ANTIGENIC DOMAINS OF THE STREPTOCOCCAL Pep M5 PROTEIN

Localization of Epitopes Crossreactive with Type 6 M Protein and Identification of a Hypervariable Region of the M Molecule

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M protein of the group A streptococcus is an elongated, alpha-helical coiled-coil molecule that extends from the bacterial cell surface as a flexible fibrillar structure (1, 2). It is a major virulence factor for the bacteria, by virtue of its property of impeding the phagocytosis of the organisms (3). >75 serologically distinct variants of the M protein have been recognized over the years and, in the human, the immunity conferred by the induced antibodies to a given M type is essentially type specific (3-7). However, immunological crossreactions occur among some of the M protein serotypes (3-7). The crossreaction between types 5 and 6 M proteins is one such example (6, 8, 9). The crossreactivity of the type 5 M protein with the type 6 M protein is retained in its peptic fragment Pep M5 (8), which represents nearly the amino-terminal half of the native M5 molecule (10).

With the exception of the nonhelical N-terminal 12-residue segment, the Pep M5 protein exhibits structural characteristics of alpha-helical coiled-coil proteins (11, 12). Based on the distribution of the nonpolar and charged amino acid residues, as well as internal homology, there are two structurally distinct regions (domains) within the coiled-coil structure of the Pep M5 molecule. The N-terminal domain of the Pep M5 protein carries a significantly higher net negative charge than its carboxy-terminal domain, and has been implicated in the antiphagocytic function of the molecule (11). In the present study, peptides derived from the two structurally distinct domains of the Pep M5 protein have been used (a) to determine whether both domains contain antigenic epitopes and (b) to identify the domains of the Pep M5 protein containing the epitope(s) crossreactive with the M6 protein.

Materials and Methods

Proteins and Peptides. Pep M5 protein was isolated by limited proteolysis of the type 5 streptococcal cells (strain B788) with pepsin, as previously described (12). Pep M6, a peptic
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Figure 1. Fractionation of the tryptic peptides of DHP Pep M6 by HPLC on a Waters \( \mu \)-Bondapak C18 reverse-phase column. Peptides were eluted with a linear gradient of 5–70% acetonitrile/0.05% trifluoroacetic acid, over 140 min. The flow rate was 1 ml/min. Fractions were pooled as indicated by the bars.

fragment of the M6 protein analogous to the Pep M5 fragment, was isolated from the type 6 streptococcal cells (strain D471) essentially by methods described for the Pep M5 protein (13). Lys M6 protein was isolated from the streptococcal strain D471 using group C phage-associated lysin as described (14). Pep M5 peptides corresponding to regions 13–64 (peptide CP5), 127–197 (peptide CP7), and 21–90 (peptide C2) of the Pep M5 protein were isolated from clostripain and chymotryptic digests, respectively, of the Pep M5 molecule, as described earlier (10, 15). In addition, a peptide corresponding to residues 130–168 of the Pep M5 protein was also isolated from a chymotryptic digest.

Arginyl Peptides of Pep M6 Protein. Arginine peptides of the Pep M6 protein were isolated by HPLC fractionation of a tryptic digest of the dihydroxypropylated (DHP)\(^4\) protein according to the procedures described earlier (10, 16) (Fig. 1).

Sequence Analysis of Pep M6 Peptides. Amino-terminal sequence analyses of the Pep M6 peptides were carried out by automated Edman degradation as previously described (10).

Antisera. New Zealand White rabbits were immunized with 100 \( \mu \)g of the Pep M5 or the Lys M6 protein in 1 ml of PBS, pH 7.4, emulsified with an equal volume of CFA, by multiple intradermal injections. The booster inoculations, given at \( \approx \)1 mo intervals, consisted of 100 \( \mu \)g of the M protein in incomplete Freund's adjuvant. The rabbits were bled 10–12 d after each immunization.

ELISA. The binding of anti-Pep M5 and the anti-Lys M6 sera to the M5 peptides was determined by ELISA, essentially by the procedure of Engvall and Perlmann (17), as previously described (18).

