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Interaction between Complement Regulators and *Streptococcus pyogenes*: Binding of C4b-Binding Protein and Factor H/Factor H-Like Protein 1 to M18 Strains Involves Two Different Cell Surface Molecules

David Pérez-Caballero,* Isabel García-Laorden,† Guadalupe Cortés,‡ Michael R. Wessels,‡ Santiago Rodríguez de Córdoba,2* and Sebastián Alberti2,3†

*Streptococcus pyogenes*, or group A *Streptococcus*, is one of the most frequent causes of pharyngitis and skin infections in humans. Many virulence mechanisms have been suggested to be involved in the infectious process. Among them is the binding to the bacterial cell surface of the complement regulatory proteins factor H, factor H-like protein 1 (FHL-1), and C4b-binding protein. Previous studies indicate that binding of these three regulators to the streptococcal cell involves the M protein encoded by the *emm* gene. M-type 18 strains are prevalent among clinical isolates and have been shown to interact with all three complement regulators simultaneously. Using isogenic strains lacking expression of the Emm18 or the Enn18 proteins, we demonstrate in this study that, in contradistinction to previously described *S. pyogenes* strains, M18 strains bind the complement regulators factor H, FHL-1, and C4b-binding protein through two distinct cell surface proteins. Factor H and FHL-1 bind to the Emm18 protein, while C4BP binds to the Enn18 protein. We propose that expression of two distinct surface structures that bind complement regulatory proteins represents a unique adaptation of M18 strains that enhances their resistance to opsonization by human plasma and increases survival of this particular *S. pyogenes* strain in the human host. These new findings illustrate that *S. pyogenes* has evolved diverse mechanisms for recruitment of complement regulatory proteins to the bacterial surface to evade immune clearance in the human host. *The Journal of Immunology, 2004, 173: 6899–6904.*

Abstract

Group A *Streptococcus*, or *Streptococcus pyogenes*, is a Gram-positive bacterium that frequently produces suppurative infections in humans (1–3), including pharyngitis and skin infections. These localized infections can be complicated by spread to deeper tissues and by the postinfectious sequelae of acute rheumatic fever and glomerulonephritis (4, 5). Recently, there has been an increase in severe, highly invasive streptococcal infections of soft tissues with high mortality (4, 6–8). *S. pyogenes* cells express one or more surface-associated fibrillar proteins, called M proteins, which are major virulence factors because they confer to the bacteria resistance to phagocytosis (9, 10). Among the different *S. pyogenes* strains, there are >100 different antigenically distinguishable M proteins (9, 11). The M proteins belong to a family of structurally related proteins that are encoded by genes within the *mgA* regulon in the streptococcal chromosome. For most *S. pyogenes* strains, this gene cluster includes not only the *emm* gene encoding M (Emm) protein, but also one or two additional genes encoding structurally related proteins designated Emn and Mrp (12, 13). The Emm proteins have affinity for several plasma proteins, including fibrinogen, IgG, IgA, and the complement regulatory proteins, factor H, factor H-like protein 1 (FHL-1),4 and C4b-binding protein (C4BP) (12, 14, 15). It has been suggested that bacterial binding of these plasma proteins contributes to the antiphagocytic properties conferred by most Emm proteins.

The interaction between a complement regulatory protein and *S. pyogenes* was first reported by Horstmann et al. (16). They demonstrated that M6 and other M serotypes (M5, M19, M24, and M28) bind factor H through the conserved regions (B or C repeats) in the Emm protein. The binding of this complement regulatory protein resulted in reduced activation by the alternative pathway on the bacterial surface, which could decrease phagocytosis by macrophages and polymorphonuclear leukocytes. Binding of factor H was, therefore, proposed as the mechanism by which Emm proteins exert their antiphagocytic effect (16). Later studies in an M4 strain demonstrated that C4BP bound to the N-terminal region of the Emm4 protein (17) and that binding of C4BP could confer bacterial protection against classical pathway activation (14). More recently, FHL-1, another complement regulator that results from recent, FHL-1, another complement regulator that results from the alternative splicing of the factor H gene, has been shown to bind to streptococcal cells throughout the N-terminal region of the Emm protein (15).

