IDENTIFICATION OF AN ENDOGENOUS MEMBRANE ANCHOR-CLEAVING ENZYME FOR GROUP A STREPTOCOCCAL M PROTEIN

Its Implication for the Attachment of Surface Proteins in Gram-positive Bacteria

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M protein of group A streptococci, an α-helical coiled-coil fibrillar molecule found on the surface of the organism (1), is responsible for the antiphagocytic property of these bacteria (2). Antigenic variation (3) and type-specific immunity are contingent upon epitopes located within the NH_2-terminal half of the M molecule (distal to the cell wall) (4). Amino acid sequences that are conserved among different M proteins are located in the COOH-terminal half (5, 6) and contain epitopes recently shown to be responsible for non-type-specific immunity against streptococcal colonization (7, 8). The attachment region of the molecule, predicted from DNA sequence, is located at the COOH-terminal end and is composed of a 6 amino acid charged tail at the COOH terminus followed by 19 hydrophobic amino acids suspected to be a membrane anchor and an adjacent proline and glycine-rich region situated within the peptidoglycan layer of the cell wall (1, 9, 10).

M protein may be released from the streptococcal cell after treatment with muralytic enzymes (11) that solubilize the cell wall. When performed in the presence of 30% raffinose, the resulting protoplasts are stabilized and the soluble components, composed mainly of cell wall carbohydrate and M protein, may be separated from the protoplasts by centrifugation (11). At pH 6.1, conditions that result in the complete removal of the cell wall with the muralytic enzyme lysin (12), an examination of the streptococcal protoplasts revealed a significant quantity of the M molecule still attached. This indicated to us that both a released and bound form of the M protein could be identified and that the M molecule may be attached directly to the cytoplasmic membrane. Though suggestive from the hydrophobic characteristics of the COOH-terminal 19 amino acids and 6 charged amino acids at the COOH terminus of the M protein sequence (a characteristic conserved among other surface proteins from gram-positive bacteria [13]), the exact mechanism by which M protein, or these other surface proteins, are bound to the cell membrane has not been directly determined.

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Recently, we reported the purification and characterization of the COOH-terminal cell wall-associated region of the M protein (9). After proteolytic digestion of the surface exposed portion of the M molecule, the region embedded within the cell wall, and thus protected from proteolysis, was released by solubilizing the cell wall with a muralytic enzyme. By amino acid sequence analysis, the NH_2 terminus of this wall-associated segment was found to start at residue 298 of the 441 DNA-predicted amino acids for the M molecule (10). Amino acid composition of this fragment showed that the COOH-terminal 19 hydrophobic amino acids and charged tail were missing (9), indicating that M protein released after cell wall digestion may be a consequence of the cleavage of this hydrophobic anchor. These findings suggested that an endogenous enzyme may be responsible for this activity (9).

In the present study, a thiol and pH-dependent enzyme with the capacity to release M protein from isolated protoplasts is identified. We show that under certain pH conditions the streptococcal cell wall may be removed without releasing M protein from the resulting protoplasts. The M protein remains bound to the membrane even after treatment with sodium carbonate, pH 11.2, and Triton X-114, indicating that attachment is limited to the cytoplasmic membrane through a hydrophobic anchor. We find that a change in the pH environment of the M protein-charged protoplasts results in the release of the M molecules that partition into the hydrophilic phase of Triton X-114. Evidence is presented that the enzyme responsible for M protein release is membrane bound.

Materials and Methods

Bacterial Strains. M type 6 streptococcal strain D471 and M^- mutant T28/51/4-4 (14) are from The Rockefeller University collection.

M Protein. ColiM6.1 protein is the purified product of the *emm*-6.1 gene expressed in *Escherichia coli* (15).

Preparation of Protoplasts. All experiments were performed using protoplasts prepared from an overnight culture of streptococci grown in Todd-Hewitt broth. For some experiments, organisms were centrifuged and washed twice in 100 mM PBS, pH 6.1. After adjusting to OD at 650 nm to 1.0, the cells were concentrated 30-fold in 50 mM phosphate buffer containing 30% (wt/vol) D-raffinose (raffinose), 5 mM EDTA, pH 6.1. Group C streptococcal phage-associated lysin (lysin), prepared as described (16), was added to the suspension yielding a final concentration of 128 U/ml, and incubated at 37°C for 45 min to remove the cell wall. Preparations examined by phase-contrast microscopy revealed protoplasts typically seen as individual phase dense spherical organisms as previously described (17). Protoplasts, prepared in this way, have been shown to yield streptococcal membranes virtually devoid of cell wall carbohydrate (12).

Protoplasts were also prepared in 50 mM phosphate buffer containing 5 mM EDTA and 30% (wt/vol) raffinose at pH 7.0, 7.4, or 8.0 and at pH 4.0, 5.0, or 5.5 in 50 mM sodium acetate buffer containing 30% raffinose and 5 mM EDTA. Unless otherwise stated, protoplasts were prepared from M^+ strain D471 in 50 mM sodium acetate buffer at pH 5.5 containing 30% raffinose and 5 mM EDTA (raffinose buffer) and washed (16,000 *g* for 5 min) in the same buffer.

