LUND UNIVERSITY

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

Carlsson, Fredric; Sandin, Charlotta; Lindahl, Gunnar

Published in:
Molecular Microbiology

DOI:
10.1111/j.1365-2958.2005.04527.x

2005

Link to publication

Citation for published version (APA):
Carlsson, F., Sandin, C., & Lindahl, G. (2005). Human fibrinogen bound to Streptococcus pyogenes M protein inhibits complement deposition via the classical pathway. *Molecular Microbiology*, *56*(1), 28-39. https://doi.org/10.1111/j.1365-2958.2005.04527.x

Total number of authors:
3

General rights
Unless other specific re-use rights are stated the following general rights apply:
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.
• Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: https://creativecommons.org/licenses/

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

LUND UNIVERSITY

PO Box 117
221 00 Lund
+46 46-222 00 00

Molecular Microbiology (2005) **56**(1), 28–39 doi:10.1111/j.1365-2958.2005.04527.x

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

Fredric Carlsson,† Charlotta Sandin† and Gunnar Lindahl*
Department of Medical Microbiology, Dermatology and Infection, Lund University, Sölvegatan 23, SE-22362 Lund, Sweden.

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.

Accepted 13 December, 2004. *For correspondence. E-mail gunnar.lindahl@mmb.lu.se; Tel. (+46) 4617 3244; Fax (+46) 4618 9117. †These authors contributed equally to this work.

© 2005 Blackwell Publishing Ltd

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;

