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# Evasion of Phagocytosis through Cooperation between Two Ligand-binding Regions in *Streptococcus pyogenes* M Protein

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## Abstract

The M protein of *Streptococcus pyogenes* is a major bacterial virulence factor that confers resistance to phagocytosis. To analyze how M protein allows evasion of phagocytosis, we used the M22 protein, which has features typical of many M proteins and has two well-characterized regions binding human plasma proteins: the hypervariable NH<sub>2</sub>-terminal region binds C4b-binding protein (C4BP), which inhibits the classical pathway of complement activation; and an adjacent semivariable region binds IgA-Fc. Characterization of chromosomal *S. pyogenes* mutants demonstrated that each of the ligand-binding regions contributed to phagocytosis resistance, which could be fully explained as cooperation between the two regions. Deposition of complement on *S. pyogenes* occurred almost exclusively via the classical pathway, even under nonimmune conditions, but was down-regulated by bacteria-bound C4BP, providing an explanation for the ability of bound C4BP to inhibit phagocytosis. Different opsonizing antisera shared the ability to block binding of both C4BP and IgA, suggesting that the two regions in M22 play important roles also under immune conditions, as targets for protective antibodies. These data indicate that M22 and similar M proteins confer resistance to phagocytosis through ability to bind two components of the human immune system.

Key words: innate immunity • group A streptococcus • phagocytosis resistance • complement • IgA

## Introduction

A pathogenic microorganism infecting a nonimmune host must evade attack from the innate immune system before infection can be established (1). For extracellular pathogens, such as many bacteria, it may be particularly important to evade phagocytosis by neutrophils, which are rapidly recruited to the site of an infection (2). Indeed, many bacterial pathogens express surface structures that interfere with phagocytosis by neutrophils (2–4). A classical example of such an antiphagocytic surface molecule is the M protein of *Streptococcus pyogenes* (group A streptococcus), which allows this pathogen to grow rapidly in human blood (5). Here, we analyze the mechanism by which M protein allows *S. pyogenes* to evade phagocytosis.

*S. pyogenes* is a gram-positive bacterium causing a variety of diseases, including acute pharyngitis, skin infections, a toxic shock syndrome, and rheumatic fever (6). The surface M protein of *S. pyogenes* plays a key role in conferring

phagocytosis resistance (5), but the hyaluronic acid capsule expressed by many strains also makes an important contribution (7). The M protein is a dimeric coiled coil with an NH<sub>2</sub>-terminal hypervariable region (HVR) that exhibits extensive sequence variability between strains, allowing classification of clinical *S. pyogenes* isolates into ~120 different M types (8).

Antibodies directed against the HVR block the antiphagocytic property of M protein, implying that the HVR plays an important role in conferring phagocytosis resistance (5, 9, 10). However, the extreme sequence variability in the HVR has made it difficult to explain how this region can have the specific function to inhibit phagocytosis. To explain this apparent paradox, it has been suggested that the HVR causes repulsion of phagocytes by having a negative charge, a mechanism that might allow extensive sequence variability (5, 11). In agreement with this hypothesis, M protein is known to inhibit binding of *S. pyogenes* to non-activated phagocytes (12). However, evidence has accu-

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Abbreviations used in this paper: C4BP, C4b-binding protein; HVR, hypervariable region; TH, Todd-Hewitt.

mulated that the HVR also has a specific ligand-binding function. In particular, the HVRs of many M proteins specifically bind the human complement regulator C4b-binding protein (C4BP), a ~570-kD plasma protein that inhibits the classical pathway of complement activation by accelerating the decay of the C3 convertase of this pathway and by acting as a cofactor to factor I in the degradation of C4b (13–17). Importantly, >50% of clinical *S. pyogenes* isolates bind C4BP, and all available evidence indicates that this property is due to expression of an M protein in which the HVR binds C4BP (13–15, 18).

In a previous paper, we showed that binding of C4BP to the HVR can partially explain the ability of an M protein to confer phagocytosis resistance (19), but the mechanism by which the M protein confers full resistance to phagocytosis remained unclear. Moreover, the contribution of bacteria-bound C4BP to phagocytosis resistance was puzzling, because C4BP specifically down-regulates the classical pathway of complement activation (16, 17), which is commonly believed not to be activated under nonimmune conditions. The data reported here provide explanations for both of these problems.

Our studies were focused on the M22 protein (19), which has properties typical of many M proteins and is commonly expressed by clinical isolates of *S. pyogenes* (20). In addition to an HVR that binds C4BP, the M22 protein has an adjacent semivariable region that binds human IgA-Fc, another common M protein ligand (21–24). Because the IgA-binding region varies in sequence between different M proteins (23), we hypothesized that this region is a target for protective antibodies and contributes to phagocytosis resistance.

The studies described here show that the C4BP- and IgA-binding regions of the M22 protein cooperate in conferring phagocytosis resistance and can fully account for the antiphagocytic property of M22. Moreover, bacteria-bound C4BP down-regulated complement activation, that occurred via the classical pathway even under nonimmune conditions, providing an explanation for the ability of bound C4BP to inhibit phagocytosis. Thus, a coherent picture is now emerging of the mechanisms, by which M22 and related M proteins confer phagocytosis resistance.

## Materials and Methods

**Bacterial Strains.** *S. pyogenes* AL168 is an OF<sup>+</sup> strain of serotype M22. Like other OF<sup>+</sup> strains of *S. pyogenes*, this strain has three adjacent genes encoding members of the M protein family, the *mrp*, *emm*, and *enm* genes (25). The strain referred to here as M22 positive is strain AL168*mrp22<sup>-</sup>emm22<sup>+</sup>*, which expresses the M22 (Sir22) protein, but not the Mrp22 protein, which also contributes to phagocytosis resistance (25). The isogenic strain referred to as M22 negative lacks expression of the M22 and Mrp22 proteins (25). The *Escherichia coli* XL-1 strain and the pUC19 vector were used for subcloning. *S. pyogenes* was grown without shaking in Todd-Hewitt (TH) broth (Difco) or TH supplemented with 0.2% yeast extract in 5% CO<sub>2</sub> at 37°C.

**Mutations in the Chromosomal *emm22* Gene That Specifically Block the Ability of *S. pyogenes* to Bind IgA or C4BP.** A mutant ( $\Delta 14$ ) of strain AL168 lacking the ability to bind IgA was constructed by

the introduction of an in frame deletion in the *emm22* gene (26), corresponding to 14 amino acid residues (67–80) in the IgA-binding region (27). A ~900-nucleotide fragment from the *emm22* gene, including the region to be deleted and ~430 nucleotides on either side, was PCR amplified from AL168 DNA, using forward and reverse primers with BamHI and EcoRI sites, respectively. After ligation into pUC19, the construct was transformed into *E. coli* XL-1, and transformants were screened by PCR for the presence of the insert. One positive clone was verified by DNA sequencing. The deletion was introduced by a second PCR using forward and reverse primers complementary to regions downstream and upstream of the desired deletion, respectively, thereby amplifying the entire construct, including the vector, except the sequence to be deleted. The PCR product was ligated with blunt ends and transformed into *E. coli* XL-1, and transformants were screened by PCR for inserts with the desired deletion and verified by DNA sequencing.