Effect of pH Change on the Release of M Protein. After lysin extraction, the protoplasts were washed three times in raffinose buffer, pH 5.5, to remove the lysin enzyme. The washed protoplasts were then suspended to the initial volume of the same buffer and divided into two equal parts and centrifuged. One of the aliquots was resuspended in the same volume of raffinose buffer, pH 5.5, and another in 50 mM phosphate buffer containing 30% raffinose and 5 mM EDTA, pH 7.4. Protoplasts were then incubated in the above buffers separately for 60 min at 37°C. Similarly protoplasts prepared at pH 7.4 were washed three times in raffinose buffer,

pH 5.5, centrifuged, and resuspended in raffinose buffers, pH 7.4 or 5.5, and incubated at 37°C for 60 min. Protoplasts were sedimented (16,000 *g* for 5 min) and the amount of M protein in the supernatants was quantitated by k-ELISA as described below.

Membrane Preparation. Protoplasts were prepared at pH 5.5 as described above, washed in raffinose buffer, and hypotonically lysed in 5 mM acetate buffer containing 1 mM PMSF, 0.1 mM *N-p*-tosyl-L-lysine chloro-methyl ketone (TLCK)[1] and 2 mM *p*-hydroxymercuriphenylsulfonic acid (pHMPS) and incubated for 30 min on ice followed by three freeze and thaw treatments. The resultant membranes were collected by centrifugation at 100,000 *g* for 1 h at 4°C, washed once in the acetate buffer and resedimented.

Sodium Carbonate Treatment. The streptococcal membrane pellet was suspended in 0.1 M sodium carbonate (pH 11.2) and incubated at 0°C for 30 min. The membranes were then sedimented and the bound M protein analyzed by Western blot as described below.

Triton X-114 Phase Separation of Bound and Released M Molecules. Sodium carbonate-treated membranes were washed once with 20 mM Tris-HCl/150 mM NaCl, pH 7.4, before Triton X-114 (TX-114) phase separation as modified from Bordier (18). Membranes were suspended in Tris buffer containing 1% (wt/vol) TX-114 and 1 mM PMSF, 0.1 mM TLCK and were incubated at 4°C for 30 min. The preparation was centrifuged at 16,000 *g* for 5 min to remove any insoluble material. The supernatant was transferred to a new tube and incubated at 37°C for 5 min to induce the condensation of TX-114 and centrifuged at room temperature for 5 min at 16,000 *g*. The resulting heavy detergent enriched TX-114 phase and the lighter detergent depleted aqueous phase were separated and the latter readjusted to a concentration of 1% TX-114. The unsolubilized membrane pellet was retreated twice with TX-114 as described above. The released form of the M protein was phase separated similarly after removing the raffinose by dialysis in 20 mM Tris-HCl/150 mM NaCl buffer, pH 7.4, containing 1 mM PMSF, 0.1 mM TLCK. Electrophoresis and Western blot analysis of the membranes and detergent rich and poor fractions were as described below.

Kinetics of M Protein Release from Protoplasts in Lysin-free Raffinose Buffer. Protoplasts prepared in raffinose buffer, pH 5.5, were washed in the same buffer and resuspended in raffinose buffer, pH 7.4, and incubated at 37°C for 150 min. At timed intervals, a 200-μl aliquot of the protoplast suspension was removed, centrifuged, and the quantity of M protein was estimated as described below.

The Effect of Specific Chemical Reagents on the Release of M Protein from Protoplasts. Protoplasts prepared in raffinose buffer, pH 5.5, were washed three times, resuspended in raffinose buffer, pH 5.5, and the protoplasts in 200-μl aliquots were sedimented. Protoplasts were then resuspended in raffinose buffer without EDTA, pH 7.4, containing various concentrations (1–15 mM) of PMSF, TLCK, zinc chloride, cadmium acetate, calcium chloride, lithium acetate, magnesium chloride, PHMB, pHMPS, dithiothreitol (DTT), 1,10-phenanthroline, EDTA, and EGTA and incubated at 37°C with rotation for 30 min (16 rpm). At the end of the incubation period, the protoplasts were sedimented and the supernatants assayed for the released M protein both by Western blot analysis and quantitative k-ELISA as described below.

Reversible and Irreversible Action of Inhibitors on the Release of M Protein. After zinc chloride, cadmium acetate, calcium chloride, PHMB and pHMPS treatment as mentioned above, the protoplasts were thoroughly washed with raffinose buffer, pH 5.5, resuspended in the intial volume of raffinose buffer, pH 7.4, and incubated once again at 37°C for 60 min with rotation. At the end of the incubation, each sample was centrifuged and the amount of released M protein was determined by k-ELISA.

Location of the Membrane Anchor-Cleaving Enzyme (MACE). Protoplasts derived from the M^- strain were prepared and washed in raffinose buffer, pH 5.5, and served as an enzyme source (M^-E^+). Protoplasts derived from the M^+ strain were similarly prepared and suspended in 2 mM PHMB in raffinose buffer for 20 min and washed five times in raffinose buffer, pH 5.5. These protoplasts served as the bound M protein substrate source (M^+E^-). After ad-

[1] *Abbreviations used in this paper:* DTT, dithiothreitol; GPI, glycosyl-phosphatidylinositol; MACE, membrane anchor-cleaving enzyme; PHMB, *p*-hydroxymercuribenzoate; pHMPS, *p*-hydroxymercuriphenylsulfonic acid; PI, phosphatidylinositol; TLCK, *N-p*-tosyl-L-lysine chloro-methyl ketone; VSG, variable surface glycoprotein.

justing the optical density of the protoplasts from each strain to 1.0 (at 650 nm), they were sedimented and resuspended in 1/30 of the original volume in raffinose buffer, pH 7.4. The M^+E^- and M^-E^+ protoplasts were then mixed in different proportions in a final volume of 1.0 ml. M^+E^- and M^-E^+ protoplasts were also incubated independently. To determine whether the enzyme is released in the supernatant of M^-E^+ protoplasts, they were suspended in raffinose buffer, pH 7.4, and incubated at 37°C with rotation for 1 h. The protoplasts were then sedimented and the supernatant was mixed with an equal volume of M^+E^- protoplasts. Each reaction mixture was incubated with rotation at 37°C for 1 h.