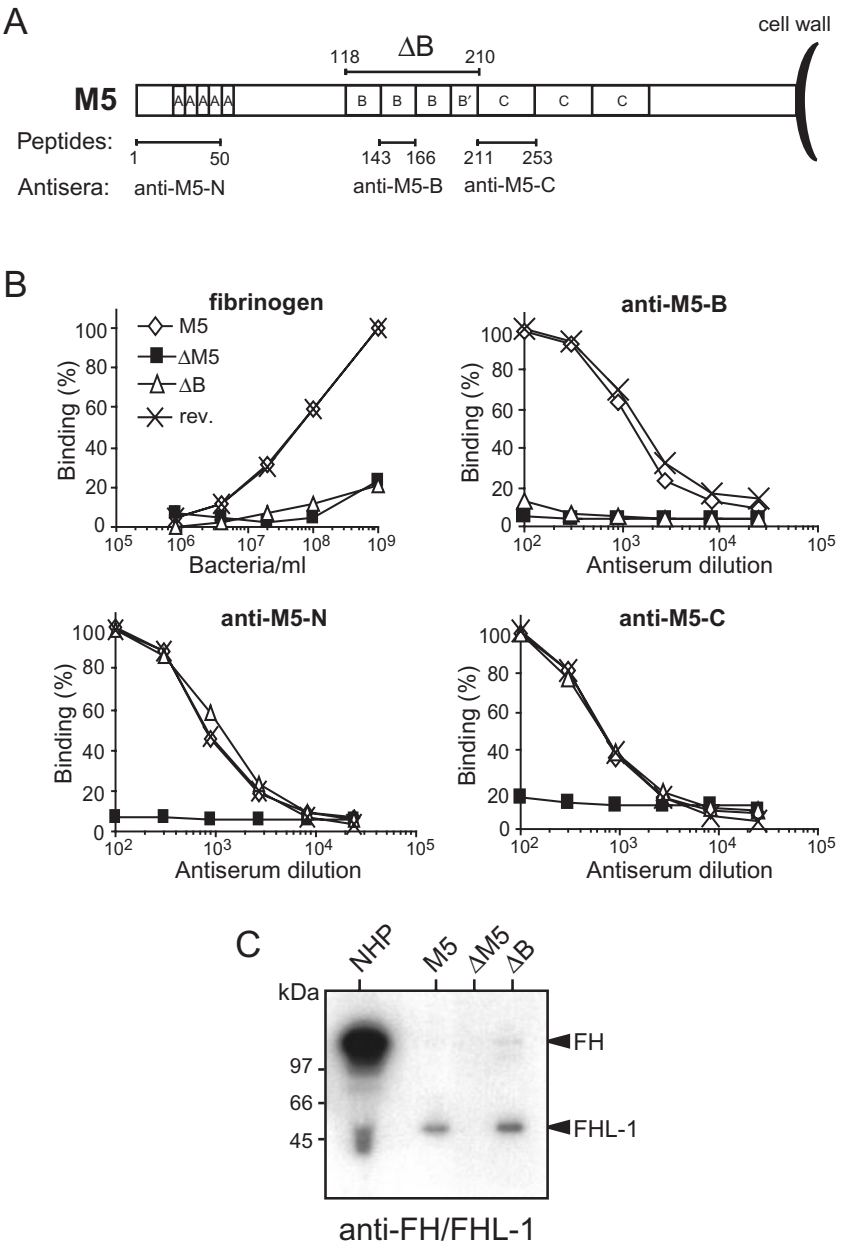

Fig. 1. Schematic representation of the M5 protein and characterization of the ΔB mutant.
A. The M5 protein has three types of repeats, designated A, B and C. The position of the ΔB deletion, which covers the fibrinogen-binding B-repeat region, is indicated. Peptides derived from the indicated regions were used to raise rabbit antisera. Numbers refer to residue positions in the processed form of the M5 protein.
B. Binding properties of the ΔB mutant. This strain was compared with the parental wild type strain (M5), a mutant lacking M protein (ΔM5) and a revertant derived from the ΔB mutant (rev.). These four strains were analysed for ability to bind radiolabelled Fg and for reactivity with the three antisera indicated. Data from three experiments with duplicate samples are presented as means, but SDs are excluded, for clarity.
C. The ΔB mutant retains ability to bind the human plasma protein FHL-1, a splice variant of factor H (FH). The bacterial strains indicated were incubated in whole human plasma, followed by elution of bound plasma proteins and Western blot analysis with antiserum to FH/FHL-1 as the probe. In normal human plasma (NHP), the antiserum mainly detects FH, which occurs in much higher concentration than FHL-1, but the bacteria selectively bind FHL-1, which binds equally well to the ΔB mutant as to the wild type M5 strain. Similar results were obtained in several experiments.

Kehoe, 1994; Ringdahl *et al*., 2000). The interaction between M protein and Fg is of high affinity (Whitnack and Beachey, 1985a; Ringdahl *et al*., 2000; Nyberg *et al*., 2004), and analysis of an M1 strain indicates that the number of binding sites on the bacterial surface is ~2.2×10^5 cell^{-1} (Nyberg *et al*., 2004). Binding of M protein to Fg has been reported to occur with a stoichiometry of ~1:1 (Whitnack and Beachey, 1985a), and the binding site in Fg is probably located in the D fragment (Runehagen *et al*., 1981; Whitnack and Beachey, 1985b).

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 ΔB, 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 ΔB. These strains were compared with the parental M5 strain and with a mutant (ΔM5) lacking the entire M5 protein. As expected, the ΔB and ΔM5 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 ΔB strain was used in functional assays, it was essential to analyse whether the ΔB 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 ΔB mutation eliminated reactivity with antibodies against the B-repeat region (Fig. 1B, upper right), but the ΔB 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 ΔB 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 ΔB 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 ΔB 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 ΔB mutant as to the wild type strain, but did not bind to the ΔM5 strain, that lacks the entire M5 protein (Fig. 1C). Thus, the amino-terminal region of the surface-exposed ΔB protein retained ligand-binding ability, strongly indicating that its structure was not distorted.

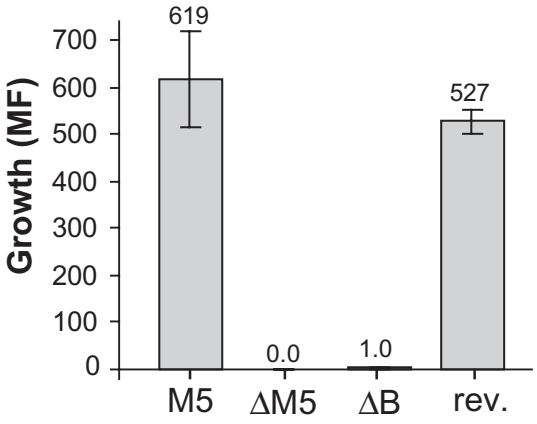

Fig. 2. Phagocytosis resistance promoted by the M5 protein requires the Fg-binding B-repeat region. The bacterial strains indicated were analysed for ability to multiply during a 3 h incubation period in non-immune human blood. Growth is presented as mean multiplication factor (MF) ± SD. The isogenic strains analysed were the wild type M5 strain (M5), its M-negative mutant (ΔM5), the mutant strain lacking the Fg-binding B-repeat region (ΔB) and a wild type revertant (rev.) derived from the ΔB mutant. Results from four experiments with three different blood donors.

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 ΔB mutant was completely unable to grow, like the ΔM5 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.

The M5 protein inhibits complement deposition via the classical pathway

Complement deposition was analysed after incubation of bacteria in fresh human plasma from non-immune donors. Because our interest was focused on Fg, it was essential to perform these experiments with plasma, rather than with serum, which is usually used for analysis of comple-

ment deposition. Hirudin, a specific thrombin inhibitor (Markwardt, 1993), was used for anticoagulation (Thern *et al.*, 1998), because the commonly used heparin may affect complement activation (Sahu and Pangburn, 1993). The deposition of complement was analysed with a mAb directed against C3d, which detects deposition, whether it has occurred via the classical or the alternative pathway [here, the classical pathway is considered to include the C1q-dependent and MBL-dependent pathways, both of which result in formation of the classical pathway C3 convertase (Volanakis and Frank, 1998)].