The present studies were stimulated by the observation that strains of the M18 serotype vary significantly in their capacity to...
bind C4BP, but not in the binding of FHL-1 (18), despite both complement regulators having been reported to interact with the same N-terminal hypervariable region of the Emm proteins studied previously. To investigate the molecular basis of the interaction between S. pyogenes surface proteins and C4BP or FHL-1, we have characterized the 

Materials and Methods

Bacterial strains and culture conditions

The S. pyogenes M18 strains 87–282, 346, SS77, 9003, and SS344 have been described previously (18). The capsule and M18 mutants derived from 87 to 282 strain (TX72, 282KZ, and TX74) were also previously described (19, 20). S. pyogenes M1 strain 8763 was previously described and does not bind C4BP (18). Streptococci were grown in Todd-Hewitt broth supplemented with 0.2% yeast extract or on solid medium containing Todd-Hewitt broth supplemented with 0.2% yeast extract supplemented with 1.8% Bacto Agar and 5% (v/v) defibrinated sheep blood. Escherichia coli was grown in Luria-Bertani broth. Erythromycin was used in the cloning experiments at 100 µg/ml for E. coli and 1 µg/ml for S. pyogenes. Cell concentration was determined using a Petroff-Hauser counting chamber (Thomas Scientific).

Proteins and Abs

Human C4BP and factor H were purified from EDTA-plasma, as described (21, 22). FHL-1 was partially purified free of factor H from EDTA-plasma using a heparin-Sepharose column and an anionic exchange chromatography. A fraction containing factor H activity was concentrated using a Q-Sepharose Fast Flow column (Amersham Biosciences). Human C4BP and factor H were purified from EDTA-plasma, as described (21–23). The 35H9 anti-human factor H mAb (IgM isotype) recognized both factor H and FHL-1 proteins (18).

DNA and RNA manipulations

Plasmid DNA was isolated using the Wizard Miniprep Kit (Promega, Madison, WI), according to the manufacturer’s instructions. S. pyogenes genomic DNA was prepared, as described previously (24). Restriction endonuclease digestion, DNA ligations, transformation of CaCl2 competent E. coli, agarose gel electrophoresis, and Southern hybridizations (ECL kit; Amersham Biosciences) were performed using standard techniques (25). S. pyogenes competent cells were prepared and transformed, as described (24). Total cellular RNA was isolated from S. pyogenes grown to 2.0 using the Qiagen miniRNA prep (Promega, Madison, WI), according to the recommended procedure.

The DNA sequence of the emn18 and the enn18 genes in M18 strains was determined by automated sequence analysis (ABI PRISM 3700) of a PCR product generated using the primers GASM1/GASM2 (26) and GASN1/GASN2, respectively.

Polymerase chain reactions

Primers used in PCR and RT-PCR experiments are listed in Table I. PCRs were performed using a 5-min hot start and the following reaction conditions: 94°C for 1 min, 48°C for 1 min, and 68°C for 1 min for 35 cycles, and final extension step at 68°C for 10 min (GeneAmp PCR 2400 System; PerkinElmer, Wellesley, MA). RT-PCR was performed using the Access RT-PCR kit (Promega) with a 45-min reaction at 48°C using 15 ng of total cellular RNA as template, followed by PCR for 35 cycles under the conditions described above.

Generation of the Emn18 protein mutant from S. pyogenes TX72

To derive an Emn18 mutant from S. pyogenes acapsular strain TX72, a 771-bp internal fragment of the enn18 gene was amplified by PCR from S. pyogenes genomic DNA using primers Emn18FB and Emn18RB. The PCR product was cloned into the temperature-sensitive E. coli-streptococcal shuttle vector pJRS233 (24) digested with BamHI to yield plasmid pJENN18. This plasmid was introduced into strain TX72 by electroporation, and erythromycin-resistant transformants were isolated at the nonpermissive temperature (37°C), as described previously (24). Growth of transformants at the nonpermissive temperature resulted in the integration of plasmid through the enn18 homologous DNA sequences found on both the plasmid and the chromosomal DNA and in formation of inactive enn18 alleles.