The rate of the release of M protein in a mixture of M^-E^+ and M^+E^- protoplasts was determined by mixing them at a ratio of 4:1 (M^-E^+/M^+E^-) and incubating at 37°C with rotation. At intervals, an aliquot was removed and centrifuged supernatants were analyzed for the presence of released M protein by quantitative k-ELISA.

Immunoreagents. Polyclonal sera against ColiM6.1 protein (the purified product of an *E. coli* strain containing the cloned M6 protein gene [15]) were prepared as described (19) and affinity purified on ColiM6.1 protein linked to glutaraldehyde-activated affinity absorbent resin (4). Polyclonal antibody to a synthetic peptide representing residues 1-21 of the native M6 protein was prepared and affinity purified as described (3). Protein A-purified mAb 1.S45.1, specific for the streptococcal group A-specific carbohydrate determinant (*n*-acetyl glucosamine $\beta1\rightarrow3$ rhamnose) (20) was a gift from Dr. D. G. Braun (Basel Institute of Immunology, Switzerland). Monospecific polyclonal antiserum to the group A-specific determinant was prepared as described previously (11). M protein-specific mAb 10B6 has an epitope located within the conserved region of the M molecule between residues 275 and 289 (21).

Capture k-ELISA for M Protein and Group Carbohydrate. A capture k-ELISA was used to quantitate the amount of M protein and group A-specific carbohydrate in streptococcal digests. For M protein quantitation, microtiter plates were sensitized with 100 μl/well of purified anti-ColiM6 antibody (4 μg/ml) at 37°C for 3 h and overnight at 4°C. After five washes in PBS/Brij 35 (19), 100 μl of serial twofold dilutions of purified ColiM6 protein (starting at 2 μg/ml) was added in triplicate to the microtiter wells. This served as the standard curve for M protein quantitation. Similarly, samples containing M protein were diluted and 100 μl was added in triplicate to the remaining wells of the antibody-sensitized plates. After incubation for 3 h at 37°C to allow reaction with the bound antibody, plates were washed five times in PBS-Brij (19). 100 μl of 1:1,000 dilution of M6 mAb 10B6 (1.7 mg/ml) was added to each well and incubated for 3 h at 37°C. After washing in PBS-Brij, 100 μl of alkaline phosphatase-conjugated anti-mouse IgG (1:1,000 dilution; Sigma Chemical Co., St. Louis, MO) was added to each well and allowed to react for 18 h at room temperature. The plates were again washed and developed as described (19). 1 min after substrate addition, wells were automatically monitored every 2 min with intermediate shaking to determine the reaction rate using an ELIDA-5 plate reader (Physica Inc., New York, NY) as described (19). Values were calculated based on the absorbance/hour of the samples compared with the standard curve plotted by third order regression analysis.

For estimation of group A specific carbohydrate (*n*-acetyl glucosamine $\beta1\rightarrow3$ rhamnose), a capture k-ELISA similar to that used for M protein quantitation was performed with specific modifications. The microtiter wells were first sensitized with polyclonal affinity-purified rabbit anti-group A-specific carbohydrate antibody (3.2 μg/ml). For a standard curve representing the complete release of the cell wall carbohydrate (12, 22, 23), a lysin extract of strain D471 digested at pH 6.1 for 60 min was serially diluted and 100 μl added to the wells in triplicate. Samples of cell wall digests were diluted and 100 μl was added to remaining wells of the sensitized plate also in triplicate. Group A-specific mAb 1.S45.1, 1:500 dilution (100 μl/well, 1.38 mg/ml), was added in the third step to identify the bound carbohydrate. After processing by the method described above for M protein, the absorbance values obtained for the unknown samples were compared with those of the standard curve.

SDS-PAGE and Western Blot. Western blots of proteins separated on 12% SDS-polyacrylamide gels were analyzed with M protein-specific mAb 10B6 and peptide-specific antibody to the NH_2-terminal sequence 1-21 of M6 protein as described (9, 21).

Results

Identification of a Bound and Released Form of the M Molecule. M protein can be released from the streptococcus by removing the cell wall with lysin, a phage-associated muralytic enzyme that solubilizes the peptidoglycan (16, 23). When performed in 50 mM phosphate buffer, pH 6.1, containing 30% raffinose, the stabilized protoplasts can be separated from the soluble wall components by centrifugation (11). By this procedure, M protein is found in the extract along with digested cell wall components (11). However, even after several washes in raffinose buffer, almost an equal amount of M protein is found to remain associated with the protoplasts as estimated by Western blot.

To determine if the release of M protein during lysin extraction is independent of cell wall removal, the lysin extraction was performed under different pH conditions in the presence of 30% raffinose. After extraction, the amount of cell wall carbohydrate released in relation to released M protein was determined by capture k-ELISA. At pH 4.0 (not shown) and 5.0 the release of group carbohydrate was <10% of that liberated at the optimal pH of 6.1, whereas at pH 5.5, 6.1, 7.0, 7.4, and 8.0 >80% of the wall carbohydrate was found to be released from the streptococcal cell (Fig. 1). In contrast, the maximum release of M protein was observed from pH 6.1 to 8.0 (~25 μg/ml), while at pH 5.5 the release was only 0.25 μg/ml. Thus, at pH 5.5, lysin is able to release >80% of the cell wall from the streptococcus without releasing significant amounts of M protein from the resulting protoplasts.

