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Human fibrinogen bound to *Streptococcus pyogenes* M protein inhibits complement deposition via the classical pathway

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Summary

Human fibrinogen (Fg) binds to surface proteins expressed by many pathogenic bacteria and has been implicated in different host-pathogen interactions, but the role of bound Fg remains unclear. Here, we analyse the role of Fg bound to *Streptococcus pyogenes* M protein, a major virulence factor that confers resistance to phagocytosis. Studies of the M5 system showed that a chromosomal mutant lacking the Fg-binding region was completely unable to resist phagocytosis, indicating that bound Fg plays a key role in virulence. Deposition of complement on *S. pyogenes* occurred via the classical pathway even under non-immune conditions, but was blocked by M5-bound Fg, which reduced the amount of classical pathway C3 convertase on the bacterial surface. This property of M protein-bound Fg may explain its role in phagocytosis resistance. Previous studies have shown that many M proteins do not bind Fg, but interfere with complement deposition and phagocytosis by recruiting human C4b-binding protein (C4BP), an inhibitor of the classical pathway. Thus, all M proteins may share ability to recruit a human plasma protein, Fg or C4BP, which inhibits complement deposition via the classical pathway. Our data identify a novel function for surface-bound Fg and allow us to propose a unifying mechanism by which M proteins interfere with innate immunity.

Introduction

Human fibrinogen (Fg) is abundant in plasma and is also present in the extracellular matrix, where it contributes to inflammation and wound healing by promoting the recruitment of inflammatory cells that express Fg-binding surface proteins (Nieuwenhuizen et al., 2001; Flick et al., 2004). Surface proteins that bind Fg are also expressed by many bacteria, such as the important human pathogens *Streptococcus pyogenes*, *Staphylococcus aureus* and *Streptococcus agalactiae*, in which Fg-binding proteins have been implicated in adhesion to the extracellular matrix (Foster and Höök, 1998; Ponnuraj et al., 2003), resistance to phagocytosis (Whitnack and Beachey, 1982; Schubert et al., 2002) and triggering of vascular leakage (Herwald et al., 2004). However, the biological role of bacterial Fg-binding proteins remains unclear. Here, we analyse the role of Fg bound to *S. pyogenes* M protein, a major virulence factor that inhibits phagocytosis (Fischetti, 1989).

*Streptococcus pyogenes* (group A streptococcus) is a common human pathogen that causes several diseases, including acute pharyngitis and the streptococcal toxic shock syndrome (Bisno et al., 2003). Like other microorganisms, *S. pyogenes* may be phagocytosed by neutrophils after surface deposition of complement (Fischetti, 1989; Weineisen et al., 2004), but phagocytosis is prevented by M protein and the hyaluronic acid capsule (Lancefield, 1962; Fischetti, 1989; Wessels et al., 1991). In particular, all clinical isolates of *S. pyogenes* express M protein, which plays a key role for the ability of this pathogen to infect its host (Ashbaugh et al., 2000). The surface-localized and variable M protein inhibits complement deposition, a property that can at least partially explain its antiphagocytic function (Jacks-Weis et al., 1982; Whitnack and Beachey, 1982; Carlsson et al., 2003). For many M proteins, this capacity to inhibit complement deposition can be explained by the ability to bind human C4b-binding protein (C4BP), a high-MW plasma protein that inhibits the classical pathway of complement activation (Thern et al., 1995; Lindahl et al., 2000; Berggård et al., 2001; Carlsson et al., 2003). This finding was surprising, because complement deposition on *S. pyogenes* has been believed to occur via the alternative pathway (Fischetti, 1989; Bisno et al., 2003), but may be explained by the demonstration that *S. pyogenes* activates the classical pathway even under non-immune conditions (Carlsson et al., 2003). However, many M proteins do not bind C4BP (Thern et al., 1995; 1998) and their molecular function has remained unclear.