Binding of C4BP, factor H, and FHL-1 to streptococcal M18 strains in EDTA-plasma

Binding of the three regulators to S. pyogenes in EDTA-plasma was performed essentially as described by Kottarsky et al. (27). Briefly, 2 × 108 bacteria were washed in PBS, pelleted, and incubated with 50 µl of EDTA-plasma diluted four times in PBS for 2 h at room temperature with agitation. After incubation, samples were centrifuged and the pellet was washed twice with 1.5 ml of PBS containing 0.05% Tween 20. Bound proteins were eluted with 50 µl of SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.01% bromphenol blue) and subjected to Western blot analysis using the mAb 35H9 to identify factor H and FHL-1, or a rabbit polyclonal Ab to identify C4BP.

C4BP-binding assays on wild-type and mutants of 87–282 strain

To determine the C4BP association constant and the number of binding sites in the different strains, binding of 125I-labeled C4BP (125I-C4BP) to S. pyogenes cells was performed, as described above, using 2.5 × 106 bacteria of TX72, 282KZ, TX74 and incubating with increasing amounts of 125I-C4BP. Nonspecific binding (<5%) was determined using cells of 8763 strain. Free nonincorporated C4BP was determined by subtracting the bound 125I-C4BP from the initially added 125I-C4BP.

Results

S. pyogenes strains of the M18 serotype are frequently found in clinical isolates from patients with pharyngitis, invasive infections, and acute rheumatic fever (3, 6, 28). Although some M18 strains bind all three complement regulators, factor H, FHL-1, and C4BP, we have identified a number of M18 strains with reduced capacity to bind C4BP. These differences in the capacity to bind C4BP are in contrast to the apparently identical capacity of all M18 strains to bind factor H and FHL-1. Fig. 1 illustrates the binding of C4BP, factor H, and FHL-1 to five selected M18 strains (87–282, 346, 9003, SS77, and SS344). Although all five M18 strains show similar capacity to bind factor H and FHL-1, the capacity to bind C4BP varies dramatically among these M18 strains. Strain 346 binds C4BP very efficiently; 87–282, SS77, and 9003 show intermediate binding; and SS344 shows no capacity to bind C4BP.

To determine whether the differences in C4BP binding among these M18 strains are a consequence of sequence variations in the N-terminal hypervariable region of the Emm18 protein, we sequenced the enn18 gene in all five M18 strains. These analyses identified two types of sequence variation: a Gly to Asp substitution at amino acid position 21 in the SS77 and SS344 strains and an insertion of 42 aa after amino acid position 203 in the 87–282 strain that is not present in the other four strains (Fig. 1b). However, neither of these enn18 sequence variations explains the differences found in the capacity to bind C4BP. Although both SS77 and SS344 contain the Gly to Asp substitution, only SS77 binds C4BP. Similarly, among the strains without the 42-aa insertion, there are strains with high C4BP-binding capacity (346), strains
showing intermediate binding (9003 and SS77), and strains that do not bind C4BP (SS344).

Our binding studies suggest that the C4BP-binding differences among the M18 strains are not a consequence of different levels of expression of the \( \text{emm18} \) gene because all M18 strains show identical capacity to bind factor H or FHL-1 (Fig. 1).

To verify that binding of C4BP to M18 strains was not mediated by the Emm18 protein, we performed binding assays using the 87–282 strain and its isogenic mutants TX72, 282KZ, and TX74. The TX72 strain is a mutant lacking the capsule, the 282KZ strain is an Emm18 protein-deficient mutant, and TX74 is a double mutant lacking both capsule and M protein (19, 20).