To analyse whether complement was deposited via the classical or alternative pathway on M5-negative bacteria, the ΔM5 strain was incubated in normal human plasma, in plasma supplemented with EDTA, which blocks both the classical and alternative pathways, or in plasma supplemented with Mg-EGTA, which blocks only the classical pathway (Fine *et al.*, 1972) (Fig. 3A). The deposition of C3d that occurred in normal human plasma was completely inhibited by both EDTA and Mg-EGTA, indicating that complement deposition on ΔM5 occurred exclusively via the classical pathway.

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-

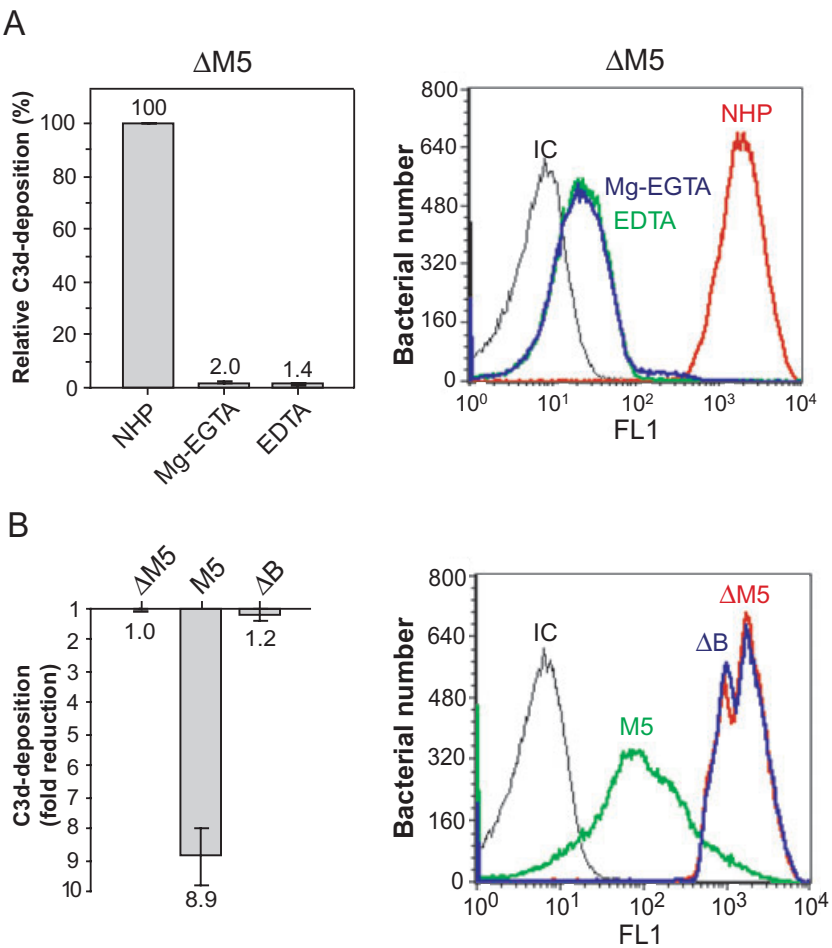

Fig. 3. Complement deposition on *S. pyogenes* occurs via the classical pathway but is inhibited by the M5 protein.
A. Deposition of C3d on the M-negative ΔM5 strain is inhibited by both Mg-EGTA and EDTA. Incubation in non-immune human plasma at 37°C for 10 min. Deposition on the ΔM5 strain in normal 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.

