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Location of the Complement Factor H Binding Site on Streptococcal M6 Protein

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The surface M protein of group A streptococci binds factor H, a regulatory protein of the alternative complement pathway, which may contribute to the antiphagocytic activity of the M molecules. To locate the factor H binding domain in the α-helical coiled-coil structure of the M molecule, the M protein was cleaved with pepsin at pH 5.8, which separates the molecule approximately in half. Western blot (immunoblot), amino acid sequence, and mass spectrometric analyses revealed that factor H bound to a 14.6-kDa C-terminal fragment of the M molecule. Competitive inhibition of factor H binding to the 14.6-kDa fragment with M protein peptides localized the binding site to amino acids 256 to 292. This segment is located within the surface-exposed region of the M6 protein, identified as the C-repeat region, whose sequence is conserved among heterologous M and M-like molecules. These studies also identified a second pepsin-susceptible site with the sequence ELAK located within the cell wall-associated region of the M molecule.

Opsonization through the deposition of complement C3b on the surface of particles via the alternative pathway is amplified by C3 convertase (C3b,Bb). The deposition of C3b is controlled by specific inhibitors in serum such as decay-accelerating factor and factor H, which prevent C3 activation by (i) rendering C3b susceptible to inactivation by factor I, (ii) restricting the conversion of C3b,Bb by competing for factor B, and (iii) accelerating the dissociation of C3 and C5 convertase (33). Factor H, a 150-kDa β-globulin that is present in serum at 500 μg/ml, controls fluid-phase as well as surface-bound C3b (33).

M protein, a fibrillar molecule on the surface of group A streptococci (36), functions to enable the organism to resist phagocytosis. The restricted deposition of C3b on M^+ streptococci was previously reported (21): an uneven distribution of C3b on the surface of M^+ streptococci was observed, compared with the even distribution on M^- organisms. The recent finding by Horstmann et al. (19) that M protein specifically binds factor H explains these results and may help define the antiphagocytic action of the M molecule. It is proposed that when the streptococcus contacts serum, the factor H bound to the M molecule inhibits or reverses the formation of C3b,Bb complexes and helps to convert C3b to the inactive form (iC3b) on the bacterial surface, preventing C3b-dependent phagocytosis. Horstmann et al. (19) also found that in addition to purified M protein, M^+ streptococci of all five different serotypes tested were able to bind factor H. This result suggests that the binding of factor H may be directed to a conserved structural characteristic found within the M-protein molecule. In this report, we localize the site on the M6 protein responsible for factor H binding and in doing so also identify a second pepsin-susceptible site on the M6 molecule located below the surface of the cell wall.

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MATERIALS AND METHODS

Factor H. Factor H was purified from human serum as previously described (19).

Pepsin digestion. Recombinant M6 protein (rM6) (13) was separated into N- and C-terminal halves by cleavage with pepsin at suboptimal pH (5.8) as detailed previously (26).

Peptides. Peptides were synthesized and purified by reverse-phase high-pressure liquid chromatography as described previously (24).

Western blotting (immunoblotting) and purification by electroelution. The fragment of the M molecule that binds factor H was identified by Western blotting and purified by electroelution. Fragments of the rM6 protein generated after pepsin digestion were separated by sodium dodecyl sulfate (SDS)–12% polyacrylamide gel electrophoresis (PAGE) and electrotransferred onto a nitrocellulose membrane as previously described (13). To identify the factor H-binding fragment, the blot was washed in 67 mM phosphate-buffered saline (PBS; pH 7.2) containing 0.5% Tween 20 (PBS-Tween), reacted with factor H (100 μg/ml in PBS) for 18 h, and washed. The bound factor H was located with anti-factor H monoclonal antibody 5B6 (at a 1:500 dilution), reacted for 3 h, washed, and detected by alkaline phosphatase-conjugated anti-mouse antibody (7). Fragments representing the N- and C-terminal halves of the M6 molecule were identified on duplicate blots, using monoclonal antibodies directed to epitopes within these regions of the protein (24, 25).