To introduce the  $\Delta 14$  mutation into the chromosome of *S. pyogenes*, the mutated *emm22* gene was transferred by PCR from pUC19 to pJRS233, a shuttle vector that exhibits temperature-sensitive replication in *S. pyogenes* (28). The derivative of pJRS233 was electroporated into AL168*mrp22<sup>-</sup>emm22<sup>+</sup>*, and the desired recombinant was isolated essentially as described previously (25, 28). A clone of the desired type was identified, and the sequence of the entire mutated chromosomal *emm22* gene, including the promoter region, was verified by DNA sequencing.

A strain ( $\Delta 7$ ) expressing an M22 variant with a seven-residue deletion in the C4BP-binding region has been described previously (19). A strain carrying both of the  $\Delta 7$  and  $\Delta 14$  mutations was constructed essentially as described for the  $\Delta 14$  strain, except that the ~900-nucleotide fragment was PCR-amplified from chromosomal DNA derived from the  $\Delta 7$  strain. The sequence of the mutated chromosomal gene, including the promoter region, was verified by DNA sequencing. This double mutant is referred to as  $\Delta 7\Delta 14$ . A revertant (rev.) strain, in which the wild-type *emm22* gene was reintroduced into the  $\Delta 7\Delta 14$  mutant, was constructed as described previously (19).

**Purified Proteins and Synthetic Peptides.** Human IgA was purchased from Cappel Organon-Teknika. Purified human C4BP was provided by B. Dahlbäck (Lund University, Lund, Sweden). The IgG-Fc-binding reagents protein A and protein G were purchased from Amersham Biosciences. The M22 (Sir22) protein was purified as described previously (26). Synthetic ~50-residue peptides derived from different M protein regions were prepared with a COOH-terminal Cys residue, not present in the M protein, allowing dimerization and strongly enhancing ligand-binding (15, 29). The 53-residue peptide M22-N and the 50-residue peptide Sap22 were derived from the C4BP- and IgA-binding regions of M22, respectively (15, 27). The 54-residue C22 peptide was derived from residues 131–182 in the conserved C-repeat region of M22, with addition of the sequence Tyr-Cys at the COOH-terminal end; the Tyr residue was added to facilitate radiolabeling. The 53-residue M5-N peptide includes the NH<sub>2</sub>-terminal part of the processed form of the M5 protein (15). These ~50-residue peptides were purchased from the Department of Clinical Chemistry, Malmö General Hospital and were dimerized as described previously (15). The 19-residue synthetic peptide M22 (1–19), derived from the NH<sub>2</sub>-terminal sequence of the processed form of the M22 protein, was conjugated to ovalbumin via an added COOH-terminal Cys residue (Medprobe).

**Antisera and mAbs.** Rabbit antisera to dimerized preparations of the synthetic peptides M22-N, Sap22, and C22 were prepared as described previously (15, 19). Rat antisera against a synthetic

peptide, M22 (181–201), derived from the conserved C-repeat region of the M22/M4 proteins, were prepared as described previously (23). Mouse antiserum against the synthetic peptide M22 (1–19) was raised by immunizing NMRI mice twice with 20  $\mu$ g OVA-peptide conjugate in Freund's adjuvants. Mouse mAbs against human C3d, C4c, and C4d were from Quidel Corp. The mouse anti-C4BP mAb67 (30) was a gift from B. Dahlbäck. Mouse IgG1 (Quidel Corp.) was used as a control because all monoclonal antibodies used were of this isotype. FITC-conjugated goat F(ab')<sub>2</sub> anti-mouse IgG (DakoCytomation) was used for detection. Rabbit antisera against the overlapping M22-N and Sap22 peptides were made specific for the homologous peptide by passing 2-ml samples repeatedly through 1-ml HiTrap columns (Amersham Biosciences) containing 5 mg of immobilized heterologous peptide.

**Affinity Chromatography.** 5 mg of dimerized preparations of the M22-N, Sap22, and M5-N peptides were immobilized in 1-ml HiTrap columns, as described previously (15). 1 ml of human plasma, prepared with hirudin (final concentration, 140 U/ml; Calbiochem) as anticoagulant, was diluted 1:5 in PBS and applied to each column, which was washed with 10 ml PBS. The columns were eluted as described previously (15, 29) and eluates were dialyzed against PBS.

**Binding Assays.** Binding of <sup>125</sup>I-labeled human C4BP and IgA to whole streptococci was analyzed as described previously (25). Binding is expressed as a percentage of that observed with the positive control, which was ~60% for both C4BP and IgA. Binding of rat antibodies to whole bacteria was detected with radiolabeled protein A (31). Binding (<3%) in control tubes with preimmune rat serum was deduced.

The reactivity of different rabbit antisera with the M22 protein and with synthetic peptides was analyzed in microtiter wells coated with 1  $\mu$ g/ml of pure M22 or 2  $\mu$ g/ml of peptide, as indicated, and detected with radiolabeled protein G, essentially as described previously (15, 19).

The ability of antisera to inhibit the interaction between M22 and radiolabeled C4BP or IgA was analyzed in microtiter wells

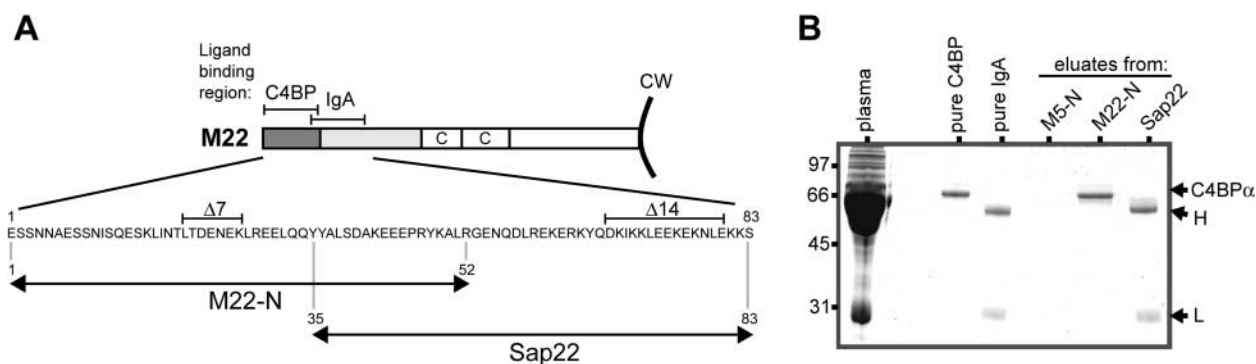
coated with pure M22 protein, as described previously (19). Inhibition is expressed as a percentage of the binding observed in the absence of antiserum, which was ~25% for each ligand.

The ability of whole bacteria to inhibit binding between pure M22 protein and mouse anti-M22 (1–19) was analyzed essentially as described previously (32). In brief, washed bacteria were used to inhibit the binding of antibodies (diluted 100-fold) to microtiter wells coated with 1  $\mu$ g/ml M22. After washes, bound mouse IgG was detected by the addition of rabbit anti-mouse IgG, followed by radiolabeled protein G.