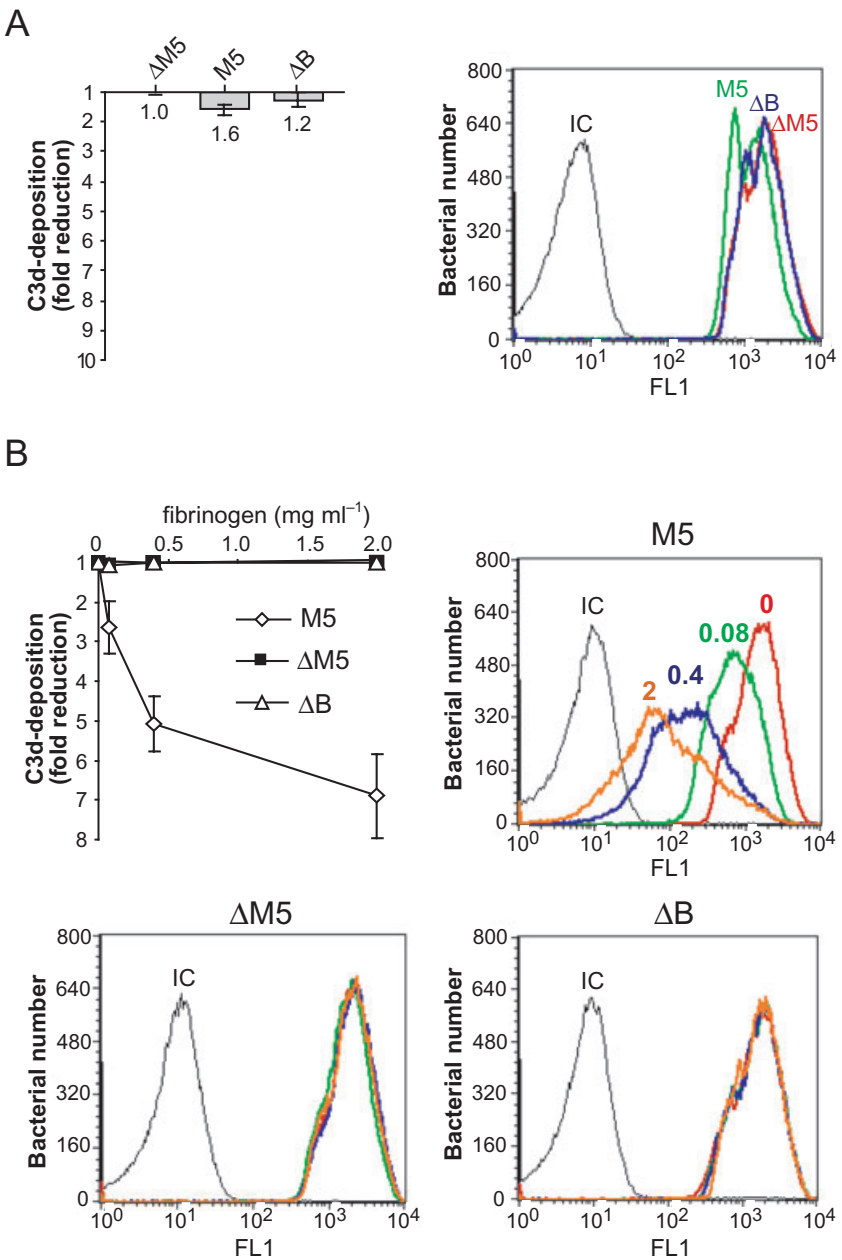

Fig. 4. Inhibition of classical pathway complement deposition by the M5 protein requires bound Fg.
A. Deposition of C3d on the three strains indicated, after incubation in non-immune human serum at 37°C for 30 min. Expression of M5 only slightly reduced C3d deposition on *S. pyogenes* (compare with data 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; Thern *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.

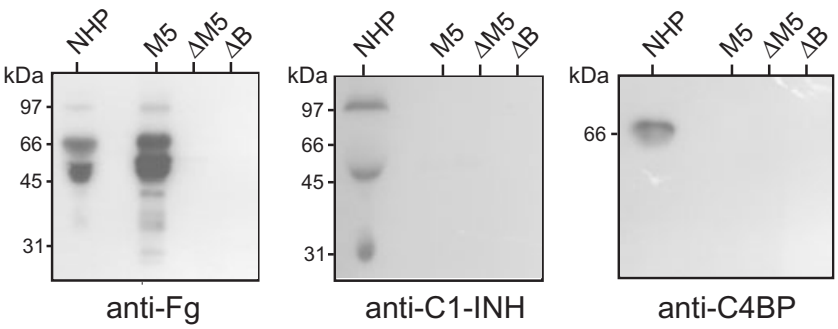

Fig. 5. Fg bound to *S. pyogenes* M5 protein does not cause recruitment of a known complement inhibitor. The three bacterial strains M5, ΔM5 and ΔB were incubated in human plasma, followed by elution of bound proteins and Western blot analysis of the eluates with antiserum against Fg or against either 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.

