Hic, a novel surface protein of *Streptococcus pneumoniae* that interferes with complement function*

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SUMMARY

The important human pathogen *Streptococcus pneumoniae* was found to absorb factor H, an inhibitor of complement, from human plasma. We identified the gene encoding a novel surface protein, factor H-binding inhibitor of complement (Hic), in the *pspC* locus of type 3 pneumococci. Unlike PspC proteins in other serotypes, Hic is anchored to the cell wall by means of an LPXTG motif, and the overall sequence homology to various PspC proteins is low. However, the NH₂-terminal region showed significant homology to the NH₂-terminal region of several PspC proteins. A fragment of Hic, covering this homologous region, was expressed as a GST fusion protein. GST:Hic39-261 bound radiolabelled factor H and inhibited binding of factor H to pneumococci of different serotypes. Interaction kinetics between GST:Hic39-261 and factor H were studied with surface plasmon resonance and showed a high affinity binding (Kₐ=5 x 10⁷, Kᵩ=2.3 x 10⁻⁸). Mutant pneumococci lacking Hic showed no absorption of factor H in human plasma and no binding of radiolabelled factor H, suggesting that Hic is responsible for factor H-binding in type 3 pneumococci. Factor H-dependent inhibition of the alternative pathway was not diminished by presence of GST:Hic39-261. In addition, an intrinsic inhibitory effect of Hic is suggested.
INTRODUCTION

Despite the availability of effective antibiotics and a polyvalent capsular polysaccharide vaccine, \textit{Streptococcus pneumoniae} remains a significant cause of morbidity and mortality, causing conditions such as otitis media, community-acquired pneumonia, septicemia and meningitis. Infants, the elderly, and immunocompromised patients are particularly susceptible to pneumococcal infection.

The polysaccharide capsule of the pneumococcus has long been recognized as the major virulence determinant (1). Virulence varies with capsular serotype, but experiments with conversion of serotypes clearly demonstrate that other factors than the capsule play a significant role (2). A number of non-capsular virulence factors have also been extensively examined. Although their relative contribution to pneumococcal virulence remains unclear, it is apparent that proteins such as PspA\textsuperscript{1}, pneumolysin, and PsaA play a role in pneumococcal virulence (3-5).

The classical and alternative pathways of complement are part of the innate immune system, and constitute an important line of defense against pneumococcal infection (6,7). There are many strategies by which bacteria can interfere with the function of the complement system (reviewed in (8)). For example, binding of the complement regulatory protein fH to bacterial surface proteins has been described by several groups (reviewed in (9)), although the precise consequences of such a binding remain somewhat elusive. fH is a 150 kDa plasma protein composed of 20 short consensus repeats, and is the best characterized member of the factor H protein family (10). fH is a crucial protein in the regulation of complement. The critical step in the amplification loop of the alternative pathway is the formation of C3 convertase (C3bBb) from surface-deposited C3b and factor B. fH inhibits complement activation by preventing association of factor B with C3b, acting as a cofactor in C3b degradation by factor I, and promoting the dissociation of Bb from both C3 and C5 convertase.
Examples of bacterial surface structures interacting with fH include M and M-like proteins of *S. pyogenes* (11,12). Furthermore, YadA in *Yersinia enterocolitica* has been shown to inhibit complement activation by coating the bacterial surface with fH (13). Recently, two groups independently described inhibition of complement-mediated opsonophagocytosis in type 3 pneumococci. One study (14) suggested that PspA interferes with deposition of C3b and/or inhibits the alternative pathway C3 convertase. Another study (15) claimed that pneumococcal resistance to phagocytosis is mediated by hitherto unknown surface proteins binding fH. PspA does not contribute to this interaction, as a PspA-deficient mutant bound similar or even larger amounts of fH than the parent strain.

In this study we expand upon previous works on pneumococci and complement by describing a novel surface protein containing a fH-binding region. The protein was identified *in silico* by using previously described fH-binding proteins as probes, and the candidate gene was found in the chromosomal locus of PspC (also called SpsA, PbcA and CbpA). A recent paper compares different allelic variants of PspC (16) in several pneumococcal serotypes, and previous works have suggested functions for PspC such as binding of secretory IgA (17), or influence on adhesion and virulence (18). Interestingly, the protein we describe differs substantially from all previously described PspC alleles, except for the amino terminal region. Our data show that this region is responsible for fH binding in type 3 pneumococci, and suggest that fH binding to other serotypes is mediated by PspC regions homologous to this domain.

**EXPERIMENTAL PROCEDURES**

*Bacterial strains*

Strains of *S. pneumoniae* used in this study are described in Table 1. In unencapsulated strains PR201, PR212, PR215, and PR218 the whole capsule locus is deleted and substituted with a
kanamycin (Km) resistance cassette. Pneumococci were grown at 37°C in TSB (Difco) or on TSA (TSB with agar) supplemented with 3% horse blood. Where appropriate, kanamycin (500 µg/ml) or chloramphenicol (3 µg/ml) was added. Bacteria used for binding assays were grown in Todd-Hewitt broth (Difco), supplemented with 0.2% Yeast extract (Difco). *Escherichia coli* strain DH5α was grown in Luria-Broth (Difco) or on LB-agar, supplemented with ampicillin (50 µg/ml) when containing pGEX.