**Phagocytosis Assays.** The assays were performed essentially as described previously (33). Overnight cultures of bacteria in TH were diluted 1:50 in TH and grown to  $A_{620} = 0.15$  without agitation at 37°C in 5% CO<sub>2</sub>. After dilution in TH, 25  $\mu$ l of bacterial suspension, containing ~50 CFU, was mixed with 250  $\mu$ l of freshly drawn human blood, using hirudin (final concentration, 140 U/ml; Calbiochem) as anticoagulant (25), and the tubes were rotated at 37°C for 3 h. Bacterial titers in the inoculum and after incubation were analyzed by the pour plate method. Because growth may vary between assays performed on different occasions and with different blood donors, growth is expressed as a fraction of that seen with the wild-type control strain. The average increase in titer ("multiplication factor") for the wild-type strain is given in the figure legends. The opsonizing effect of rabbit antiserum was analyzed by the addition of a 50- $\mu$ l sample to a phagocytosis system, using preimmune rabbit serum as control.

The phagocytosis assays used blood from "nonimmune" human donors (i.e., blood that allowed growth of the M22-expressing strain and, therefore, did not contain opsonizing anti-M22 antibodies). Among several donors tested in preliminary experiments, blood from most donors allowed growth of the M22-expressing strain, and blood from three such donors was used in each set of phagocytosis experiments, with similar results for each donor.

**Analysis of Complement Deposition on *S. pyogenes* by Flow Cytometry.** Overnight cultures of bacteria in TH were diluted 1:20 in 10 ml TH and grown to  $A_{620} = 0.4$  without agitation at 37°C in



**Figure 1.** Schematic representation of the M22 protein and characterization of ligand-binding regions by affinity chromatography. (A) The M22 protein can be divided into an NH<sub>2</sub>-terminal hypervariable region (dark gray; HVR), a semivariable region (light gray), and a conserved COOH-terminal region with two C-repeats. The locations of two well-characterized regions binding human C4BP and IgA-Fc, respectively, are indicated. A less well-defined region binding human IgG-Fc (not depicted) is most likely located on the COOH-terminal side of the IgA-binding region (26, 27). The C4BP- and IgA-binding regions can be studied as ~50-residue synthetic peptides, designated M22-N and Sap22, respectively, for which the amino acid sequences are indicated, except that the peptides also include a COOH-terminal Cys residue to allow dimerization (15, 29). The positions of a 7-residue deletion in the C4BP-binding region ( $\Delta 7$ ) and a 14-residue deletion in the IgA-binding region ( $\Delta 14$ ) are indicated. CW, streptococcal cell wall. (B) Affinity chromatography demonstrating that the C4BP- and IgA-binding regions of M22 bind their ligands with high specificity. Whole human plasma was applied to columns containing the immobilized peptides M22-N or Sap22. A control column contained the M5-N peptide, derived from an M protein that does not bind C4BP or IgA. Proteins bound to the columns were eluted and analyzed by SDS-PAGE under reducing conditions. The Coomassie-stained gel shows whole human plasma, reference samples of pure human C4BP and IgA, and eluates from the columns, as indicated. The ~70-kD band in the M22-N eluate was identified as the C4BP  $\alpha$ -chain, and the two bands in the Sap22 eluate were identified as the heavy (H) and light (L) chains of IgA.



5% CO<sub>2</sub>. After two washes in 10 ml PBS, 300- $\mu$ l samples of bacterial suspensions ( $\sim 5 \times 10^6$  bacteria) were mixed with 240  $\mu$ l freshly prepared nonimmune human serum and incubated at 37°C for 30 min. The nonimmune serum was obtained from nonimmune human blood, defined as described in *Phagocytosis Assays*, and at least two different sera were used in each analysis, with similar results. Activation via the classical pathway was blocked with 10 mM EGTA + 2.5 mM MgCl<sub>2</sub>, and activation via the classical and alternative pathways was blocked with 10 mM EDTA. The incubated bacteria were washed twice with 1.5 ml PBS and resuspended in PBS containing mAb (total volume, 300  $\mu$ l; mAb concentration, 20  $\mu$ g/ml) at room temperature for 10 min. After washing with 1.5 ml PBS, the bacteria were resuspended in 300  $\mu$ l FITC-conjugated goat F(ab')<sub>2</sub> anti-mouse IgG that had been diluted 1:10 in PBS, and incubated for 10 min at room temperature. Finally, the bacteria were washed with PBS and resuspended in 800  $\mu$ l PBS for flow cytometry analysis on an Epics II flow cytometer (Coulter Electronics). The instruments forward scatter, side scatter, and fluorescence 1 were set at 157, 26, and 663 V, respectively, and the forward scatter discriminator was set at 10.

## Results

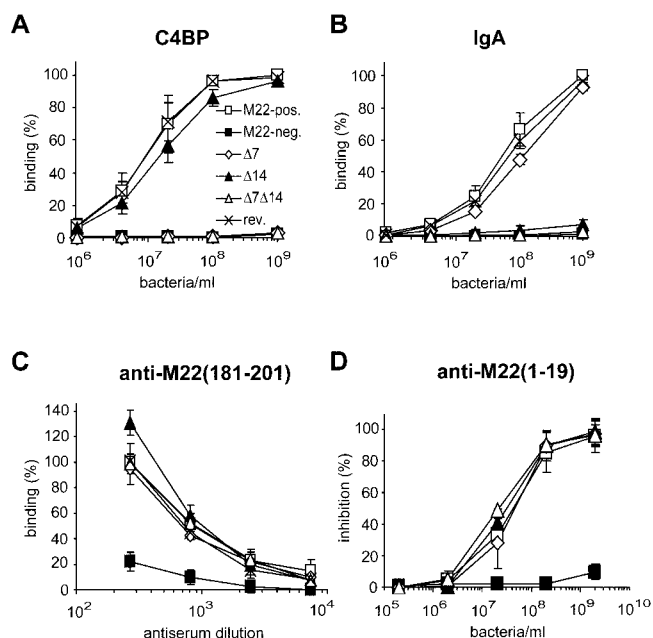
**Ligand-binding Regions of the M22 Protein.** The C4BP- and IgA-binding regions of M22 can be studied as  $\sim 50$ -residue synthetic peptides, which retain ability to bind the corresponding ligands (15, 27, 29). These ligand-binding peptides were designated M22-N and Sap22, respectively (Fig. 1 A). The defined binding regions and the corresponding synthetic peptides have overlapping amino acid sequences (27, 29), but the two binding sites in M22 are non-overlapping (i.e., C4BP does not inhibit binding of IgA, and vice versa; reference 13). The IgA-binding region binds serum IgA and secretory IgA of both subclasses (27, 29).

The two peptides specifically bind their ligands in human serum (15, 29). Because an important aspect of the work reported here involved phagocytosis assays with human blood, a situation where the bacteria are exposed to whole plasma, the binding specificity of the two peptides was further analyzed under these conditions. Whole human plasma was applied to columns containing immobilized peptide and bound proteins were eluted and analyzed by SDS-PAGE (Fig. 1 B). Anticoagulation was achieved with hirudin, a specific thrombin inhibitor (34), to avoid possible problems associated with the use of citrate or EDTA plasma. Protein eluted from the M22-N column was pure C4BP, with a major  $\sim 70$  kD band identified as the C4BP  $\alpha$ -chain by reactivity with specific antiserum (15). Material eluted from the Sap22 column was pure IgA, with major bands identified as  $\alpha$  heavy and light chains, respectively (29). In contrast, no plasma protein was retained in a column containing the control peptide M5-N, derived from an M protein that does not bind C4BP or IgA (15). Thus, the two M22-derived peptides specifically bound C4BP and IgA, respectively, among all proteins in whole human plasma, confirming the high binding specificity of the regions.

