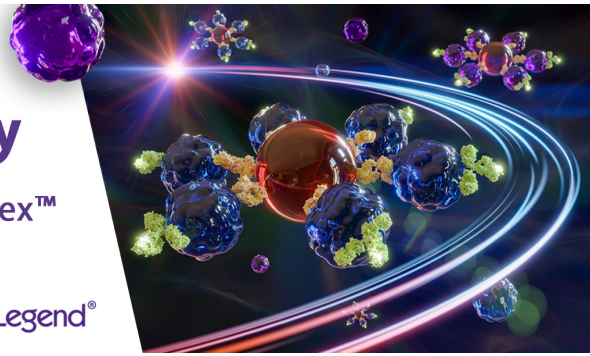


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J Immunol (2002) 168 (4): 1886–1894.

<https://doi.org/10.4049/jimmunol.168.4.1886>

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Streptococcus pneumoniae is an important cause of upper and lower respiratory tract infections, meningitis, peritonitis, bacterial arthritis, and sepsis. Here we have studied a novel immune evasion mechanism of serotype 3 pneumococci, which are particularly resistant to phagocytosis. On their surfaces the bacteria express the factor H-binding inhibitor of complement (Hic), a protein of the pneumococcal surface protein C family. Using radioligand binding, microtiter plate assays, surface plasmon resonance analysis, and recombinant constructs of factor H, we located the binding site of Hic to short consensus repeats (SCRs) 8–11 in the middle part of factor H. This represents a novel microbial interaction region on factor H. The only other ligand known so far for SCRs 8–11 of factor H is C-reactive protein (CRP), an acute phase protein that binds to the pneumococcal C-polysaccharide. The binding sites of Hic and CRP within the SCR8–11 region were different, however, because CRP did not inhibit the binding of Hic and required calcium for binding. Binding of factor H to Hic-expressing pneumococci promoted factor I-mediated cleavage of C3b and restricted phagocytosis of pneumococci. Thus, virulent pneumococci avoid complement attack and opsonophagocytosis by recruiting functionally active factor H with the Hic surface protein. Hic binds to a previously unrecognized microbial interaction site in the middle part of factor H. *The Journal of Immunology*, 2002, 168: 1886–1894.

Streptococcus pneumoniae (pneumococcus) is a Gram-positive diplococcus and a frequent cause of otitis media, sinusitis, pneumonia, peritonitis, meningitis, bacterial arthritis, and sepsis (1). Despite the use of vaccines and antibiotic therapy, pneumococci still cause significant morbidity and mortality worldwide, particularly in infants, elderly, and immunocompromised individuals. Pneumococci are also part of the normal flora in the respiratory tract. The reasons why some pneumococcal strains are more virulent than others are still largely unknown.

According to the structures of their capsular surface polysaccharides, pneumococci are divided in over 90 serotypes (2). Of these, about 20 are frequently isolated from clinical samples, all encapsulated (3). The capsule is a virulence factor that protects pneumococci from the immune system, but other mechanisms are also important (4). Pneumolysin and pneumococcal surface protein

A (PspA)³ are the best-known noncapsular virulence factors to date (5–7).

Serotype 3 pneumococci are among the most common serotypes in clinical samples. They account for 7–8% of pneumococci isolated from invasive infections (8, 9). Serotype 3 pneumococci are known to be particularly resistant to phagocytosis (10). Recently it was shown that a surface protein of *S. pneumoniae* serotype 3 binds to the complement (C) factor H (fH) (11). This protein was tentatively named as the factor H-binding inhibitor of complement (Hic). Hic is an ~70-kDa protein that penetrates the cell wall of pneumococcus. The chromosomal *hic* gene encodes a protein of 612 aa that, in its N-terminal part (residues 1–60), is homologous to pneumococcal surface protein C (PspC) proteins of other pneumococci. The factor H-binding site on Hic has been located to the N-terminal amino acid residues 39–261 of the molecule (11). Recently, binding of fH to PspC of serotype 2 strain D39 has also been reported (12).