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Complement inhibition by M protein-bound fibrinogen

We have noted that M proteins that do not bind C4BP bind Fg, and vice versa. For example, highly purified preparations of the M4, M22 and M60 proteins bind C4BP but not Fg (Stenberg et al., 1992; Thern et al., 1995; 1998), while the M1, M5 and M6 proteins bind Fg (Whitnack et al., 1984; Åkesson et al., 1994) but not C4BP (Thern et al., 1995; Johnsson et al., 1996). This situation led us to hypothesize that C4BP and Fg play similar roles in S. pyogenes pathogenesis. Using the M5 system, we show here that M protein-bound Fg indeed inhibits complement deposition via the classical pathway, a property that may explain the ability of M5 to inhibit phagocytosis. Our findings identify a novel function for surface-bound Fg and suggest that all M proteins recruit a human plasma protein to inhibit complement deposition via the classical pathway.

Results

Experimental system

The M5 protein, shown schematically in Fig. 1A, is a dimeric coiled-coil protein attached to the S. pyogenes cell wall via the carboxy-terminal region (Fischetti, 1989; Kehoe, 1994; Navarre and Schneewind, 1999). Human Fg binds in the B-repeat region, which is composed of 3.6 imperfect 25-residue tandem repeats (Miller et al., 1988; 2005 Blackwell Publishing Ltd, Molecular Microbiology, 56, 28–39
The Fg-binding region of M5 is essential for phagocytosis resistance

To analyse the role of Fg bound to the M5 protein, we constructed an *S. pyogenes* mutant, designated \( \Delta B \), in which the chromosomal gene encodes an M5 protein without the Fg-binding B-repeat region (Fig. 1A). To exclude that any phenotype of this strain was caused by a secondary mutation inadvertently introduced during strain construction, we also constructed a revertant strain (‘rev.’), in which the wild-type *emm5* allele was reintroduced into the chromosome of \( \Delta B \). These strains were compared with the parental M5 strain and with a mutant (\( \Delta M5 \)) lacking the entire M5 protein. As expected, the \( \Delta B \) and \( \Delta M5 \) strains lacked ability to bind Fg, while the wild-type M5 strain and the revertant bound Fg equally well (Fig. 1B, upper left).

Before the \( \Delta B \) strain was used in functional assays, it was essential to analyse whether the \( \Delta B \) protein was expressed normally on the bacterial surface. For this purpose, we used several antisera raised against synthetic peptides derived from different parts of the M5 protein (Fig. 1A). The \( \Delta B \) mutation eliminated reactivity with antibodies against the B-repeat region (Fig. 1B, upper right), but the \( \Delta B \) mutation did not affect reactivity with antibodies directed against the amino-terminal region or against the C-repeat region (Fig. 1B, lower panels), indicating that the \( \Delta B \) protein was expressed normally on the bacterial surface and that its overall structure was not distorted.

A ligand-binding assay provided further evidence that the structure of the \( \Delta B \) protein was intact. This assay was based on the previous finding that the human plasma protein factor H-like protein 1 (FHL-1) binds to the amino-terminal region (residues 1–103) of the M5 protein (Johnsson et al., 1998). Although FHL-1 is a shorter splice variant of factor H (FH), which occurs in higher concentration than FHL-1 in plasma, *S. pyogenes* almost exclusively binds FHL-1 in plasma, i.e. under physiological conditions (Johnsson et al., 1998). To analyse whether the \( \Delta B \) mutation affected the ability of the M5 protein to bind FHL-1, we performed an absorption experiment, in which whole bacteria were incubated with fresh human plasma, followed by elution of bound human proteins and analysis of eluates by Western blot. This analysis indicated that FHL-1 bound equally well to the \( \Delta B \) mutant as to the wild type strain, but did not bind to the \( \Delta M5 \) strain, that lacks the entire M5 protein (Fig. 1C). Thus, the amino-terminal region of the surface-exposed \( \Delta B \) protein retained ligand-binding ability, strongly indicating that its structure was not distorted.

The ability of the different strains to resist phagocytosis was analysed in the Lancefield bactericidal assay (Lancefield, 1957), which measures phagocytosis by neutrophils in non-immune human blood, i.e. blood lacking opsonizing antibodies (Fischetti, 1989; Schnitzler et al., 1995). For the wild-type M5 strain, the average increase in titre during the 3 h incubation period was 619, corresponding to a generation time of ~19 min, and similar results were obtained with the revertant strain (Fig. 2). In contrast, the \( \Delta B \) mutant was completely unable to grow, like the \( \Delta M5 \) strain. The inability of these strains to grow in blood was not a result of a general effect on growth, because all strains grew equally well in human plasma (data not shown). These data show that the Fg-binding B-repeat region of M5 plays an essential role in phagocytosis resistance.