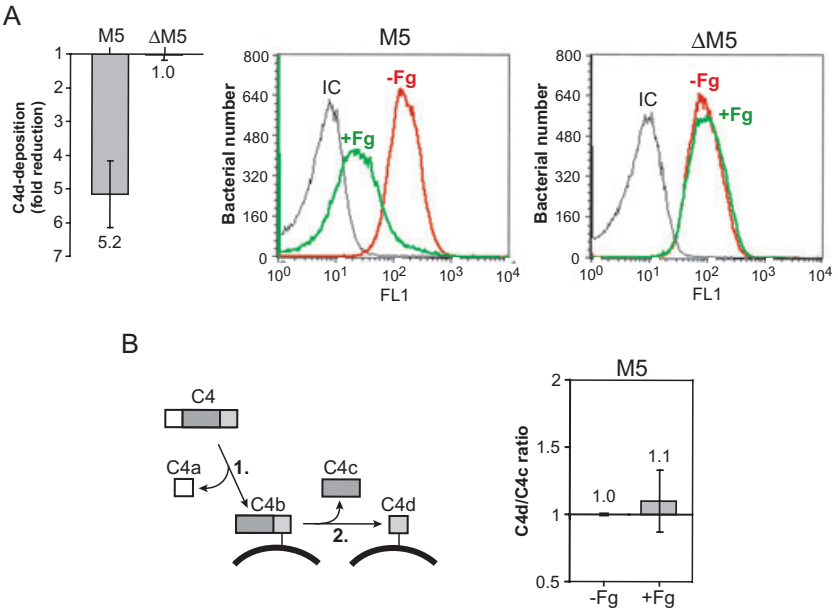

Fig. 6. Fg bound to the M5 protein inhibits formation of the classical pathway C3 convertase on the bacterial surface.
A. Wild type M5 bacteria and the M-negative strain ΔM5 were incubated in non-immune human serum, with or without the addition of pure Fg (1 mg ml^{-1}), as indicated, and the bacteria were analysed for surface deposition of the C4d fragment, which reflects formation of the classical pathway C3 convertase. The added Fg, which bound to surface-localized M5 protein, caused a 5.2-fold reduction in C4d deposition on the M5 strain, but did not reduce C4d deposition on the ΔM5 strain. Incubation at 37°C for 10 min. Results (means ± SD) from three experiments with different serum donors. Representative histograms are included. IC, isotype control.
B. Analysis of surface deposition of C4b and its constituent parts C4c and C4d. Left: activation of the classical pathway causes surface deposition of C4b (which together with C2a may form the classical pathway C3 convertase, C4b,2a). C4b can be degraded into C4d, which remains surface-bound, and C4c, that is released. Thus, degradation of C4b causes an increased C4d/C4c ratio on the surface. Right: wild type M5 bacteria were incubated in non-immune human serum, with or without the addition of pure Fg (1 mg ml^{-1}), as indicated. Bacteria were analysed by FACS® for presence of C4d and C4c on the surface, using specific mAbs. These mAbs recognize C4d and C4c, whether they are part of intact C4b or are present as free fragments. Addition of Fg did not affect the C4d/C4c ratio on the M5 strain, implying that Fg did not promote degradation of C4b. Incubation at 37°C for 10 min. Results (means ± SD) from four experiments using three different serum donors.

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 (Johnsson *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

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

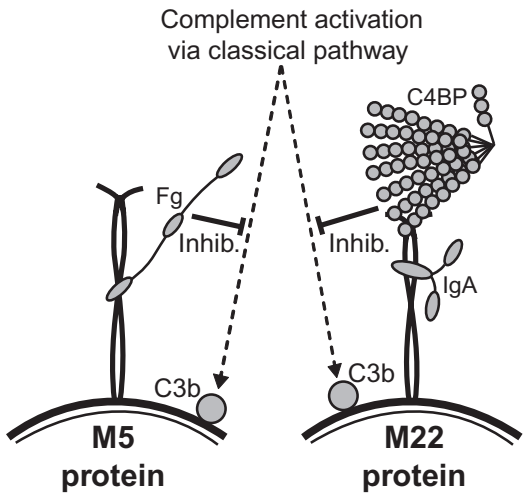

Fig. 7. Working model for the inhibition of complement deposition by M proteins. Complement is activated via the classical pathway by *S. pyogenes,* potentially resulting in surface deposition of C3b. However, M protein (a dimeric coiled-coil protein) inhibits this deposition of C3b by recruiting a human 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.

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% CO_2 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; Ringdahl *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 *Bam*HI and *Sal*I 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. pyogens* (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 ^{125}I-labelled human Fg was analysed as described (Thern *et al*., 1998). Binding of polyclonal rabbit antibodies to streptococci was detected with ^{125}I-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 (~5×10^6 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 $MgCl_2$ 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.

Acknowledgements

We thank Karin Berggård and Annika Andreasson for valuable help. This work was supported by the Swedish Research Council, Lund University Hospital, the Royal Physiographic Society in Lund, and the Trusts of Golje, Kock and Österlund.

References

Åkesson, P., Schmidt, K.H., Cooney, J., and Björck, L. (1994) M1 protein and protein H: IgGFc- and albumin-binding streptococcal surface proteins encoded by adjacent genes. *Biochem J* **300:** 877–886.

Ashbaugh, C.D., Moser, T.J., Shearer, M.H., White, G.L., Kennedy, R.C., and Wessels, M.R. (2000) Bacterial determinants of persistent throat colonization and the associated immune response in a primate model of human group A streptococcal pharyngeal infection. *Cell Microbiol* **2:** 283–292.

Beachey, E.H., Seyer, J.M., and Dale, J.B. (1987) Protective immunogenicity and T lymphocyte specificity of a trivalent hybrid peptide containing NH_2-terminal sequences of types 5, 6, and 24 M proteins synthesized in tandem. *J Exp Med* **166:** 647–656.