Factor H is an important fluid-phase regulator of the alternative pathway of complement (AP). It is a soluble 150-kDa protein composed of 20 short consensus repeat (SCR) domains. Each SCR contains ~60 amino acids held in a domain structure by two disulfide bridges. Factor H has an important function in protecting host cells from C attack. It regulates the AP by inhibiting the binding of factor B to C3b, acting as a cofactor for factor I-mediated cleavage of C3b (cofactor activity) and accelerating the decay of the AP C3 convertase C3bBb (decay-accelerating activity) (13). By controlling the key steps in the AP amplification cycle, fH

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Received for publication October 11, 2001. Accepted for publication December 14, 2001.

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¹ This work was supported by the Life 2000 program of the Academy of Finland, the Sigrid Jusélius Foundation, the Helsinki University Central Hospital Funds, the University of Helsinki, Deutsche Forschungsgemeinschaft (Project Zi432/5-1), Deutscher Akademischer Austauschdienst, and Thüringer Ministerium für Wissenschaft, Forschung und Kunst and Swedish Research Council (Project 7480).

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³ Abbreviations used in this paper: PspA, pneumococcal surface protein A; C, complement; fH, complement factor H; Hic, factor H-binding inhibitor of complement; PspC, pneumococcal surface protein C; AP, alternative pathway of complement; SCR, short consensus repeat; FHL-1, factor H-like protein 1; CRP, C-reactive protein; NHS, normal human serum; PMN, polymorphonuclear leukocyte; VBS, Veronal-buffered saline; GVB, VBS plus 0.1% gelatin.

also inhibits activation that has been initiated via the classical or the lectin pathway. Surfaces rich in anionic glycosaminoglycans or sialic acids promote the binding of fH to surface-bound C3b, resulting in suppressed C activation (14–16).

Certain microbes use binding of fH as a mechanism to avoid C attack. For example, the group A streptococcal M protein binds to the SCR7 of fH (17, 18). Serum-resistant strains of *Borrelia burgdorferi* strains were recently observed to bind to both the C-terminal and N-terminal parts of fH (19–21). Also, the Por1A protein and the sialylated lipo-oligosaccharide of *Neisseria gonorrhoeae* can bind fH (22, 23). In general, the binding of fH may confer serum resistance to bacteria and prevent their opsonophagocytosis (24).

The major functional region on fH for cofactor and decay-accelerating activities has been located to the SCR domains 1–4. Although the AP regulatory site is in the N terminus, fH has at least three binding sites for C3b: at the N-terminal domains 1–4, in the middle region, and in the most C-terminal SCR20 (25–27). In addition, as many as three polyanion binding sites have been demonstrated on fH at domain 7, around domain 13, and in domains 19–20 (28–31).

In addition to the full-size fH, the fH gene also gives rise to an alternatively spliced product, the factor H-like protein 1 (FHL-1). FHL-1 consists of the seven most N-terminal SCR domains of fH plus four unique amino acids at the C-terminal end. The plasma concentration of FHL-1 is only 50 $\mu\text{g}/\text{ml}$, i.e., $\sim 10\%$ of that of fH. Although FHL-1 does not appear to discriminate between AP activating and nonactivating surfaces, it has similar cofactor activity as fH (32). No C regulatory activities have yet been assigned to the factor H-related proteins 1–5, which are encoded by separate genes (31).

In addition to its well-known ligands, C3b and polyanions, fH binds to C-reactive protein (CRP) (33–35). CRP is the prototype acute-phase protein in humans. Its normal plasma level is below 1 $\mu\text{g}/\text{ml}$, but during acute inflammation, infection, or tissue damage, the CRP level rises several hundred-fold within 24–48 h. CRP was identified on the basis of its binding to the C-polysaccharide of certain pneumococcal strains. By binding to complement C1q, CRP activates the classical pathway (36, 37). Recently, we localized the binding sites of CRP to SCR7 of fH and FHL-1 and to SCRs 8–11 of fH (35). This suggests that CRP may also regulate C activation at the C3b level (33, 35, 38).