Characterization of M Protein as an Integral Membrane Protein. TX-114 phase separation was used to determine the hydropathic characteristics of the released form of the M molecule. By this technique, the M protein was found to partition into the detergent poor (DP) aqueous phase (Fig. 2). Sodium carbonate treatment has been used to distinguish between peripheral and integral membrane proteins (24). Streptococcal membranes prepared after cell wall removal at pH 5.5 and treated with 100 mM sodium carbonate (pH 11.2) retained their M protein that could be released after membrane solubilization with 1% SDS (Fig. 2). Extraction of sodium carbonate-treated membranes with TX-114 does not release the M molecules that are retained within the insoluble membrane pellet (Fig. 2).

The membrane-bound form of the M protein was found to be composed of two

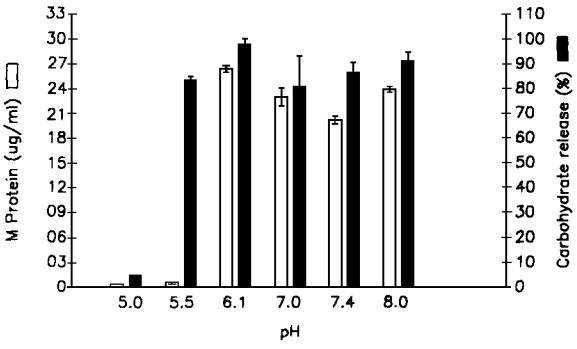

FIGURE 1. Effect of pH on the release of M protein and cell wall carbohydrate during protoplast formation. M+ streptococci were suspended with lysin in 30% raffinose buffer at various pH conditions. After 45 min incubation at 37°C, the suspension was centrifuged and the supernatant was analyzed by k-ELISA for M protein and group carbohydrate release.

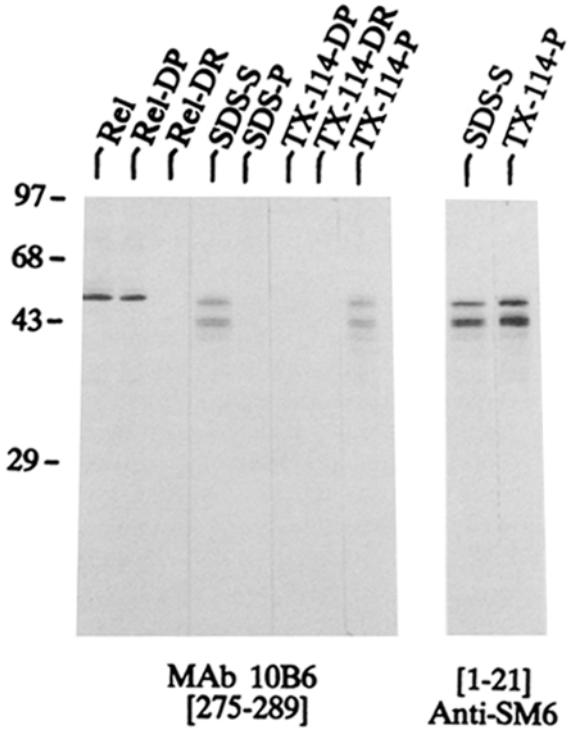

FIGURE 2. SDS-PAGE and Western blot analysis of released and membrane bound forms of M protein. M protein released from protoplasts at pH 7.4 (Rel) was partitioned with TX-114. The detergent-poor (Rel-DP) and detergent-rich (Rel-DR) fractions were probed with M protein specific monoclonal 10B6 whose epitope is mapped at residues 275–289. M protein–charged membranes washed with sodium carbonate were solubilized in 1% SDS, centrifuged, and analyzed. The soluble proteins (SDS-S) and insoluble pellet (SDS-P) were reacted with monoclonal 10B6. Sodium carbonate washed membranes were also treated with TX-114, centrifuged, and the supernatant was partitioned into detergent-poor (TX-114-DP) and detergent-rich (TX-114-DR) fractions. These along with the membrane pellet (TX-114-P) were reacted with the 10B6 monoclonal. The membrane form of the M protein in the SDS-S and the TX-114-P were reacted with antibodies to synthetic peptide 1–21 of the native M6 molecule (Anti-SM6). Molecular mass standards (kD) include, phosphorylase B, 97; BSA, 68; ovalbumin, 43; carbonic anhydrase, 29.

major bands, one slightly smaller than the released form (~55 kD) and the other at 43 kD (Fig. 2). Both molecular forms react with peptide-specific antibody directed to residues 1–21 of the native molecule, ruling out the possibility that the reduction in size is due to a cleavage at the NH_2-terminal end. Because both forms are membrane bound, the differences observed between them may be the result of variation at the COOH-terminal end.

Effect of Change in pH on M Protein Release. To examine if the release of M protein from the protoplasts is pH dependent and not lysin dependent, protoplasts were prepared in raffinose buffer at pH 5.5 and washed three times in this buffer without lysin. When the protoplasts were then sedimented and resuspended in raffinose buffer at pH 7.4 and further incubated for 30 min without lysin, the bound M protein was released from the protoplasts (Fig. 3). However, when protoplasts were first prepared at pH 7.4 (resulting in the release of M protein along with wall components), washed, resuspended, and incubated in raffinose buffer at pH 5.5 for up to 30 min at 37°C, the further release of M protein was negligible (Fig. 3). These results indicate that the release of M protein is not mediated by the lysin enzyme itself but by a pH-dependent endogenous factor.