**DNA methods, cloning, and sequencing**

By PCR SOEing (19), a chloramphenicol transferase cassette was flanked by sequences found up-and downstream of *hic*. PR218 was transformed with this construct. By double cross-over mutagenesis the *hic* gene was consequently replaced with the *cat* cassette, generating the *hic*-deficient mutant FP13.

Oligonucleotides HICf1 (5’-TGGGATCCCAGAGAAGGAGGTACTAC-3’) and HICr1 (5’-GGAGCCTGAATTCGACGAAG-3’), containing *Bam*HI and *Eco*RI restriction sites respectively, were used in a polymerase chain reaction (PCR) to amplify DNA corresponding to amino acids 39 to 261 in Hic. The PCR was performed with Taq polymerase (Gibco BRL), and consisted of 30 cycles at 94°C for 1 min., 50°C for 1 min., and 72°C for 1 min., followed by a final extension at 72°C for 7 min. Template was prepared by resuspending bacterial colonies in water, boiling for 5 min., and removal of bacterial debris by centrifuging at 13000 x g. The PCR-amplified fragment was gel-purified with Sephaglas Bandprep (Pharmacia Biotech), digested with *Bam*HI and *Eco*RI (Pharmacia Biotech) and ligated with likewise digested vector pGEX-5X-3 (Pharmacia Biotech) using T4 DNA ligase (Pharmacia Biotech). Plasmid pGEX-5X-3:*hic*(39-261) was then electroporated into DH5α *E. coli* according to the GST gene fusion system protocol (Pharmacia Biotech). Transformants were screened for presence of insert by plasmid mini-preps.
and restriction enzyme digestion. The clone used for overexpression of the fusion protein GST:Hic\textsuperscript{39-261} was verified by purifying the plasmid and sequencing the complete insert. Fusion protein was affinity purified according to the instructions in the GST gene fusion system manual (Pharmacia Biotech).

\textit{Ligand binding and protein methods}

Plasma absorption experiments were performed with log-phase pneumococci (OD\textsubscript{600} \sim 0.4). Bacteria were washed twice in PBS, pH 7.4, containing 0.05 % Tween 20 (PBST), and the bacterial concentration was adjusted to 2 \times 10^{10} cells/ml. 100 \mu l of bacteria were incubated for 1 h with 100 \mu l of human plasma. Bacteria were washed five times with PBST, and bound proteins were eluted from the cells with 100 \mu l 0.1 M glycine/HCl, pH 2.0. The pH of the eluted material was adjusted to \sim 7 with 1 M Tris. C3-deficient serum was obtained from a patient with selective and complete C3 deficiency (C3 < 1 mg/liter), kindly provided by Dr. G. Eggertsen, Huddinge Hospital, Sweden.

Protein samples were separated by SDS-PAGE (20) containing 8-12% acrylamide. Proteins were blotted onto an Immobilon-P\textsuperscript{TM} PVDF-membrane (Millipore) as described (21). Rabbit polyclonal antiserum against fH diluted 1:1000 was the source of primary antibodies. Horseradish peroxidase-conjugated anti-rabbit goat antibodies (Bio-Rad, Bio-Rad Laboratories, CA) were used as secondary antibodies, and detection of immuno-reactive bands was performed by chemiluminescence as described (22).

Slot blot, Western blot and bacterial binding assays were performed with fH (Sigma), radiolabelled with \textsuperscript{125}I using the Iodobeads kit (Pierce). Unincorporated \textsuperscript{125}I was removed by gel filtration on Sephadex G-25 (Pharmacia Biotech). Slot blots were performed by applying 5, 1, 0.02 and 0.004 \mu g of purified protein onto nitrocellulose membranes in a slot blot apparatus (Schleicher &
Schuell). The membrane was blocked for 4 x 20 min. in PBST containing 0.25% (w/v) gelatin (Difco). Radiolabelled fH (200000 cpm/ml) was then added, and the membrane was incubated for 1 h at room temperature. The membrane was washed for 4 x 20 min. in PBST, 0.5 M NaCl, and membrane-associated radioactivity was visualized by exposure onto a phosphoimaging plate (Fuji Photo Film). Western blot membranes were treated in the same way. Binding assays with pneumococci were performed as described (23), using log-phase bacteria (OD$_{600}$ ~0.4).

