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Role of the Hypervariable Region in Streptococcal M Proteins: Binding of a Human Complement Inhibitor[1]

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Antigenic variation allows pathogenic microorganisms to evade the immune system of the infected host. The variable structure must play an important role in pathogenesis, but its function is in most cases unknown. Here, we identify a function for the surface-exposed hypervariable region of streptococcal M5 protein, a virulence factor that inhibits phagocytosis. The hypervariable region of M5 was found to bind the human complement inhibitor FHL-1 (factor H-like protein 1), a 42-kDa plasma protein. Plasma absorption experiments with M5-expressing bacteria showed that the interaction with FHL-1 occurs also under physiologic conditions. Studies of another extensively characterized M protein, M6, indicated that this protein also has a binding site for FHL-1 in the hypervariable region. The complement-inhibitory function of FHL-1 was retained after binding to streptococci, suggesting that bound FHL-1 protects bacteria against complement attack. All available data now indicate that FHL-1, or another human complement inhibitor, binds to the hypervariable region of M proteins. These findings provide insights into the forces that drive antigenic variation and may explain why the hypervariable region of M protein is essential for phagocytosis resistance. Moreover, these data add to a growing body of evidence that human complement inhibitors are major targets for pathogenic microorganisms. ***The Journal of Immunology,*** **1998, 161: 4894–4901.**

The Gram-positive bacterium *Streptococcus pyogenes* (group A streptococcus) is an important human pathogen that causes diseases of the throat and skin. These acute infections may be followed by rheumatic fever and other postinfectious complications that probably are due to autoimmune phenomena (1). During the last decade, much interest has also been focused on *S. pyogenes* as the cause of a toxic shock-like syndrome with high mortality (2).

Among the virulence factors of *S. pyogenes*, particular attention has been paid to the M proteins, which are fibrillar surface structures that inhibit phagocytosis (3, 4). The M proteins exhibit antigenic variation and confer protective immunity that is type specific (5). The structure that is subject to antigenic variation is the N-terminal region, which protrudes into the environment. This region encompasses ~50 to 100 amino acid residues and shows extensive sequence variation between different clinical isolates, but not within a single strain. There are probably >100 different serologic types of M protein, each identified by its hypervariable region. The structural variation within a single type is surprisingly limited, possibly because the existing types have been selected as the most fit variants (6, 7).

Although much is known about the genetic mechanisms that give rise to antigenic variation in microbial virulence factors (8, 9), the exact function of the variable structure is rarely known. However, it can be assumed that the variable structure plays an important role during infection, since it apparently is a major target for the immune system and is not lost by mutation. Here, we describe studies aimed at defining the function of the hypervariable region in M proteins.

A clue to the role of the hypervariable region in M proteins was provided by studies with the human complement inhibitor C4BP[3] (10–12). Several hypervariable regions were found to bind C4BP with high affinity, although the binding regions showed no or little amino acid residue identity. Importantly, bacteria-bound C4BP was found to retain its inhibitory function, suggesting that C4BP contributes to virulence (10). However, many other M proteins, including the extensively studied M5 and M6 proteins, do not bind C4BP (10, 11). Thus, the binding of C4BP is not a general property of hypervariable regions, and the role of this region has remained unclear.

Although the M5 and M6 proteins do not bind C4BP, they bind another human complement inhibitor, the plasma protein factor H (FH) (13–16). However, this property cannot explain the function of the hypervariable region of these M proteins, since FH was reported to bind in the conserved part of the M protein (15, 17). On the other hand, the M5 and M6 proteins were recently found to bind yet another complement inhibitor, the plasma protein FHL-1 (factor H-like protein 1), a naturally occurring splice variant of FH (16, 18). We now report that FHL-1 binds to the hypervariable region of the M5 protein and probably also to the corresponding region of M6. This finding was surprising, but fits with other data

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[3] Abbreviations used in this paper: C4BP, complement factor 4b-binding protein; FH, complement factor H; FHL-1, complement factor H-like protein 1; SCR, short consensus repeat; PBSAT, PBS with 0.02% sodium azide and 0.05% Tween-20; VSG, variant surface glycoprotein; RCA, regulator of complement activation; ^{125}I-FHL-1, ^{125}I-labeled FHL-1.

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indicating that FHL-1 has properties not shared by FH (19). Bacteria-bound FHL-1 was found to retain its complement-inhibitory function, indicating that it is important in pathogenesis. Thus, the studies with FHL-1 and the previous studies of C4BP now make it possible to attribute a specific function to the hypervariable region of many M proteins: the binding of a human complement inhibitor.

