THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 47, Issue of November 24, pp. 37257–37263, 2000

Printed in U.S.A.

Hic, a Novel Surface Protein of *Streptococcus pneumoniae* That Interferes with Complement Function*

Received for publication, May 26, 2000, and in revised form, August 28, 2000
Published, JBC Papers in Press, August 30, 2000, DOI 10.1074/jbc.M004572200

Robert Janulczyk‡§, Francesco Iannelli¶, Anders G. Sjöholm‖, Gianni Pozzi¶, and Lars Björck‡

From the Departments of ‡Cell and Molecular Biology, Section for Molecular Pathogenesis, ‖Laboratory Medicine, Section of MIG, Lund University, 22100 Lund, Sweden and the ¶Department of Molecular Biology, University of Siena, 53100 Siena, Italy

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 NH_2-terminal region showed significant homology to the NH_2-terminal region of several PspC proteins. A fragment of Hic, covering this homologous region, was expressed as a glutathione *S*-transferase (GST) fusion protein. GST:Hic$^{39–261}$ bound radiolabeled factor H and inhibited binding of factor H to pneumococci of different serotypes. Interaction kinetics between GST:Hic$^{39–261}$ and factor H were studied with surface plasmon resonance and showed a high affinity binding ($K_A = 5 \times 10^7$, $K_D = 2.3 \times 10^{-8}$). Mutant pneumococci lacking Hic showed no absorption of factor H in human plasma and no binding of radiolabeled 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 the presence of GST:Hic$^{39–261}$. In addition, an intrinsic inhibitory effect of Hic is suggested.

Despite the availability of effective antibiotics and a polyvalent capsular polysaccharide vaccine, *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 factors other 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,[1] pneumolysin, and pneumococcal surface antigen A 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 Ref. 8). For example, binding of the complement regulatory protein fH to bacterial surface proteins has been described by several groups (reviewed in Ref. 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 *Streptococcus 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 previously 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 an 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

* This work was supported by Swedish Medical Research Council Project 7480, the Medical Faculty, Lund University, the Foundations of Kock, Lundberg, and Österlund, the Göran Gustavsson Foundation for Research in Natural Sciences and Medicine, and Active Biotech AB, and by grants from CNR (P. F. Biotecnologie) and MURST (Cofinanziamento 1999). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF252857.

§ To whom correspondence should be addressed: Dept. of Cell and Molecular Biology, Section for Molecular Pathogenesis, Lund University, Solvegatan 39, 22100 Lund, Sweden. Tel.: 46-46-2224489; Fax: 46-46-157756; E-mail: Robert.Janulczyk@medkem.lu.se.

[1] The abbreviations used are: PspA, pneumococcal surface protein A; fH, complement factor H; 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; PAGE, polyacrylamide gel electrophoresis; aa, amino acid.

paper (16) compares different allelic variants of PspC 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 I. In unencapsulated strains PR201, PR212, PR215, and PR218 the whole capsule locus is deleted and substituted with a kanamycin (Km) resistance cassette.[2] 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 (19), a chloramphenicol transferase cassette was flanked by sequences found upstream 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′-TGGGATCCCAGAGAAGGAGGTAACTAC-3′) and HICr1 (5′-GGAGCCTGAATTCGACGAAG-3′), containing *Bam*HI and *Eco*RI restriction sites respectively (underlined), were used in a PCR to amplify DNA corresponding to amino acids 39–261 in Hic. The PCR was performed with *Taq* polymerase (Life Technologies, Inc.) 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 removing bacterial debris by centrifuging at 13,000 × *g*. The PCR-amplified fragment was gel-purified with Sephaglas Bandprep (Amersham Pharmacia Biotech), digested with *Bam*HI and *Eco*RI (Amersham Pharmacia Biotech), and ligated with likewise digested vector pGEX-5X-3 (Amersham Pharmacia Biotech) using T4 DNA ligase (Amersham Pharmacia Biotech). Plasmid pGEX-5X-3:*hic*-(*39–261*) was then electroporated into DH5α *E. coli* according to the GST gene fusion system protocol (Amersham 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$^{39-261}$ 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 (Amersham Pharmacia Biotech).