Surface plasmon resonance was performed by coupling affinity-purified anti-GST antibodies (Biacore) onto sensor chip CM5 (Biacore) by standard amine coupling, according to the manufacturers instructions. ~10000 resonance units (RU) of antibodies were coupled. Each cycle of analysis was then commenced by immobilization of 500-1500 RU of GST or GST:Hic$_{39-261}$ (10µg/ml), followed by injection of fH (2 - 0.127 µM). The kinetic studies were performed in PBST. Each cycle was terminated by the regeneration of the chip with 10 mM glycine pH 2.2. Global analysis of data was performed with multiple models in BiaEvaluation 3.0.

Hemolysis assay
A previously described assay for the measurement of fH-mediated inhibition of the alternative pathway was employed (24). Rabbit erythrocytes (National Institute of Veterinary Medicine, Uppsala, Sweden) were washed and suspended at 5 x 10$^8$ cells/ml. The serum of a patient with homozygous C2-deficiency (25) was used as a source of complement. Highly purified fH was also kindly provided by Dr. L. Truedsson. The hemolytic reaction was performed in veronal-buffered saline with 16 mM EGTA, 4 mM Mg$^{2+}$ and 0.1 % gelatine. The concentration of C2-deficient serum producing about 80% hemolysis in the assay system was determined in preliminary experiments. Final incubation mixtures (200 µl) contained C2-deficient serum diluted 1/12 with or without additional proteins (fH, GST, or GST:Hic$_{39-261}$, at 30, 90 or 60 µM,
respectively) and 2.5 x 10^7 rabbit erythrocytes (50 µl). The erythrocytes were added 5 min after
the other reagents. After 5-40 min reactions were stopped by the addition of 750 µl cold VBS, 10
mM EDTA. Samples were centrifuged at 3000 rpm for 5 min., and the supernatants were
removed for measurement of light absorbance at 412 nm.

**Bioinformatics**

Sequence comparisons were performed with MacVector 6.5.3 (Oxford Molecular, Oxford, United
Kingdom). Database searches utilized the Entrez server at The National Institute for
Biotechnology Information. The sequence of PspC from the type 4 pneumococcus was obtained

**RESULTS**

*Binding of complement factor H by S. pneumoniae*

Previous observations in our laboratory indicated that *S. pneumoniae* is capable of absorbing
proteins from human plasma (unpublished results). To better investigate the nature of these
interactions, a series of strains (Table 1), both encapsulated and unencapsulated, was incubated
with plasma. After washing, plasma proteins bound to the pneumococci were eluted and
separated by polyacrylamide gel electrophoresis (PAGE), showing that strains D39 (type 2,
encapsulated), and all four unencapsulated strains (serotypes 2, 3, 3, and 19) absorbed a protein
with an estimated molecular mass of 140 kDa (Fig. 1, STAIN). In repeated experiments PR218,
an unencapsulated derivative (Pearce, Iannelli and Pozzi, manuscript in preparation) of Avery
strain A66, consistently showed the most prominent absorption of the 140 kDa protein. The
protein absorbed by PR218 was subjected to trypsin-in-gel digestion, and six internal fragments
were sequenced by Edman degradation. These sequences showed 100% identity to various
regions in human complement factor H. A replica of the gel in Fig. 1 was transferred to a membrane by electroblotting, and probed with a rabbit anti-fH antiserum. The antiserum reacted with the aforementioned 140 kDa band, a band of similar size in plasma, and purified fH (Fig. 1, BLOT). There was also a weak reactivity with a band corresponding to ~50 kDa. When subjected to NH₂-terminal sequencing, the band was identified as human immunoglobulin heavy chains, suggesting specific or unspecific binding of immunoglobulins to pneumococci, and subsequent crossreactivity with the secondary goat anti-rabbit antibodies.

To exclude that binding of fH was secondary to complement activation and deposition of C3 at the bacterial surface, the plasma absorption with strain D39 was repeated using a C3-deficient serum. The protein reacting with anti-fH antibodies was present in equal amounts when comparing results from absorptions of normal human plasma and the C3-deficient serum (data not shown), showing that fH binding is independent of C3.

A group of *S. pneumoniae* strains was examined for binding of radiolabelled fH. Most strains showed significant binding of fH, although the degree of binding varied considerably between strains (Table 2). Encapsulated strains generally bound somewhat less than the corresponding unencapsulated strains. To investigate whether the binding was mediated by proteinaceous structures, binding of fH to strain PR218 was examined following treatment of bacteria with different proteases (data not shown). Binding was almost completely abolished by pretreatment with papain. Trypsin also caused a major decrease, whereas pepsin had a moderate effect (<50% decrease). These results confirm a previous observation (15) that fH binding can be abolished by pretreatment of bacteria with trypsin.
Identification and sequence analysis of a candidate gene encoding a fH-binding protein

A recent paper (15) suggests that type 3 pneumococci are resistant to complement activation and phagocytosis by virtue of fH binding to proteinaceous surface structures. Previously described bacterial surface proteins known to interact with fH include streptococcal M proteins (11) and YadA (13) from Y. enterocolitica. Nucleotide and amino acid sequences of YadA, M1, M1.1 (26), M6 (27), and the M-like protein H (28), were used to search a pneumococcal (type 4 strain JNR. 7/87) genome database obtained from The Institute for Genomic Research (http://www.tigr.org).