Table I. Oligonucleotide primers used for PCR and RT-PCR

<table>
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<tr>
<th>Name</th>
<th>Sequence (5′–3′)</th>
<th>Gene</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAS1</td>
<td>5′-TATT (G/C)GCTTAGAAATTAA-3′</td>
<td>( \text{emm18} )</td>
<td>26</td>
</tr>
<tr>
<td>GAS2</td>
<td>5′-GCAAGTCTTTGAGCCTGTTTT-3′</td>
<td>( \text{emm18} )</td>
<td>26</td>
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<td>GAS3</td>
<td>5′-CTCAGCGGCACGCCCTTTACT-3′</td>
<td>( \text{emm18} )</td>
<td>−283/−262*</td>
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<tr>
<td>GAS4</td>
<td>5′-GCTCTTGGACCCCTTACAA-3′</td>
<td>( \text{emm18} )</td>
<td>1115–1135*</td>
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<td>Emm18FB</td>
<td>5′-CGGCTACTTTGATCCACAG-3′</td>
<td>( \text{emm18} )</td>
<td>226/246* /BamHI site</td>
</tr>
<tr>
<td>Emm18RB</td>
<td>5′-GGTAAATTGGGATCCCTCC-3′</td>
<td>( \text{emm18} )</td>
<td>977/997* /BamHI site</td>
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<tr>
<td>NF</td>
<td>5′-AGAAGAAAATCGCGCGCT-3′</td>
<td>( \text{emm18} )</td>
<td>214/231*</td>
</tr>
<tr>
<td>NR2</td>
<td>5′-TGGGTTGCTGCTTCACCTG-3′</td>
<td>( \text{emm18} )</td>
<td>1002/1022*</td>
</tr>
</tbody>
</table>

* Nucleotide position relative to the start codon of \( S. \text{pyogenes} \) \( \text{emm18} \) gene (accession number X80494).

FIGURE 1. Binding of factor H, FHL-1, and C4BP to different M18 strains. a, Cells of the strains 87–282, 346, SS77, 9003, and SS344 were incubated with EDTA-plasma. The proteins bound to the streptococcal surface were eluted, separated by SDS-PAGE, and subjected to a Western blot using a mouse mAb (35H9) that recognizes both factor H and FHL-1, or a rabbit polyclonal antiserum against human C4BP. b, Alignment of Emm18 amino acid sequences. The complete amino acid sequences of Emm18 protein of all five M18 strains are shown. The 87–282 strain was used as the reference sequence. A gap in the sequences of 346, SS77, 9003, and SS344 strains was introduced at position 204 to accommodate an insertion of 42 aa present in the 87–282 strain.
The two mutants that lack Emm18 protein expression, 282KZ and TX74, failed to bind factor H or FHL-1 (Fig. 2). These results are consistent with previous data indicating that both proteins have binding sites in the Emm18 protein (15, 29). Factor H binds to the B or C repeat region, depending on the M serotype (15, 29), and FHL-1 to the N-terminal hypervariable region (15). Therefore, lack of binding to factor H and FHL-1 is consistent with lack of expression of the Emm18 protein on the cell surface of the 282KZ and TX74 strains. In marked contrast to these results, both Emm18-deficient mutant strains bound C4BP (Fig. 2), a finding that implies that the lack of Emm18 protein does not impair the binding of C4BP to the streptococcal surface. Whereas data from other S. pyogenes serotypes indicate that both C4BP and FHL-1 interact with the same N-terminal hypervariable region of the Emm proteins (15, 17), results of the present study strongly suggest that FHL-1 and C4BP bind to different molecules on the streptococcal surface in M18 strains. Moreover, because mutants lacking Emm18 protein are able to bind C4BP, we also conclude that in M18 strains the binding site for C4BP on the streptococcal surface is located in a molecule different from the Emm18 protein. As expected, lack of the capsule had no effect on the capacity to bind C4BP, FHL-1, or factor H by the 87–282 streptococcal strain.