Ligand Binding and Protein Methods—Plasma absorption experiments were performed with log phase pneumococci (A_{600} ~0.4). Bacteria were washed twice in phosphate-buffered saline, pH 7.4, containing 0.05% Tween 20 (PBST), and the bacterial concentration was adjusted to 2×10^{10} cells/ml. 100 μl of bacteria were incubated for 1 h with 100 μl of human plasma. Bacteria were washed five times with PBST, and bound proteins were eluted from the cells with 100 μl of 0.1 M glycine HCl, pH 2.0. The pH of the eluted material was adjusted to ~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^{TM} polyvinylidene difluoride 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) were used as secondary antibodies, and detection of immunoreactive bands was performed by chemiluminescence as described (22).

Slot blot, Western blot, and bacterial binding assays were performed with fH (Sigma), radiolabeled with ^{125}I using the IODO-BEADS kit (Pierce). Unincorporated ^{125}I was removed by gel filtration on Sephadex G-25 (Amersham Pharmacia Biotech). Slot blots were performed by applying 5, 1, 0.02, and 0.004 μg of purified protein onto nitrocellulose membranes in a slot blot apparatus (Schleicher & Schuell). The membrane was blocked for 4 × 20 min in PBST containing 0.25% (w/v) gelatin (Difco). Radiolabeled fH (200,000 cpm/ml) was then added, and the membrane was incubated for 1 h at room temperature. The membrane was washed 4 times for 20 min in PBST, 0.5 M NaCl, and membrane-associated radioactivity was visualized by exposure onto a phosphoimaging plate (Fuji Film). Western blot membranes were treated in the same way. Binding assays with pneumococci were performed as described (23), using log phase bacteria (A_{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 manufacturer's instructions. ~10,000 resonance units of antibodies were coupled. Each cycle of analysis was then commenced by immobilization of 500–1500 resonance units of GST or GST:Hic$^{39-261}$ (10 μg/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 5×10^8 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% gelatin. 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 2.5×10^7 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 of cold veronal-buffered saline, 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, UK). Data base searches utilized the Entrez server at The National Institute for Biotechnology Information. The sequence of PspC from the type 4 pneumococcus was obtained from The Institute for Genomic Research website.

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.[3] To better investigate the nature of these interactions, a series of strains (Table I), both encapsulated and unencapsulated, was incubated with plasma. After washing, plasma proteins bound to the pneumococci were eluted and separated by SDS-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[4] 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 previously mentioned 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 NH_2-terminal sequencing, the band was identified as human immunoglobulin heavy chains, suggesting specific or unspecific binding of immunoglobulins to pneumococci and

[2] F. Iannelli, B. J. Pearce, and G. Pozzi, unpublished data.

[3] R. Janulczyk, F. Iannelli, A. G. Sjöholm, G. Pozzi, and L. Björck, unpublished results.

[4] B. J. Pearce, F. Iannelli, and G. Pozzi, manuscript in preparation.

TABLE I
S. pneumoniae strains

Bacterial strains	Relevant properties	Source or ref.
A66	Capsulated clinical strain (type 3)	38, 39
PR218	Km^R. Unencapsulated derivative of A66	Footnote 2 data
FP13	Cm^R, Km^R, Hic^-. Mutant of PR218 in which *hic* is deleted and substituted with a *cat* cassette.	This work
D39	Capsulated clinical strain (type 2)	38, 40
Rx1	Unencapsulated derivative of D39	41
PR201	Km^R. Unencapsulated derivative of D39	Footnote 2
FP7	*pspC* deletion mutant of Rx1 (in-frame deletion of nucleotides 124–1338, GenBank™ accession number AF067128)	This work
G54	Capsulated clinical strain (type 19f)	39
PR212	Km^R. Unencapsulated derivative of G54	Footnote 2
3496	Capsulated (type 3)	39
PR215	Km^R. Unencapsulated derivative of 3496	Footnote 2

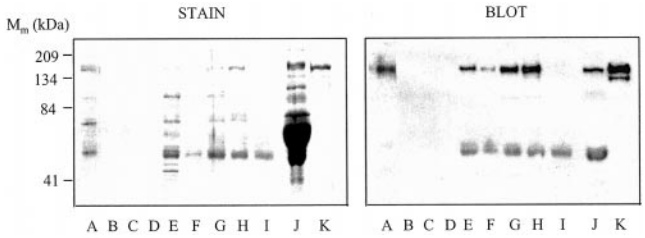


FIG. 1. **Pneumococcal absorption of plasma proteins.** SDS-PAGE analysis (*STAIN*) of plasma proteins absorbed by and eluted from D39 (*A*), HB565 (*B*), 3496 (*C*), G54 (*D*), PR201 (*E*), PR212 (*F*), PR215 (*G*), PR218 (*H*), and FP13 (*I*). *Lanes J* and *K* contain diluted plasma and fH, respectively.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