**Characterization of *S. pyogenes* Mutants Affected in the Binding of C4BP and/or IgA.** The role of the C4BP- and IgA-binding regions in phagocytosis resistance was analyzed

with isogenic bacterial mutants, specifically affected in binding of one or both of the ligands.

A mutant ( $\Delta 7$ ) specifically lacking the ability to bind C4BP, due to a seven-residue deletion in the C4BP-binding region (Fig. 1 A), has been described previously (19). For studies of the IgA-binding region, we constructed the  $\Delta 14$  strain, which has a 14-residue deletion in the COOH-terminal part of the IgA-binding region (Fig. 1 A). The  $\Delta 14$  and  $\Delta 7$  deletions were designed to correspond to 14 and 7 residues, respectively, to leave intact a possible 7-residue periodicity in the coiled coil M protein (5). The double mutant  $\Delta 7\Delta 14$  carried both deletions. To avoid possible effects of plasmids, the different deletions were introduced into the streptococcal chromosome by homologous recombination. To exclude the possibility that any property of the strains was due to inadvertent introduction of a secondary mutation during strain construction, a revertant strain (rev.) was constructed, in which the



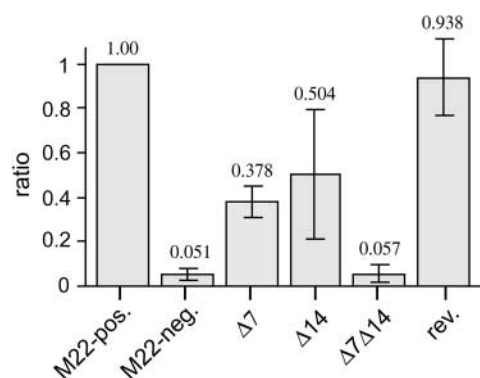
**Figure 2.** Effect of short chromosomal deletions in the gene encoding the M22 protein on the ligand-binding and immunological properties of *S. pyogenes* strains. (A and B) Washed suspensions of bacteria were analyzed for ability to bind radiolabeled C4BP or IgA, as indicated. The two strains designated  $\Delta 7$  and  $\Delta 14$  carried the corresponding deletions (Fig. 1 A) and the  $\Delta 7\Delta 14$  strain carried both deletions. M22-positive and -negative control strains were included, as indicated. A third control (rev.) was derived from the  $\Delta 7\Delta 14$  strain by reintroduction of the wild-type allele. (C) Surface expression of the M22 protein in the different mutants analyzed with rat antibodies against the M22 (181–201) peptide, derived from the conserved C-repeat region. Washed bacteria were incubated with the antiserum, diluted as indicated, and bound antibodies were identified by incubation with radiolabeled protein A. Binding for the M22-positive control at the lowest antiserum dilution was defined as 100%. (D) Immunological characterization of the most NH<sub>2</sub>-terminal part of the M22 protein in the different mutant strains. The binding between mouse antibodies against the M22 (1–19) peptide and pure M22, immobilized in microtiter wells, was inhibited with whole bacteria of different strains as indicated. Symbols are explained in A. The data in A–D are based on three different experiments with duplicate samples and are presented as means  $\pm$  SD.

wild-type *emm22* allele was reintroduced into the double mutant  $\Delta 7\Delta 14$ .

The ability to bind C4BP and IgA was analyzed for the strain expressing the M22 protein, for its mutant lacking M22, and for the isogenic mutants described above (Fig. 2, A and B). As expected, the M22-negative strain completely lacked ability to bind C4BP and IgA. The  $\Delta 14$  mutation eliminated binding of IgA, but had little or no effect on the binding of C4BP, implying that this mutation specifically affected the IgA-binding domain and did not affect surface expression of M22. Similarly, the  $\Delta 7$  mutation abolished binding of C4BP, but not of IgA, in agreement with previous observations (19). The double mutant  $\Delta 7\Delta 14$  completely lacked ability to bind both ligands, whereas the revertant strain bound C4BP and IgA equally well as the wild type. These results indicated that the  $\Delta 14$  and  $\Delta 7$  mutations specifically affected the ability to bind C4BP or IgA, respectively, making them suitable for further analysis.

To analyze whether the different mutations affected the level at which the M22 protein is expressed on the bacterial surface, the bacterial mutants were analyzed for ability to react with antibodies raised against an internal peptide, M22 (181–201), derived from the conserved C-repeat region of the M22 protein (Fig. 2 C). These antibodies showed similar reactivity with all strains except the M22-negative strain, indicating that the mutant M22 proteins were expressed normally on the bacterial cell surface. This conclusion was confirmed by analysis of the ability of the strains to bind a third M22 ligand, IgG-Fc, which may bind at a site located between the IgA-binding region and the conserved C-repeat region (26). All strains except the M22-negative strain had similar IgG-Fc-binding ability, confirming that the M22 protein was expressed at normal levels in the mutants (unpublished data).

The ability of the  $\Delta 7$  mutant to bind IgA and of the  $\Delta 14$  mutant to bind C4BP indicated that the global structure of the NH<sub>2</sub>-terminal part of the M22 protein was not distorted in these mutants. However, it could not be excluded that the combination of the two single mutations in  $\Delta 7\Delta 14$  caused a change in structure of the NH<sub>2</sub>-terminal region, affecting the ability of M22 to confer phagocytosis resistance. To analyze this problem, we used mouse antibodies raised against a synthetic peptide derived from the first 19 residues of M22, a region present in all of the mutants. This region of M22 was of particular interest, because the most NH<sub>2</sub>-terminal part of M protein has been implicated in phagocytosis resistance (5, 9). Binding of the anti-M22 (1–19) antibodies to pure M22 protein was inhibited by bacteria expressing the wild-type M22 protein, but not by M22-negative bacteria, as expected (Fig. 2 D). Moreover, binding was inhibited equally well by the three strains  $\Delta 7$ ,  $\Delta 14$ , and  $\Delta 7\Delta 14$ . These data indicate that the structure of the most NH<sub>2</sub>-terminal region of M22 was not distorted in any of the mutants, implying that they were all suitable for analysis of the role of the ligand-binding regions in phagocytosis resistance.



**Figure 3.** Role of the C4BP- and IgA-binding regions for ability of the M22 protein to confer phagocytosis resistance. The six strains described in Fig. 2 were analyzed for ability to grow in whole human blood during a 3-h period. Anticoagulation was achieved with hirudin. Bacterial growth is expressed as a ratio of the growth observed for the strain expressing the wild-type M22 protein, for which the average factor of growth (multiplication factor) in these experiments was 395. Data are based on eight experiments with three different blood donors and presented as means  $\pm$  SD.