To test whether the Emm18 protein had any influence on binding of C4BP, we performed detailed binding assays to determine the dissociation constants for the interaction between C4BP and the four isogenic streptococcal strains (wild-type 87–282 and the three mutant M18 strains TX72, 282KZ, and TX74) (Fig. 3). We observed similar dissociation constants for all four strains, illustrating that lack of the Emm18 protein does not change significantly the characteristics of the binding site for C4BP in the M18 streptococcal cell surface. This result indicates that the contribution of the Emm18 protein to the binding of C4BP in the wild-type 87–282 strain is negligible. Interestingly, however, lack of expression of the M18 protein on the cell surface (mutants 282KZ and TX74) resulted in a significant 2- to 3-fold increase in the number of C4BP molecules bound to the streptococcal surface. It is possible that lack of the relatively abundant cell surface Emm protein facilitates the binding of a large molecule such as C4BP to other surface molecules and results in an apparent increase in the number of binding sites. Alternatively, it could be, as has been observed in some Gram-negative bacteria, that lack of expression of a membrane protein results in an increased expression of other proteins in the cell surface (30).

We also conducted binding assays to investigate whether C4BP and factor H/FHL-1 bound independently to the surface of the strain 87–282. These experiments clearly demonstrated that there was no competition, and confirmed the existence of two separate binding sites for C4BP and factor H/FHL-1 on strain 87–282 (data not shown).

Results of these experiments suggest that other molecules on the streptococcal cell surface, different from the Emm18 protein, are responsible for the interaction between C4BP and M18 strains. Therefore, we considered whether other members of the M protein family might be binding partners for C4BP. As is shown in Fig. 4, in the M18 strains included in this study the mga regulon contains only two genes encoding for two emm-like proteins, Emm18 and Enn18. M18 strains have no mrp18 gene, as it was previously reported (13). Enn18 has been reported to bind human IgG3, but its role in interaction with complement regulatory proteins has not been studied (31).

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Sequence analysis of the \textit{enn18} gene from the M18 strains (87–282, 346, 9003, SS77, and SS344) did not detect any difference between them or with the \textit{enn18} sequence deposited in GenBank (accession X80494) independently if they bound C4BP or not. However, determination of the \textit{enn18} expression by RT-PCR revealed that all M18 strains produced similar amounts of the \textit{enn18} mRNA, except strain SS344, which exhibited no capacity to bind C4BP and transcribed less \textit{enn18} mRNA, suggesting that the Enn protein might be involved in C4BP binding to M18 strains.

To investigate the role of the Enn protein in the binding of C4BP to M18 strains, we interrupted by insertion-duplication mutagenesis the \textit{enn18} gene required for the synthesis of the Enn18 protein. Because the lack of capsule had no effect on the binding of C4BP to \textit{S. pyogenes}, we used the acapsular mutant TX72, in which the efficiency of transformation is higher than in the encapsulated strain 87–282, to generate an Enn18 mutant. A schematic representation of the chromosome of parent strain TX72 and its Enn18-deficient mutant TX76 is shown in Fig. 5a. Southern blot analysis of the TX76 chromosome, using as probe an \textit{enn18} internal fragment, confirmed that two incomplete copies of the \textit{enn18} gene were generated by the integration of the plasmid (Fig. 5b). To further demonstrate that interruption of \textit{enn18} gene abolished its expression, we used RT-PCR to estimate the levels of \textit{enn18} gene transcription in 87–282 and the four isogenic mutants (282KZ, TX72, TX74, and TX76). We detected \textit{enn18} gene transcripts in all strains, including the Emm18 mutant strains 282KZ and TX74. However, inactivation of \textit{enn18} resulted in the interruption of the gene transcription in TX76 (Fig. 5c, lower panel). As expected, \textit{enn18} gene transcription was only interrupted in the Emm18 mutant strains 282KZ and TX74, but not in the wild-type strain 87–282, in the capsule-deficient mutant TX72, or in the Enn18-deficient mutant TX76 (Fig. 5c, upper panel).