**Fig. 2.** Phagocytosis resistance promoted by the M5 protein requires the Fg-binding B-repeat region. The bacterial strains indicated were analysed for ability to multiply during a 3 h incubation period in non-immune human blood. Growth is presented as mean multiplication factor (MF) ± SD. The isogenic strains analysed were the wild type M5 strain (M5), its M-negative mutant (\( \Delta M5 \)), the mutant strain lacking the Fg-binding B-repeat region (\( \Delta B \)) and a wild type revertant (rev.) derived from the \( \Delta B \) mutant. Results from four experiments with three different blood donors.
Complement inhibition by M protein-bound fibrinogen

The effect of the intact M5 protein and of the B-repeat region on complement deposition was evaluated by incubating the three strains ΔM5, M5 and ΔB in normal plasma (Fig. 3B). As compared with the ΔM5 strain, C3d deposition on the M5 strain was reduced 8.9-fold, implying that the M5 protein inhibits complement deposition via the classical pathway. In contrast, no reduction was observed for the ΔB strain, indicating that the Fg-binding B-repeat region is required for the ability of M5 to inhibit complement deposition. Together with the phagocytosis assays described above (Fig. 2), these data indicate that the B-repeat region of M5 confers phagocytosis resistance by inhibiting complement deposition via the classical pathway.

Inhibition of classical pathway complement deposition by the M5 protein requires bound Fg

The finding that complement deposition via the classical pathway was inhibited by the wild-type M5 protein, but not by the ΔB protein, suggested that bound Fg was involved in the inhibition. The role of bound Fg was directly evalu-

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**Fig. 3.** Complement deposition on *S. pyogenes* occurs via the classical pathway but is inhibited by the M5 protein. A. Deposition of C3d on the M-negative ΔM5 strain is inhibited by both Mg-EGTA and EDTA. Incubation in non-immune human plasma at 37°C for 10 min. Deposition on the ΔM5 strain in normal human plasma (NHP) was defined as 100%. B. Deposition of C3d on *S. pyogenes* is inhibited by surface expression of the M5 protein but not by expression of the ΔB protein. The three bacterial strains indicated were incubated in non-immune human plasma for 30 min; similar data were obtained at 10 min. Deposition on the ΔM5 strain was defined as 1.0. A and B show results from three FACS experiments with different plasma donors, with means ± SD, and representative histograms are included. IC, isotype control.
ated by analysing deposition in non-immune serum and in serum supplemented with pure human Fg. In serum, the amount of C3d deposited on the wild type M5 strain was only slightly reduced, as compared with the ΔM5 and ΔB strains (Fig. 4A), indicating that the M5 protein has little or no effect on complement deposition in the absence of Fg. The key role of Fg was confirmed by the demonstration that addition of pure Fg caused a dose-dependent inhibition of deposition on the M5 strain (Fig. 4B, upper panels). At physiological concentrations of Fg (2 mg ml$^{-1}$), complement deposition was reduced as much as observed in whole human plasma (Fig. 3B), and deposition was considerably decreased even at 25-fold lower concentration of Fg (0.08 mg ml$^{-1}$). In contrast, addition of Fg did not decrease deposition on the ΔM5 and ΔB strains, which do not bind Fg, implying that Fg must be surface-bound to protect against complement attack (Fig. 4B). Addition of pure fibronectin, which may contaminate Fg preparations (Engvall et al., 1978) and binds to some M proteins (Cue et al., 2001), did not decrease complement deposition on the M5 strain, excluding that the effects observed were caused by contaminating fibronectin (data not shown). Thus, the B-repeat region of M5 inhibits deposition of complement via the classical pathway by recruiting host Fg, which must be surface-bound to exert this function.