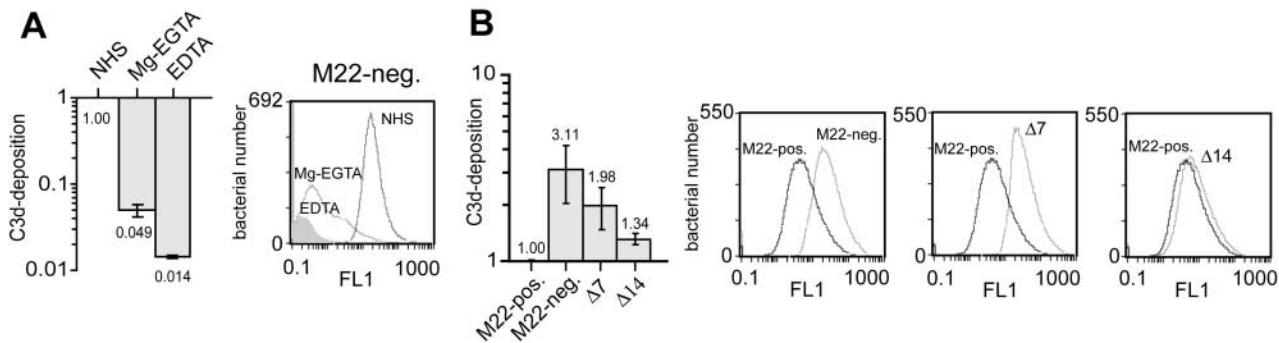
*The C4BP- and IgA-binding Regions of M22 Cooperate in Conferring Phagocytosis Resistance.* The contribution of the two regions to phagocytosis resistance was analyzed in the classical bactericidal assay, which measures the ability of streptococci to grow in whole human blood (33). However, hirudin rather than heparin was used for anticoagulation, as described previously (25), because heparin may interfere with complement activity.

The growth of the M22-negative control strain in whole blood was reduced 20-fold compared with the M22-positive control strain, whereas growth of the  $\Delta 7$  and the  $\Delta 14$  strains was reduced 2.6-fold and 2-fold, respectively (Fig. 3). Thus, a mutation affecting only one of the ligand-binding regions had a limited but significant effect on phagocytosis resistance. The effect of the  $\Delta 7$  mutation was smaller in these experiments than in previously reported tests (19), possibly due to variability in the whole-blood phagocytosis system.

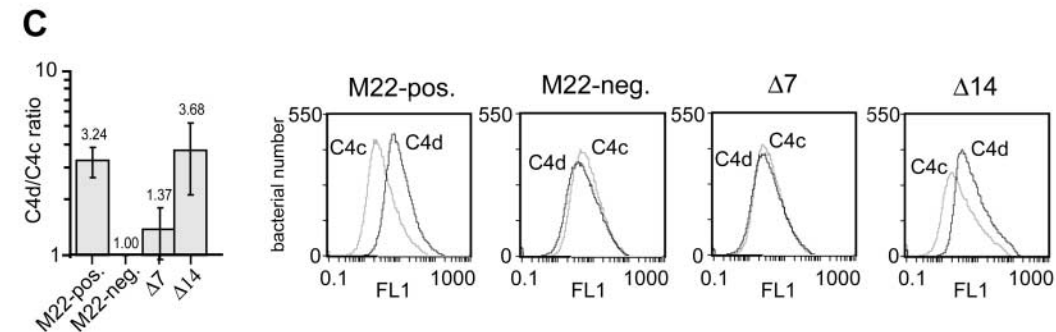
The growth of the double mutant  $\Delta 7\Delta 14$  was reduced to the same level as the M22-negative strain (Fig. 3). Thus, the ability of the M22 protein to confer phagocytosis resistance may be fully explained as cooperation between the C4BP- and IgA-binding regions. Moreover, comparison of the single mutants with the double mutant indicates that each of the two ligand-binding regions makes an important contribution to phagocytosis resistance. Importantly, the revertant strain (rev.) had regained full resistance, demonstrating that the lack of growth of the  $\Delta 7\Delta 14$  double mutant was indeed due to the two mutations affecting the M22 protein. The reduced growth of the mutants in the phagocytosis system was not due to a general effect on growth, because all strains grew equally well in human plasma (unpublished data).

*S. pyogenes Activates Complement via the Classical Pathway but Activation Is Down-regulated by Bacteria-bound C4BP.* It seemed possible that bacteria-bound C4BP contributed to phagocytosis resistance by down-regulating the deposition

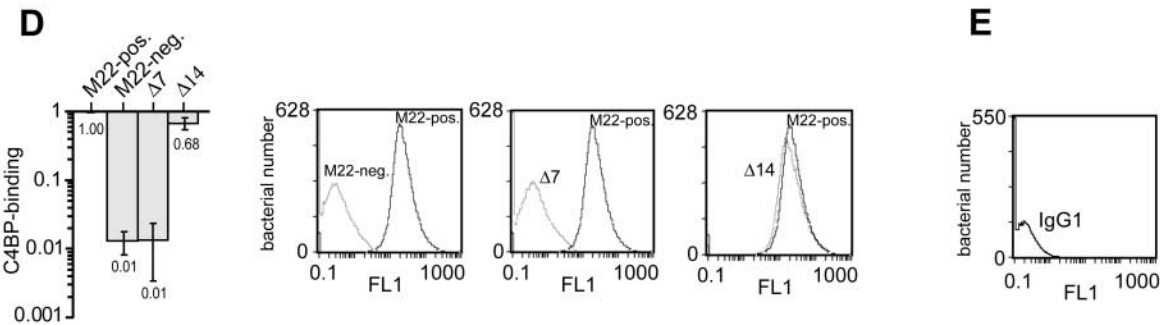
# C3d-deposition



# Deposition of C4c and C4d



# C4BP-binding



**Figure 4.** Analysis of complement deposition on the surface of *S. pyogenes*. Bacteria were incubated for 30 min in nonimmune human serum and analyzed by FACS® for bound complement components as indicated. Strains used were *S. pyogenes* expressing the M22 protein (M22-pos.), its M22-negative mutant (M22-neg.), and the two mutants  $\Delta 7$  and  $\Delta 14$ . For each type of analysis, results obtained in at least three different experiments are shown to the left as means  $\pm$  SD, and representative histograms are shown to the right. Data obtained with a subclass control are shown in E. (A) Deposition of C3d on M22-negative *S. pyogenes* incubated in normal human serum (NHS), in serum with EDTA, or in serum with Mg-EGTA. Deposition in normal human serum was defined as 1.00 (left). (B) Deposition of C3d on the M22-positive strain and its mutants. Deposition of C3d on the M22-positive strain was defined as 1.00. (C) Complement activation via the classical pathway, analyzed with mAbs against C4c and C4d. The C4d/C4c ratio observed for the M22-negative strain was defined as 1.00. (D) Binding of C4BP, analyzed with the anti-C4BP mAb67. Binding of C4BP to the M22-positive strain was defined as 1.00. (E) Control with an IgG1 mAb.

of complement (19). In agreement with this hypothesis, bacteria-bound C4BP was found to retain its cofactor activity (13). However, the role of C4BP remained unclear, because phagocytosis was analyzed with blood from non-immune donors (i.e., in the absence of opsonizing antibodies; reference 19), when the classical pathway of complement activation was not expected to be activated. This

situation prompted us to analyze complement deposition on *S. pyogenes* under nonimmune conditions, with particular focus on the role of bacteria-bound C4BP and deposition via the classical pathway.