TABLE II
Binding of radiolabeled fH to pneumococci

Strain	Type	fH binding (%)[a]	
		Encaps.	Unencaps.
D39/PR201	2	2.3	3.6
3496/PR215	3	15.4	26.6
HB565/PR218	3	27.6	40.8
G54/PR212	19	33.9	35.9

[a] Four pairs of encapsulated (encaps.)/unencapsulated (unencaps.) strains were examined. Binding is expressed as the percentage of added radioactivity. Results are means of two experiments with duplicate samples.

subsequent cross-reactivity 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 radiolabeled fH. Most strains showed significant binding of fH, although the degree of binding varied considerably between strains (Table II). 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 data base obtained from The Institute for Genomic Research. The highest scoring homology for all the probes was found in a 2106-base pair open reading frame, 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 *S. 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-base pair open reading frame, 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 data base 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

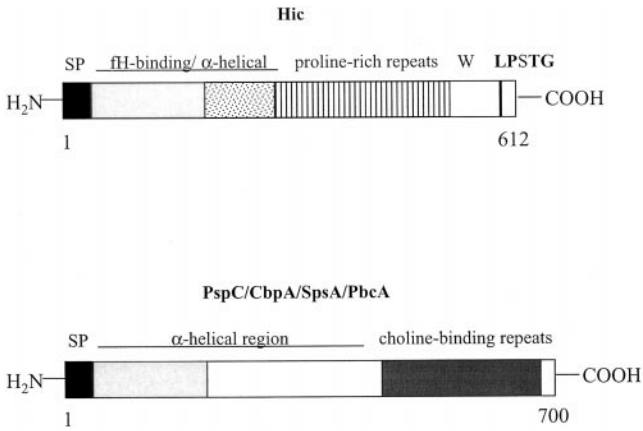


FIG. 2. **Comparison of Hic and PspC.** Schematic representation of Hic and PspC. The signal peptide (*SP*) and the wall spanning region (*W*) are indicated.

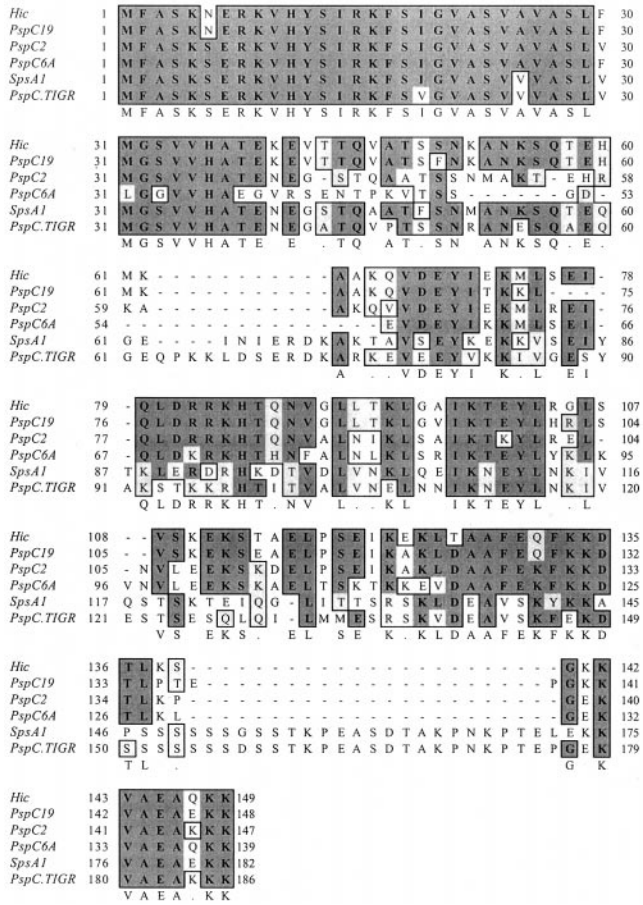


FIG. 3. **Sequence comparison of Hic and allelic variants of PspC.** ClustalW alignment of the NH_2-terminal region from Hic (serotype 3) and allelic variants of PspC, including SpsA. Identical and similar residues are shown in *dark* and *light shading*, respectively. Numbers in the protein names indicate 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.

