or concentrated with a Diaflo concentrator fitted with a YM05 filter (Amicon Corp., Danvers, Mass.). Pooled and concentrated fractions were dialyzed against 0.05 M sodium acetate (pH 5.0), and applied to a 1-ml fast-protein liquid chromatography column (Mono S; Pharmacia Fine Chemicals, Piscataway, N.J.) equilibrated in the same buffer. After the column was washed with 5 volumes of the starting buffer, the peptide was eluted with a 30.0-ml linear NaCl gradient from 0.00 to 0.2 M. Purity was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis with anti-SM6(381–398) antibody as described above.

Analytical methods. For amino acid analysis, peptides were hydrolyzed in 6 N HCl at 110°C for 22 h and derivatized with ethanol-triethylamine-water-phenylisothiocyanate (7:1:1:1) in a Picotag Work Station (Waters Associates, Inc., Milford, Mass.) and analyzed with a Novapak C18 column (Waters) and a Waters 840 Data Module. Amino acid sequence analysis was performed by automated Edman degradation in a model 470A gas phase sequencer (Applied Biosystems, Foster City, Calif.). The phenylthiohydantoin amino acids were identified by high-pressure liquid chromatography on a C18 column with either a 1084B analyzer (Hewlett-Packard Co., Rockville, Md.) or a model 120A PTH analyzer (Applied Biosystems). For amino sugar analysis, peptides were hydrolyzed in 4 N HCl at 100°C for 7 h. They were then derivatized and analyzed as described above for amino acid analysis with a Novapak C18 column (Waters). All analytical procedures were performed by the Rockefeller University Central Biotechnology Facility.

Synthetic peptides. Synthetic peptides SM6(308–327), SM6 (339–352), and SM6(381–398) were prepared by the solid-phase method of Barany and Merrifield (1) and purified by high-pressure liquid chromatography on a Brownlee C8 reverse-phase column (Brownlee Laboratories, Santa Clara, Calif.) with a gradient of acetonitrile in 0.05% trifluroacetic acid. A cysteine residue was synthesized at the carboxy terminus for linkage to a carrier molecule. The sequence was verified by amino acid composition and sequence analysis as described above.

Coupling of synthetic peptide to carrier protein. The peptides were coupled to the carrier protein ovalbumin through the amidated C-terminal cysteine of the peptide. A heterobifunctional reagent *N*-succinimidyl-3(2-pyridyldithic)propionate (Pharmacia) was used for thiolation of ovalbumin (Worthington) according to the manufacturer's instructions. In general, 5.0 mg of purified synthetic peptide in 200 μl of 0.01 M phosphate-buffered saline (pH 7.5) was coupled to 8.0 mg of functionalized ovalbumin in 1.7 ml of saline. The

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       (C-terminal end of helical central rod region)

265  Leu Thr Ala Glu Leu Asp Lys Val Lys Glu[GLU LYS GLN ILE  Mab 10B6
279  SER ASP ALA SER ARG GLN GLY LEU ARG ARG ASP]Leu Asp Ala
293  Ser Arg Glu Ala Lys Lys Gln Val Glu Lys Ala Leu Glu Glu
307  Ala[ASN SER LYS LEU ALA ALA LEU GLU LYS LEU ASN LYS GLU  SM6(308-327)
321  LEU GLU GLU SER LYS LYS LEU]Thr Glu Lys Glu Lys Ala Glu
335  Leu Gln Ala Lys[LEU GLU ALA GLU ALA LYS ALA LEU LYS GLU  SM6(339-352)
349  GLN LEU ALA LYS]Gln Ala Glu Glu Leu Ala Lys Leu Arg Ala

       (Proline/glycine region)

363  Gly Lys Ala Ser Asp Ser Gln Thr
371  Pro Asp Ala Lys
375  Pro Gly Asn Lys Val Val
381 [PRO GLY LYS GLY GLN ALA
387  PRO GLN ALA GLY THR LYS                                  SM6(381-398)
393  PRO ASN GLN ASN LYS ALA]
399  Pro Met Lys Glu Thr Lys Arg Gln Leu
408  Pro Ser Thr Gly Glu Thr Ala Asn Pro

       (Membrane anchor region)

417  Phe Phe Thr Ala Ala Ala Leu Thr Val Met Ala Thr Ala Gly
431  Val Ala Ala Val Val Lys Arg Lys Glu Glu Asn
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FIG. 1. Amino acid sequence of the C-terminal region of M6 protein (16) arranged to highlight a portion of the helical central rod region (residues 265 through 362), the proline- and glycine-rich region (residues 363 through 416), and the membrane anchor (residues 417 through 441) with the charged tail represented by the last six residues. Uppercase letters in boldface type indicate the sequence recognized by monoclonal antibody 10B6 (19) and the position and sequence of the synthetic peptides used to prepare antibody probes.

mixture was then rotated initially for 2 h at room temperature and then overnight at 4°C.