Because the pneumococcal Hic protein and CRP both bind to fH, we wanted to compare the binding sites of Hic and CRP on fH and to examine the functional consequences of the Hic-fH interaction. We found that Hic binds to SCRs 8–11 of fH, showing that this is a novel binding site on fH exploited by microbes. CRP binds to the same region but to a distinct epitope in a calcium-dependent manner. These studies establish the middle SCR8–11 part of fH as a functionally important region with multiple ligands. By binding fH and preventing opsonophagocytosis, the *pspC* locus-encoded surface protein Hic apparently provides an important virulence factor for serotype 3 *Streptococcus pneumoniae*.

Materials and Methods

C components and CRP

Human factor H and factor I were purchased from Calbiochem (La Jolla, CA). C3 was purified and C3b generated with factors B and D in the presence of Mg^{2+} ions as described previously (39, 40). Recombinant fH constructs SCR1–6, 1–7, 8–11, 8–20, and 15–20 were cloned and produced in the baculovirus expression system as described previously (27, 41–43). Human CRP was obtained from Sigma-Aldrich (St. Louis, MO).

The mouse mAbs 131X and 196X against human fH were produced as previously described (25). Purified fH, SCR1–6, 1–7, 8–11, 8–20, 15–20,

19–20, C3b, Hic-GST, and Hic were radiolabeled with iodine (^{125}I ; NEN, Boston, MA) using the Iodogen method (Pierce, Rockford, IL).

Normal human serum (NHS) was obtained from healthy laboratory personnel. NHS was heat inactivated by incubation at 56°C for 30 min. Polymorphonuclear leukocytes (PMNs) were isolated from the peripheral blood (supplemented with 10 mM EDTA) of healthy donors. The blood sample was placed at 37°C , and after 1 h the buffy coat was collected. The buffy coat was centrifuged through Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. The erythrocyte-PMN fraction was collected and erythrocytes were lysed with H_2O . The PMNs were washed twice with Veronal-buffered saline (VBS; 142 mM NaCl, 1.8 mM sodium barbital, 3.3 mM barbituric acid (pH 7.4)) and used in the phagocytosis assay.

Pneumococcal strains and Hic protein

Three pneumococcal strains were used in the binding assays. HB565 is an encapsulated Hic-expressing strain and a derivative of the A66 strain (11). PR218 is an unencapsulated mutant of HB565, and FP13 is an unencapsulated mutant of PR218 that does not express Hic. For assays, the bacteria were grown in Todd-Hewitt broth (Difco, Detroit, MI) with 0.2% yeast extract (Difco) supplement until mid-log phase (OD_{650} 0.4).

A fragment of Hic, covering amino acids 39–261, was expressed as a GST fusion protein as previously described (11). To obtain GST-free Hic (Hic $^{39-261}$), the fusion protein was cleaved with factor Xa while bound to the matrix according to the instructions of the manufacturer (Amersham Pharmacia Biotech). Purified GST from *Schistosoma japonicum* was obtained by growing the PGEX-4T-1 plasmid (Amersham Pharmacia Biotech) in *Escherichia coli* HB101 cells and inducing protein production by 0.6 mM isopropyl- β -D-thiogalactopyranoside (Calbiochem). After sonication, GST was purified by attachment to Glutathione-Sepharose (Amersham Pharmacia Biotech), and after washing the protein was eluted with 20 mM glutathione (reduced form; Sigma-Aldrich) in 100 mM Tris-HCl (pH 9.0; Sigma-Aldrich).