Berggård, K., Johnsson, E., Morfeldt, E., Persson, J., Stålhammar-Carlemalm, M., and Lindahl, G. (2001) Binding of human C4BP to the hypervariable region of M protein: a molecular mechanism of phagocytosis resistance in *Streptococcus pyogenes. Mol Microbiol* **42:** 539–551.

Bisno, A.L., Brito, M.O., and Collins, C.M. (2003) Molecular basis of group A streptococcal virulence. *Lancet Infect Dis* **3:** 191–200.

Brown, J.S., Hussell, T., Gilliland, S.M., Holden, D.W., Paton, J.C., Ehrenstein, M.R., *et al.* (2002) The classical pathway is the dominant complement pathway required for innate immunity to *Streptococcus pneumoniae* infection in mice. *Proc Natl Acad Sci USA* **99:** 16969–16974.

Carlsson, F., Berggård, K., Stålhammar-Carlemalm, M., and Lindahl, G. (2003) Evasion of phagocytosis through cooperation between two ligand-binding regions in *Streptococcus pyogenes* M protein. *J Exp Med* **198:** 1057–1068.

Courtney, H.S., Liu, S., Dale, J.B., and Hasty, D.L. (1997) Conversion of M serotype 24 of *Streptococcus pyogenes* to M serotypes 5 and 18: effect on resistance to phagocytosis and adhesion to host cells. *Infect Immun* **65:** 2472–2474.

Cue, D., Lam, H., and Cleary, P.P. (2001) Genetic dissection of the *Streptococcus pyogenes* M1 protein: regions involved in fibronectin binding and intracellular invasion. *Microb Pathog* **31:** 231–242.

Engvall, E., Ruoslahti, E., and Miller, E.J. (1978) Affinity of fibronectin to collagens of different genetic types and to fibrinogen. *J Exp Med* **147:** 1584–1595.

Fine, D.P., Marney, S.R., Jr, Colley, D.G., Sergent, J.S., and Des Prez, R.M. (1972) C3 shunt activation in human serum chelated with EGTA. *J Immunol* **109:** 807–809.

Fischetti, V.A. (1989) Streptococcal M protein: molecular design and biological behavior. *Clin Microbiol Rev* **2:** 285–314.

Fischetti, V.A., Horstmann, R.D., and Pancholi, V. (1995) Location of the complement factor H binding site on streptococcal M6 protein. *Infect Immun* **63:** 149–153.

Flick, M.J., Du, X., Witte, D.P., Jirouskova, M., Soloviev, D.A., Busuttil, S.J., *et al.* (2004) Leukocyte engagement of fibrin(ogen) via the integrin receptor $\alpha_M\beta_2$/Mac-1 is critical

for host inflammatory response *in vivo*. *J Clin Invest* **113:** 1596–1606.

Foster, T.J., and Höök, M. (1998) Surface protein adhesins of *Staphylococcus aureus*. *Trends Microbiol* **6:** 484–488.

Herwald, H., Cramer, H., Mörgelin, M., Russell, W., Sollenberg, U., Norrby-Teglund, A., *et al.* (2004) M protein, a classical bacterial virulence determinant, forms complexes with fibrinogen that induce vascular leakage. *Cell* **116:** 367–379.

Horstmann, R.D., Sievertsen, H.J., Knobloch, J., and Fischetti, V.A. (1988) Antiphagocytic activity of streptococcal M protein: selective binding of complement control protein factor H. *Proc Natl Acad Sci USA* **85:** 1657–1661.

Horstmann, R.D., Sievertsen, H.J., Leippe, M., and Fischetti, V.A. (1992) Role of fibrinogen in complement inhibition by streptococcal M protein. *Infect Immun* **60:** 5036–5041.

Jacks-Weis, J., Kim, Y., and Cleary, P.P. (1982) Restricted deposition of C3 on M+ group A streptococci: correlation with resistance to phagocytosis. *J Immunol* **128:** 1897–1902.

Johnsson, E., Thern, A., Dahlbäck, B., Hedén, L.O., Wikström, M., and Lindahl, G. (1996) A highly variable region in members of the streptococcal M protein family binds the human complement regulator C4BP. *J Immunol* **157:** 3021–3029.

Johnsson, E., Berggård, K., Kotarsky, H., Hellwage, J., Zipfel, P.F., Sjöbring, U., and Lindahl, G. (1998) Role of the hypervariable region in streptococcal M proteins: binding of a human complement inhibitor. *J Immunol* **161:** 4894–4901.

Jones, K.F., and Fischetti, V.A. (1988) The importance of the location of antibody binding on the M6 protein for opsonization and phagocytosis of group A M6 streptococci. *J Exp Med* **167:** 1114–1123.

Kantor, F.S., and Cole, R.M. (1959) A fibrinogen precipitating factor (FPF) of group A streptococci. *Proc Soc Exp Biol Med* **102:** 146–150.