Rabbit immunization. New Zealand White rabbits were bled and immunized intradermally with 200 to 400 μg of the purified synthetic peptide-coupled ovalbumin emulsified in Freund complete adjuvant (1:1) at multiple sites. Rabbits were boosted intradermally at monthly intervals after the first immunization with 200 μg of the respective coupled peptide in Freund incomplete adjuvant (1:1). All rabbits were bled 3 weeks after the first immunization and 7 to 10 days after each booster. All sera were filter sterilized and stored at 4°C. The sera were screened for antibodies to the immunizing peptide and native M6 protein by an enzyme-linked immunosorbent assay as described previously (7, 10).

Extraction of group-specific polysaccharide of streptococci with nitrous acid. To expose the antigenic epitopes of the M protein buried within the group carbohydrate, whole streptococci (strain D471) were extracted with nitrous acid (31). Briefly, a washed pellet from an overnight 50 ml culture in Todd-Hewitt broth was suspended in 4 ml of 0.01 M of phosphate-buffered saline (pH 7.0). Then 0.5 ml of 4 N sodium nitrite and an equal quantity of glacial acetic acid were added to the bacterial suspension and mixed under constant slow rotation for 15 min, at which time the mixture became frothy and yellow. Nitrous acid-extracted bacteria were then washed three times in phosphate-buffered saline, and the bacterial pellet was collected after centrifugation at 7,000 × *g* for 10 min.

Bacterial dot-blot immunoassay. The reactivity of surface-exposed epitopes of the M protein of group A streptococci was determined with synthetic peptide-specific antisera, anti-SM6(308–327), anti-SM6(339–352), and anti-SM6(381–398), each in a final dilution of 1:500, by a bacterial dot-blot immunoassay as described previously (19).

RESULTS

Extraction of the cell wall-associated region of M protein. Because of the large number of lysines and arginines within the M sequence (16), trypsin effectively digests the M protein exposed on the surface of the streptococcal cell (31). We assumed, however, that the region of the M protein embedded within the cell wall would be protected from tryptic digestion. Thus, after treating the streptococcal cells with trypsin at a concentration of 100 μg/ml, the cell wall peptidoglycan was solubilized with phage-associated lysin to release the wall-associated M molecule. The M-protein fragments released after this process were identified with a monoclonal antibody and peptide-specific antibodies directed to sequences within the C-terminal region of the molecule (Fig. 1).

Western blots of pooled fractions of partially purified wall-associated peptides after CM-cellulose chromatography exhibited multiple protein bands in the molecular weight (MW) range of 16,000 to 25,000 when reacted with anti-SM6(308–327), anti-SM6(339–352), and anti-SM6(381–398) antisera, with the smallest-MW fragment exhibiting the highest yield (Fig. 2). While no native M molecule was seen in the digest (about MW 55,000), the doublet of two closely spaced peptides of approximately 35,000 MW probably represents a small quantity of partially digested M protein associated with some cell wall fragments, since this peptide doublet reacted weakly with anti-*N*-acetylglucosamine antibody (Fig. 2) but did not bind wheat germ agglutinin (data not shown). No reaction with either anti-*N*-acetylglucosamine antibody (Fig. 2) or wheat germ agglutinin (data not shown) was observed with any of the 16,000- to 25,000-MW fragments. Monoclonal antibody 10B6, whose epitope is located 33 residues N-terminal to SM6(308–327) at residues 275 through 289 (Fig. 1) (19), did not react with the 16,000 to 25,000-MW protein bands (data not shown). These data indicate that the smallest trypsin-resistant fragment reactive with antibodies to epitopes located from amino acid 308 to 398 within the C-terminal region of the M molecule is almost 16,000 MW.