The highest scoring homology for all the probes was found in a 2106 bp open reading frame (ORF), encoding a putative protein of 702 amino acids (aa). The degree of homology between the pneumococcal protein and the fH-binding proteins used for searching was low, but significant, when comparing the full sequences. However, there were several limited regions of markedly higher homology. A GENBANK search with this putative protein identified it as an allele of PspC, also denoted SpsA, CbpA, or PbcA (16). PspC was then conversely used to search the Streptococcus pyogenes genome sequencing project, and the highest scoring match was found to encode the M1 protein.

We chose to sequence the chromosomal locus of the pspC gene in Avery strain A66 (type 3) and its derivative PR218. Serotype 3 strains strongly resist phagocytosis (29), and PR218 shows a prominent absorption of fH from human plasma. The locus contained a 1836 bp ORF, encoding a putative protein of 612 aa. The gene was tentatively named hic, for factor H binding inhibitor of complement (GenBank accession number AF252857). The Hic protein (schematically depicted in fig. 2, top) contains a proline-rich region consisting of 24 repeats of 11 amino acids. Near the COOH-terminus there is a consensus sequence LPSTGS, typical of Gram-positive cell wall-anchored proteins (30). This sequence is followed by a hydrophobic COOH-terminal tail. The Hic sequence was used to search the streptococcal Genome Project database, and identified M
protein as the best match. Several pneumococcal surface proteins were found to be homologous to Hic. SpsA from type 2 and type 47 pneumococci contains a region highly homologous to Hic. SpsA binds secretory IgA and its secretory component (17). CbpA (18), an adhesin and virulence determinant in type 2 pneumococci, also contains this region, as does PbcA (unpublished sequence from GENBANK). SpsA2, CbpA, and PbcA together form the D39-lineage of PspC alleles (16). The 149 aa long NH$_2$-terminal region of Hic, including the predicted 37 aa leader peptide, was aligned with the corresponding region in PspC proteins from serotypes 1, 2, 4, 6A, and 19 (Fig. 3). No particular function has previously been suggested for this region in the PspC proteins. Interestingly, the remainder of Hic showed no significant homology to the PspC proteins except for shorter stretches in the proline-rich region of PspC. In contrast to Hic (Fig. 2, bottom), the PspC proteins contain a series of repeats with choline-binding motifs, a different mechanism for surface attachment. Computer predictions (31,32) of the secondary structure resulted in strong predictions of $\alpha$-helical structure in the NH$_2$-terminal region of both Hic (aa 40 - 270) and PspC (aa 50 - 250).

**Construction and properties of a Hic-deficient mutant strain**

To investigate the possible contribution of Hic to pneumococcal fH-binding, the unencapsulated strain PR218 (serotype 3), showing the most pronounced absorption of fH from human plasma, was chosen for further studies. Splicing by overlap extension (gene SOEing) (19) was used to flank an antibiotic resistance cassette with sequences found up- and downstream of the hic gene. PR218 was transformed with this construct and the hic gene was deleted by double cross-over mutagenesis. The resulting strain, FP13, grew as well as the parent strain in ordinary growth media. The deletion of hic was confirmed by PCR experiments.
The mutant strain was subjected to plasma absorption experiments identical to those described above. In contrast to the parent strain (Fig. 1), no band at the position of fH was detected with the anti-fH antiserum. Furthermore, the binding of radiolabelled fH to wild type and mutant bacteria was examined using serial dilutions of bacteria (Fig. 4). Unlike the parent strain PR218, the mutant strain FP13 showed background levels of fH binding even at the highest bacterial concentration. The possible contribution of PspC to pneumococcal fH binding was also examined by constructing a mutant derivative of D39, where the pspC gene has been truncated so that the NH₂-terminal amino acids are missing in PspC. This mutant, called FP7, was used in plasma absorption experiments, and no band reacting with the anti-fH antibodies was eluted (data not shown).