Materials and Methods

Bacterial strains, plasmids, and culture conditions

S. pyogenes strain M5 Manfredo (20) was from Dr. M. Kehoe (University of Newcastle-upon-Tyne, U.K.). The *S. pyogenes* M6 strain JRS4 and its isogenic derivatives, JRS145 and JRS251, were from Dr. June R. Scott (Emory University, Atlanta, GA). Strain JRS4 is referred to here as M6. In strain JRS145, the gene encoding protein M6 is deleted; this strain is referred to here as ΔM6. Strain JRS251, herein referred to as M6ΔC, expresses an M6 deletion mutant lacking the entire central C repeat region; however, this protein is expressed on the bacterial surface, since the wall attachment region is intact (21). *Escherichia coli* LE392 was the host for construction of plasmids and for purification of streptococcal proteins. pKEJ1 is a pBR322 derivative carrying the *emm*5 gene of strain M5 Manfredo (11, 20). pSIR2202 is a pK19-derivative carrying the *emm*22(*sir*22) gene, which encodes the Emm22(Sir22) protein (22). The *E. coli-S. pyogenes* shuttle vector pLZ12Spec has been described (23). Shuttle vectors were electroporated into streptococci as described (24). *S. pyogenes* strains were grown in Todd-Hewitt (TH) broth (Difco, Detroit, MI) in 5% CO_2 at 37°C. Strains harboring derivatives of pLZ12Spec were grown in the presence of spectinomycin at 100 μg/ml for streptococci or 20 μg/ml for *E. coli*. The nomenclature for streptococcal genes and proteins used here follows that of Whatmore et al. and Thern et al. (6, 12).

Deletion of the emm*5 gene in strain M5 Manfredo*

The temperature-sensitive shuttle vector pJRS233 (25) was used to delete the entire *emm*5 gene. Two PCR-amplified fragments were prepared first. One of these fragments corresponded to the 3′ part of the *mga*5 gene (located upstream) and part of the intergenic region. The other PCR fragment corresponded to the 5′ part of the *scpA*5 gene (located downstream) and part of the intergenic region. These PCR fragments were ligated into pJRS233, and the kanamycin resistance cassette ΩKm2 (26) was introduced between the two PCR fragments. The resulting plasmid was used to isolate a mutant of strain M5 Manfredo, in which the *emm*5 gene had been replaced with the ΩKm2 cassette, as described (12, 26). The structure of the mutant was verified by PCR analysis. The *mga*5 gene, which positively regulates *emm*5, was shown to be intact in the mutant, as demonstrated by expression of M5 after introduction of the *emm*5 gene on a plasmid. The M5 protein binds fibrinogen (4), and the mutant was found to completely lack fibrinogen-binding ability. Moreover, the strain was shown to lack reactivity with anti-M5 antiserum. This strain is designated herein as ΔM5.

Construction of streptococcal strains expressing chimeric proteins

Isogenic streptococcal strains expressing M5 or Emm22, or one of the chimeric proteins M5-Emm22 or Emm22-M5, were constructed by transforming strain ΔM5 with derivatives of plasmid pLZ12Spec carrying the corresponding four genes. The *emm*5 gene was introduced into pLZ12Spec as described (16). The *emm*22(*sir*22) gene was recovered as a 2.2-kbp *Hin*dIII fragment of pSIR2202 and ligated into pLZ12Spec digested with *Hin*dIII. The chimeric gene *emm*5-*emm*22 was derived from pLZ12Spec carrying the *emm*5 gene, by exchanging the 3′ end of the *emm*5 gene, located downstream of the *Bgl*II site (11), with a *Bgl*II fragment of pSIR2202 encoding the 3′ part of the *sir*22 gene. The chimeric gene *emm*22-*emm*5, which includes the region of *emm*5 located downstream of the *Bgl*II site, was constructed as described (11), recovered on an *Eco*R/*Sph*I fragment, and ligated into pLZ12Spec. The structure of the chimeric genes was verified by DNA sequencing. Surface expression of the four proteins in the ΔM5 strain was verified by analyzing the ability of the transformants to bind various ligands for which the binding sites in the streptococcal proteins are known. As expected, the strain expressing M5 bound fibrinogen (4); the strain expressing Emm22 bound C4BP and IgG (11, 22); the strain expressing M5-Emm22 bound IgG; and the strain expressing Emm22-M5 bound C4BP and fibrinogen.

Purified proteins and antisera

Streptococcal proteins were purified after expression of the corresponding genes in *E. coli* carrying the appropriate plasmids. The Emm22 and Emm22-M5 proteins were purified from osmotic shock lysates, as described (11, 22). (Note that the Emm22 protein was previously referred to as Sir22 (12, 22)). The M5 and M5-Emm22 proteins were purified from whole cell lysates, using affinity chromatography on immobilized fibrinogen or IgG, respectively (11, 22). Small amounts of contaminating proteins in the latter two preparations were removed by Sephacryl S-300 HR gel filtration (Pharmacia, Uppsala, Sweden). Recombinant FHL-1, fitted with a histidine tag, was expressed in the baculovirus system and purified as described (27). Purified FH, factor I, and C3b used for cofactor assays were purchased from Advanced Research Technologies (San Diego, CA). Two other preparations of highly purified FH were the kind gifts of Drs. Andreas Hillarp and Lennart Truedsson (Lund University). Human fibrinogen was from IMCO (Stockholm, Sweden). Sheep anti-human FH serum was from The Binding Site (Birmingham, U.K.).