Characterization of the cell wall-associated fragment. The 16,000-MW fragment was purified by fast-protein liquid chromatography. Immunoblot analysis of the purified preparation revealed that the 16,000-MW peptide was composed of two closely spaced major and minor peptides reactive with anti-SM6(381–398) (Fig. 3) as well as with anti-SM6(308–327) and anti-SM6(339–352) (data not shown), in-

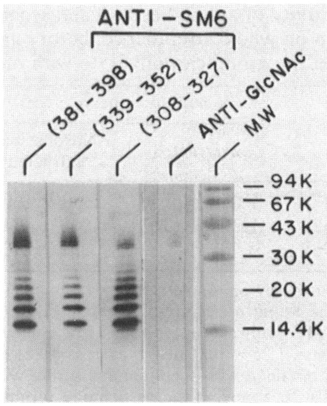

FIG. 2. Western blot analysis of partially purified (CM-cellulose) cell wall-associated region of the M6 protein. After trypsin digestion to remove surface-exposed M protein, the wall-associated region of the M molecule was released by digesting the cell wall with phage lysin and partially purified on CM-cellulose. M-protein bands were visualized by antisera raised against synthetic peptides to sequences in the wall-associated region, anti-SM6(381–398), anti-SM6 (339–352), and anti-SM6(308–327). A duplicate section of the gel was blotted and analyzed separately with anti-*N*-acetylglucosamine antibody (ANTI-GlcNAc) (11) to check for the presence of cell wall carbohydrate. The weakly reactive 35,000-MW peptide doublet was visible only after 60 min of incubation in the developer compared with 10 to 12 min for other blots. See Fig. 1 for the sequence and location of synthetic peptides. MW standards are given in thousands.

dicating that both contain intact epitopes for the three antibody preparations.

Amino acid sequence analysis. N-terminal sequence analysis of the two peptides was performed to determine their location within the M6 sequence (16). The simultaneous sequence of the two peptides was facilitated by the fact that they differed in concentration, allowing for the identification of the major and minor sequences. The results localized the N terminus of the larger peptide at lysine-298 of the M molecule and positioned the smaller peptide five amino acids C terminal to this site at alanine-303 (Fig. 1 and 4). Apparently, trypsin cleaved between the adjacent lysines at residues 297 and 298 to release the major peptide and at lysine-302 to release the minor peptide. The fact that other lysines were located C terminal to these sites (at residues 310, 316, 319, 325, 326, and below; Fig. 1) but were not attacked by the trypsin lends support to the fact that this region was protected from attack due to its location within the cell wall.

To rule out the possibility that these two fragments have a conformation which allows them to be resistant to trypsin, the purified wall-associated peptides were subjected to trypsin digestion under the same conditions used in the initial digestion. Western blot analysis of samples taken at timed intervals revealed that these peptides were completely digested after 5 min of contact with the trypsin (data not shown).

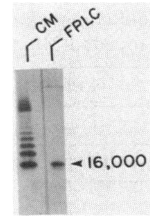

FIG. 3. Western blot analysis of the purified 16,000-MW cell wall-associated M6 peptide. Peptide was purified by fast-protein liquid chromatography (FPLC). A partially purified preparation (CM-cellulose [CM]) of the cell wall-associated M6 peptides and purified (FPLC) cell wall-associated M6 peptide was visualized with anti-SM6(381–398). The arrow marks the position of the 16,000-MW peptide.

Amino acid composition. Since the starting position of each peptide was determined by sequence analysis, the amino acid composition of the peptide mixture was used to determine the approximate location of their C-terminal end. The amino acid composition found in the purified preparation was compared with predicted compositions derived from the M6 sequence (16), beginning at residue 298 (determined with and without the residues in the membrane anchor and charged tail). The composition for the peptides was nearly identical to that predicted without the membrane anchor and charged tail (Table 1). The lack of two phenylalanines, one methionine, four valines, and seven alanines, all of which are located in the membrane anchor region (16) (Fig. 1), further emphasized the absence of this region from the isolated wall-associated peptides.

Amino sugar analysis. Amino sugar analysis was used to determine whether the cell wall-associated M6 fragments are covalently linked to either the glycan moiety of the peptidoglycan (*N*-acetylglucosamine or *N*-acetylmuramic acid) or the group carbohydrate (*N*-acetylglucosamine). Results revealed the presence of less than 0.5 residue per mol of glucosamine or muramic acid (Table 1).