Mapping of the factor H-binding region of Hic

We decided to investigate the binding properties of the non-repeat region of Hic, including the part shared by Hic, PspC, CbpA, SpsA, and PbcA. Therefore, the hic region encoding the NH₂-terminal part of Hic (aa 39 - 261) was cloned into the vector pGEX, resulting in a fusion with the gene encoding GST. Control sequencing of the insert verified the presence of the partial hic gene, showing 100 % identity with the DNA sequence from strain A66. GST:Hic³⁹⁻²⁶¹ and GST were overexpressed, affinity purified, and analysed by SDS-PAGE (Fig. 5A, STAIN). Although partly degraded, the main protein band had the expected mass (54 kDa). An identical gel was blotted to a membrane, which was then incubated with radiolabelled fH, washed, and subjected to autoradiography. fH bound to GST:Hic³⁹⁻²⁶¹, but not to GST (Fig. 5A, BLOT). The fusion protein and the GST control were also applied in serial dilutions onto a nitrocellulose membrane. The membrane was probed with radiolabelled fH, which showed binding to the fusion protein, and no binding to GST (Fig. 5B). Furthermore, the fusion protein was used in a competitive
binding assay to investigate whether GST:Hic\textsuperscript{39-261} could compete with fH binding to PR218 bacteria. The result (Fig. 5C) shows that the Hic domain of the fusion protein blocks bacterial fH binding, whereas GST alone does not affect binding. Similar experiments were performed with \textit{S. pneumoniae} strains 3496, G54, PR215, and HB565. Also in these experiments GST:Hic\textsuperscript{39-261} blocked the binding of fH to the tested strains, whereas GST had no effect (data not shown). The G54 strain is of serotype 19, and the similarity between Hic and PspC of a type 19 pneumococcus (see Fig. 2B) implicates that fH binding to non type 3 strains is mediated by the PspC region homologous to Hic.

The interaction between GST:Hic\textsuperscript{39-261} and fH was further examined by surface plasmon resonance. Anti-GST antibodies were coupled to a carboxymethylextran chip, followed by the immobilization of GST:Hic\textsuperscript{39-261} as the ligand. Highly purified fH was used as the analyte at various concentrations. fH interacted with GST:Hic\textsuperscript{39-261} over the whole range of concentrations (0.1 - 2 µM), showing partial saturation at the highest fH concentration. The experiment was repeated three times, with independent couplings onto the chips. A representative experiment with consecutive regenerations of one chip is shown (Fig. 6A). As a control, GST was similarly immobilized on an anti-GST antibodies chip. No binding of fluid phase fH was obtained (data not shown). A global analysis of data was performed to determine rate constants of association/dissociation and constants of association and dissociation (Fig. 6B), applying the standard Langmuir 1:1 model.

\textit{Hic and complement inhibition}

From a functional point of view, an important question is whether the binding of Hic to fH affects the complement inhibitory function of fH. To address this issue we adapted a previously described methodology where complement-mediated lysis of rabbit erythrocytes in serum is
inhibited by the addition of fH (24). By using a C2-deficient serum as a source of complement, any influence from the classical pathway was excluded. Initial experiments were performed and showed a dose-dependent inhibition of alternative-pathway mediated hemolysis when fH was added (data not shown). By increasing the concentration of fH in the reaction threefold (relative to fH in serum), a complete inhibition of hemolysis was achieved. Smaller fH increments resulted in partial inhibition of hemolysis. The effect of GST:Hic\(^{39-261}\), GST, or fH and GST:Hic\(^{39-261}\) (1:2 molar ratio) on hemolysis was investigated. A preincubation step (5 min) allowed some time for equilibration of the fH and GST:Hic\(^{39-261}\) interaction. A kinetic study of hemolysis showed that the presence of GST:Hic\(^{39-261}\) in the reaction did not decrease the fH-mediated inhibition of complement activation. Rather, the simultaneous presence of fH and GST:Hic\(^{39-261}\) resulted in increased inhibition. Interestingly, GST:Hic\(^{39-261}\) may have an intrinsic complement inhibitory effect, as hemolysis was partially inhibited when the fusion protein alone (with no surplus fH) was added (Fig. 7).

DISCUSSION

Complement-dependent opsonophagocytosis is a crucial defense against infection with *Streptococcus pneumoniae* (33). Two recent studies discuss how type 3 pneumococci may subvert the normal function of the complement system. One study (14) showed that the alternative pathway of complement is essential for efficient clearance of bacteria, and that pneumococcal interference with complement activation is a virulence determinant. Based on comparisons of PspA-negative mutants with wild type bacteria the researchers claim that PspA blocks recruitment of the alternative pathway, by an unknown mechanism. Another study (15) describes the binding of complement factor H to trypsin-sensitive structures at the surface of pneumococci, independent of previous activation of complement. Notably, the study rules out
any major contribution of PspA with respect to binding of fH. The findings indicate that factor H binding proteins might constitute an independent virulence factor.