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 α-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 (19) was used to flank an antibiotic resistance cassette with sequences found upstream and downstream of the *hic* gene. PR218 was transformed with this construct, and the *hic* gene was deleted by double crossover 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.

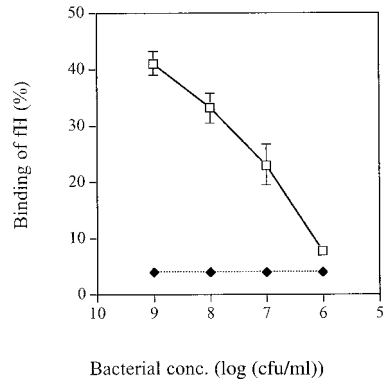


FIG. 4. **Binding of radiolabeled factor H to a Hic-deficient mutant.** Serial dilutions of PR218 (□) and the Hic-deficient mutant FP13 (♦) were incubated with radiolabeled fH. Binding is expressed as the percentage of added radioactivity. The data points are averages of three experiments with duplicate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

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 radiolabeled 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 405 NH_2-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 NH_2-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:Hic^{39-261} and GST were overexpressed, affinity-purified, and analyzed by SDS-PAGE (Fig. 5*A, STAIN*). Although partially degraded, the main protein band had the expected mass (54 kDa). An identical gel was blotted to a membrane, which was then incubated with radiolabeled fH, washed, and subjected to autoradiography. fH bound to GST:Hic^{39-261} but not to GST (Fig. 5*A, BLOT*). The fusion protein and the GST control were also applied in serial dilutions onto a nitrocellulose membrane. The membrane was probed with radiolabeled fH, which showed binding to the fusion protein, and no binding to GST (Fig. 5*B*). Furthermore, the fusion protein was used in a competitive binding assay to investigate whether GST:Hic^{39-261} could compete with fH binding to PR218 bacteria. The result (Fig. 5*C*) shows that the Hic domain of the fusion

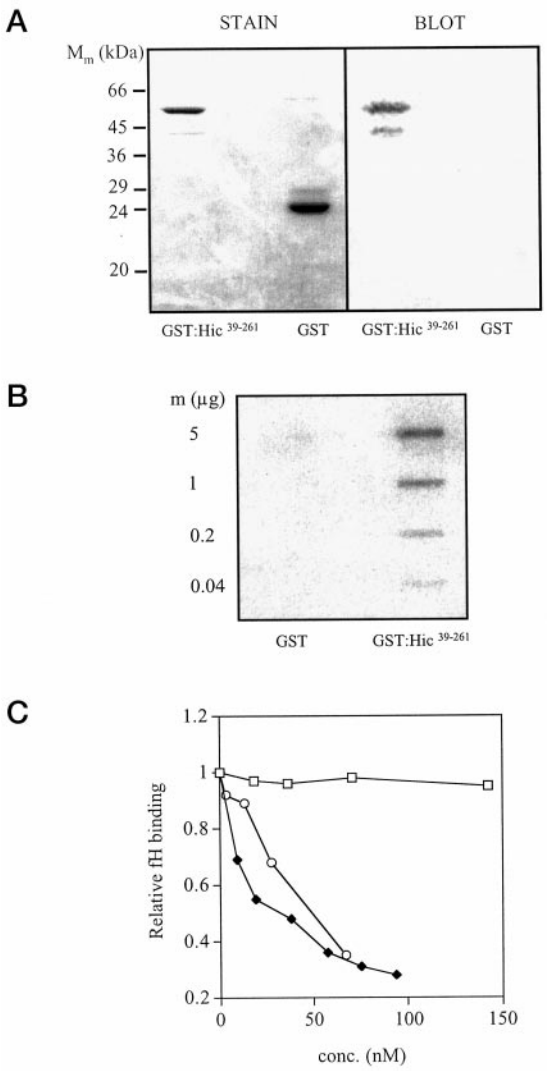
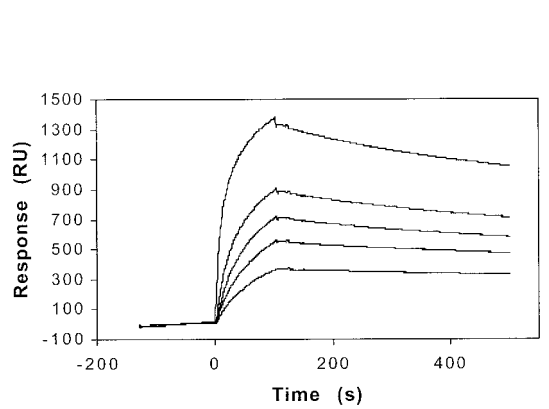