Absorption of plasma with streptococci and elution of bound proteins

Bacteria in an overnight culture were harvested, washed, and suspended in PBS to 10^{10} bacteria/ml. Bacterial samples of increasing size, pelleted in microfuge tubes, were mixed with 300 μl of pooled human plasma diluted 4× in PBS, and the mixtures were incubated for 1 h at room temperature. The samples had been supplemented with EDTA (0.34 M) to prevent complement activation. After centrifugation, supernatants were collected, and the presence of material reacting with anti-FH serum was analyzed by rocket electrophoresis (28). In elution experiments, incubations were performed essentially as described above. However, for technical reasons elutions could not be performed with very small samples of bacteria, unlike the absorption tests. Therefore, the elution analysis was performed in such a way that several bacterial samples of the same size were incubated with varying amounts of plasma. The ratios (μl plasma)/(number of bacteria) in the three samples analyzed in the elution experiment covered the same range as in the absorption analysis (see Fig. 1). For the elution analysis, three samples of pelleted bacteria (100 μl, corresponding to ~10^{10} bacteria) were mixed with different volumes of EDTA-plasma, diluted 2x× in PBS. After incubation for 1 h at room temperature, the samples were centrifuged and the pellets were washed with 1.5 ml of PBSAT (PBS with 0.02% sodium azide and 0.05% Tween-20). Bound proteins were eluted with 300 μl of 0.1 M glycine-HCl, pH 1.8, for 20 min at room temperature, and the eluates were neutralized.

Binding assays

The ability of whole streptococcal cells to bind ^{125}I-FHL-1 was determined as described (16). Binding of ^{125}I-FHL-1 to purified streptococcal proteins was analyzed after immobilization of the bacterial proteins in microtiter wells. The wells were coated overnight at 4°C with 50 μl of a protein solution (1 μg/ml in PBS). After washing and blocking with cold PBSAT, 50 μl of ^{125}I-FHL-1 (~16,000 cpm) was added to each well, and the plates were incubated for 2 h at 4°C. After washing with PBSAT, the radioactivity associated with each well was determined. Unspecific binding (<0.6%) was determined in control wells and has been subtracted. The purified streptococcal proteins had a similar ability to bind to the plastic wells, as shown by control experiments (data not shown).

Cofactor activity of FHL-1 after binding to bacteria

A pellet containing 2 μl of washed bacteria (~2 × 10^8 bacteria) was resuspended with 18 μl of TBS (50 mM Tris, 150 mM NaCl, pH 7.3) containing 2 μg of pure FHL-1. After incubation for 90 min at room temperature, unbound FHL-1 was removed by washing with 450 μl of TBST (TBS with 0.05% Tween-20). To determine the cofactor activity of bound FHL-1, ^{125}I-labeled C3b (~350,000 cpm, corresponding to 9 ng) and factor I (44 ng) were added in a total volume of 20 μl. After incubation for 2 h at 37°C, the tubes were centrifuged and the supernatants were recovered. For analysis of C3b degradation, samples (~30,000 cpm) were subjected to SDS-PAGE under reducing conditions, and the gels were dried and autoradiographed. As a positive control, pure FHL-1 without bacteria was used as the cofactor, using the same amount of FHL-1 as that added to the bacteria.

Competitive inhibition experiments

The procedures were similar to those previously described (16). The wells of microtiter plates were coated by incubation with a solution of M5 (2 μg/ml) or FH (10 μg/ml). After blocking, radiolabeled ligand (FHL-1 or

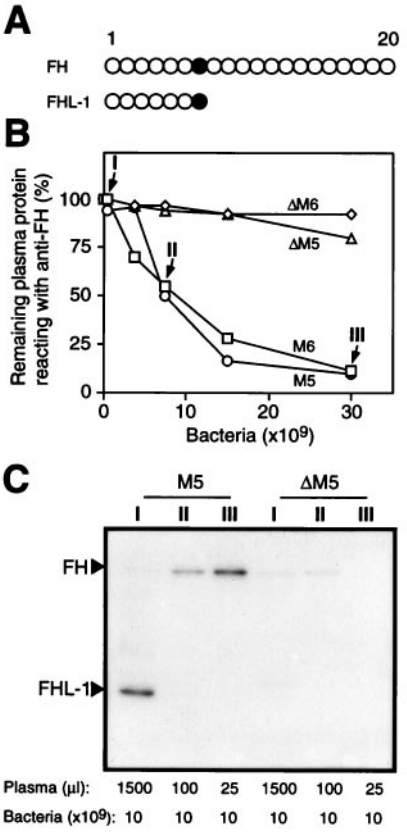

FIGURE 1. Absorption of FH and FHL-1 from plasma by whole streptococci. *A*, Schematic representation of FH and its naturally occurring splice variant FHL-1. Each circle represents one SCR module. SCR7, which is required for the binding to M protein, is shaded. The cofactor activity requires SCR1–4. *B*, Absorption of plasma with M5- or M6-expressing streptococcal strains and with isogenic mutants (ΔM5 and ΔM6) lacking expression of M5 or M6. Bacterial samples of increasing size were incubated with a fixed amount of pooled human EDTA-plasma, and the absorbed plasma samples were analyzed for remaining material reacting with anti-FH. Each data point represents one sample. These experiments were performed three times with similar results. *C*, Elution analysis of plasma proteins bound to streptococci. Plasma and bacteria were mixed in the same ratios as in the three samples marked I–III in *B*. For technical reasons, the elution analysis was performed with samples containing fixed numbers of bacteria, which were incubated with different amounts of EDTA-plasma. Bound proteins were eluted, and the eluates were subjected to Western blot analysis under reducing conditions using anti-FH serum to identify FH and FHL-1. After incubation with radiolabeled protein G, bound Abs were detected by autoradiography. These experiments were performed five times, with similar results.