**Fig. 4.** Inhibition of classical pathway complement deposition by the M5 protein requires bound Fg.
A. Deposition of C3d on the three strains indicated, after incubation in non-immune human serum at 37°C for 30 min. Expression of M5 only slightly reduced C3d deposition on *S. pyogenes* (compare with data for plasma in Fig. 3B). Deposition of C3d on the ΔM5 strain was defined as 1.0.
B. Addition of pure human Fg to serum blocks complement deposition on the M5 strain, but not on the non-Fg-binding ΔM5 and ΔB strains. Bacteria were incubated in non-immune serum at 37°C for 30 min, with addition of pure Fg, as indicated. Deposition of C3d on ΔM5 in the absence of Fg was defined as 1.0. Each set of data in A and B represents means ± SD from three experiments with different serum donors. Representative histograms are included. IC, isotype control.
Binding of Fg to S. pyogenes does not cause recruitment of a known complement inhibitor

A possible mechanism, by which bacteria-bound Fg inhibits complement deposition via the classical pathway, would involve recruitment of one of the human plasma proteins that inhibit this pathway, C1 inhibitor (C1-INH) and C4BP (Morgan and Harris, 1999). This hypothesis was analysed by incubation of the M5 strain and its two mutants ΔM5 and ΔB in whole human plasma, followed by elution of bound proteins and analysis by Western blot (Fig. 5). As expected, Fg was present in the eluate from the M5 strain, but not from the ΔM5 or ΔB strains, while C1-INH and C4BP were absent from all eluates, implying that none of these human proteins was causing the Fg-dependent inhibition of complement deposition.

Fg bound to the M5 protein inhibits formation of the classical pathway C3 convertase

Deposition of complement via the classical pathway may be inhibited through reduced formation of the classical pathway C3 convertase (which is composed of complement components C4b and C2a) or through enhanced decay and degradation of convertase that has been formed (Morgan and Harris, 1999). To analyse how Fg bound to M protein inhibits deposition, we analysed surface deposition of C4d, a fragment of the classical pathway C3 convertase that remains on the surface even after decay and degradation of this convertase (Volanakis and Frank, 1998; Morgan and Harris, 1999). The results (Fig. 6A) indicate that presence of Fg strongly reduced deposition of C4d on M5 bacteria but not on ΔM5 bacteria. These data confirm that bacteria-bound Fg inhibits deposition via the classical pathway and suggest that it acts by inhibiting formation of the C3 convertase.

To further explore the mechanism, by which Fg reduces the amount of complement deposited on the bacterial surface, we analysed surface deposition of C4b (Fig. 6B). During formation of the classical pathway C3 convertase (C4b,2a), C4b is first deposited on the surface (Fig. 6B, left part). If C4b is not part of an intact C3 convertase, it will be degraded, with release of the C4c fragment, while C4d remains surface-bound (Morgan and Harris, 1999). The C4d/C4c ratio on the bacterial surface can therefore be used to analyse whether an inhibitor blocks deposition of C4b or promotes degradation of this molecule (Fig. 6B). If the inhibitor blocks deposition, the C4d/C4c ratio will not be affected (although the total number of deposited C4b molecules has decreased), but if the inhibitor promotes degradation, the ratio will increase. The data in Fig. 6B (right) show that the presence of Fg did not affect the C4d/C4c ratio on M5-expressing bacteria, indicating that C4b remains intact in the presence of Fg. Altogether, the data in Fig. 6 indicate that bacteria-bound Fg inhibits formation of the classical pathway C3 convertase rather than promoting its decay or degradation. However, it cannot be excluded that bound Fg promotes release of the entire C4b molecule from the bacterial surface by an unknown mechanism.