Absorption of Anti-M5 Serum with Type 6 Streptococci. This was carried out using heat-killed type 6 streptococci (19). Anti-M5 serum was mixed with the heat-killed type 6 streptococci (type 6 cells from 100 ml of an overnight culture per milliliter of the M5 antiserum) and the suspension was rotated at 4°C for 2 h. The cells were removed by centrifugation and the supernatant was filter sterilized through a 0.45 \( \mu \)m filter.

Results

Binding of Anti-Pep M5 Antibodies to Pep M5 Peptides. The coiled-coil structure of the Pep M5 protein contains two distinct domains (11). To localize the antigenic epitopes within the coiled-coil structure of the Pep M5 protein, two peptides from its N-terminal domain comprising residues 13–64 and 21–90, and two peptides from its C-terminal domain comprising residues 127–197 and 130–168, were examined by ELISA for their ability to react with rabbit anti-Pep M5 sera. The positions of these peptides within the two structurally distinct domains of the molecule are schematically indicated in Fig. 2. Binding of the Pep M5 peptides was examined with sera from four different rabbits. As can be seen in

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| Abbreviations used in this paper: | DHP, dihydroxypropylated; LMM, light meromyosin. |
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Figure 2. Schematic diagram of the Pep M5 protein, the pepsin-derived N-terminal half of the type 5 M protein. The N-terminal 12-residue segment is nonhelical, whereas the remainder of the molecule has a coiled-coil structure. The location of the two structurally distinct domains A and B within the coiled-coil structure of the Pep M5 molecule, as well as the location within these domains of the peptides selected for the binding studies, are shown.

Table 1

Binding of Anti-Pep M5 and Anti-Lys M6 Antibodies Against Pep M5 Protein and Its Peptides as Measured by ELISA

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Anti-Pep M5</th>
<th>Anti-Lys M6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>604 807 598</td>
<td>599 605 839</td>
</tr>
<tr>
<td>Pep M5 protein*</td>
<td>1.73 1.87</td>
<td>1.66 1.39</td>
</tr>
<tr>
<td>Pep M5 peptides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Region 13–64</td>
<td>1.58 1.26</td>
<td>1.26 1.15</td>
</tr>
<tr>
<td>Region 21–90</td>
<td>1.49 1.46</td>
<td>1.35 1.11</td>
</tr>
<tr>
<td>Region 127–197</td>
<td>0.89 0.15</td>
<td>1.04 0.90</td>
</tr>
<tr>
<td>Region 130–168</td>
<td>1.07 0.10</td>
<td>0.41 0.43</td>
</tr>
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* Pepsin-derived fragment of the M5 protein comprising approximately the N-terminal half of the molecule.
† Values <0.02.

Table 1, anti-Pep M5 sera from all four rabbits exhibited binding to peptides from both the N- and C-terminal domains of the Pep M5 protein. However, the extent of reactivity of the different sera with the peptides from the two domains varied. While the reactivity of all the sera with peptides from the N-terminal domain was comparable, some sera reacted much more strongly than others with the peptide(s) from the C-terminal domain. These results clearly indicate that antigenic epitopes are present in both the N- and C-terminal domains of the Pep M5 protein.

Crossreaction of Anti-Lys M6 Sera with Pep M5 Peptides. Sera raised against the Lys M6 protein exhibited marked crossreaction with the Pep M5 protein (Table 1). To determine whether an M6-crossreactive epitope resides within the N- and/or C-terminal domain of the Pep M5 protein, we tested the M5 peptides described above for their binding with the rabbit anti-Lys M6 sera. Although all three rabbit anti-Lys M6 sera crossreact with the Pep M5 protein, unlike the anti-Pep M5 sera, the anti-Lys M6 sera reacted only with the C-terminal peptides 127–197 and 130–168 of the Pep M5 protein, but not with the N-terminal peptides (Table 1). In addition, whereas anti-Lys M6 serum 605 reacted strongly with region 130–168, a subfragment of region 127–197, the other two anti-Lys M6 sera exhibited significantly less binding with this region. These results therefore demonstrate that the C-terminal region 127–197 of the Pep M5 protein...
contains one or more M6 crossreactive epitopes, some of which are within region 130–168. The lower reactivity of the anti-Lys M6 sera 839 and 841 with region 130–168 is suggestive of either the loss of a crossreactive determinant, as a result of cleavage at the 168–169 peptide bond, or the loss of a crossreactive epitope within region 169–197 of the Pep M5 protein.