Ligand blotting analysis of fH deletion mutant binding to Hic

Hic-GST was run into a 10% SDS-PAGE gel under nonreducing conditions and electrotransferred onto a nitrocellulose membrane. Nonspecific binding sites were blocked by incubating the membrane for 1 h at 22°C in 5% fat-free milk in PBS. The membrane was washed and incubated with radiolabeled constructs of fH (SCRs 1–6, 1–7, 8–20, and 15–20) in VBS for 72 h at 4°C . The blot was washed with VBS and the binding of proteins was analyzed after an exposure of the membrane to an x-ray film (Super RX; Fuji Photo Film, Tokyo, Japan). In a reverse setting, the fH constructs SCR1–6 (590 ng), SCR1–7 (510 ng), SCR8–11 (420 ng), SCR8–20 (390 ng), and SCR15–20 (580 ng) were run into a 12.5% SDS-PAGE gel under nonreducing conditions and transferred onto a nitrocellulose membrane. After blocking with 5% fat-free milk, the membrane was washed and incubated with radiolabeled Hic-GST (8 μg ; specific activity, 2.6×10^5 cpm/ μg) in 10 ml 1/3 VBS for 72 h at 4°C . After washing, the binding interactions were analyzed as described above.

Radioligand binding assay

A microtiter-well binding assay was used for the analysis of protein-protein interactions. The proteins (fH; SCRs 1–6, 1–7, 8–11, 8–20, and 15–20; CRP; C3b; and BSA; 10 $\mu\text{g}/\text{ml}$ in VBS, 60 $\mu\text{l}/\text{well}$) were coated on microtiter wells (Nunc Polysorp, Wiesbaden, Germany) by an overnight incubation at 4°C . The wells were washed with VBS and nonspecific binding sites were blocked by incubation (15 min at room temperature) with 0.1% gelatin in VBS (GVB). Radioactive Hic or Hic-GST (20,000 cpm/well; specific activities, 1.7×10^6 cpm/ μg and 2.6×10^5 cpm/ μg , respectively) was added to the wells (60 $\mu\text{l}/\text{well}$) and incubated for 1 h at room temperature. The wells were washed for five times with VBS and detached for measurement of radioactivity with a gamma counter. The binding percentage was counted as percentage of bound cpm (well) per offered total cpm. The wells coated with BSA were examined as controls. All analyses were performed three times in duplicate.

The competition assays were performed by mixing varying amounts of CRP (10–300 $\mu\text{g}/\text{ml}$), unfractonated heparin (Heparin 5000 IU/ml, Lövens Kemiske Fabrik, Ballerup, Denmark; 30–300 IU/ml), or the mAbs 196X or 131X (30–100 $\mu\text{g}/\text{ml}$) with the radiolabeled Hic or Hic-GST during the overlay stage. The CRP binding experiments were performed in the presence of CaCl_2 (final concentration, 1 mM). In controls, to exclude calcium-dependent binding interactions, 10 mM EDTA was added to the assay buffers.

The ability of the SCR8–11 construct to inhibit the binding of Hic to SCR8–20 was tested by mixing SCR8–11 (10 $\mu\text{g}/\text{ml}$) with ^{125}I -Hic-GST (20,000 cpm; 75 ng/well) in the fluid phase before addition to the SCR8–

20-coated well. In a reverse setting, SCR8–11 was coated to the well, and a mixture of SCR8–20 (10 $\mu\text{g/ml}$) and ^{125}I -Hic-GST was added.

Binding assay with whole bacteria

Pneumococcal strains were grown until mid-log phase and washed three times with GVB. The bacteria (2×10^{10} cells/ml, 4×10^8 cells/reaction) were incubated with the radioactive proteins (CRP, fH, SCR1–7, or SCR8–20; specific activities, 1×10^7 cpm/ μg , 3×10^6 cpm/ μg , 1×10^6 cpm/ μg , and 6×10^5 cpm/ μg , respectively; $\sim 20,000$ cpm of each) in GVB for 10 min at 37°C with gentle mixing. After incubation, the reaction mixture (40 μl) was centrifuged ($10,000 \times g$, 3 min) through 20% sucrose (BDH Laboratory Supplies, Poole, U.K.) in GVB. To separate the pellets, the sucrose-containing tubes were cut, and radioactivities in the pellets and the supernatants were measured in a gamma counter. The ratios of bound to total activity were calculated.