Discussion

The work reported here throws new light on the role of Fg in S. pyogenes infections, a subject that has remained controversial. Early work suggested that Fg bound to M protein inhibits association of S. pyogenes with phagocytes, possibly by inhibiting complement deposition (Whitnack and Beachey, 1982), but direct evidence that M proteins exploit bound Fg to confer phagocytosis resistance has been lacking, and it is now known that many M proteins do not bind Fg (O'Toole et al., 1992; Stenberg et al., 1992; Podbielski et al., 1996; Tern et al., 1998). Moreover, functional studies in the M6 system led to the conclusion that bound Fg had little or no effect on complement deposition or phagocytosis resistance (Fischetti, 1989; Horstmann et al., 1992). Although a previous study in the M5 system indicated that the B-repeat region had a limited effect on phagocytosis resistance (Ringdahl et al., 2000), the results of that study were unclear, because the plasmid-complemented strains used grew very poorly in blood. In contrast, our studies with the chromosomal ΔB mutant show that the B-repeat region of M5 plays a key role in phagocytosis resistance.

**Fig. 5.** Fg bound to S. pyogenes M5 protein does not cause recruitment of a known complement inhibitor. The three bacterial strains M5, ΔM5 and ΔB were incubated in human plasma, followed by elution of bound proteins and Western blot analysis of the eluates with antiserum against Fg or against either of two inhibitors of the classical pathway, C1-INH or C4BP. Each Western blot included a sample of normal human plasma (NHP) and eluates from the three strains, as indicated. The M5 strain bound Fg, as expected, but none of the strains bound any of the two complement inhibitors. However, the inhibitors were identified in NHP.
Interestingly, the analysis described here indicates that Fg bound to the M5 protein promotes phagocytosis resistance by inhibiting complement deposition via the classical pathway. Previous studies have shown that the M22 protein binds human C4BP, thereby reducing complement deposition via the classical pathway and promoting phagocytosis resistance (Berggård et al., 2001; Carlsson et al., 2003). Thus, an M protein may recruit either C4BP or Fg to inhibit complement deposition via the classical pathway. Because all purified M proteins analysed so far bind either Fg or C4BP, the available data allow us to propose a unifying model for M protein function (Fig. 7).

According to this model, S. pyogenes triggers formation of the classical pathway C3 convertase even under non-immune conditions, as reported also for some other Gram-positive pathogens (Wessels et al., 1995; Brown et al., 2002; Ren et al., 2004). This complement activation under non-immune conditions may be caused by the binding of ‘natural antibodies’, that trigger the C1q-dependent pathway (Brown et al., 2002), or to binding of mannose-binding lectin (MBL), that triggers the lectin pathway. Of note, MBL binds to bacteria of several species, including S. pyogenes (Neth et al., 2000).

The model proposed here predicts that complement deposition is inhibited by a human plasma protein recruited to the surface-localized M protein. Some M proteins, such as the M5 protein, prevent complement deposition by binding Fg, while other M proteins, such as M22, bind C4BP. The role of M protein-bound C4BP can be readily explained, because bound C4BP retains its complement inhibitory function and protects bacteria from complement deposition (Thern et al., 1995; Carlsson et al., 2003), but the mechanism by which Fg inhibits complement deposition remains to be defined. Our data indicate that M protein-bound Fg inhibits formation of the classical pathway C3 convertase on the bacterial surface, an effect that could be the result of steric hindrance. However, bound Fg does not inhibit binding of the plasma protein FHL-1 to the amino-terminal region of M5 (Johnson et al., 1998), arguing against steric hindrance.

The properties of the M5 and M22 proteins indicate that they inhibit complement deposition by different mechanisms (Fig. 7). These two M proteins also exhibit another important difference, as demonstrated by analysis under immune conditions, i.e. in the presence of opsonizing antibodies. It is well-known that antibodies directed

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against the amino-terminal hypervariable region (HVR) of M protein are opsonizing (Beachey et al., 1987; Jones and Fischetti, 1988). For the M22 protein, this is logical, because opsonizing antibodies block binding of C4BP, which binds to the HVR (Johnsson et al., 1996; Morfeldt et al., 2001; Carlsson et al., 2003). However, for the M5 protein the situation is paradoxical, because Fg binds to the B-repeats, while the HVR is a target for opsonizing antibodies. The reasons for this apparent paradox are not clear, and the biological function of the HVR in M5 is not known. Work is in progress to study this complex problem.