**Sequence Homology Between Pep M5 and Pep M6 Proteins.** The fact that segment 127–197 of the type 5 M protein contains epitopes that are crossreactive with the M6 protein is clearly suggestive of similarity in sequence and/or structure of a region in the M6 protein to that of the second domain of the Pep M5 protein. To gain further insight into this aspect, sequence analyses were carried out on the arginine peptides of the Pep M6 protein, the pepsin-derived N-terminal half of the M6 protein (Fig. 3). The sequence of peptide DHP-T5 provided a 20 residue overlap with the N-terminal sequence of the whole molecule and extended the N-terminal sequence of the protein to 63 residues. Peptide DHP-T3 lacks arginine and hence represents the C-terminal peptide of the Pep M6 protein. These amino acid sequences of the DHP-peptides of the Pep M6 protein were later verified by comparison with the amino acid sequence deduced from the DNA sequence of the M6 genome (20), but for an inversion of residues 29 and 30 of peptide DHP-T6. The reason for this inversion is not clear at present.

Comparison of the 63 residue N-terminal sequence of the M6 protein with the N-terminal region of the Pep M5 protein revealed that 42% of the residues within these regions of the two proteins are identical (Fig. 4A). A visual examination of the amino-terminal 52-residue sequence of peptide Pep M6/DHP-T6 revealed that it is highly homologous with region 135–186 of the Pep M5 protein (Fig. 4B). 69% of the residues between the two segments compared are identical. More significantly, the homology between residues 16–52 of Pep M6/DHP-T6 and region 150–186 of the Pep M5 protein are even
FIGURE 4. Comparison of the amino-terminal sequences of Pep M6 and its arginyl peptides to various regions of the Pep M5 protein. The amino acid residues are indicated by the single-letter code, and residues identical between the sequences compared are boxed.

more extensive. 31 of the 37 residues between the two segments are identical (84% identity). These results clearly suggest that, as in the Pep M5 protein, structurally distinct domains may be present in the Pep M6 protein. Similar sequence comparisons with the Pep M6 peptides DHP-T3 and DHP-T4 revealed that peptide DHP-T4 exhibits nearly 42% identity with residues 111–134 of the Pep M5 protein (Fig. 4D). On the other hand, the sequence of the Pep M6 peptide DHP-T3 matched up very well with the C-terminal 15 residues of the Pep M5 protein (Fig. 4C). The sequence of peptide DHP-T3 was identical to residues 183–197 of the Pep M5 protein except for the substitution of an aspartic acid in place of asparagine at position 189 of Pep M5 protein, and an extra residue (alanine) after leucine 197 of the Pep M5 protein.

Absorption of the M6 Crossreactivity of the Pep M5 Antisera with Type 6 Streptococci. Binding studies of the Pep M5 peptides with the anti-M6 sera clearly demonstrated the presence of M6-crossreactive epitopes within the C-terminal domain of the Pep M5 protein. To determine whether the C-terminal domain of the Pep M5 protein contains other antigenic epitopes in addition to the M5-crossreactive epitopes, serum from one of the rabbits (serum 604) was absorbed with the type 6 streptococci, and the absorbed sera reexamined for binding to the M5 peptides. As can be seen from Table II, and consistent with the results
observed in Table I, the activity with the peptides from the N-terminal domain of Pep M5 protein was unaffected, whereas the binding with the C-terminal peptides 127–197 and 130–168 was markedly reduced. Thus, the C-terminal domain of the Pep M5 protein contains type-specific antigenic epitopes in addition to the M6 crossreactive epitopes.