Analysis of complement deposition on the surface of *S. pyogenes* was performed by flow cytometry, after incubation of bacteria in nonimmune human serum (Fig. 4).



Complement activation via the classical or alternative pathways results in surface deposition of C3b and fragments of C3b, all of which can be identified with an anti-C3d mAb (16). As expected, C3d was deposited on M22-negative bacteria incubated in nonimmune serum, and deposition was abolished by the addition of EDTA, which inhibits both pathways (Fig. 4 A). Interestingly, C3d-deposition was reduced 20-fold also in the presence of Mg-EGTA, which blocks only the classical pathway. Thus, deposition of complement on the M-negative strain occurred almost exclusively via the classical pathway.

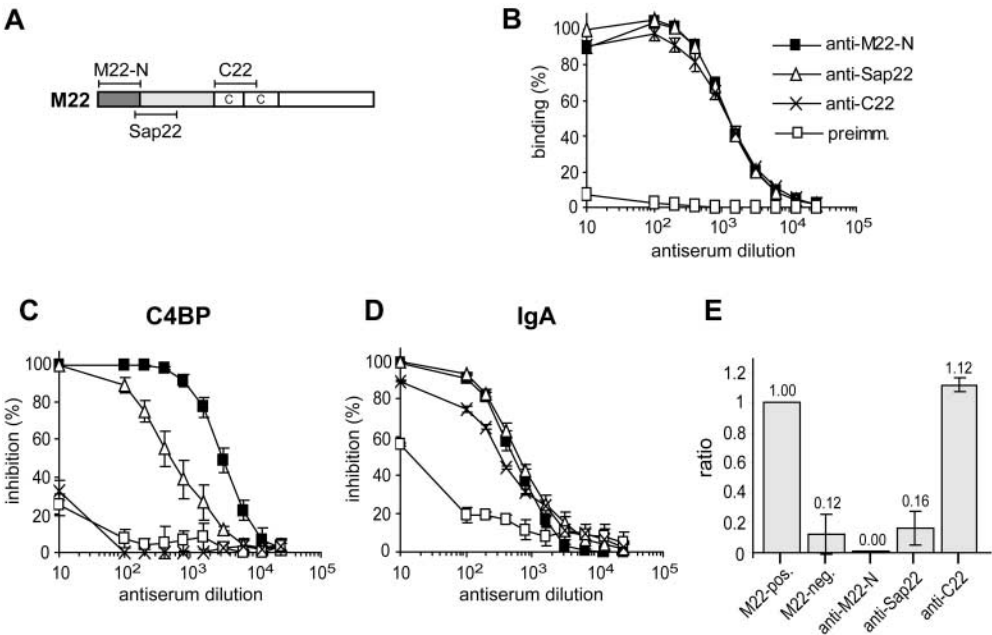
Deposition of C3d was approximately threefold higher on the M22-negative strain than on the M22-positive strain, implying that expression of the M22 protein inhibits most of the complement deposition occurring via the classical pathway (Fig. 4 B). Deposition of C3d on the  $\Delta 7$  strain was increased approximately twofold, but this result was not significantly different from the approximate threefold increase seen for the M22-negative strain. Complement deposition on the  $\Delta 14$  strain was only slightly increased, indicating that the IgA-binding region has little or no effect on complement deposition. These data indicate that the ability of the M22 protein to inhibit surface deposition of complement under nonimmune conditions may be entirely due to the ability of M22-bound C4BP to inhibit activation via the classical pathway.

Activation of the classical pathway results in surface deposition of C4b, which in the presence of C4BP and factor I is degraded to C4c and C4d. The C4c part is released into the medium, whereas C4d remains bound to the surface (16, 17). Thus, the C4d/C4c ratio will be increased if the

classical pathway is down-regulated by C4BP (35). The deposition on *S. pyogenes* of these complement components was analyzed with specific mAbs. The C4d/C4c ratio was approximately threefold higher on the M22-positive strain (Fig. 4 C), implying that the M22 protein inhibited activation of complement occurring via the classical pathway under nonimmune conditions. The ratio observed for the  $\Delta 7$  mutant was only slightly higher than for the M22-negative control, and the difference was not statistically significant, indicating that the ability of M22 to down-regulate the classical pathway was due to its ability to bind C4BP. In contrast, the  $\Delta 14$  mutant was not affected in the ability to inhibit complement deposition via the classical pathway, indicating that the IgA-binding region inhibits phagocytosis by a different mechanism. These data support the conclusion that the C4BP-binding region of the M22 protein causes down-regulation of the classical pathway.

To analyze whether the bacterial strains bound C4BP under the conditions of the FACS<sup>®</sup> analysis, binding of C4BP was analyzed with the anti-C4BP mAb67, which is directed against an epitope in C4BP located outside the M protein binding site (30). In agreement with the results obtained with radiolabeled C4BP (Fig. 2), binding of C4BP was reduced  $\sim 100$ -fold for the M22-negative strain and for the  $\Delta 7$  mutant, whereas binding to the  $\Delta 14$  strain was almost unaffected (Fig. 4 D).

**Antibodies Directed against the C4BP- and IgA-binding Regions Are Opsonizing.** The role of the ligand-binding regions of M22 under immune conditions was analyzed with specific antisera. Attempts to raise rabbit antisera that specifically inhibited binding of only one ligand were unsuccessful.



**Figure 5.** Ability of polyclonal antisera, directed against different parts of M22, to inhibit ligand binding and opsonize for phagocytosis. (A) Localization in the M22 protein of the three peptides M22-N, Sap22, and C22 used to raise rabbit antisera. (B) Reactivity of antipeptide antisera with pure M22 protein immobilized in microtiter wells. Bound Abs were detected with radiolabeled protein G. The sera had been adjusted to have similar titer by appropriate dilution in preimmune serum. (C and D) Ability of antipeptide antisera to inhibit binding of radiolabeled C4BP or IgA to pure M22 protein immobilized in microtiter wells. Symbols are explained in B. The data in B–D are based on three experiments with duplicate samples and are presented as means  $\pm$  SD. (E) Phagocytosis experiments. The antipeptide antisera were analyzed for ability to opsonize the M22-

positive strain. As controls, the M22-positive strain and an isogenic M22-negative mutant were analyzed for growth in the presence of preimmune rabbit serum. Bacterial growth was determined after incubation in whole human blood for 3 h, using hirudin as anticoagulant, and is expressed as a ratio of the growth observed for the strain expressing the M22 protein, for which the average factor of growth (multiplication factor) in these experiments was 461. Each analysis is based on at least three separate experiments with three different blood donors and the data are presented as means  $\pm$  SD.

cessful, apparently because antibodies to one region sterically inhibit the binding of both ligands (see the last paragraph in Results). Therefore, analysis under immune conditions was performed with sera raised against the two peptides M22-N and Sap22, of which the anti-M22-N serum has been studied previously (19). The analysis also used an antiserum raised against a 54-residue peptide designated C22, derived from the conserved C-repeat region, which is not believed to influence phagocytosis resistance (Fig. 5 A; reference 36).