The effect of CRP on the binding of fH SCR8–20 to the Hic-positive and -negative strains was analyzed by mixing various amounts of CRP (final concentration 0–100 $\mu\text{g/ml}$) with radiolabeled SCR8–20 (20,000 cpm) in the presence of either CaCl_2 (final 1 mM) or EDTA (final 10 mM). The mixtures were incubated with pneumococci (4×10^8 cells) in 40 μl GVB for 30 min at 37°C . After incubation, the reaction mixtures were centrifuged through 20% sucrose, and the radioactivities were determined as described above.

Surface plasmon resonance analysis of Hic-fH interactions

Surface plasmon resonance measurements were performed using the Biacore 2000 instrument and analyzed with the BIAevaluation 3.0 software (Biacore, Uppsala, Sweden). Hic, Hic-GST, and GST were immobilized on carboxylated dextran CM5 chips (Biacore) by using the amine coupling procedure according to the protocol of the manufacturer. Binding analyses were done using 1/3 or 1/2 VBS (75 mM NaCl or 50 mM NaCl, respectively), pH 7.4, at a flow rate of 5 $\mu\text{l/min}$. The Hic-, Hic-GST-, and GST-coupled chips were used for localizing the fH domains required for Hic binding. Before injecting into the Biacore flow cell, fH and recombinant constructs of fH were dialyzed against the flow buffer. The protein concentrations of the reagents were measured using the BCA Protein Assay (Pierce, Rockford, IL). The final concentrations of proteins in the Hic binding assay were as follows: fH, 67 $\mu\text{g/ml}$; SCR1–7, 64 $\mu\text{g/ml}$; SCR8–11, 93 $\mu\text{g/ml}$; SCR8–20, 64 $\mu\text{g/ml}$; and SCR15–20, 115 $\mu\text{g/ml}$. As controls, all binding tests were also performed using a blank chip that was activated and deactivated without any coupled proteins. After each binding experiment, the surface was regenerated by 30 μl of 3 M NaCl in acetate buffer, pH 4.6 (regeneration buffer).

Cofactor assay for C3b inactivation

The functional activity of the pneumococcus-bound fH was tested by using a factor I-cofactor assay. The factor I-mediated cleavage of C3b was analyzed essentially as described previously (44). Purified C3b was radiolabeled to an initial specific activity of 2.3×10^7 cpm/ μg . The three pneumococcal strains were incubated with fH (final concentration, 30 ng/ml to 3 $\mu\text{g/ml}$) in GVB for 30 min at 37°C . After washing three times with GVB, factor I (20 $\mu\text{g/ml}$) and ^{125}I -C3b (200,000 cpm) were added, and the mixture (in 40 μl GVB) was incubated for 1 h at 37°C . Experiments performed in the absence of factor I and/or fH were taken as negative controls. After incubation, the samples were centrifuged and the supernatants were heated to 95°C in a reducing buffer (containing 2.5% 2-ME) and run in a 10% SDS-PAGE gel. The gels were fixed with 5% acetic acid for 30 min, dried, and autoradiographed with the Fujifilm BAS 2500 instrument (Fuji Photo Film). The conversion of C3b to iC3b was analyzed as the cleavage of the C3b- α' chain into 67-, 43-, and 41-kDa fragments.

The ability of pneumococci to bind fH directly from serum was tested by incubating PR218 (unencapsulated, Hic-positive) and FP13 (unencapsulated, Hic-negative) strains in 0.5–5% heat-inactivated serum (30 min, 37°C). In a control, polyclonal anti-fH Ab (diluted 1/100; Calbiochem) was added to inhibit fH binding. The bacteria were washed three times with VBS and mixed with factor I (20 $\mu\text{g/ml}$) and ^{125}I -C3b (200,000 cpm). Tests performed in the absence of factor I and/or fH served as negative controls. The samples were incubated for 1 h at 37°C and analyzed as described above.