The present investigation describes a novel pneumococcal surface protein, responsible for the binding of fH in type 3 pneumococci. The gene encoding the protein (Hic) is found in the locus of pspC, a possible virulence determinant and protective antigen, but shows highly atypical characteristics compared to previously described pspC alleles. In a study where a number of pspC alleles were sequenced (16) the type 3 strain was considered PspC-negative, since there was no protein reacting with PspC-antibodies and PCR experiments failed to amplify the pspC gene. Apart from a region in the NH$_2$-terminal part of the protein, there is little sequence homology between Hic and PspC. Furthermore, Hic is anchored to the cell wall by the presence of an LPXTGX motif while PspC harbors the choline binding motif. In this respect, Hic is similar to the M protein in S. pyogenes, also known to bind fH (11). A Hic-deficient mutant failed to absorb fH from human plasma, in contrast to the wild type strain. This mutant did not bind radiolabelled fH, indicating that most or all of the fH binding to PR218 bacteria is due to the presence of Hic at the pneumococcal surface. Although an extensive screening was not performed, most of the strains examined showed binding of fH, suggesting that this phenotype is not confined to type 3 strains. Furthermore, the recombinant fH-binding fragment of Hic could compete with binding of radiolabelled fH to pneumococci of different serotypes. PspC may confer fH binding to pneumococci by virtue of the region similar to Hic, but it cannot be excluded that other surface structures interact with the same part of fH as Hic does. Surface plasmon resonance experiments with the fusion between GST and the NH$_2$-terminal Hic region (GST:Hic$^{39-261}$) and fH showed that the interaction has high affinity.

The presence of a similarity region in the NH$_2$-terminal part of various PspC proteins and Hic, offers the interesting possibility that this rather variable region, contained in the recombinantly
expressed Hic fragment, is responsible for factor H binding in many pneumococcal strains. It has previously been shown that PspC is a protective antigen, and the NH2-terminal region probably has to undergo significant genetic variation in order to avoid eliciting specific antibodies. This, however, does not preclude specific binding of fH. In comparison, the M5 and M6 proteins of *S. pyogenes* have been shown to bind factor H-like protein 1 by their hypervariable region (12). Similarly, several M-like proteins bind another complement regulatory protein, C4 binding protein (34), by the hypervariable NH2-terminal region. We found that a type 2 mutant strain that expressed an NH2-terminally truncated form of PspC failed to absorb fH from plasma, unlike the parent strain D39. Although not conclusive, since the truncation also involved a part of PspC that shows no homology with Hic, this experiment supports the idea that fH binding could be mediated by the NH2-terminal regions of both PspC and Hic.

Many studies have showed that interference with the function of the complement system is a highly relevant aspect of pneumococcal virulence. More specifically, the binding of fH has been shown to correlate with resistance to opsonophagocytosis. A previous study showed that type 3 pneumococci, despite C3b deposition on both cell wall and capsule, strongly resist phagocytosis (29). Our data show that hic, a highly atypical pspC allele, encodes the major fH-binding protein of type 3 pneumococci. The complement inhibitory function of fH is not impaired in the presence of Hic. By accumulating an active complement inhibitor at the pneumococcal surface, Hic may act alone or in concert with PspA to block deposition of C3b and concomitant opsonophagocytosis. As previously described, a putative C3 proteinase in types 3, 4 and 14 pneumococci (35) should have similar effects. The present findings also indicated that Hic could have an intrinsic capacity to inhibit the alternative pathway. One possibility is that complex formation with Hic potentiated the inherent function of fH, as fH was present in the diluted C2-deficient serum used in the hemolytic assay. Interactions between Hic and other components of
the alternative pathway may also be considered, perhaps by binding to short consensus repeats. In conclusion, pneumococci appear highly prone to interfere with the complement system. Binding of fH, by different mechanisms, has also been described for group A streptococci (11), Y. enterocolitica (13), and N. gonorrhoeae (36,37) and may represent a widespread theme in bacterial adaptation to the human host.

FOOTNOTES

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1 Abbreviations used are: fH, complement factor H; PspA, pneumococcal surface protein A; PsA, pneumococcal surface antigen A; PspC, pneumococcal surface protein C; CbpA, choline-binding protein A; SpsA, Streptococcus pneumoniae secretory IgA binding protein; GST, glutathione S-transferase; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis

2 F. Iannelli, B. J. Pearce, and G. Pozzi, unpublished
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FIGURE LEGENDS

Fig. 1. Pneumococcal absorption of plasma proteins. SDS-PAGE analysis (STAIN) of plasma proteins absorbed by and eluted from D39 (A), HB565 (B), 3496 (C), G54 (D), PR201 (E), PR212 (F), PR215 (G), PR218 (H), and FP13 (I). Lanes J and K contain diluted plasma and fH, respectively. Proteins from an identical gel were transferred by electroblotting to a PVDF membrane. A fH-antiserum was used to identify immunoreactive bands (BLOT).

Fig. 2. Comparison of Hic and PspC. Schematic representation of Hic and PspC. The signal peptide (SP) and the wall spanning region (W) are indicated.

Fig. 3. Sequence comparison of Hic and allelic variants of PspC. ClustalW alignment of the NH2-terminal region from Hic (serotype 3) and allelic variants of PspC, including SpsA. Identical and similar residues are shaded in dark and light, respectively. Numbers in the protein names indicate the serotype of the strain from which the sequence was obtained. PspC.TIGR is from a serotype 4 strain. GenBank/EMBL accession numbers are as follows: PspC2, AF068646; PspC6A, AF068645; PspC19, AF068648; SpsA1, Y10818.