The major factor H-reactive fragment, M6(228–357), was isolated after electrophoresis of the rM6 pepsin digest by preparative SDS-PAGE as described by Pancholi and Fischetti (32). Briefly, the portion of the gel containing the smallest factor H-binding fragment (Fig. 1C) was excised and placed in a Bio-Rad electroelution device, and the fragment was eluted from the gel. The eluted fragment was precipitated, then washed with ice-cold acetone, and suspended in distilled water. The protein concentration was determined by the bicinchoninic acid technique, and the solution was stored frozen at −70°C in aliquots.

Monoclonal antibodies. M6 protein-specific monoclonal antibodies directed to epitopes within the N- and C-terminal regions of the M molecule were reported earlier (24). Factor H-specific monoclonal antibodies were prepared by techniques employed to prepare M protein-specific monoclonal antibodies (25), using purified human serum factor H as the immunogen (19). Clones reactive with factor H in enzyme-linked immunosorbent assay (ELISA) and Western blotting were purified by two rounds of limiting dilutions. As with all monoclonal antibodies used in previous studies, the factor H monoclonal antibodies were purified on protein A from liquid cultures (25).

Competition ELISA. Microtiter plates were sensitized with 100 μl of 1 μg of the electroeluted M6(228–357) fragment per ml. Synthesized M6 peptide (100 μg/ml), predetermined by serial dilution to give maximal inhibition, was added to each well along with 2 μg of factor H in 50 μl of PBS, and the mixture incubated for 3 h at 37°C. The wells were washed with PBS-Tween, a 1:1,000 dilution of anti-factor H monoclonal antibody was added, and the mixture was incubated for 3 h at 37°C. Plates were washed with PBS-Tween, goat anti-mouse alkaline phosphatase conjugate was added, and the reaction was developed as described above. Positive control wells contained factor H without peptide. Wells without peptides but containing all other reagents served as background controls. All assays were performed in triplicate, and results are expressed as means.

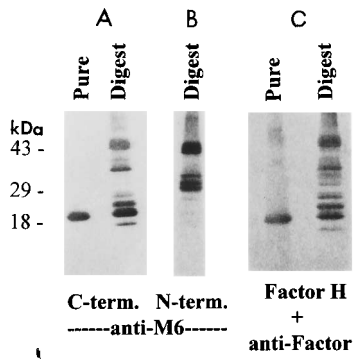

FIG. 1. Western blot analysis of the pepsin-digested rM6 protein. After pepsin digestion, fragments of the rM6 protein were separated by SDS-PAGE and electrotransferred to nitrocellulose. Individual lanes were cut and processed separately. To identify the N- and C-terminal halves of the M molecule, strips were reacted with N-terminally specific (A) and C-terminally specific (B) monoclonal antibodies. To determine which fragments have the capacity to bind factor H, a nitrocellulose strip was reacted first with factor H and then with anti-factor H monoclonal antibodies. As can be seen, only polypeptides that react with the C-terminally specific monoclonal bound factor H (compare panels A and C). The smallest factor H-binding fragment was purified by gel excision and electroelution and incorporated in the blot to determine its ability to bind factor H. The broad band above the 43-kDa marker in the digest is undigested rM6 protein.

Analytical tests. N-terminal sequence analysis was performed on the electroeluted fragment transferred onto a polyvinylidene difluoride (PVDF) membrane as previously described (32). The molecular mass of the factor H-binding fragment was determined by mass spectrometry in the laboratory of Mass Spectrometry and Gas Phase Ion Chemistry of The Rockefeller University, using a modification of the matrix-assisted laser desorption technique (2).

RESULTS

Reactivity of factor H to N- and C-terminal halves of the M molecule. To determine the region of the M molecule which was able to bind factor H, the M6 protein was digested with pepsin at pH 5.8, which cleaves the molecule at about its center, resulting in two major cleavage products, a number of partially cleaved molecules (9, 11), and some uncleaved M protein. Fragments representing the N- and C-terminal halves of the M molecule may be identified from this mixture after separation by SDS-PAGE and Western blotting using N- and C-terminal-specific monoclonal antibodies (Fig. 1A and B). Western blots of the whole pepsin digest reacted first with factor H and then with anti-factor H monoclonal antibodies was used to determine which fragment contained the factor H binding site. As seen in Fig. 1C, factor H bound to the fragments of the M molecule which were also reactive with the C-terminally specific monoclonal antibody (Fig. 1A) (24, 25). Fragments reactive with the N-terminally specific antibody showed no factor H binding. The undigested intact M molecule (broad band above the 43-kDa marker in Fig. 1) reacted with both M antisera and factor H.