To allow direct comparisons, the three antisera were appropriately diluted in preimmune rabbit serum, resulting in similar reactivity with the M22 protein (Fig. 5 B). Binding of C4BP to the M22 protein was inhibited by the anti-M22-N serum and approximately sixfold less well also by the anti-Sap22 serum, but not by the anti-C22 serum (Fig. 5 C). In contrast, binding of IgA was inhibited by all three antisera (Fig. 5 D). The surprising ability of the anti-C22 serum to inhibit binding of IgA was not due to cross-reactivity of the C22 peptide with the IgA-binding region (unpublished data), but was probably due to steric hindrance. Preimmune rabbit serum caused some inhibition of IgA-binding, possibly because rabbit IgA competes with human IgA for binding to M22.

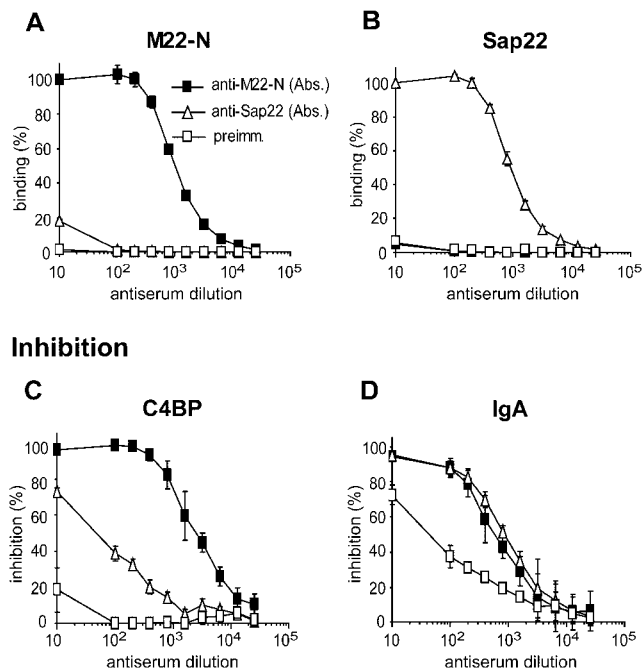
In phagocytosis experiments, each of the anti-M22-N and anti-Sap22 antisera efficiently opsonized the M22-expressing strain, reducing growth to the level of the M22-negative control (Fig. 5 E). In contrast, the anti-C22 antibodies lacked opsonizing capacity. Thus, antisera that blocked binding of both C4BP and IgA promoted phagocytosis.

**Antibodies Specific for the C4BP- or IgA-binding Region Inhibit Binding of Both Ligands.** Because the M22-N and Sap22 peptides have overlapping sequences (Fig. 1 A), it was not surprising that antisera against these peptides inhibited binding of both ligands (Fig. 5). Indeed, each of these sera reacted not only with the homologous peptide but also with the heterologous peptide (unpublished data). Antisera specific for either of the two ligand-binding regions were prepared by absorption of the anti-M22-N and anti-Sap22 antisera with the heterologous peptide, as described in Materials and Methods. Such absorptions made the antisera almost completely specific for the homologous peptides (Fig. 6, A and B). Nevertheless, these absorbed antisera retained the ability to inhibit binding of both ligands to the M22 protein (Fig. 6, C and D), most likely due to steric hindrance, and they retained the ability to reduce growth in blood (unpublished data). Thus, polyclonal antibodies specific for one ligand-binding region blocked binding of both ligands and promoted phagocytosis.

## Discussion

Among pathogenic bacteria, *S. pyogenes* is unique in its ability to grow rapidly in nonimmune whole human blood. This property is to a large extent due to the expression of surface M protein, which inhibits phagocytosis by neutrophils (5). Although *S. pyogenes* is usually not found in the blood of infected individuals, but causes infections on mu-

## Binding



**Figure 6.** Antibodies specific for either of the C4BP- or IgA-binding regions in M22 inhibit binding of both ligands. (A and B) Antisera against either of the M22-N and Sap22 peptides were absorbed with the heterologous peptide and analyzed for reactivity with the indicated peptide, which was immobilized in microtiter wells. (C and D) Ability of the absorbed sera to inhibit binding of radiolabeled C4BP or IgA to pure M22 protein immobilized in microtiter wells.

cosal surfaces or in wounded tissue, the ability of M protein to inhibit phagocytosis is probably an important virulence mechanism also under these conditions, because phagocytes will be recruited to the site of an infection. Interactions between M proteins and human plasma proteins are also expected to occur on mucosal surfaces, where plasma exudative responses contribute to a first-line defense system (37). Indeed, it seems likely that the most important interactions between *S. pyogenes* and the immune system occur on mucosal surfaces.

To analyze the molecular mechanisms by which M protein confers phagocytosis resistance, we used the M22 protein, in which the HVR binds C4BP and the semivariable region binds IgA. The very high specificity with which these regions bind their human ligands strongly suggests that the interactions are biologically significant. Importantly, it was possible to construct M22 mutants specifically affected in binding of only one of the ligands, allowing analysis of the role of each ligand in phagocytosis resistance. Moreover, a double mutant had immunological and ligand-binding properties, indicating that it could be viewed as the sum of the two single mutants. Characterization of these different mutants demonstrated that each ligand-binding region made a major contribution to phagocytosis resistance, whereas full resistance required the

activity of both regions. The mutant lacking ability to bind both ligands was as sensitive to phagocytosis as an M-negative strain, implying that phagocytosis resistance conferred by the M22 protein can be fully explained as cooperation between the C4BP- and IgA-binding domains. Because M22 has properties typical of many M proteins, it seems likely that binding of C4BP and IgA contributes to phagocytosis resistance in many strains of *S. pyogenes*. For example, the purified M4, M28, and M60 proteins have binding properties very similar to those of the M22 protein (13, 38). This situation raises the question of the role of C4BP and IgA in phagocytosis resistance.

Efficient phagocytosis of a bacterial pathogen under non-immune conditions requires opsonization by complement (3), implying that a pathogen may evade phagocytosis by interfering with complement deposition. Therefore, binding of C4BP to the surface of *S. pyogenes* can most simply be explained as a mechanism that allows evasion of complement attack (13, 19). However, the role of bacteria-bound C4BP has remained unclear, because this complement regulator specifically inhibits activation of the classical pathway, which is commonly believed not to be activated under nonimmune conditions. The data reported here resolve this paradox, because deposition of complement on the M-negative *S. pyogenes* strain occurred almost exclusively via the classical pathway even under nonimmune conditions but was reduced by the C4BP-binding region of M22. Thus, bacteria-bound C4BP limits classical pathway activation occurring under nonimmune conditions, a finding that may explain why bound C4BP contributes to the protection of *S. pyogenes* against phagocytosis.