To examine the release of M protein from the protoplasts with relation to time, protoplasts prepared in raffinose buffer, pH 5.5, were centrifuged and resuspended in raffinose buffer, pH 7.4. At timed intervals, aliquots were removed, centrifuged, and supernatants were assayed for M protein. A continuous increase occurred in

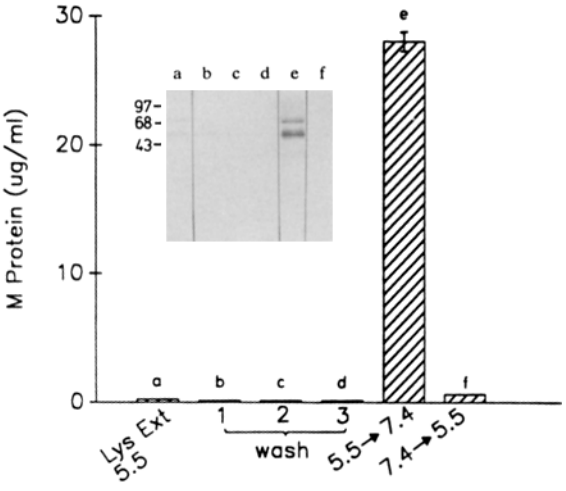

FIGURE 3. Effect of change in pH on the release of M protein. The supernatant of protoplasts prepared at pH 5.5 and three washes in raffinose buffer were examined for M protein release by k-ELISA. The washed protoplasts were then suspended in raffinose buffer at pH 7.4, incubated for 30 min, centrifuged, and the supernatant was analyzed for M protein. The supernatant of protoplasts prepared at pH 7.4 and incubated at pH 5.5 was also examined for M protein release. Inset shows a Western blot of the supernatants of the samples (*a–f*) analyzed by k-ELISA. Molecular weight markers are as in Figure 2.

the amount of M protein released into the supernatant with a maximum level achieved at ~60 min (Fig. 4). No significant increase in the amount of M protein was observed after this time. Western blot analysis revealed a concomitant decrease in the amount of M protein remaining on protoplasts obtained at each time interval with negligible amounts found after 60 min (data not shown).

Identification of an M Protein-releasing Enzyme. Specific chemical reagents were used to determine their effect on the release of M protein from the protoplasts. Protoplasts, prepared and washed in raffinose buffer at pH 5.5, were resuspended in raffinose buffer, pH 7.4, containing such reagents. By this technique, the release of M protein could be inhibited with 10 mM zinc chloride, 10 mM cadmium acetate, 10 mM $CaCl_2$, 0.1–2 mM PHMB and pHMPS (Table I). Lithium acetate (10 mM), magnesium chloride (10 mM), TLCK (5 mM), PMSF (5 mM), or phenanthroline did not significantly influence the release of M protein from the protoplasts nor did EDTA and EGTA (Table I). In the presence of 5 mM DTT, the release of M protein was found to increase 50% above control protoplasts incubated without DTT (Table I).

Reversible and Irreversible Enzyme Inhibitors. Protoplasts prepared at pH 5.5 and pretreated with Zn^{2+}, Cd^{2+}, or Ca^{2+} then washed free of these inhibitors and rein-

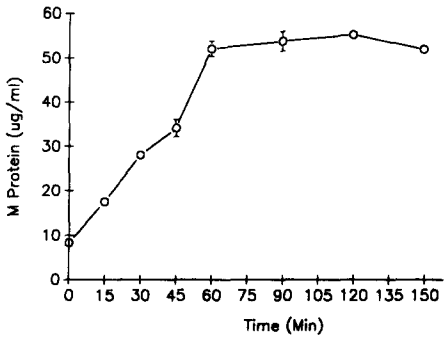

FIGURE 4. Time course of the release of M protein from protoplasts. Protoplasts prepared in raffinose buffer at pH 5.5 were washed and suspended in raffinose buffer 7.4. Samples removed at timed intervals were centrifuged and the supernatant was examined by k-ELISA for M protein.

TABLE I

Effect of Specific Chemical Reagents on the Release of M Protein

Reagent	M protein	
	A*	B‡
	μg/ml	
Control	28.6 ± 1.03	
5 mM PMSF	26.1 ± 0.72	
1 mM TLCK	24.0 ± 0.83	
10 mM $ZnCl_2$	0.5 ± 0.05	35.3 ± 4.84
10 mM CdOAc	4.4 ± 0.56	25.5 ± 1.75
10 mM $CaCl_2$	2.2 ± 0.08	27.9 ± 2.69
10 mM EDTA	35.3 ± 2.04	
10 mM EGTA	32.5 ± 5.21	
5 mM $MgCl_2$	34.2 ± 2.77	
10 mM LiOAc	37.5 ± 4.76	
1 mM PHMB	8.8 ± 1.71	1.7 ± 0.19
2 mM pHMPS	8.1 ± 0.56	3.2 ± 0.17
5 mM dithiothreitol	43.7 ± 2.34	
5 mM 1,10-phenanthroline	27.1 ± 0.49	

Values are mean ± SEM of triplicate samples.

* A, Protoplasts were suspended in raffinose buffer, pH 7.4, containing the chemical reagents, incubated at 37°C for 30 min, centrifuged, and supernatant was analyzed for M protein.