M5; ~16,000 cpm) was added together with the purified protein indicated. The wells were incubated for 2 h at 4°C and then washed, and the radioactivity associated with each well was determined.

Results

Selective binding of FHL-1 to the M5 and M6 proteins

The human complement regulators FH and FHL-1 are plasma proteins that cause degradation of C3b by acting as cofactors for the protease factor I (27, 29–32). In addition, both FH and FHL-1 reduce the formation of C3b by causing decay of the C3 convertase of the alternative pathway (29, 31, 33). The 150-kDa FH protein is composed of 20 short consensus repeat (SCR) modules (Fig. 1*A*) and has a plasma concentration of ~400 μg/ml, while the 42-kDa FHL-1 protein, which is composed of the first 7 SCRs of FH, has a molar concentration that is 2 to 10 times lower (19). Previous work has shown that both of these plasma proteins bind to the streptococcal M5 and M6 proteins (13–17).

To study the ability of FH/FHL-1 to bind to surface-exposed M proteins in a physiologic setting, samples of plasma were absorbed with increasing amounts of bacteria expressing the M5 or M6 protein (Fig. 1*B*). The plasma was supplemented with EDTA to prevent complement activation. After absorption, the plasma samples were analyzed for remaining FH and FHL-1 (detected together as material reacting with anti-FH antiserum). The M5 and M6 strains efficiently bound plasma proteins reacting with anti-FH, but isogenic strains without M protein lacked this ability.

Since the absorption analysis did not distinguish between binding of FH and FHL-1, proteins that had bound to M5 bacteria were eluted, and the eluates were analyzed for presence of FH and FHL-1 by Western blot. The samples that were analyzed corresponded to arrows I through III in Figure 1*B* and represent samples with very different ratios of bacteria to plasma. For technical reasons (see *Materials and Methods*), the elution analysis was performed with samples containing fixed numbers of bacteria, which were incubated with different amounts of plasma (Fig. 1*C*). The analysis showed that FHL-1 was present in eluate I, while the other two eluates contained little or no FHL-1 (Fig. 1*C*, *left*). The absence of FHL-1 from eluates II and III is most likely due to the small amounts of plasma used, allowing too little FHL-1 to bind in these samples. As expected, the binding of FHL-1 in sample I required expression of the M5 protein, as shown by a control experiment with the mutant lacking M protein (Fig. 1*C*, *right*).

Surprisingly, the presence of FH in the eluates did not parallel that of FHL-1. Thus, FH was not present in eluate I, but was found in increasing amounts in the other two eluates. The lack of binding of FH in sample I can be explained by the presence of fibrinogen, which is found in high concentration in plasma and binds to M protein, thereby blocking the binding of FH, but not of FHL-1, to the M protein (see below). In samples II and III, in which relatively small volumes of plasma were used for the absorption, the bacteria caused depletion of fibrinogen, allowing binding of FH (data not shown).

Elution analysis with the M6 strain and its isogenic M6-deficient mutant gave results similar to those obtained with the M5 system (data not shown). Thus, the elution experiments indicated that the M5 and M6 proteins bind FHL-1, rather than FH, when bacteria are exposed to a large volume of plasma. Since this situation corresponds to the initial stages of a streptococcal infection, when bacteria enter the bloodstream or encounter plasma proteins transudated into the respiratory pathway (34), the emphasis of the following work was placed on studies of FHL-1. Pure FHL-1 and the M5 system were used for this work.

FHL-1 binds to the hypervariable N-terminal region of the M5 protein

In agreement with the results obtained in elution experiments, pure FHL-1 was found to bind to M5-expressing bacteria, but not to the isogenic M5-deficient strain (Fig. 2*A*).

Chimeric proteins were used to localize the region in M5 that binds FHL-1. Different parts of M5 were combined with another M protein, the Emm22 protein (12, 22), which does not bind FHL-1 (see below). The Emm22 protein was chosen as the fusion partner, since the dimeric coiled-coil structure, that is a typical feature of M proteins, may be necessary for the binding of ligands (35, 36).