Wheat germ agglutinin, as a lectin binding specifically to *N*-acetylglucosamine, was also used to detect the presence of this sugar in association with the wall-associated peptides. Western blots of both the partially purified (from CM-cellulose) and purified peptides were reacted with wheat germ agglutinin followed by rabbit anti-wheat germ agglutinin antibody. The absence of reactivity (data not shown) further indicated the absence of *N*-acetylglucosamine in the peptide preparation. Similarly, the absence of reactivity of any of the 16,000 to 25,000-MW protein bands in the partially purified preparation (Fig. 2) and the purified wall-associated peptides (data not shown) with anti-*N*-acetylglucosamine antibody also substantiated the above results.

Localization of embedded antigenic epitopes of the M protein after nitrous acid extraction of cell wall polysaccharide. Nitrous acid extraction of the group carbohydrate was used

	297						303							310
M6	**LYS**	**LYS**	**GLN**	**VAL**	**GLU**	**LYS**	**ALA**	**LEU**	**GLU**	**GLU**	**ALA**	**ASN**	**SER**	**LYS**
Major peptide		Lys	Gln	Val	Glu	Lys	Ala	Leu	Glu	Glu	Ala			
Minor peptide							Ala	Leu	Glu	Glu	Ala	Asn	X	Lys

FIG. 4. Alignment of the N-terminal amino acid sequence of the purified cell wall-associated M6 peptides (major and minor) with the amino acid sequence of the M6 protein (M6, uppercase letters, boldface type) segment at residues 297 through 310. Two overlapping sequences beginning at lysine-298 (major peptide) and alanine-303 (minor peptide) are shown.

TABLE 1. Amino acid composition of the purified cell wall-associated M6 peptide[a]

Amino acid	No. of residues per molecule		
	Predicted		Found
	With membrane[b] anchor	Without membrane[c] anchor	
Ala	26	19	20.0
Arg	3	2	2.9
Asx	9	8	5.8
Glx	28	26	25.0
Gly	7	6	8.4
His	0	0	0.0
Ile	0	0	0.0
Leu	14	13	14.4
Lys	24	22	21.1
Met	2	1	1.6
Phe	2	0	0.0
Pro	8	8	6.9
Val	7	3	2.0
Ser	5	5	6.0
Thr	9	6	4.2
Trp	0	0	ND[d]
Tyr	0	0	0.0

[a] Amino sugar content was <0.5 residue per mol.
[b] Calculated from residues Lys-298 to Asn-441 in the M6 sequence.
[c] Calculated from residues Lys-298 to Pro-416 in the M6 sequence.
[d] ND, Not determined.

to determine whether some of the epitopes of the M protein, which were embedded within the carbohydrate moiety, could be uncovered. Nitrous acid-extracted and unextracted streptococci were reacted with peptide-specific antibodies [anti-SM6(308–327), anti-SM6(339–352), and anti-SM6(381–398)] in a bacterial dot-blot immunoassay. None of the antibodies showed reactivity with control unextracted cells (Table 2). Of these peptide-specific antibodies tested, only anti-SM6(308–327) showed reactivity with the nitrous acid-extracted streptococci when compared with reactivity in the unextracted cells ($P < 0.001$). This suggests that nitrous acid extraction removed sufficient quantities of the group carbohydrate to expose the epitope at residues 308 through 327 buried within this moiety of the cell wall. This treatment did not expose the epitopes for the other two antibodies, indicating that they are located deeper within the wall.

DISCUSSION

Trypsin was used to digest the exposed M protein from the streptococcal cell wall (31). After the cell wall was solubilized with a muralytic enzyme (11, 13), antibodies to synthetic peptides of sequences within the C-terminal region of the M molecule were used to identify the released wall-associated fragment of the protein. The 16,000-MW fragment, which was identified as the smallest trypsin-resistant fragment of the M protein, was purified and characterized. Because of its location within the cell wall matrix, this fragment was resistant to attack by trypsin despite the presence of several arginine and lysine residues within the sequence, with one lysine as close as seven residues C terminal to the last sensitive site.