A number of previous studies on M proteins and the complement system have been focused on the alternative pathway, which is commonly assumed to be the main pathway for complement deposition under non-immune conditions. In particular, it has been reported that some M proteins bind human FH, which inhibits complement deposition via the alternative pathway (Horstmann et al., 1988; Fischetti et al., 1995). Because bacteria-bound FH retains its complement regulatory activity, it was proposed that bound FH protects bacteria from complement deposition via the alternative pathway and thereby confers phagocytosis resistance (Horstmann et al., 1988). However, studies of bacterial mutants suggest that bound FH is not required for phagocytosis resistance (Perez-Casal et al., 1995; Kotarsky et al., 2001). The role of FH is also called into question by the finding that M proteins selectively bind the splice variant FHL-1, rather than FH, under physiological conditions (Johnsson et al., 1998). The binding site for FHL-1 was localized to the amino-terminal region of the M5 protein, suggesting that bound FHL-1 might contribute to phagocytosis resistance (Johnsson et al., 1998), but the available data do not support this hypothesis (Kotarsky et al., 2001). Thus, the role of bacteria-bound FHL-1/FH remains unclear.

The conclusion, that M proteins inhibit complement deposition via the classical pathway, does not exclude that they contribute to phagocytosis resistance and virulence also in other ways. For example, the M5 protein has been implicated in adhesion to human epithelial cells, in addition to its antiphagocytic function (Courtney et al., 1997). The other M protein discussed above, the M22 protein, not only binds C4BP but also binds IgA, which contributes to phagocytosis resistance by an unknown mechanism (Carlsson et al., 2003) (Fig. 7). Moreover, it has recently been reported that the Fg-binding M1 protein is released from bacteria by proteases, allowing it to form soluble Fg-containing complexes that affect vascular permeability (Herwald et al., 2004). However, the in vivo significance of this phenomenon is unclear, because enzymatic release of M1 may be blocked by bound Fg (Nyberg et al., 2004). Another study of the M1 protein led to the proposal that it actually acts intracellularly (Staali et al., 2003), but many other reports agree that M proteins act extracellu-

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**Fig. 7.** Working model for the inhibition of complement deposition by M proteins. Complement is activated via the classical pathway by S. pyogenes, potentially resulting in surface deposition of C3b. However, M protein (a dimeric coiled-coil protein) inhibits this deposition of C3b by recruiting a human plasma protein, which acts by reducing the formation or activity of the classical pathway C3 convertase. Some M proteins, such as M5, bind fibrinogen (Fg), while other M proteins, such as M22, recruit the ~570 kDa C4b-binding protein (C4BP), an inhibitor of the classical pathway. Importantly, the inhibition of complement deposition promotes resistance to phagocytosis. As indicated, the M22 protein (and many other M proteins) also binds IgA, which contributes to phagocytosis resistance by an unknown mechanism (Carlsson et al., 2003). Note that other S. pyogenes surface structures, not shown here, may also affect complement deposition and phagocytosis resistance.

larly (Whitnack and Beachey, 1982; Manjula et al., 1985; Schnitzler et al., 1995; 1999; Weineisen et al., 2004).

As mentioned above, many M proteins, such as M22, do not bind Fg. However, binding studies with whole bacteria have shown that virtually all clinical isolates of S. pyogenes, including M22-expressing strains, bind Fg (Kantor and Cole, 1959; Kronvall et al., 1979; Stenberg et al., 1992). In many strains this property depends on the expression of a Fg-binding M protein, but in other strains the Fg-binding ability is caused by another member of the M protein family, the Mrp protein (O’Toole et al., 1992; Stenberg et al., 1992; Podbielski et al., 1996; Thern et al., 1998). Interestingly, this Fg-binding protein contributes to phagocytosis resistance (Podbielski et al., 1996; Thern et al., 1998). Because all Mrp-expressing strains analysed so far also express a C4BP-binding M protein (Stenberg et al., 1992; Thern et al., 1995), such strains might exploit both Fg and C4BP for evasion of phagocytosis.