Characterization of the factor H-binding fragment. We selected the smallest factor H-binding fragment, with an apparent molecular mass of 19 kDa, for further study (Fig. 1C, digest). The fragment was purified by excision from the SDS-gel and electroelution, and we verified that it still bound factor H (Fig. 1C, pure). The N-terminal sequence of the purified fragment was determined after transfer onto a PVDF membrane. The results revealed the sequence AKKDEGNKV, which begins at residue 228 of the reported sequence of the native M6 molecule (17) (Fig. 2). This identifies the pepsin cleavage site for the M6 molecule to be between Leu-227 and Ala-228. Mass spectrometric analysis was used to determine the size of the binding fragment in order to locate its precise C terminus. The analysis indicated that the fragment had a molecular weight of 14,626. An examination of the complete M6 sequence revealed that a fragment beginning at Ala-228 and predicted to have a molecular weight of approximately 14,626 would end at residue Leu-357. This site also has an ELAK sequence identical to the ELAK sequence located at the pepsin site in the center of the M molecule (Fig. 2), indicating that the binding site for factor H is located between residues 228 and 357 of the M6 molecule.

Localization of the factor H binding sequence. Peptide inhibition analysis was used to determine the sequence within the M6(228–357) fragment responsible for factor H binding. Of the M6 synthetic peptides tested, overlapping peptides sM6(256–277) and sM6(272–292) could block ≥89% of factor H binding to the M6(228–357) fragment (Table 1). In contrast, peptides located in other regions of the M protein inhibited ≤38% of factor H binding to the M6 fragment. These results indicate that the factor H binding region is located between Lys-256 and Ala-292 of the mature M6 protein. Alignment of residues 256 to 292 with known M protein sequences revealed

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1    RVFPRGTVENPDKARELLNKYDVENSMLQANNDKLTTENNNLTDQNKNLT
51   TENKNLTDQNKNLTTENKNLTDQNKNLTTENKELKAEENRLTTENKGLTK
101  KLSEAEEEAANKERENKEAIGTLKKTLDETVKDKIAKEQESKETIGTLKK
151  TLDETVKDKIAKEQESKETIGTLKKTLDETVKDKIAKEQESKETIGTLKK
201  ILDETVKDKIAREQKSKQDIGALKQEL
                              ↑
                           Pepsin
Seq. AKKDEGNKV......
     :::::::::
228  AKKDEGNKVSEASRKGLRRDLDASREAKKQVEKDLANLTAELDKVKEEKQ
278  ISDASRQGLRRDLDASREAKKQVEKALEEANSKLAALEKLNKELEESKKL 14.626 kDa
328  TEKEKAELQAKLEAEAKALKEQLAKQAEEL
                               ↑
                            Pepsin

358  AKLRAGKASDSQTPDAKPGNKVVPGKGQAPQAGTKPNQNKAPMKETKRQL
408  PSTGETANPFFTAAALTVMATAGVAAVVKRKEEN
```

FIG. 2. Identification of the factor H binding region (fragment 228–357) of the M6 molecule after pepsin digestion. The complete M6 sequence is shown separated into the fragments released after pepsin digestion at pH 5.8. The N-terminal sequence of the factor H-binding fragment (Seq.) is shown aligned over the N-terminal region of one of the C-terminal fragments. Pepsin cleaved within the ELAK sequence, separating the molecule between L-227 and A-228. A second ELAK cleavage site was identified by mass spectroscopy of the 14.626-kDa binding fragment to be between L-357 and A-358.