Sequence analysis of the purified wall-associated fragment indicated that the last trypsin-susceptible sites were at lysine-297 and -302. This places a small segment of the helical central rod region (residues 298 through 362) as well as the proline- and glycine-rich region (residues 363 through 416) of the M protein within the cell wall (12). Evidence

TABLE 2. Reactivity of antibodies to wall-associated regions of the M protein on whole streptococci before and after the extraction of the group carbohydrate with nitrous acid

Antibody	Reactivity[a] (mean ± SE)		Differential reactivity
	Unextracted	Nitrous acid extracted	
Anti-SM6 (308–327)	1.08 ± 0.07	2.50 ± 0.07	1.42[b]
Anti-SM6 (339–352)	1.20 ± 0.16	1.41 ± 0.14	0.21[c]
Anti-SM6 (381–396)	0.46 ± 0.05	0.39 ± 0.05	−0.07[c]

[a] A dot-blot immunoassay on whole type 6 streptococci (strain D471) was used to determine the extent of reactivity of the antisera (20). Densitometer readings are expressed in arbitrary units; 10^5 peak height units of densitometer reading (1 peak height = 1.25×10^{-4} mV) was considered 1 U. An average of four different values obtained with a preimmune serum was subtracted from each value obtained with a corresponding immune serum. Values are from four different readings.
[b] $P < 0.001$.
[c] $P < 0.5$ (Student's t test).

suggesting that the short helical rod segment is located within the group carbohydrate moiety of the cell wall is based on the reactivity of a peptide-specific antibody [anti-SM6(308–327)] only after nitrous acid extraction of the group carbohydrate (Table 2 and Fig. 5). The epitope for anti-SM6(339–352), also located in the helical region, was not exposed after nitrous acid extraction, suggesting that either not all the carbohydrate was removed, as previously found by Swanson et al. (31), to expose this epitope, or this region is situated within the peptidoglycan moiety of the wall. Assuming a 0.149-nm rise per amino acid in an alpha helix (18, 27), the 65 residues of the buried helical region (residues 298 through 362) would extend about 9.8 nm from the peptidoglycan, suggesting that the carbohydrate moiety of the cell wall is approximately 9.8 nm thick. This is close to the 12- to 15-nm value derived from measurements of the cell wall from electron micrographs (31), thus supporting the view that the helical region at residues 298 through 362 of the M molecule is located within the group carbohydrate moiety of the wall.

While some attempts have been made to identify the cell wall anchor region in staphylococcal protein A (15, 29, 30), the actual location and position of the wall-associated region have not been determined directly. Thus, conclusive evidence is not available concerning the mechanism by which gram-positive organisms attach some of their surface proteins to the cell. The reported covalent interaction of protein A with the cell wall (15, 29, 30) has not been confirmed here with M protein. Amino sugar analysis of the purified wall-associated peptide and wheat germ agglutinin lectin immunoblot analysis suggested that the C-terminal region of the M molecule is not covalently linked to either the group carbohydrate or glycan moiety of the cell wall peptidoglycan. Since the cross-bridge in *Streptococcus pyogenes* peptidoglycan is L-Ala-L-Ala (13), the absence of additional alanines from the amino acid composition of the wall-associated fragment (Table 1), which would be contributed by the peptide portion of the peptidoglycan, rules out any linkage through this moiety. Thus, although previous studies suggested a covalent interaction between the M protein and the cell wall (11), it is likely that the C-terminal region of the M protein is not covalently linked to but is intercalated within the highly cross-linked peptidoglycan (25). The nearly regular placement of prolines and glycines within this region suggests that some sort of specific structure may be necessary for this interaction (12).