The chimeric protein M5-Emm22 includes the N-terminal 103 amino acids of M5, corresponding to the hypervariable region of the protein (3, 37), whereas the reciprocal construct Emm22-M5 includes the 347 C-terminal amino acids of the M5 protein (Fig. 3*A*). The four proteins shown in Figure 3*A* were expressed by

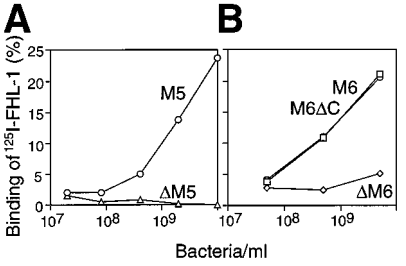

FIGURE 2. Binding of ^{125}I-FHL-1 to different strains of *S. pyogenes* as a function of bacterial concentration. *A*, Binding of FHL-1 to the M5-expressing strain and its isogenic M-negative mutant, ΔM5. *B*, Binding of FHL-1 to the M6 strain and two isogenic derivatives. M6 designates the wild-type strain, M6ΔC is a mutant expressing M6 lacking the central C repeat region, and ΔM6 is a mutant lacking the whole M6 protein. These experiments were performed twice with similar results.

transformants of the M5-deficient strain, and the four transformants were studied for ability to bind FHL-1 (Fig. 3*B*). Only strains expressing the M5 or the M5-Emm22 protein bound FHL-1, indicating that the FHL-1 binding region of M5 is located in the N-terminal hypervariable part of the protein.

The binding properties of the four proteins were further analyzed in experiments employing highly purified preparations. Western blot analysis showed that the M5 and M5-Emm22 proteins bound FHL-1, while the Emm22 and Emm22-M5 proteins lacked this binding property (Fig. 3*C*). Finally, the ability to bind FHL-1 was analyzed after immobilization of the four purified proteins in microtiter wells (Fig. 3*D*). Only the M5 and M5-Emm22 proteins bound FHL-1. Together, these experiments show that FHL-1 binds to the N-terminal hypervariable region of M5.

Competitive inhibition experiments with different ligands of the M5 protein

The M5 protein not only binds FHL-1, but also binds FH (16). Moreover, M5 binds fibrinogen via the B repeat region (4). Possible effects of FH and fibrinogen on the binding of FHL-1 were studied in inhibition experiments.

The binding of radiolabeled FHL-1 to M5 was inhibited by unlabeled FHL-1, as expected, but not by fibrinogen (Fig. 4*A*). Thus, the binding of fibrinogen to the B repeat region does not block the binding of FHL-1 to the N-terminal region. The binding of FHL-1 to M5 was inhibited very poorly by FH, suggesting that these two structurally related ligands may bind to different sites in M5.

The interaction between FH and the M5 protein was also analyzed (Fig. 4*B*). This binding was inhibited by FH, as expected, but