The findings reported here are of relevance not only for bacterial pathogenesis and innate immunity but also for the normal function of human Fg. In addition to its role in haemostasis, Fg plays an important proinflammatory role, by enhancing the recruitment to inflammatory sites of leukocytes, which have Fg-binding surface proteins (Nieuwenhuizen et al., 2001; Flick et al., 2004). However, the
finding that Fg bound to M protein inhibits complement activation via the classical pathway suggests that Fg bound to human cells might also have an anti-inflammatory effect, by reducing spontaneous deposition of complement triggered via the classical pathway (Manderson et al., 2001). This hypothesis has interesting implications for the pathogenesis of inflammatory and autoimmune diseases.

In summary, our data indicate that human Fg bound to S. pyogenes M5 protein promotes phagocytosis resistance by inhibiting complement deposition via the classical pathway. This finding identifies a previously unrecognized function for surface-bound Fg and defines a novel mechanism by which pathogenic bacteria evade innate immunity. It will be of interest to analyse whether other bacterial pathogens that bind Fg also exploit this ligand to confer protection against complement attack via the classical pathway.

Experimental procedures

Bacterial strains

The S. pyogenes M5 Manfredo strain is an OF− strain expressing the M5 protein (Miller et al., 1988). This strain is referred to here as M5. An isogenic M-negative mutant of the M5 strain, denoted ΔM5, has been described (Johnsson et al., 1998). S. pyogenes was grown in Todd-Hewitt broth supplemented with 0.2% yeast extract (THY) without agitation in 5% CO2 at 37°C. The Escherichia coli XL-1 strain was used for subcloning.

Construction of a chromosomal ΔB mutant

A chromosomal mutant (ΔB) of the M5 strain expressing an M5 protein lacking the Fg-binding B repeat region (Kehoe, 1994; Ringdalh et al., 2000), was constructed by the introduction of an in-frame deletion in the emm5 gene corresponding to 93 amino acid residues (118−210) in the processed form of the protein (Miller et al., 1988) (Fig. 1A), using previously described procedures (Perez-Casal et al., 1993; Carlsson et al., 2003). A ~3.7 kb fragment, including the emm5 gene and large parts of the two surrounding genes (mga and scpA), was polymerase chain reaction (PCR) amplified from genomic wild type DNA, using forward and reverse primers with BamHI and SalI sites respectively. The fragment was ligated into pUC19 and after transformation into E. coli XL-1 transformants were screened for the presence of the insert by PCR. The deletion was introduced by a second PCR, employing forward and reverse primers complementary to DNA downstream and upstream of the desired deletion, respectively, therefore 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. Transformants were screened by PCR for inserts with the desired deletion and one positive clone was verified by DNA sequencing. To introduce the deletion into the chromosome of S. pyogenes, the mutated emm5 gene was transferred to pJRS233, a shuttle vector exhibiting temperature-sensitive replication in S. pyogenes (Perez-Casal et al., 1993). This derivative of pJRS233 was electroporated into the ΔM5 strain, and a desired recombinant clone was isolated as described (Perez-Casal et al., 1993). The sequence of the entire mutated chromosomal emm5 allele, including the promoter region, was verified by DNA sequencing.

A revertant strain, in which the wild-type emm5 allele was reintroduced into the chromosome of the ΔB mutant by allelic exchange was constructed essentially as described (Berggård et al., 2001).

Purified proteins and peptides

Purified human Fg and fibronectin were purchased from American Diagnostica (Stamford, CT) and Sigma-Aldrich (St. Louis, MO) respectively. Protein G was from Amersham Biosciences (Uppsala, Sweden). Synthetic peptides corresponding to different regions of the M5 protein (Fig. 1) were purchased from the Department of Clinical Chemistry, Malmö General Hospital (Malmö, Sweden) or Neosystems S.A. (Strasbourg, France). A carboxy-terminal cysteine residue, not present in the M5 protein, was included in each peptide, to allow dimerization or conjugation to a carrier protein. Tyrosine residues were incorporated adjacent to the carboxy-terminal cysteine residue in the M5-N and M5-C peptides to facilitate radiolabelling, if needed (Morfeldt et al., 2001).