ELISA for C activation and opsonization

An ELISA was used for analyzing C component deposition on whole pneumococci. The pneumococcal strains PR218 and FP13 were grown to mid-log phase, washed with GVB, and resuspended in GVB (10^{10} bacteria/ml). A total of 9×10^8 bacteria were incubated in the presence of 10 mM MgEGTA with 10% NHS in GVB or GVB alone (final volume, 100 μl) for 10 min at 37°C with gentle mixing. The bacteria were washed with ice-cold

GVB containing Pepstatin A and Antipain (both from Sigma-Aldrich), 1 $\mu\text{g/ml}$ each, and resuspended in the same buffer. The bacterial suspensions (50 $\mu\text{l/well}$) were incubated for 3 h at 37°C on a microtiter plate (Nunc Polysorp). After incubation, the plate was washed with VBS/0.05% Tween. Abs (rabbit anti-human C3c; DAKO A/S, Glostrup, Denmark; or mouse anti-human iC3b; Quidel, La Jolla, CA) were diluted in GVB (1/1000 and 5 $\mu\text{g/ml}$, respectively) and incubated on the plates for 1 h at room temperature. After washing with VBS/Tween, the conjugated secondary Abs (HRP goat anti-rabbit IgG and HRP rabbit anti-mouse IgG diluted 1/2000 in GVB; both from Jackson ImmunoResearch Laboratories, West Grove, PA) were added and incubated for 1 h at room temperature. The wells were washed and the substrate (8 mg *o*-phenylenediamine tablets, Dako; in 12 ml H_2O and 5 μl of H_2O_2) was added. The OD (492 nm) was determined using a Multiskan 340 MCC spectrophotometer (Labsystems, Helsinki, Finland).

Phagocytosis assay

The phagocytosis assay was modified from Neeleman et al. (45). The unencapsulated pneumococcal strains PR218 (Hic-positive) and FP13 (Hic-negative) were grown in Todd-Hewitt Broth supplemented with 0.2% yeast extract and FITC (20 $\mu\text{g/ml}$; Sigma-Aldrich) to mid-log phase, washed with GVB, and resuspended in GVB. Bacteria (5×10^6) were incubated with NHS (0–50%) in the presence 10 mM MgEGTA at 37°C on an agitator (opsonization). Freshly isolated PMNs (2.5×10^5 cells) were added after 10 min. Phagocytosis was allowed for 12 min, after which ice-cold GVB was added. After washing three times, the cells were resuspended in VBS and examined immediately by flow cytometry (FACScan 440, BD Biosciences, San Jose, CA). The data were analyzed with the Lysys II software supplied by BD Biosciences.

Results

Binding of fH and fH recombinant constructs to pneumococci

First, we studied the binding of fH and its deletion mutants to the three pneumococcal strains, which differed in their Hic expression and encapsulation. The strains HB565 (encapsulated, Hic-positive), PR218 (unencapsulated, Hic-positive), and FP13 (unencapsulated, Hic-negative) were incubated with radiolabeled fH, SCR1–7, or SCR8–20 (7, 20, and 30 ng/reaction, respectively) and centrifuged through 20% sucrose. As shown in Fig. 1, fH and the SCR8–20 construct bound more strongly to the Hic-positive strains (HB565 and PR218) than to the Hic-negative strain (FP13). No such Hic-dependent differences were seen in the binding of SCR1–7. The encapsulated strain HB565 bound less SCR8–20 (33.2%) than the unencapsulated strain PR218 (51.7%), but still the binding was much stronger than to the Hic-negative strain FP13 (5.3%). In general, the level of SCR8–20 binding was clearly higher than that of fH. This may indicate that the binding site for Hic is more exposed on the SCR8–20 construct than on fH. In support of this assumption, factor H has recently been suggested to have a convoluted tertiary structure (46).