Our previous studies of M protein extracted from the cell

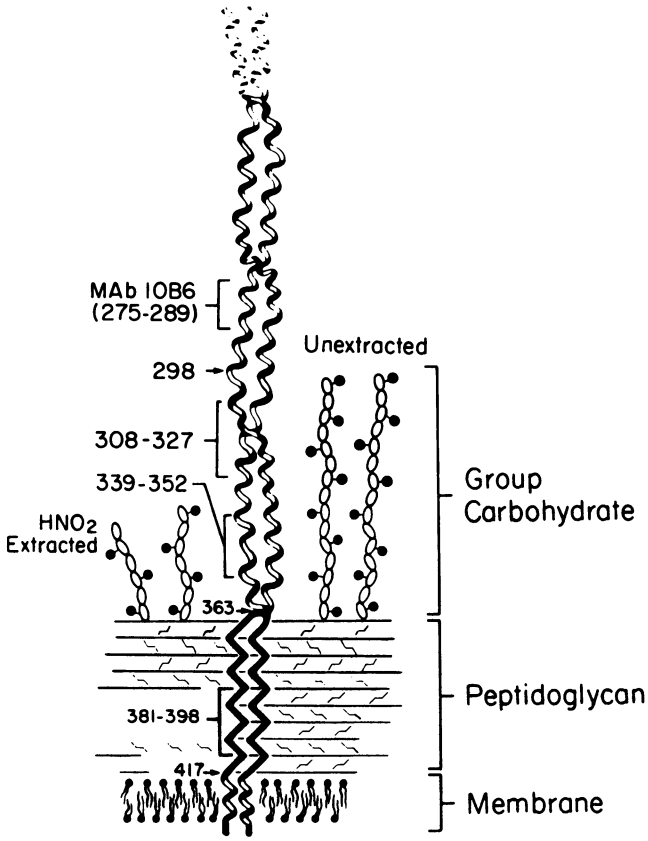

FIG. 5. Tentative model of the C-terminal region of the M6 molecule illustrating the alpha-helical coiled-coil central rod region and wall and membrane anchor regions and their association with the streptococcal cell wall (adapted from Fischetti et al. [12]). The right side of the figure represents the cell wall prior to extraction with nitrous acid (unextracted). The left side represents the cell wall after nitrous acid extraction (HNO_2) to remove some of the group carbohydrate (31). Numbers represent the relative position of amino acids in the sequence: 308–327, 339–352, and 381–398 indicate the positions of the sequences used to generate synthetic peptide antibodies (see Fig. 1 for detailed sequence). Monoclonal antibody (MAb) 10B6 (residues 275 through 289) is the epitope site of this monoclonal antibody (19). The group carbohydrate is composed of a rhamnose backbone (⬭⬭) with *N*-acetylglucosamine side chains (●).

wall with phage lysin (11) supports the noncovalent attachment of the M protein, since the majority of the released native M molecule was found not to be associated with wall fragments. This was based on a lack of reactivity of the isolated M molecule with *N*-acetylglucosamine specific antibody, while a minor proportion of M molecules was reactive with this antibody (11). This latter fraction could not be degraded further with additional muralytic enzyme treatment. Similarly, in the present study, the 35,000-MW peptide doublet (Fig. 2) reacted weakly with anti-*N*-acetylglucosamine antibody. These findings suggest the presence of a region of the streptococcal cell wall with a high degree of cross-linking (25, 26) that is resistant to muralytic enzyme, thus trapping a portion of the M molecule within this resistant core. In *Bacillus subtilis*, an enzyme-resistant fraction of the cell wall has been described (6) and attributed to the cell wall ends, which are older and thus are likely to be more cross-linked. The location of the muralytic enzyme-resistant core in the streptococcal cell has not as yet been described.

Based on amino acid composition, the wall-associated fragment isolated in these studies was found to be devoid of the C-terminal membrane anchor region found in the native M molecule (10, 16). This may be due to the fact that the organisms used for the isolation of the wall-associated region were in the stationary phase of growth and that the membrane anchor may be necessary to prevent release of the mature M protein during active logarithmic growth. Since M protein is not found free in the growth media of streptococci (10), its association with the cell wall may be closely regulated. M protein is synthesized during cell division at the newly forming partial septum (31; our unpublished data), where the cell wall is likely to be less cross-linked. Thus, we postulate that the membrane anchor may be necessary to keep the M molecule attached to the cell until such time as the peptidoglycan is cross-linked around the proline- and glycine-rich region, anchoring the molecule to the cell. The membrane anchor, no longer necessary, would then be cleaved.

The C-terminal region of the M6 protein molecule has strong sequence homology to the C-terminal region of staphylococcal protein A (5, 16) and the C-terminal end of protein G, an immunoglobulin-binding protein from group G streptococci (5, 14). This suggests a common mechanism by which these gram-positive organisms attach some of their cell wall proteins to the cell. The conservation of phenylalanine located between the cell wall and the membrane anchor region of M protein, protein A, and protein G sequences, suggests that this amino acid may play a central role for the enzyme which cleaves the membrane anchor. Our current studies are designed to verify this hypothesis.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI 11822 from the National Institutes of Health.

We thank Mary Windels and Tracy Guinta for their technical assistance in raising antisera against synthetic peptide and Emil C. Gotschlich and Kevin F. Jones for their helpful comments on the manuscript.

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