‡ B, Protoplasts were prepared as in A, washed free of chemical inhibitors and reincubated at 37°C for 60 min in raffinose buffer, pH 7.4.

cubated in raffinose buffer, pH 7.4, were found to again release M protein effectively (Table I). In contrast, PHMB- or pHMPS-treated protoplasts exhibited minimal M protein release under the same conditions (Table I). Thus, while divalent cations such as Zn^{2+}, Cd^{2+}, and Ca^{2+} act as reversible inhibitors of the releasing enzyme, the inhibitory effect of PHMB or pHMPS is irreversible.

Location of the Releasing Enzyme in Streptococcal Cells. To determine if the enzyme activity is membrane bound and not released in the protoplast supernatant, an assay system was designed in which protoplasts from an M^- streptococcal strain served as a source of enzyme (M^-E^+) and protoplasts from an M^+ strain, treated with PHMB to irreversibly inactivate the endogenous enzyme, were a source of bound M protein (M^+E^-). The M^-E^+ and M^+E^- protoplasts prepared at pH 5.5 were mixed at various ratios and incubated for 1 h in raffinose buffer, pH 7.4. Maximum release of M protein was found to occur at a ratio of 4:1 (M^-E^+/M^+E^-) (Fig. 5), suggesting that the quantity of M protein released is enzyme dependent. The time course of the release using a 4:1 ratio (M^-E^+/M^+E^-) is also shown in Fig. 5 (inset).

When M^-E^+ protoplasts were first incubated in raffinose buffer, pH 7.4, alone for 60 min at 37°C and the supernatant was added to the M^+E^- protoplast suspension and incubated for an additional 60 min at 37°C, no release of M protein was observed (Fig. 5). This indicates that the M protein-releasing enzyme is bound to the protoplast membrane and not secreted.

Discussion

The association of M protein to the cytoplasmic membrane became apparent when streptococcal protoplasts were examined after cell wall removal with the muralytic

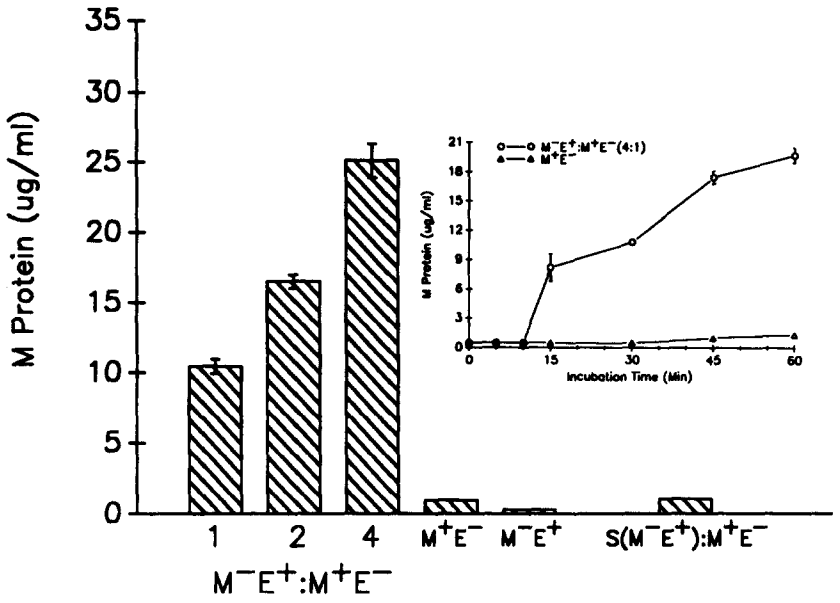

FIGURE 5. Membrane association of the M protein-releasing enzyme. As an enzyme source, protoplasts from M^- streptococci (M^-E^+) (prepared at pH 5.5) were mixed at various ratios (1:1, 2:1, 4:1) at pH 7.4 with PHMB-treated protoplasts prepared from M^+ streptococci (M^+E^-). M^+E^- and M^-E^+ protoplasts incubated alone showed no release as did the supernatant from M^-E^+ protoplasts [$S(M^-E^+)$] mixed 1:1 with M^+E^- protoplasts. Inset shows the time course of M protein release from protoplasts mixed 4:1 (M^-E^+/M^+E^-) ratio and no release from PHMB treated M^+E^- protoplasts incubated alone.

enzyme lysin. Lysin, an acetyl-muramyl-L-alanine amidase (25), is active against group A streptococcal cell walls over a broad pH range (from pH 5.5–8.0) with peak activity at pH 6.1 (23), the pH routinely used to remove the streptococcal cell wall for membrane preparation (12, 16, 22). Using a capture k-ELISA to identify the released components after lysin extraction under various conditions, >80% of the cell wall could be removed at pH 5.5. However, at this pH, nearly all the surface M protein remains firmly bound to the resulting protoplasts. When the protoplasts are transferred to raffinose buffer at pH 7.4, M protein is released into the supernatant until it can no longer be detected on the protoplasts by 60 min. Because the protoplasts are washed free of lysin before the shift to pH 7.4, this confirms that the release is mediated by an endogenous factor and not the lysin preparation itself.

Thiol blocking agents and certain divalent cations were found to inhibit the release of the M protein from protoplasts, while DTT was stimulatory. This supports the view that the endogenous factor is likely a thiol-dependent membrane anchor-cleaving enzyme (MACE). Because MACE activity was not found in the supernatant of protoplasts incubated at pH 7.4 and protoplast-protoplast contact was required for the release of M protein from MACE-inactivated protoplasts implies that the enzyme is not secreted but bound to the membrane and exposed on the outer surface. The initial delay observed in the release of M protein from the inacti-

vated protoplasts (Fig. 5, inset) may be a function of the need to cleave a certain number of M molecules to afford closer contact between protoplasts for efficient cleavage (26).