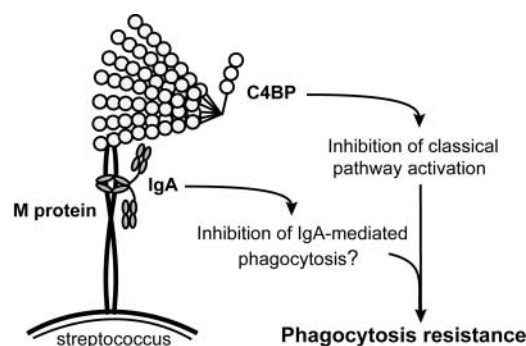
The M22 protein reduced total complement deposition approximately threefold, but not completely. Therefore, it could be argued that the residual complement deposited on M22-expressing bacteria might be sufficient to permit phagocytosis. However, studies of *Neisseria gonorrhoeae* have indicated that a fourfold reduction in complement deposition, as measured in vitro, is sufficient to confer full resistance to complement (39), and a similar situation may prevail in *S. pyogenes*. Moreover, the inability of M22 to completely block complement deposition may explain why two regions in M22 are required to confer full resistance to phagocytosis.

The conclusion that *S. pyogenes* activates the classical pathway is in agreement with accumulating evidence that this pathway is continuously activated at low levels, even under nonimmune conditions and may be further activated by pathogens (40, 41). Indeed, the classical pathway may be more important than the alternative pathway for clearance of some infections under nonimmune conditions (41–43). Among the mechanisms that cause activation of the classical pathway under nonimmune conditions, particular interest has been focused on “natural IgM,” which is present in nonimmune individuals and recognizes pathogens (44–46). Other mechanisms for activation of the classical pathway under nonimmune conditions include direct interaction of bacteria with complement component C1 and activation of the mannose-binding lectin pathway (47). The latter

mechanism may be of particular relevance to *S. pyogenes*, which binds the mannose-binding lectin (48).

The function of the IgA-binding domain in phagocytosis resistance is less readily explained than that of the C4BP-binding region. Indeed, it may appear paradoxical that the ability of *S. pyogenes* to bind a component of the adaptive immune system confers phagocytosis resistance under non-immune conditions. However, the molecular mechanism by which IgA-binding M proteins interact with IgA throws light on this problem. The IgA-binding region of different M proteins binds at the  $\alpha 2$ – $\alpha 3$  interface in the Fc part of IgA (49), at a site similar to that used by the human IgA receptor CD89, which is expressed on phagocytes and promotes IgA-mediated phagocytosis (50, 51). Consequently, an IgA-binding M protein inhibits the binding of IgA–Fc to CD89, thereby inhibiting IgA effector function (49). With regard to *S. pyogenes* infections, a possible scenario is, therefore, that IgA-binding M proteins block IgA-mediated phagocytosis promoted by “natural” IgA antibodies under nonimmune conditions (49). This hypothesis is supported by the observation that normal human subjects have natural IgA antibodies against a large number of *S. pyogenes* antigens (52).

According to the arguments put forward in the previous paragraphs, the C4BP- and IgA-binding regions in M22 and similar M proteins allow *S. pyogenes* to evade opsonization by two mechanisms: (a) the C4BP-binding region down-regulates activation of complement via the classical pathway and (b) the IgA-binding region may interfere with IgA-mediated opsonization (Fig. 7). It is noteworthy that each of the ligand-binding regions must be inactivated to confer full sensitivity to phagocytosis, implying that both regions make important contributions to phagocytosis resistance. This observation is in agreement with many re-



**Figure 7.** Model for the role of human C4BP and IgA in resistance to phagocytosis conferred by M22 and related M proteins. The two ligands bind to separate sites in the NH<sub>2</sub>-terminal part of M22, which is a dimeric coiled coil. The size and shapes of the molecules are drawn approximately to scale (references 17, 60–62). The C4BP molecule has seven identical  $\alpha$ -chains and one short  $\beta$ -chain composed of complement control protein modules. The M protein binds to C4BP at the module 1–2 interface in the  $\alpha$  chain (references 30, 63). The binding site for M protein in IgA is located at the  $\alpha 2$ – $\alpha 3$  interface in the Fc part (reference 49). According to the model, bacteria-bound C4BP protects against complement-mediated opsonization, which occurs via the classical pathway even under nonimmune conditions. The ability to bind IgA–Fc may allow the bacteria to interfere with phagocytosis promoted by natural IgA.

ports that efficient phagocytosis of pathogens requires opsonization by both complement and immunoglobulin (53).

The studies reported here were focused on the C4BP- and IgA-binding regions of M22, because these well-characterized regions are located in the variable NH<sub>2</sub>-terminal part of M22 and bind their ligands with high specificity. However, the M22 protein also has a less well-defined IgG-binding region, most likely located COOH-terminally of the IgA-binding region (26, 27). Little is yet known about the properties and function of this IgG-binding region, but it does not appear to contribute to phagocytosis resistance in nonimmune human blood, because the double mutant  $\Delta 7\Delta 14$ , which had lost the ability to bind C4BP and IgA but still bound IgG, was as sensitive to phagocytosis as a strain lacking the entire M protein.

The model presented in this paper can explain how M22 and similar M proteins confer phagocytosis resistance, but many M proteins (e.g., the extensively studied M5 and M6 proteins) do not bind C4BP and IgA and must use different mechanisms to inhibit phagocytosis. Although the plasma proteins fibrinogen, factor H, and factor H-like protein 1 have been implicated in phagocytosis resistance conferred by these M proteins (54–56), their role remains unclear. The complexity of phagocytosis resistance is emphasized by the finding that the genetic background of a strain influences M protein function (57, 58). For example, the M22 protein does not confer phagocytosis resistance in the M5 background and vice versa (58). This phenomenon limits the possibility to use heterologous hosts or hybrid M proteins for analysis of M protein function.

An important aspect of the work reported here is that phagocytosis resistance was analyzed under both nonimmune and immune conditions. Analysis under nonimmune conditions showed that each of the C4BP- and IgA-binding regions contributed to phagocytosis resistance. Similarly, analysis under immune conditions demonstrated that opsonizing antibodies could be raised against either of the overlapping synthetic peptides derived from the two ligand-binding regions. The opsonizing ability of these antibodies may be explained by their ability to inhibit binding of both ligands. Interestingly, the ability to block binding of both ligands was retained by antibodies made specific for one region by absorption, indicating that antibodies to one region sterically block binding of both ligands. This finding implies that sequence changes in one region may allow escape from opsonizing antibodies directed against that region, which could explain the known sequence variability in the two ligand-binding regions (14, 23). Together, these data indicate that the two ligand-binding regions play important roles under both nonimmune and immune conditions.

Polyclonal antibodies directed against the C-repeat region of M22 did not opsonize, in agreement with earlier papers indicating that the C-repeat region does not contribute to phagocytosis resistance (10, 36). These nonopsonizing antibodies directed against the C-repeat region actually blocked the binding of IgA, probably due to steric effects, but their lack of opsonizing capacity may be at least

partially explained by their lack of effect on the binding of C4BP.

In summary, the data reported here indicate that M22 and similar M proteins confer phagocytosis resistance through cooperation between two variable regions that bind human C4BP and IgA, respectively, two components of the humoral immune system. Interestingly, an unrelated streptococcal surface protein, the  $\beta$  protein of group B streptococcus, also has separate binding sites for IgA and a human complement regulator, in that case the plasma protein factor H (59). Thus, the ability of a bacterial surface protein to simultaneously interact with human IgA and a human complement regulator may be a virulence mechanism of general significance. Development of methods to inhibit these interactions could provide novel approaches to the therapy and prevention of streptococcal infections.

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