Fig. 4. Binding of radiolabelled factor H to a Hic-deficient mutant. Serial dilutions of PR218 (□) and the Hic-deficient mutant FP13 (♦) were incubated with radiolabelled fH. Binding is
expressed as the percentage of added radioactivity. The data points are averages of three experiments with duplicate samples. Standard deviation is indicated by error bars.

**Fig. 5. Binding of factor H to Hic.** (A) SDS-PAGE analysis of GST and recombinant Hic. Two identical gels were prepared and either stained (STAIN) or blotted. The membrane from the blot was incubated with radiolabelled fH (BLOT).

(B) A serial dilution (5, 1, 0.2 and 0.04 µg) of GST and recombinant Hic was applied onto a nitrocellulose membrane which was incubated with radiolabelled fH.

(C) A competitive binding assay was performed by incubating PR218 bacteria (10^9 CFU/ml) with radiolabelled fH in the presence of increasing concentrations of unlabelled fH (○), GST (▲), and GST:Hic 39-261 (◆).

**Fig. 6. Kinetic analysis of the interaction between Hic and factor H by surface plasmon resonance.** (A) One representative sensorgram (out of three) is shown. The concentration of the analyte (fH) was 2000, 667, 333, 167 and 83 nM, and the time scale has been adjusted so that 0 s represents the injection starting point.

(B) Kinetic constants from a global analysis of the data with a standard Langmuir 1:1 model. Values are mean ± SD from three different experiments.

**Fig. 7. Hic and factor H inhibit alternative pathway hemolysis.** Rabbit erythrocytes were incubated with C2-deficient serum and a kinetic study of hemolysis was performed. The influence of added fH, GST, and/or GST:Hic 39-261 on hemolysis was studied. Numbers represent hemolysis as a fraction of maximum hemolysis in the control (90-95%). Data shown are from one representative experiment (n=3). The curves represent control reaction (▲), reaction with added fH (◆), GST:Hic 39-261 (■), GST (▲), and a combination of fH and GST:Hic 39-261 (○).
Table 1. *Streptococcus pneumoniae* strains

<table>
<thead>
<tr>
<th>Bacterial Strains</th>
<th>Relevant Properties</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A66</td>
<td>Capsulated clinical strain (type 3)</td>
<td>(38,39)</td>
</tr>
<tr>
<td>PR218</td>
<td>Km(^R). Unencapsulated derivative of A66</td>
<td>Iannelli, Pearce, and Pozzi (unpublished)</td>
</tr>
<tr>
<td>FP13</td>
<td>Cm(^R), Km(^R), Hic(^-). Mutant of PR218 in which hic is deleted and substituted with a cat cassette.</td>
<td>This work</td>
</tr>
<tr>
<td>D39</td>
<td>Capsulated clinical strain (type 2)</td>
<td>(38,40)</td>
</tr>
<tr>
<td>Rx1</td>
<td>Unencapsulated derivative of D39</td>
<td>(41)</td>
</tr>
<tr>
<td>PR201</td>
<td>Km(^R). Unencapsulated derivative of D39</td>
<td>Iannelli, Pearce, and Pozzi (unpublished)</td>
</tr>
<tr>
<td>FP7</td>
<td><em>pspC</em> deletion mutant of Rx1 (in-frame deletion of nucleotides 124 to 1338, GenBank accession no. AF067128)</td>
<td>This work</td>
</tr>
<tr>
<td>G54</td>
<td>Capsulated clinical strain (type 19f)</td>
<td>(39)</td>
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<tr>
<td>PR212</td>
<td>Km(^R). Unencapsulated derivative of G54</td>
<td>Iannelli, Pearce, and Pozzi (unpublished)</td>
</tr>
<tr>
<td>3496</td>
<td>Capsulated (type 3)</td>
<td>(39)</td>
</tr>
<tr>
<td>PR215</td>
<td>Km(^R). Unencapsulated derivative of 3496</td>
<td>Iannelli, Pearce, and Pozzi (unpublished)</td>
</tr>
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</table>
**Table 2.** Binding of radiolabelled fH to pneumococci.

<table>
<thead>
<tr>
<th>strain</th>
<th>type</th>
<th>encaps.</th>
<th>unencaps.</th>
</tr>
</thead>
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<tr>
<td>D39 / PR201</td>
<td>2</td>
<td>2.3</td>
<td>3.6</td>
</tr>
<tr>
<td>3496 / PR215</td>
<td>3</td>
<td>15.4</td>
<td>26.6</td>
</tr>
<tr>
<td>HB565 / PR218</td>
<td>3</td>
<td>27.6</td>
<td>40.8</td>
</tr>
<tr>
<td>G54 / PR212</td>
<td>19</td>
<td>33.9</td>
<td>35.9</td>
</tr>
</tbody>
</table>

* Four pairs of encapsulated/unencapsulated strains were examined.

Binding is expressed as the percentage of added radioactivity.