Membrane anchor-cleaving enzymes have been isolated from both bacteria and eukaryotes, including mammalian tissues (27, 28). However, irrespective of their origin, these enzymes have a specificity for the membrane anchor of eukaryotic cell surface proteins (27, 28). Despite their appearance in bacterial cells, such enzymes have not been reported to act on their surface proteins. These enzymes have been shown to be either phospholipase C (PLC) or phospholipase D (PLD) with a substrate specificity for either phosphatidylinositol (PI), phosphatidylinositol phosphates (PI-phosphates), or glycosyl-phosphatidylinositol (GPI) (28). Many properties of the MACE observed in the present study (Table I) are similar to those reported for these anchor degrading enzymes (29–31). The inability of phenanthroline, EDTA, or EGTA to inhibit the action of MACE and the fact that its activity is independent of Ca^{2+} (Table I) indicates that it more closely resembles PLC and not PLD (28–31).

The mechanism by which M protein or other surface proteins of gram-positive bacteria are anchored to the cell is not known. The presence of 19 hydrophobic amino acids at the COOH-terminal end of the M molecule is sufficient in length to span the cytoplasmic membrane (1) and thus may act to anchor the M molecule to the cell. However, this mechanism has not been directly proven. It is also believed that proteins such as staphylococcal protein A may be covalently linked to the cell wall matrix (32); however, direct evidence for this type of interaction has also not been confirmed (32). Our previous report on the purification and characterization of the cell wall-associated region of the M protein indicates that it is not covalently linked to the cell wall peptidoglycan or group carbohydrate moieties (9). The results of the present study support and extend this idea and indicate that the M molecule is anchored to the cell solely through the cytoplasmic membrane. The fact that M protein is not extracted from the membrane after treatment with sodium carbonate at pH 11.2 (24), confirms that the M protein is an integral membrane molecule and not peripherally associated. Furthermore, since M protein could not be extracted from the membrane with TX-114 but could be released with SDS indicates that the bound form is tightly associated with the membrane. The released form was found to partition into the aqueous phase of TX-114, suggesting that lack of a region sufficiently hydrophobic to allow partitioning into the detergent rich fraction.

Our finding that the COOH-terminal 19 hydrophobic amino acids and charged tail of the M molecule, which are predicted from its DNA sequence (10), were not part of the released form of M protein after cell wall removal (9), suggests that the release of M protein from the membrane is in some way associated with the cleavage of the COOH-terminal hydrophobic region. Two mechanisms may accomplish this: (*a*) The M molecule is bound directly to the membrane through the 19 hydrophobic amino acids and charged tail and the MACE cleaves this anchor to release the M molecule or, (*b*) the hydrophobic tail is removed during M protein assembly on the cell surface resulting in a post-translational event that links the M protein to a new membrane anchor complex, and the MACE releases the M protein from this complex. The latter idea is similar to that found with the attachment of cell surface proteins in eucaryotes via a GPI complex (29). For example, from sequence analysis of the variable surface glycoprotein (VSG) of *Trypanosoma brucei* (33), and biosyn-

thesic studies of VSG (29), it is clear that the VSG mRNA encodes for a short (17–23 amino acid) COOH-terminal hydrophobic tail that is cleaved after biosynthesis of the VSG protein and replaced with a GPI moiety. This moiety then serves to anchor the glycoprotein to the trypanosome membrane. The membrane form of VSG can be converted to a water-soluble secretory form by the action of GPI-specific phospholipase C that releases the VSG from the GPI-anchor (30, 31). Similar occurrences have been found in several other eucaryotic surface proteins (28, 29).

In support of the latter hypothesis for M protein attachment, the COOH-terminal sequence of six surface proteins from streptococci and staphylococci was compared with the corresponding region of three selected GPI anchored proteins (all predicted from DNA sequence) (29) (Fig. 6). In general, sequences that signal GPI attachment are not identical among different GPI anchored proteins but show a recognizable pattern of specific amino acids (29), a characteristic also seen in the recognition sequences required in the cleavage of NH_2-terminal signal sequences (34). Results of the sequence comparison revealed identity or conservation of amino acids between the bacterial proteins (with consensus sequence LP × TG) and the proposed cleavage and GPI attachment sequence of the three GPI anchored molecules (Fig. 6), all three of which are cell adhesion proteins (29). The conservation of a serine and/or threonine flanking the proposed cleavage site preceded by a proline and a hydrophobic amino acid is particularly significant (Fig. 6, arrow). In addition, GPI anchored proteins feature a run of hydrophobic amino acids, beginning ~10 residues after the cleavage point, which usually extends to the COOH terminus (29), a characteristic also found in the bacterial surface proteins with the addition of charged residues at the terminus in the latter molecules (Fig. 6). The cleavage site proposed for the GPI anchored proteins correlates almost exactly with the predicted cleavage site on the M molecule based on the amino acid composition of the COOH-terminal end of the released form of M protein (9).