Discussion

Several previous studies have demonstrated immunologic crossreactions between types 5 and 6 M proteins (6, 8, 9). Det M6, the M6 protein extracted with nonionic detergent and comprising the amino-terminal half of the M6 protein, has been shown (6) to bind with unabsorbed hyperimmune rabbit sera prepared against the type 5 streptococci. Some of the monoclonal antibodies to the type 5 M protein (Pep M5) have been shown to crossreact with types 6 and 19 M proteins (8). More recently, Jones et al. (9) demonstrated that two of the monoclonal antibodies to the M6 protein crossreact with the type 5 M protein. The crossreactive relationships observed among the various M protein serotypes could be a reflection of sequence similarities between these molecules and/or similarity in the higher orders of their structures.

Our earlier studies have demonstrated that the streptococcal M protein has an alpha-helical coiled-coil structure (2, 12). Our more recent studies on the Pep M5 protein, the N-terminal half of the type 5 M protein, have demonstrated that, based on the distribution of the nonpolar and charged residues as well as internal sequence homology, there are two distinct domains within the coiled-coil structure of the Pep M5 protein (11). While the distribution of nonpolar residues within region 13–121 (the amino-terminal domain) is atypical of other coiled-coil proteins, the periodicity in region 122–196 (the carboxy-terminal domain) is more typical of that found in other coiled-coil proteins, such as the myosin rod region, keratin, desmin, and vimentin, rather than the prototypical tropomyosin molecule. Furthermore, the amino-terminal domain carries a significantly higher net negative charge than the carboxy-terminal region, suggesting that the former region, which is distal to the bacterial cell surface (2, 21) may play a role in the biological function of the Pep M5 molecule (11). Within these two regions, the molecule exhibits distinct internal homology.

The results of the present study demonstrate that both structurally distinct
domains of the Pep M5 protein contain antigenic epitopes. Region 127–197 of the Pep M5 protein, representing essentially the complete C-terminal domain of the molecule, has been found to contain one or more epitopes crossreactive with the M6 protein. In addition, some of the crossreactive epitopes have been localized within region 130–168 of the Pep M5 protein. Sequence comparisons clearly demonstrate that region 150–186 of the Pep M5 protein, representing nearly the second half of the C-terminal domain of the molecule, exhibits as much as 84% identity with a segment of the Pep M6 protein. Within the segment 150–168 of the Pep M5 protein, 16 of the 19 residues are identical with residues in region 16–34 of Pep M6 peptide DHP-T6. Thus, it appears likely that one of the M6-crossreactive epitopes of the Pep M5 protein is located within residues 150–168 of the molecule. It may be added here that segment 150–168 of the Pep M5 protein is an internally homologous segment based on its high homology with region 175–193 of the Pep M5 molecule (10). Furthermore, region 16–34 of peptide Pep M6/DHP-T6 is also located in a highly repetitive region of the M6 molecule (20).

Sequence comparisons also reveal virtual identity of the C-terminal segment 183–197 of the Pep M5 protein with the C-terminal hexadecapeptide of the Pep M6 protein, suggesting that either whole or part of this sequence may constitute another crossreactive epitope between the M5 and M6 proteins. In view of the immunologic crossreactivity of region 127–197 of the Pep M5 protein with anti-M6 antibodies, and the sequence homology of the final two-thirds of the C-terminal domain of the Pep M5 protein with sequences from the Pep M6 protein, one or more epitopes crossreactive with the M5 and M6 proteins may be placed close to the pepsin-susceptible site of the M5 protein.

The present results also demonstrate that, although the location of the site of pepsin cleavage along the length of the M protein molecule, as reflected in the molecular weights of their pepsin-derived fragments, is different for the M5 and M6 proteins (2, 10, 13), the sequence around the cleavage site is essentially identical in both proteins. This is certainly suggestive of a conformational similarity of this region within the respective parent protein that renders these regions susceptible to proteolysis with pepsin. Limited proteolysis studies with alpha-helical coiled-coil proteins, as well as proteins in general, have been of great value in detecting structural and functional domains or regions of these molecules. For example, the rod portion of the myosin molecule can be proteolytically cleaved into distinct subfragments S-2 and light meromyosin (LMM), each of which retains specific functions of the parent molecule (22, 23). Such cleavage is closely related to the mechanical properties of the molecule. It has been suggested that the junction between S-2 and LMM may contain a molecular hinge that lets the rod bend (23). Analogously, as previously suggested of the streptococcal M proteins (13), the pepsin-derived Pep M fragment may indeed be considered a functional domain of the M protein molecule, with the pepsin-cleavage site representing the boundary of the second domain of the M molecule.