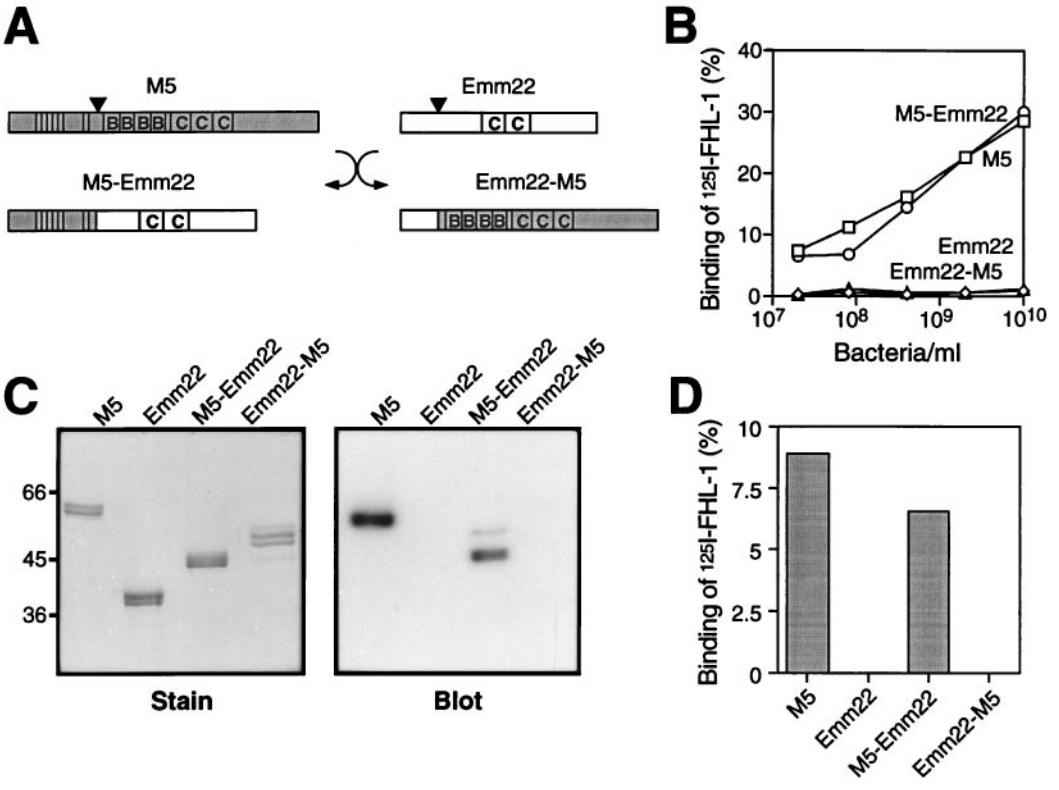

FIGURE 3. Ability of chimeric streptococcal proteins to bind FHL-1. *A*, Schematic representation of the processed forms of the two M proteins M5 (450 amino acid residues) and Emm22 (324 residues) and two chimeric proteins derived from these M proteins. The letters B and C indicate the positions of different types of repeats; the M5 protein also has short A repeats in the N-terminal region, as indicated by vertical lines. The chimeric protein M5-Emm22 includes the N-terminal 103 amino acid residues of M5 and the C-terminal 267 residues of Emm22. The chimeric protein Emm22-M5 includes the N-terminal 57 residues of Emm22 and the C-terminal 347 residues of M5. *B*, Binding of ^{125}I-FHL-1 to *S. pyogenes* strain ΔM5 transformed with plasmids encoding M5 (□), Emm22 (◇), M5-Emm22 (○), or Emm22-M5 (△). *C*, Western blot analysis. Highly purified preparations of the four proteins shown in *A* were subjected to SDS-PAGE under reducing conditions. The blotting membrane was incubated with FHL-1 (1 μg/ml), followed by sheep anti-human FH serum (diluted ×1000). After washing and incubation with ^{125}I-protein G, bound Abs were detected by autoradiography. No reaction was observed if the incubation step with FHL-1 was omitted. Molecular mass markers are in kilodaltons. *D*, Highly purified preparations of the four proteins shown in *A* were used to coat microtiter wells. The immobilized streptococcal proteins were analyzed for ability to bind ^{125}I-FHL-1. Each of the experiments shown in *B*, *C*, and *D* was performed three times with similar results.

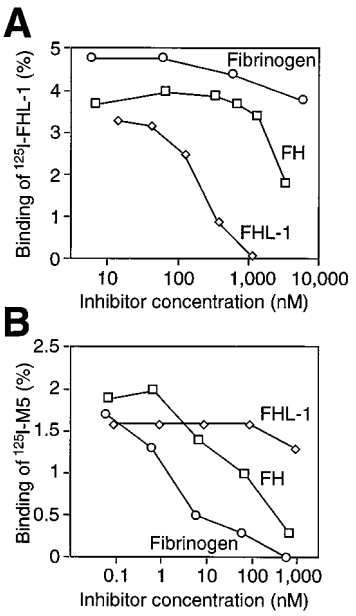

FIGURE 4. Competitive inhibition tests. *A*, Analysis of the interaction between FHL-1 and M5. The binding of ^{125}I-FHL-1 to M5 immobilized in microtiter wells was inhibited with different proteins, as indicated. *B*, Analysis of the binding between FH and M5. The binding of ^{125}I-M5 to FH immobilized in microtiter wells was inhibited with different proteins. These experiments were performed twice with similar results.

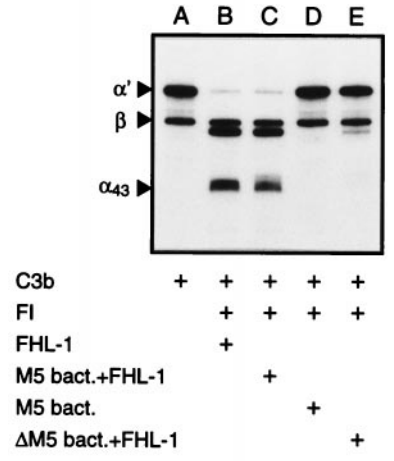

FIGURE 5. Cofactor activity of FHL-1 bound to the M5 strain. *Lane A*, ^{125}I-labeled C3b. The α' and β polypeptides are seen. *Lane B*, ^{125}I-labeled C3b incubated with factor I and FHL-1. *Lane C*, ^{125}I-labeled C3b incubated with factor I and with FHL-1 bound to the M5 strain. The bacteria were preincubated with FHL-1 and carefully washed before being use as a cofactor. *Lane D*, ^{125}I-labeled C3b and factor I incubated with the M5 strain. *Lane E*, ^{125}I-labeled C3b and factor I incubated with the non-M5-expressing strain ΔM5, which was preincubated with FHL-1. The same amount of FHL-1 was used for the samples analyzed in *lanes A*, *B*, *C*, and *E*. This experiment was performed twice with similar results.

was inhibited only weakly, if at all, by FHL-1. However, the binding of FH to M5 was completely inhibited by fibrinogen, suggesting that FH and fibrinogen have overlapping binding sites in the B repeat region of M5. The ability of fibrinogen to inhibit the binding of FH is in agreement with data reported for the M6 protein (38) and may explain the surprising result with FH obtained in the elution analysis (Fig. 1*C*).

Taken together, the inhibition experiments indicate that the major binding sites for FHL-1 and FH are located in separate regions of M5 and that FH may bind in the B repeat region of M5. This conclusion is in good agreement with the elution analysis, which gave very different results for FHL-1 and FH (Fig. 1*C*). No further attempts were made to characterize the binding site(s) for FH in M5, since only FHL-1 appeared to interact with the streptococci under physiologic conditions (see Fig. 1*C*).