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                    ↓
N-CAM          IPA TLGSPSTSSSFVSLLLSAVTLLLLC
                   ::: :
CsA            APS SATTLISPLSLIVIFISFVLLI
                   ::: ::
LFA-3          IPS SGHSRHRYALIPIPLAVITTCIVLYMNVL
                   ::: ::
M Protein      LPS TGETANPFFTAAALTVMATAGVAAVVKRKEEN
                   ::: ::
Protein G      LPT TGEGSNPFFTAAALAVMAGAGALAVASKRKED
                   ::: ::
IgAbp          LPS TGETANPFFTAAAATVMVSAGMLALKRKEEN
                   ::: ::
wapA           LPS TGEQAGLLLTTVGLVIVAVAGVYFYRTRR
                   ::  ::
Protein A      LPE TGEENPLIGTTVFGGLSLALGAALLAGRRREL
                   ::  ::
Fibronectin BP LPE TGGEESTNKGMLFGGLFSILGLALLRRNKKNHKA
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FIGURE 6. Alignment of the COOH-terminal end of three GPI-anchored adhesion proteins from eukaryotes (29) (neural cell adhesion molecule [N-CAM], *Dictyostelium discoideum* contact site A [CsA] and lymphocyte function-associated [LFA-3]) and six bacterial surface proteins: group A streptococcal M protein (10), group G streptococcal protein G (41), *Streptococcus mutans* wall-associated protein A (wapA) (42), staphylococcal protein A (32), IgA binding protein (IgAbp) (43) from group A streptococci and fibronectin binding protein (fibronectin BP) (44), all predicted from DNA sequence. Sequence to the right of the space (*arrow*) is absent from the released form of M protein (9) and predicted for the other five bacterial proteins. The predicted cleavage of the three GPI-anchored proteins is based on comparison with sequences from other GPI-anchored proteins for which the cleavage site is known (29). Colons indicate amino acids found within the proposed cleavage and attachment region of GPI-anchored proteins (29), which are either identical or conserved when compared with the COOH-terminal reigon of the bacterial proteins.

PI-PLC from *Bacillus thuringensis* (kindly supplied by Martin Low, Columbia University, New York, NY) and phosphatidyl choline-PLC from *B. cereus* (type III, Sigma Chemical Co.) were unable to release M protein from PHMB-inactivated protoplasts (data not shown). This suggests that either the M protein is not anchored by a GPI complex or, since anchor degrading enzymes have been shown to be selective for certain GPI anchors (28) (for example, PI-PLC will not cleave the GPI anchor of *D. discoideum* CsA [35]), the M protein anchor may not be sufficiently conserved in structure or exposed on the membrane surface for enzymatic cleavage by these specific GPI-degrading enzymes.

The size difference found between the secreted and bound forms of the M molecule by Western blot analysis (Fig. 2), is consistent with certain GPI anchored proteins where the bound form is found to be either smaller (36) larger (37) or the same size (38, 39) as the released form by SDS-PAGE. Thus, the size differences observed between the bound and released forms as well as between the two bound forms may depend on the nature of their respective modifications. While the replacement of a hydrophobic COOH-terminal peptide domain by a GPI complex has been established for many surface proteins of eukaryotic cells as well as in yeast (28, 29), such post-translational modifications have as yet to be reported for surface protein of procaryotes. However, based on the finding of both a membrane anchor-cleaving activity for the M protein in streptococcal membranes and amino acids corresponding in sequence and position to those found in GPI-anchored proteins suggest that the M molecule may also be post-translationally modified for membrane attachment. Because the COOH-terminal hydrophobic tail found for M protein is highly conserved in other surface proteins from gram-positive bacteria (Fig. 6) (13), the mechanism by which these proteins are bound to the cell may also be conserved.

In eukaryotes, anchor degrading enzymes may function to regulate the concentration of proteins at the cell surface or control the secretion of certain molecules (28, 29). In the trypanosome, for example, the release of the VSG is suggested as the mechanism by which the organism escapes immune recognition (40). M protein is however, not usually found in the supernatant of growing streptococci, thus it is unlikely that an anchor cleaving enzyme would act to release the molecule in conventional culture broth. However, it is not known if during the process of colonization of the mucosal surfaces or during invasion it is advantageous for the streptococcus to shed all or a portion of its fiberous M protein to expose other structures more proximally situated on the streptococcal surface. Whether the ability to release M protein is a biologically significant characteristic for the pathogenesis of streptococcal infections is under investigation.

Summary

How streptococcal M protein or other surface proteins of gram-positive bacteria are anchored to the cell is poorly understood. Previously, we reported that M protein released after cell wall removal with a muralytic enzyme lacked the COOH-terminal hydrophobic amino acids and charged tail predicted from DNA sequence. An endogenous membrane anchor-cleaving enzyme has now been identified with the ability to release M protein from isolated streptococcal protoplasts. At pH 5.5 in the presence of 30% raffinose, the streptococcal cell wall may be removed with

a muralytic enzyme without releasing M protein from the resulting protoplasts indicating that the M molecule is attached through the bacterial cytoplasmic membrane. Release of M molecules occurs when the M protein-charged protoplasts are placed in raffinose buffer at pH 7.4. Although Zn^{2+}, Cd^{2+}, Ca^{2+}, PHMB, and pHMPS inhibit the activity of the releasing enzyme, the blocking activity of Zn^{2+}, Cd^{2+}, and Ca^{2+} are reversible while PHMB and pHMPS are irreversible. PHMB-treated protoplasts are unable to release M protein at pH 7.4. However, M protein is liberated from these protoplasts when mixed with those prepared from M^- streptococci serving as an enzyme source. The supernatant from M^- protoplasts is unable to release M protein from PHMB-inactivated M^+ protoplasts, confirming that the anchor-cleaving enzyme is membrane bound. Thus, the M protein releasing activity appears to be the result of a thiol-dependent anchor-cleaving enzyme. Streptococcal membranes treated with sodium carbonate and Triton X-114 still retain the M protein verifying that it is an integral membrane molecule. Evidence also is presented indicating significant sequence similarity between M protein and certain GPI-anchored proteins in the region responsible for protein anchoring.

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