FIG. 5. **Binding of factor H to Hic.** *A,* SDS-PAGE analysis of GST and recombinant Hic. Two identical gels were prepared and either stained (*STAIN*) or blotted. The membrane from the blot was incubated with radiolabeled fH (*BLOT*). *B,* a serial dilution (5, 1, 0.2, and 0.04 μg) of GST and recombinant Hic was applied onto a nitrocellulose membrane that was incubated with radiolabeled fH. *C,* a competitive binding assay was performed by incubating PR218 bacteria (10^9 colony-forming units/ml) with radiolabeled fH in the presence of increasing concentrations of unlabeled fH (○), GST (□), and GST:Hic$^{39-261}$ (♦).

protein blocks bacterial fH binding, whereas GST alone does not affect binding. Similar experiments were performed with *S. pneumoniae* strains 3496, G54, PR215, and HB565. Also in these experiments GST:Hic$^{39-261}$ 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. 2*B*) implicates that fH binding to non-type 3 strains is mediated by the PspC region homologous to Hic.

The interaction between GST:Hic$^{39-261}$ and fH was further examined by surface plasmon resonance. Anti-GST antibodies were coupled to a carboxymethyl dextran chip, followed by the immobilization of GST:Hic$^{39-261}$ as the ligand. Highly purified fH was used as the analyte at various concentrations. fH interacted with GST:Hic$^{39-261}$ 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



B

	k_a (10^4 x Ms^{-1})	k_d (10^{-4} x s^{-1})	K_A (10^7 x M^{-1})	K_D (10^{-8} x M)
Hic + fH	2.8 ± 0.24	6.1 ± 2.4	5.0 ± 1.9	2.3 ± 1.1

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 ± S.D. from three different experiments.

(Fig. 6*A*). 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. 6*B*), applying the standard Langmuir 1:1 model.

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 3-fold (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 *S. pneumoniae* (33). Two recent

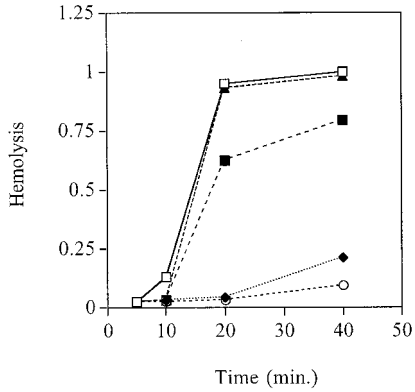


FIG. 7. **Hic and factor H inhibit alternative pathway hemolysis.** Rabbit erythrocytes were incubated with C2-deficient serum, and a kinetic study of hemolysis was performed. The influence of added fH, GST, and/or GST:Hic$^{39-261}$ on hemolysis was studied. *Numbers* represent hemolysis as a fraction 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$^{39-261}$ (○).

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 (14) 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 with 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 LP*X*TG*X* motif, whereas 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 radiolabeled 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 radiolabeled 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 similar 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 NH_2-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 NH_2-terminal region. We found that a type 2 mutant strain that expressed an NH_2-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 NH_2-terminal regions of both PspC and Hic.

Many studies have shown 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 described previously, 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 *Neisseria gonorrhoeae* (36, 37) and may represent a widespread theme in bacterial adaptation to the human host.

Acknowledgments—We acknowledge the Streptococcal Genome Sequencing Project and B. A. Roe, S. P. Linn, L. Song, X. Yuan, S. Clifton, M. McShan, and J. Ferretti. We thank Ingbritt Gustavsson and Eva Holmström for excellent technical assistance and Henrik Svensson for helpful comments on surface plasmon resonance. We are indebted to Dr. Ulf Sjöbring for fruitful discussions.

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