Kehoe, M.A. (1994) Cell-wall-associated proteins in Gram-positive bacteria. *New Compr Biochem* **27:** 217.

Kotarsky, H., Gustafsson, M., Svensson, H.G., Zipfel, P.F., Truedsson, L., and Sjöbring, U. (2001) Group A streptococcal phagocytosis resistance is independent of complement factor H and factor H-like protein 1 binding. *Mol Microbiol* **41:** 817–826.

Kronvall, G., Schönbeck, C., and Myhre, E. (1979) Fibrinogen binding structures in β-hemolytic streptococci group A, C, and G. Comparisons with receptors for IgG and aggregated β_2-microglobulin. *Acta Pathol Microbiol Scand [B]* **87:** 303–310.

Lancefield, R.C. (1957) Differentiation of group A streptococci with a common R antigen into three serological types, with special reference to the bactericidal test. *J Exp Med* **106:** 525–544.

Lancefield, R.C. (1962) Current knowledge of type-specific M antigens of group A streptococci. *J Immunol* **89:** 307–313.

Lindahl, G., Sjöbring, U., and Johnsson, E. (2000) Human complement regulators: a major target for pathogenic microorganisms. *Curr Opin Immunol* **12:** 44–51.

Manderson, A.P., Pickering, M.C., Botto, M., Walport, M.J., and Parish, C.R. (2001) Continual low-level activation of the classical complement pathway. *J Exp Med* **194:** 747–756.

Manjula, B.N., Schmidt, M.L., and Fischetti, V.A. (1985) Unimpaired function of human phagocytes in the presence of phagocytosis-resistant group A streptococci. *Infect Immun* **50:** 610–613.

Markwardt, F. (1993) Hirudin: the famous anticoagulant agent. *Adv Exp Med Biol* **340:** 191–211.

Miller, L., Gray, L., Beachey, E., and Kehoe, M. (1988) Antigenic variation among group A streptococcal M proteins. Nucleotide sequence of the serotype 5 M protein gene and its relationship with genes encoding types 6 and 24 M proteins. *J Biol Chem* **263:** 5668–5673.

Morfeldt, E., Berggård, K., Persson, J., Drakenberg, T., Johnsson, E., Lindahl, E., *et al.* (2001) Isolated hypervariable regions derived from streptococcal M proteins specifically bind human C4b-binding protein: implications for antigenic variation. *J Immunol* **167:** 3870–3877.

Morgan, B.P., and Harris, C.L. (1999) *Complement Regulatory Proteins*. San Diego, CA: Academic Press.

Navarre, W.W., and Schneewind, O. (1999) Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. *Microbiol Mol Biol Rev* **63:** 174–229.

Neth, O., Jack, D.L., Dodds, A.W., Holzel, H., Klein, N.J., and Turner, M.W. (2000) Mannose-binding lectin binds to a range of clinically relevant microorganisms and promotes complement deposition. *Infect Immun* **68:** 688–693.

Nieuwenhuizen, W., Mosesson, M.W., and De Maat, M.P. (eds) (2001) Fibrinogen. *Ann N Y Acad Sci* 936: 1–645.

Nyberg, P., Rasmussen, M., Von Pawel-Rammingen, U., and Björck, L. (2004) SpeB modulates fibronectin-dependent internalization of *Streptococcus pyogenes* by efficient proteolysis of cell-wall-anchored protein F1. *Microbiology* **150:** 1559–1569.

O'Toole, P., Stenberg, L., Rissler, M., and Lindahl, G. (1992) Two major classes in the M protein family in group A streptococci. *Proc Natl Acad Sci USA* **89:** 8661–8665.

Perez-Casal, J., Price, J.A., Maguin, E., and Scott, J.R. (1993) An M protein with a single C-repeat prevents phagocytosis of *Streptococcus pyogenes*: use of a temperature-sensitive shuttle vector to deliver homologous sequences to the chromosome of *S. pyogenes*. *Mol Microbiol* **8:** 809–819.

Perez-Casal, J., Okada, N., Caparon, M.G., and Scott, J.R. (1995) Role of the conserved C-repeat region of the M protein of *Streptococcus pyogenes*. *Mol Microbiol* **15:** 907–916.

Podbielski, A., Schnitzler, N., Beyhs, P., and Boyle, M.D. (1996) M-related protein (Mrp) contributes to group A streptococcal resistance to phagocytosis by human granulocytes. *Mol Microbiol* **19:** 429–441.

Ponnuraj, K., Bowden, M.G., Davis, S., Gurusiddappa, S., Moore, D., Choe, D., *et al.* (2003) A 'dock, lock, and latch' structural model for a staphylococcal adhesin binding to fibrinogen. *Cell* **115:** 217–228.

Ren, B., Szalai, A.J., Hollingshead, S.K., and Briles, D.E. (2004) Effects of PspA and antibodies to PspA on activation and deposition of complement on the pneumococcal surface. *Infect Immun* **72:** 114–122.