Antisera and mAbs

The polyclonal rabbit anti-M5-N serum has been described (Berggård et al., 2001). Polyclonal antisera directed against the M5-B peptide, conjugated to OVA, and against the dimerized M5-C peptide were raised as described (Morfeldt et al., 2001). Polyclonal goat anti-human FH/FHL-1 was from Calbiochem (La Jolla, CA). Mouse mAbs, of the IgG1 isotype, directed against human C3d, C4d and C4c were from Quidel Corp. (San Diego, CA). Mouse IgG1 (Quidel Corp.) was used as isotype control. Polyclonal FITC-conjugated goat F(ab′)2 anti-mouse IgG (DakoCytomation, Glostrup, Denmark) was used for detection. Polyclonal rabbit anti-human Fg and anti-human C1-inactivator were from DakoCytomation, and polyclonal rabbit anti-human C4BP was the kind gift of Dr B. Dahlbäck (Lund University, Sweden).

Binding assays with bacteria

The ability of S. pyogenes to bind 125I-labelled human Fg was analysed as described (Thern et al., 1998). Binding of polyclonal rabbit antibodies to streptococci was detected with 125I-labelled protein G, as described (Stålhammar-Carlemalm et al., 1993). Binding is expressed as a percentage of maximal binding observed for the positive control, which was ~50% for Fg and ~85% for protein G and rabbit antibodies.

Plasma absorption assay

The ability of S. pyogenes to bind human plasma proteins was analysed essentially as described (Johnsson et al., ...
1998). Overnight cultures of the different bacterial strains were washed and resuspended to 10^10 bacteria ml^{-1} in PBS. A sample (500 μl) was pelleted and resuspended in 1500 μl of human plasma, collected from a non-immune donor in EDTA-vacuette® tubes (Greiner bio-one, Kremsmünster, Austria). After incubation for 1 h at room temperature, the bacteria were washed three times with PBSAT (PBS supplemented with 0.02% azid and 0.05% Tween-20). Bound proteins were eluted with 150 μl of 3 M KSCN for 20 min at room temperature. The eluates were dialysed against PBS and analysed by Western blot.

**Phagocytosis assay**

Resistance to phagocytosis in whole human blood was analysed essentially as described (Lancefield, 1957; Carlsson et al., 2003). In brief, bacteria were grown to A_620 = 0.15 in THY, diluted in THY, and a sample (25 μl) containing ~100 colony-forming units (cfu) was mixed with 285 μl of freshly drawn human blood. Anti-coagulation was achieved with the specific thrombin-inhibitor hirudin (Refludan; Schering) at a final concentration of 140 U ml^{-1} (Thern et al., 1998). The tubes were rotated at 37°C for 3 h, and bacterial growth (multiplication factor; MF) was calculated by dividing the bacterial titre after incubation with that before incubation. Blood was drawn from non-immune human donors, i.e. donors lacking anti-M5 opsonizing antibodies.

**Analysis of complement deposition on S. pyogenes by flow cytometry**

FACS®-analysis was performed essentially as described (Carlsson et al., 2003). Washed bacteria (~5 × 10^8 cfu in 300 μl of PBS), harvested at A_620 = 0.4, were mixed with freshly prepared human plasma or serum (240 μl), and incubated at 37°C for 10 or 30 min, as indicated. Plasma was supplemented with hirudin (Refludan, 140 U ml^{-1}) to prevent coagulation. Plasma or sera from at least three different non-immune donors (defined as described in Phagocytosis assay) were used in each analysis. The incubated bacteria were washed twice with 1.5 ml of PBS, resuspended in 300 μl of PBS containing 20 μg ml^{-1} of the appropriate primary mAb and incubated at room temperature for 10 min. After washing with 1.5 ml of PBS, the bacteria were resuspended in 300 μl of FITC-conjugated goat F(ab')2 anti-mouse IgG that had been diluted 1:10 in PBS, and incubated for 10 min at room temperature. Finally, the bacteria were washed and resuspended in 800 μl of PBS for FACS®-analysis. Activation via the classical pathway was blocked with 10 mM EGTA + 2.5 mM MgCl₂ and total complement activation was blocked with 10 mM EDTA. The effect of Fg was analysed by adding pure human Fg to serum, supplemented with hirudin (Refludan, 70 U ml^{-1}) to prevent clot formation. PBS was added to the control tubes without Fg, resulting in the same final concentration of serum (33%) and bacteria in all tubes.

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