To investigate whether Enn18 is involved in binding of C4BP to M18 strains, we performed binding assays using the TX72 strain and its isogenic Emm18- and Enn18-deficient mutants TX74 and TX76, respectively (Fig. 6). As expected, lack of the capsule in mutant strain TX72 had no effect on the capacity to bind C4BP, FHL-1, or factor H. As shown above, Emm18 mutant strain TX74 failed to bind factor H or FHL-1, but bound C4BP. By contrast, Enn18 mutant strain TX76 bound factor H or FHL-1, but failed to bind C4BP. These results demonstrate that Enn18 protein mediates binding of C4BP to M18 strains.

**Discussion**

M18 strains are one of the most prevalent serotypes among clinical isolates. We found that strains of the M18 serotype vary significantly in their capacity to bind C4BP, but not in the binding of FHL-1 (18), despite both complement regulators having been reported to interact with the same N-terminal hypervariable region of the Emm proteins. Using M18 isogenic strains lacking expression of the Emm18 protein, we found that the Emm18 protein, as reported previously for other \textit{S. pyogenes} strains, mediates binding of factor H and FHL-1 on the streptococcal surface. It was surprising, however, that lack of Emm18 on the cell surface did not...
eliminate binding of C4BP, indicating that some other streptococcal surface molecule was responsible for this interaction. We considered the possibility that the E118 protein was responsible for the binding of C4BP to the streptococcal surface in these strains. This hypothesis was supported by our finding that disruption of the enm18 gene completely eliminated binding of C4BP. As expected, lack of E118 protein did not alter the binding of factor H or FHL-1 to the surface of the streptococci. Our data provide strong evidence that in M18 strains factor H and FHL-1 bind to the E118 protein, while C4BP binds to the E118 protein. Presenting distinct binding sites for factor H/FHL-1 and C4BP located in separate cell surface proteins may provide a more efficient strategy to recruit these three complement regulators simultaneously, rather than having them competing for binding sites in one single surface molecule. Further studies will be needed to characterize the C4BP binding sites in the E118 protein.

Our findings that FHL-1 and C4BP bind to different molecules on the surface of the M18 streptococcal cell, and that binding of C4BP does not require expression of the E118 protein contrast with data previously reported for other streptococcal serotypes. Elegant studies performed in an M22 strain showed that C4BP binds to the N-terminal hypervariable region of the E118 protein and not to the E22 protein (32). Results of the present investigation imply that the molecular mechanisms involved in the binding of complement regulators by the different M serotypes are not necessarily conserved, and that different M serotypes may use distinct surface proteins to bind C4BP. In this regard, it has been reported that while factor H binds to the surface of M5 strains through a binding site in the B repeats of the Emm5 protein, the same factor H uses a binding site in the C repeats of the Emm6 protein to bind to M6 strains (15, 29). Moreover, Pandiripally et al. (33) have recently shown that Fba, a protein unrelated to the M protein family, is the molecule responsible for the factor H and FHL-1 binding in an M1 strain. In addition, factor H and C4BP bind to different molecules in Neisseria gonorrhoeae: factor H binds to sialic acids or to loop 5 of porin protein 1A (34, 35), whereas C4BP binds to this pathogen by type IV Pili, Por1A (loop 5 and 7) (36, 37).

In conclusion, we have demonstrated that the complement regulators FHL-1 and C4BP bind to M18 S. pyogenes strains through two different cell surface proteins. Factor H and FHL-1 bind to the E118 protein, while C4BP binds to the E118 protein. Expression of two distinct binding sites for complement regulatory proteins may represent a unique adaptation of M18 strains that enhances their resistance to complement-mediated opsonophagocytosis. This virulence mechanism further illustrates the diverse and sophisticated strategies used by S. pyogenes for survival in the human host.

Acknowledgments

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