Results are means of two experiments with duplicate samples.
Fig. 1.
Fig. 2.

Hic

SP  fH-binding/ α-helical  proline-rich repeats  W  LPSTG  COOH

1  612

PspC/CbpA/SpsA/PbcA

SP  α-helical region  choline-binding repeats  COOH

H₂N  1  700
Fig. 3.

Hic

1 MFASKNEKRKVKHIYSIRKFSIGVAVASVAVASLF30
PspC19 1 MFASKNEKRKVKHIYSIRKFSIGVAVASVAVASLF30
PspC2 1 MFASKSERKVKHIYSIRKFSIGVAVASVAVASLV30
PspC6A 1 MFASKSERKVKHIYSIRKFSIGVAVASVAVASLV30
SpA1 1 MFASKSERKVKHIYSIRKFSIGVAVASVAVASLV30
PspC.TIGR 1 MFASKSERKVKHIYSIRKFSIGVAVASVAVASLV30

Hic

PspC19 31 MGSVHATEKEVTTQVATSTSNKANKSQTIEH60
PspC2 31 MGSVHATEENGSTQATSSNMAKTEH60
PspC6A 31 MGSVHATEENGSTQATSSNMAKTEH60
SpA1 31 MGSVHATEENGSTQATSSNMAKTEH60
PspC.TIGR 31 MGSVHATEENGSTQATSSNMAKTEH60

Hic

PspC19 61 MK-------AKOVDEYIEKMLSIE78
PspC2 61 MK-------AKOVDEYITKMLSIE75
PspC6A 59 KA-------AKOVDEYITKMLSIE76
SpA1 54 AK-------EVEYIIEKMLSIE66
PspC.TIGR 61 GE-------INIERDKAKTAYSEYKEKKVSELY86

Hic

PspC19 79 QLDRRKTQNVGELTKLGAIKTEYLRLGS107
PspC2 76 QLDRRKTQNVGELTKLGVIKTEYLHRILS104
PspC6A 77 QLDRRKTQNVGELTKLGVIKTEYLHRILS104
SpA1 67 QLDRRKTQNVGELTKLGVIKTEYLHRILS104
PspC.TIGR 91 AKSTKRRHTIVALVNELNNIKNENEKNIKV116

Hic

PspC19 105 - VSEKESTAELEPSIKAKLDAAFEQFKKD135
PspC2 105 - VSEKESTAELEPSIKAKLDAAFEQFKKD132
PspC6A 105 - VSEKESTAELEPSIKAKLDAAFEQFKKD133
SpA1 96 VNELEKSKDELPSIKAKLDAAFEQFKKD125
PspC.TIGR 117 QSTSKTEIQGRITTSSKLEDVEASVSKYKKA145

Hic

PspC19 121 ESTSSEQLQI-LEMMESRSKVDDEAVSKFEKD149
PspC2 121 ESTSSEQLQI-LEMMESRSKVDDEAVSKFEKD149
PspC6A 121 ESTSSEQLQI-LEMMESRSKVDDEAVSKFEKD149

Hic

136 TLLK-4GKK142
PspC19 133 TLPTE-4GKK141
PspC2 136 TLPK-4GKK140
PspC6A 126 TLPK-4GKK132
SpA1 146 PSSSSSSSTSSTKPEASDTAKKPNKPTLEEK175
PspC.TIGR 150 SSSSSTSSTKPEASDTAKKPNKPTLEEK179

Hic

143 VAEAQKK149
PspC19 142 VAEAQKK148
PspC2 141 VAEAKKK147
PspC6A 133 VAEAQKK139
SpA1 176 VAEAKKK182
PspC.TIGR 180 VAEAKKK186

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Fig. 4.
**Fig. 5.**

A. STAIN BLOT

- GST: Hic 39-261
- GST

B. STAIN

- GST
- GST: Hic 39-261

C. Graph

- Relative fH binding vs. conc. (nM)
  - GST
  - GST: Hic 39-261
**A**

![Graph showing time vs. response in RU](image)

**B**

<table>
<thead>
<tr>
<th></th>
<th>$k_a$ ($10^4$ x Ms$^{-1}$)</th>
<th>$k_d$ ($10^{-4}$ x s$^{-1}$)</th>
<th>$K_A$ ($10^7$ x M$^{-1}$)</th>
<th>$K_D$ ($10^{-8}$ x M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hic + fH</td>
<td>2.8 ± 0.24</td>
<td>6.1 ± 2.4</td>
<td>5.0 ± 1.9</td>
<td>2.3 ± 1.1</td>
</tr>
</tbody>
</table>

Fig. 6.
Fig. 7.
Hic, a novel surface protein of Streptococcus pneumoniae that interferes with complement function
Robert Janulczyk, Francesco Iannelli, Anders G. Sjoholm, Gianni Pozzi and Lars Bjorck
J. Biol. Chem. published online August 30, 2000

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