Binding of FHL-1 to the M6 protein: evidence for binding in the N-terminal region

Like M5, the extensively studied M6 protein binds both FH and FHL-1 and shows selective binding of FHL-1 in human plasma (see above; and Refs. 13 and 16). It has been reported that FH binds to M6 in the central C repeat region (17), which is homologous to the central C repeat region of the M5 protein (cf Fig. 3*A*). It was therefore of interest to analyze the role of the C repeat region for binding of FHL-1. Isogenic mutants of an M6 strain were used for this analysis (21).

FHL-1 bound equally well to the wild-type M6 strain and to a mutant expressing M6 protein without C repeats, but an isogenic strain lacking expression of M6 did not bind FHL-1 (Fig. 2*B*). Thus, the central C repeat region is not required for the binding of FHL-1 to M6. Moreover, the binding of FHL-1 to M6 was not inhibited by fibrinogen, a ligand that most likely binds to the B repeats of the M6 protein, which are almost identical to the fibrinogen-binding B repeats of M5 (data not shown; Ref. 4). Since the most C-terminal part of the M6 protein is hidden in the bacterial cell wall (3), these data indicate that the binding site for FHL-1 in M6 is located in the N-terminal part of the protein, as found to be the case for the M5 protein.

FHL-1 retains its cofactor activity when bound to streptococci

The cofactor activity of streptococcus-bound FHL-1 was analyzed in a C3b degradation assay (Fig. 5).

The C3b molecule is composed of two polypeptides, the α'- and β-chains (Fig. 5, *lane A*). As expected, incubation of C3b with FHL-1 and factor I caused cleavage of the α'-chain, and appearance of a 43-kDa fragment (*lane B*). When FHL-1 was replaced with M5-expressing streptococci that had been preincubated with FHL-1, degradation of the α'-chain was also observed (*lane C*). In contrast, degradation of the α'-chain did not occur with M5-deficient streptococci that had been preincubated with FHL-1 (*lane E*). The degradation of C3b was not due to a protease activity in the M5-expressing bacteria, as shown by incubation of C3b and M5 bacteria in the absence of FHL-1 (*lane D*). Together, these results indicate that FHL-1 retains its cofactor activity when bound to streptococci.

Discussion

The data reported here indicate that the human complement inhibitor FHL-1 binds to the N-terminal hypervariable region of the M5 and M6 proteins, two well-known M proteins. The hypervariable region of these M proteins has long been suspected to play an important role in pathogenesis, but its function has been elusive (3, 4, 39). The interaction with FHL-1 now makes it possible to propose a model for the function of the hypervariable region in pathogenesis (Fig. 6).

S. pyogenes is a Gram-positive bacterium, which is not sensitive to the lytic effect of complement but is attacked through surface deposition of C3b. After the initial deposition of a C3b molecule, the amplification system of the alternative pathway rapidly increases the deposition of C3b, making the bacteria sensitive to

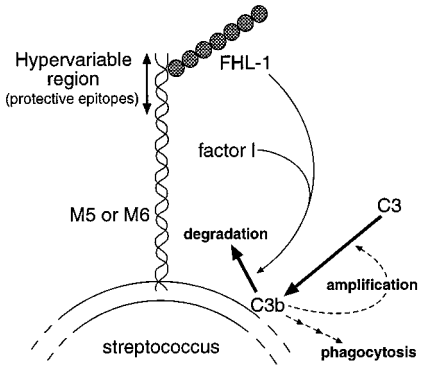

FIGURE 6. Schematic model for phagocytosis resistance in *S. pyogenes* expressing the M5 or M6 protein. The M protein, a dimer with coiled-coil structure, uses the hypervariable region to bind FHL-1. Bound FHL-1 acts as a cofactor for the factor I-mediated degradation of surface deposited C3b. If C3b is not degraded, an amplification system causes the formation of more surface deposited C3b, which promotes phagocytosis. Thus, the binding of FHL-1 prevents phagocytosis. The model predicts that the FHL-1-binding region would be a major target for immune attack, explaining the hypervariability in this region. The model may also explain why Abs to epitopes outside of the hypervariable region normally are not protective, because they would not block the binding of FHL-1 (see text).

phagocytosis (40). However, phagocytosis can be prevented by the degradation of C3b. Since streptococcus-bound FHL-1 promotes the degradation of C3b, it seems likely that bound FHL-1 contributes to phagocytosis resistance. It follows that the FHL-1-binding region in M protein would be an important target for protective Abs, a situation that would favor antigenic variation. Thus, the binding of FHL-1 to the N-terminal region of the M protein provides an explanation for the hypervariability in that region. The model may also explain why Abs to epitopes outside of the hypervariable region normally do not promote phagocytosis (39, 41). Such Abs would not block the binding of FHL-1, and degradation of C3b would continue. However, phagocytosis may require the presence of both Ig and C3b on the bacterial surface (42, 43). Therefore, only Abs directed against the FHL-1-binding region would promote phagocytosis. Thus, the model provides a molecular explanation for the occurrence of "protective epitopes," a concept that is of central importance in microbial pathogenesis and vaccine development.