A recent study by Jones et al. (9) has demonstrated the presence of variable and conserved epitopes among the various M protein serotypes. The epitope for the monoclonal M6 antibody 10B6, which is conserved among several serologically different M proteins including the type 5 M protein, was shown to be
located on the carboxyl side of the pepsin cleavage site of the M6 molecule. Together with the present results, this indicates that crossreactive epitopes are present on either side of the pepsin-susceptible site of the M protein. It appears that there are probably less structural variations among the various M protein serotypes on either side of the pepsin cleavage site.

The study by Jones et al. (9) suggested a greater antigenic variability within the N-terminal half of the different M protein serotypes. This was attributed to the amino-terminal region of the M protein being distal to the bacterial cell surface (2, 21), which might render it more exposed to the environment and hence more accessible to the host’s immune surveillance system. The present results suggest an even higher degree of variability within the N-terminal half of the Pep M fragment, i.e., the N-terminal quarter of the native M protein molecule. Thus, if the pepsin-derived, biologically active fragment of the M protein is considered the variable part of the M molecule, it appears that the N-terminal half of the Pep M protein, corresponding to the N-terminal quarter of the native M molecule, represents a hypervariable domain of the M molecule.

Summary
Pep M5, the pepsin-derived N-terminal half of the group A streptococcal type 5 M protein exhibits immunologic crossreaction with type 6 M protein, localizing some of the M6-crossreactive epitope(s) within this segment of the M5 protein. Based on the amino acid sequence of the Pep M5 protein, two structurally distinct domains have been recognized within its coiled-coil structure. We have now found that peptides derived from both the structurally distinct domains of the Pep M5 protein contain antigenic epitopes. Furthermore, only the peptides from the C-terminal domain of the Pep M5 protein crossreacted with rabbit anti-M6 sera, whereas those from the N-terminal domain did not. Consistent with this, sequence analyses of the arginyl peptides of the Pep M6 protein, the pepsin-derived N-terminal half of the M6 protein, revealed extensive homology of some of these peptides with regions within the C-terminal domain of the Pep M5 molecule. While an arginyl peptide of the Pep M6 protein exhibits 84% homology with region 150–186 of the Pep M5 protein, the C-terminal hexadecapeptide of the Pep M6 protein is virtually identical with the corresponding region of the Pep M5 protein. These results are suggestive of conformational similarities in the region around the pepsin-susceptible site within the M5 and M6 proteins. In addition, one or more epitopes of the M5 protein that are crossreactive with the M6 protein may be placed close to the pepsin-susceptible site of the M5 protein. Previous studies have suggested the N-terminal half of the M proteins to be the variable part of the molecule among the different M protein serotypes. The present results suggest that the N-terminal quarter of the M protein may represent the hypervariable domain of the M molecule.

We wish to thank Drs. Maclyn McCarty and Emil C. Gotschlich for their encouragement and continued interest in these studies. We also wish to thank Ms. Maria L. Schmidt for her excellent technical assistance, and Dr. Kevin F. Jones for his comments on the manuscript.

Received for publication 19 September 1985.
Note added in proof: In a recent study, (Scott, J. R., S. Hollingshead, and V. A. Fischetti. Homologous regions within M protein genes in group A streptococci of different serotypes. Manuscript submitted for publication) used DNA probes consisting of fragments of the structural gene for the M6 protein to locate conserved and variable regions within M molecules from different M serotypes. It was found that probes corresponding to the N-terminal half of the M6 molecule exhibit little homology with DNA from the heterologous serotypes. However, probes corresponding to the C-terminal half of the M6 molecule revealed greater conservation among different M serotypes.

References

15. Manjula, B. N., S. M. Mische, and V. A. Fischetti. 1983. Primary structure of