```
                    <----Spacer-------|---C-repeat---->

               256                273                292    % Identity
M6   (256-292)  KQVEKDLANLTAELDKVKEEKQISDASRQGLRRDLDA
                ::::::::::::::::::::::::::::::::::::::::     100 (100)
M12  (290-326)  KQVEKDLANLTAELDKVKEEKQISDASRQGLRRDLDA

                                :: :::::::: ::::::::         (90)
M55                             KEDKQISDASRKGLRRDLDA

                                ::::: ::::::::::::::         (95)
M19                             KEEKQTSDASRQGLRRDLDA

                          : ::  :  :   :: ::: ::::::::      49 (70)
M5   (265-301)  DASREAKKQLEAEQQKLEEQNKISEASRKGLRRDLDA

                          : ::  :  :   :: :::: :::::::      49 (70)
M24  (354-390)  DASREAKKQLEAEHQKLEEQNKISEASRQSLRRDLDA

                  :::               ::::: :::  : ::: :      43 (65)
M49  (140-176)  REVEKRYQEQLQKQQQLETEKQISEASRKSLSRDLEA
```

FIG. 3. Alignment of the M6(256–292) sequence with comparable regions of M12 (37), M5 (28), M24 (29), and M49 (16), with amino acid segment in parentheses. The reported M55 and M19 sequences (18) were incomplete. Amino acids identical with the M6 sequence are identified by colons. Percent identity between residues 256 and 292 of the M6 sequence is shown; percent identities between residues 273 and 292 are in parentheses.

from 43 to 100% identity (65 to 100% between 273 and 292) within comparable regions of these molecules (Fig. 3).

DISCUSSION

Pepsin at pH 5.8 separates the N-terminal A- and B-repeat regions from the C-terminal C-repeat and wall anchor regions of the native M6 protein (9, 11). A previous analysis, using trypsin to digest the cell surface-exposed segment of the M6 protein, showed that the region of the M molecule N terminal to residue Lys-298 is located on the surface of the streptococcal cell wall (31). Sequences C terminal to Lys-298 are buried within the wall and protected from digestion with trypsin and perhaps interaction with molecules in the environment (31). In the current study, we found that factor H binds to amino acids 256 to 292, located within the surface-exposed region of the M6 protein, that is identified as the C-repeat region of the molecule (17). Monoclonal antibody and sequence analyses of different M molecules indicate that the C-repeat region is highly conserved among a large number of known M proteins of group A streptococci (18, 24, 25) as well as group G streptococci (22) and M-like molecules (15, 16). At least two C repeats are found within all of the sequenced M and M-like molecules on group A streptococci (6, 15, 17, 27–30, 37).

The C-repeat region of the M6 protein is made up of two repeat blocks of 23 amino acids each (residues 235 to 257 and 277 to 299) separated by a spacer of 19 amino acids (residues 258 to 276) (Fig. 4) (4). A similar spacer sequence also separates the B repeats from the C repeats. Directly following the second C-repeat block and located below the surface of the cell wall (31) is a partial C-repeat block (14, 17). The inability of peptide sM6(248–269) to inhibit factor H binding to fragment sM6(228–357) suggests that amino acids 256 to 269 may play little role in the binding activity, further localizing the binding region to amino acids 270 to 292, encompassing the junction between the spacer and the second C repeat (Fig. 4). The fact that peptides sM6(256–277) and sM6(272–292) are both able to compete for the binding of factor H suggests that segment 272–277 plays an important role in the binding activity. Comparison of the sequences of the inhibitory peptides with comparable regions of other M sequences revealed that the majority of the identities (from 65 to 100%) are found C terminal to residue 273 (Fig. 3), surrounding the spacer–C-repeat junction. The conservation of the sequence within this factor H binding region in several M molecules lends support to the idea that the antiphagocytic property of M molecules may be a result of this binding.

Because M molecules including the C-repeat region are in a coiled-coil conformation (14, 36), residues in positions 1 and 4 of a seven-residue repeat pattern of nonpolar amino acids would be internalized in the formation of the coiled coil (34). Thus, it is likely that factor H recognizes a specific conformation on the outer face of the M protein helix rather than a linear sequence, since as seen in Fig. 3, this factor H binding sequence is not 100% identical in all known M molecules. However, the high percentage of identity within this region may be sufficient to establish the proper conformation for factor H binding. This possibility is supported by the recent report by Horstmann et al. (20), who found that factor H also binds fibrinogen (a coiled-coil molecule), even though no sequence identity with M6 region 256–293 is seen (our unpublished data). Hence, an identical linear sequence may not be essential for the formation of the factor H binding domain.