Previously, we studied several members of the M protein family and showed that the N-terminal hypervariable region of these proteins binds the human complement inhibitor C4BP, a high m.w. plasma protein (11). When these studies were performed, the C4BP-binding proteins were known to be Ig-Fc-binding members of the structurally defined M protein family (10, 44), but it was not known whether they have the antiphagocytic function that characterizes "classical" M proteins such as M5 and M6. However, a recent study indicates that this is indeed the case (12). Thus, many antiphagocytic M proteins bind either of the two human complement inhibitors, FHL-1 or C4BP, and the binding sites for these ligands are located in the hypervariable region of the M proteins. This situation suggests that FHL-1 and C4BP may have similar functions in streptococcal pathogenesis, although they have been assigned different functions in complement regulation. C4BP is generally considered an inhibitor of the C3 convertase of the classical pathway (45), while FHL-1 has been identified as an inhibitor of the C3 convertase of the alternative pathway (18, 32). However, several lines of evidence now indicate that the functions of C4BP and FHL-1 overlap (46–48), which suggests that they may be used for the same purpose by *S. pyogenes*.

Previous work has shown that FH binds to the M6 protein (13, 14), and a binding site for FH was localized to the conserved C repeat region of this M protein (17). The finding that FHL-1 binds to the hypervariable region of M5 and M6 may therefore seem surprising. However, the inhibition experiments reported here support the conclusion that the major binding sites for FHL-1 and FH are located in separate regions of the M5 protein. A possible explanation for this situation is provided by data showing that the FH molecule can fold back on itself (49), suggesting that ligand-binding regions may be hidden in the native conformation of FH, but exposed in FHL-1. Indeed, FHL-1, but not FH, has been found to promote the attachment of mammalian cells (19). The complexity of the interaction between FH and M protein is underlined by the work of Sharma and Pangburn (14), who studied M6-expressing bacteria and found evidence for a second FH-binding site in M6, in addition to the binding site in the C repeat region. It has been suggested that this second FH-binding site in M6 is located close to the N-terminal part of the C repeat region (15). However, it also seems possible that FH binds to the B repeat region, since the binding of FH to M6 to a large extent can be inhibited by fibrinogen, which most likely binds to the B repeats (4, 38). The binding of FH to M proteins was not analyzed further in this study, since FHL-1 appears to be a more important ligand under physiologic conditions.

The finding that *Streptococcus*-bound FHL-1 retains its cofactor activity was not unexpected, since the cofactor activity of FHL-1 has been localized to SCR1–4, while the M protein binding site was located to SCR7 (15, 16, 27, 50). However, it cannot be excluded that some of the cofactor activity in our assay was due to FHL-1 that had dissociated from the bacteria during the experiment. In a streptococcal infection, such dissociation may be an advantage, since an increased concentration of free FHL-1 in the bacterial microenvironment could contribute to the protection against complement attack.

Since the well-known M5 and M6 proteins use the hypervariable region to bind FHL-1, it seems likely that many M proteins have this property. This raises the question of the structural basis for the interaction with FHL-1. We propose that regions with very different sequences have similar structures, allowing them to bind FHL-1. Support for this notion comes from studies of the *Trypanosoma brucei* variant surface glycoprotein (VSG), a protein that shows extensive antigenic variation (8, 9). Studies of two VSG variable domains with little sequence identity showed that they have similar structures (51), suggesting that they may also have similar function. However, the function of the variable domain in VSGs is not known. Our identification of a ligand for the hypervariable region of the M5 and M6 proteins implies that structural analysis of these regions may be particularly rewarding for the analysis of protein-protein interactions and for studies of sequence constraints in antigenic variation. The same argument applies to the interaction between certain hypervariable regions and C4BP.

FHL-1 is a member of the RCA (regulator of complement activation) family of proteins, a complex system of complement inhibitors present in plasma and on cell membranes (31). Accumulated evidence indicates that these proteins not only act as human complement inhibitors but are also major targets for pathogenic microorganisms. Early studies in this field showed that EBV attaches to the RCA protein CR2 on B cells (52) and that *S. pyogenes* binds the plasma protein FH (13). More recent studies have shown that the RCA proteins CD46 and CD55 act as receptors for measles virus, echoviruses, *Neisseria*, *S. pyogenes*, and/or some strains of *E. coli* (53–59). The RCA protein CR1 may be a receptor for proteins expressed on the surface of *Plasmodium falciparum*-infected erythrocytes (60). The plasma protein C4BP not only binds

to many strains of *S. pyogenes* but also binds to *Bordetella pertussis* (61). Moreover, FH contributes to the serum resistance of *Neisseria gonorrhoeae* by binding to sialic acid residues on the bacterial surface (62). These examples, and the data reported here, indicate that interactions with RCA proteins are of general importance in microbial pathogenesis.

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