Ringdahl, U., Svensson, H.G., Kotarsky, H., Gustafsson, M.,

Weineisen, M., and Sjöbring, U. (2000) A role for the fibrinogen-binding regions of streptococcal M proteins in phagocytosis resistance. *Mol Microbiol* **37:** 1318–1326.

Runehagen, A., Schönbeck, C., Hedner, U., Hessel, B., and Kronvall, G. (1981) Binding of fibrinogen degradation products to *S. aureus* and to β-hemolytic streptococci group A, C and G. *Acta Pathol Microbiol Scand [B]* **89:** 49–55.

Sahu, A., and Pangburn, M.K. (1993) Identification of multiple sites of interaction between heparin and the complement system. *Mol Immunol* **30:** 679–684.

Schnitzler, N., Haase, G., Bussing, A., Kaufhold, A., Beyhs, P., and Podbielski, A. (1995) Measuring resistance to phagocytosis of group A and G streptococci: comparison of direct bactericidal assay and flow cytometry. *Med Microbiol Immunol (Berl)* **184:** 17–22.

Schnitzler, N., Haase, G., Podbielski, A., Lutticken, R., and Schweizer, K.G. (1999) A co-stimulatory signal through ICAM-β_2 integrin-binding potentiates neutrophil phagocytosis. *Nat Med* **5:** 231–235.

Schubert, A., Zakikhany, K., Schreiner, M., Frank, R., Spellerberg, B., Eikmanns, B.J., and Reinscheid, D.J. (2002) A fibrinogen receptor from group B Streptococcus interacts with fibrinogen by repetitive units with novel ligand binding sites. *Mol Microbiol* **46:** 557–569.

Staali, L., Mörgelin, M., Björck, L., and Tapper, H. (2003) *Streptococcus pyogenes* expressing M and M-like surface proteins are phagocytosed but survive inside human neutrophils. *Cell Microbiol* **5:** 253–265.

Stålhammar-Carlemalm, M., Stenberg, L., and Lindahl, G. (1993) Protein Rib: a novel group B streptococcal cell surface protein that confers protective immunity and is expressed by most strains causing invasive infections. *J Exp Med* **177:** 1593–1603.

Stenberg, L., O'Toole, P., and Lindahl, G. (1992) Many group A streptococcal strains express two different immunoglobulin-binding proteins, encoded by closely linked genes: characterization of the proteins expressed by four strains of different M-type. *Mol Microbiol* **6:** 1185–1194.

Thern, A., Stenberg, L., Dahlbäck, B., and Lindahl, G. (1995) Ig-binding surface proteins of *Streptococcus pyogenes* also bind human C4b-binding protein (C4BP), a regulatory component of the complement system. *J Immunol* **154:** 375–386.

Thern, A., Wästfelt, M., and Lindahl, G. (1998) Expression of two different antiphagocytic M proteins by *Streptococcus pyogenes* of the OF+ lineage. *J Immunol* **160:** 860–869.

Volanakis, J.E., and Frank, M.M. (eds). (1998) *The Human Complement System in Health and Disease.* New York: Marcel Dekker.

Weineisen, M., Sjöbring, U., Fällman, M., and Andersson, T. (2004) Streptococcal M5 protein prevents neutrophil phagocytosis by interfering with CD11b/CD18 receptor-mediated association and signaling. *J Immunol* **172:** 3798–3807.

Wessels, M.R., Moses, A.E., Goldberg, J.B., and DiCesare, T.J. (1991) Hyaluronic acid capsule is a virulence factor for mucoid group A streptococci. *Proc Natl Acad Sci USA* **88:** 8317–8321.

Wessels, M.R., Butko, P., Ma, M., Warren, H.B., Lage, A.L., and Carroll, M.C. (1995) Studies of group B streptococcal infection in mice deficient in complement component C3 or C4 demonstrate an essential role for complement in both innate and acquired immunity. *Proc Natl Acad Sci USA* **92:** 11490–11494.

Whitnack, E., and Beachey, E.H. (1982) Antiopsonic activity of fibrinogen bound to M protein on the surface of group A streptococci. *J Clin Invest* **69:** 1042–1045.

Whitnack, E., and Beachey, E.H. (1985a) Biochemical and biological properties of the binding of human fibrinogen to M protein in group A streptococci. *J Bacteriol* **164:** 350–358.

Whitnack, E., and Beachey, E.H. (1985b) Inhibition of complement-mediated opsonization and phagocytosis of *Streptococcus pyogenes* by D fragments of fibrinogen and fibrin bound to cell surface M protein. *J Exp Med* **162:** 1983–1997.

Whitnack, E., Dale, J.B., and Beachey, E.H. (1984) Common protective antigens of group A streptococcal M proteins masked by fibrinogen. *J Exp Med* **159:** 1201–1212.