In a recent study, the M protein gene was constructed such

TABLE 1. Competitive inhibition of binding of factor H to fragment M6(228–357), using isolated peptides

Peptide	% Inhibition[a]
140–158	11[b]
223–244	25
240–260	16
248–269	38
256–277	94
272–292	89

[a] Mean of triplicate wells run in two separate experiments. Deviation among replicates never exceeded 5%.

[b] A concentration of 100 μg/ml of each peptide was used as inhibitor.

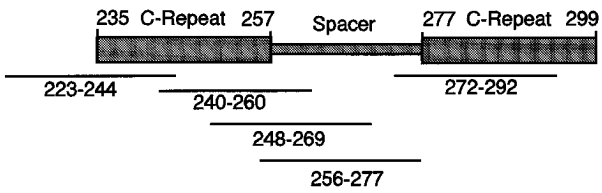

FIG. 4. Block diagram of the two cell surface-exposed C repeats of the M6 protein separated by a spacer sequence. Locations of the synthetic peptides used in the competitive inhibition of factor H binding to peptide M6(228–357) are shown.

that it contained only one C repeat without the intervening spacer (35). The streptococci expressing such an M protein were examined for their survival in a phagocytosis assay (35). Contrary to our finding that the spacer between the first and second C repeats is important for factor H binding, the other authors' results revealed that despite this deletion, the streptococci were able to resist phagocytosis. This result may be explained by the fact that the sequence preceding the first C repeat (i.e., the spacer between the last B repeat and the first C repeat), which is retained in the genetic construct (35), may be sufficiently conserved in both sequence (up to 50% identity with the spacer between the first and second C repeats) and conformation to allow factor H to also bind to this junction region. However, since our factor H-binding fragment (residues 228 to 357) is cleaved within this first spacer, the conformation at this end of the fragment may not be sufficiently conserved to allow factor H to bind, thus preventing us from identifying this binding site in the competitive inhibition assay.

The binding of factor H within the M protein C-repeat region could help explain the finding that antibodies that bind to both the B- and C-repeat segments of the M molecule on whole streptococci are unable to permit phagocytosis to occur in vitro (23). This unusual finding is despite the fact that the antibodies that bind to these regions are capable of adequately fixing complement as are antibodies directed to the N-terminal A repeats (23). However, only the A-repeat antibodies are opsonic. Perhaps factor H (an elongated molecule of 150 kDa) bound to the C-repeat region, in addition to its ability to control the deposition of C3b on the cell surface, is also able to control the deposition of C3b at the Fc portion of the antibodies bound to the B and C repeats. This would necessitate that phagocytosis proceed only via the Fc receptor, which has been shown to be inefficient (10). Factor H bound to the C-repeat domain located close to the cell surface may be unable to control those antibodies bound to the distant N-terminal hypervariable region, resulting in efficient phagocytosis.

Our studies also revealed for the first time that in addition to the ELAK pepsin-susceptible sequence found in the center of the M6 molecule, a second site with the identical sequence (beginning at residue Glu-356) was present in a segment of the M molecule found below the surface of the cell wall (31). While the second ELAK sequence is also found within the cell wall-associated region of the M5 (28), M24 (29), M12 (37), and M57 (27) proteins, the surface-exposed ELAK sequence is found only in the M6 and M5 molecules. The other M proteins have an ELEK sequence (M57 has ELAN) located at approximately the same position near the center of their respective M molecules. It is likely that the ELEK and ELAN sequences are sufficiently conserved to allow pepsin to cleave at pH of 5.8. This is partly supported by the fact that the M24 protein can be cleaved with pepsin to yield a molecule that is close to the size expected if the cleavage occurred at the ELEK site (1).

A number of important characteristics have been attributed to the C-repeat region of M protein. It has been found to harbor the epitopes necessary to separate group A streptococci into two major classes. Those serotypes responsible for most of the reported cases of rheumatic fever are associated with class I, and those that produce a serum opacity factor are in class II (5). In addition, the conserved nature of the sequence found within the exposed C repeats among over 30 M serotypes (25) is the basis for a mucosal vaccine capable of protecting against pharyngeal challenge by heterologous streptococcal types (3, 8, 12). The specific binding of complement factor H to this region defines yet another important characteristic of the C repeats.

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