Binding of ^{125}I -Hic to fH and its recombinant deletion mutants

Because the Hic-expressing bacteria bound fH and SCR8–20, we next analyzed the direct binding of recombinantly expressed Hic to fH and its recombinant deletion mutants. The binding of ^{125}I -Hic-GST to fH deletion mutants was studied by radioligand blotting. Nitrocellulose membrane-bound fH constructs were incubated with an overlay of radiolabeled Hic-GST (8 μg). After exposure to an x-ray film, bands in the SCR8–11 and 8–20 fragment-containing lanes were found to have accumulated radioactive Hic-GST (Fig. 2). No binding was seen to the lanes containing fH constructs SCR1–6, 1–7, or 15–20. In a reverse experiment, Hic-GST was run into a gel, transferred to the nitrocellulose membrane, and incubated with radiolabeled deletion mutants of fH. A clear binding reaction could be seen with SCR8–20 but not with SCR1–6, 1–7, or 15–20 (data not shown). Due to a limited amount of material, SCR8–11 was not tested in this setup.

Further analyses were performed with a microtiter-well radioligand binding assay. Factor H constructs SCR1–6, 1–7, 8–11,

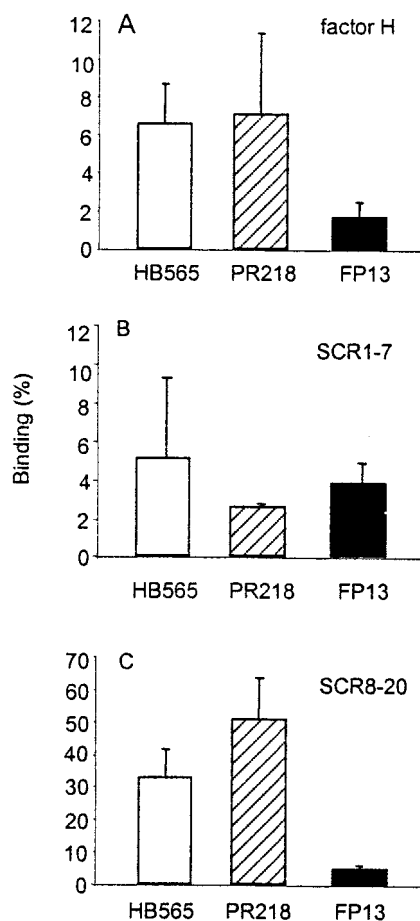


FIGURE 1. The binding of factor H, SCR1–7, and SCR8–20 to three pneumococcal strains. Three strains (2×10^{10} cells/ml, 4×10^8 cells/tube), HB565 (encapsulated, Hic⁺), PR218 (unencapsulated, Hic⁺), and FP13 (unencapsulated, Hic⁻) were incubated with ¹²⁵I-fH, -SCR1–7, or -SCR8–20 (7, 20, and 30 ng/reaction, respectively) for 30 min and spun through 20% sucrose. The binding is expressed as the percentage of radioactivity sedimenting to the pellet. The binding of fH and SCR8–20 is stronger to the Hic-positive strains than to the Hic-negative FP13 strain. No such difference is observed in the binding of SCR1–7, which remains clearly weaker than that of SCR8–20. Note the different scale in the SCR8–20 binding figure.

8–20, and 15–20 were coated on microtiter plate wells. Wells coated with BSA were analyzed as controls. After washing, the wells were incubated with radioactive Hic-GST (75 ng/well). Hic-GST showed a strong binding to SCR8–11 (average 43.4%) and 8–20 (42.8%) (Fig. 3A). The amount of Hic-GST that bound to other fH deletion mutants varied from 1.6% to 3.2%, which was in the same range as binding to BSA, which served as a negative control. When radiolabeled Hic without GST was used, a similar result was obtained, except that the binding percentages with SCR8–11 and 8–20 were 27% and 24%, respectively (data not shown). This decrease compared with Hic-GST may be due to the partial fragmentation of Hic during the GST removal process.

To test whether the binding of Hic to SCR8–20 is dose dependent, microtiter plate wells were coated with SCR8–20 (10 μ g/ml) or BSA (10 μ g/ml) as a control and incubated with varying amounts of ¹²⁵I-Hic (0.6–60 ng/well; 1,000–100,000 cpm). As shown in Fig. 3B, the binding to SCR8–20 was dependent on the dose of ¹²⁵I-Hic input.

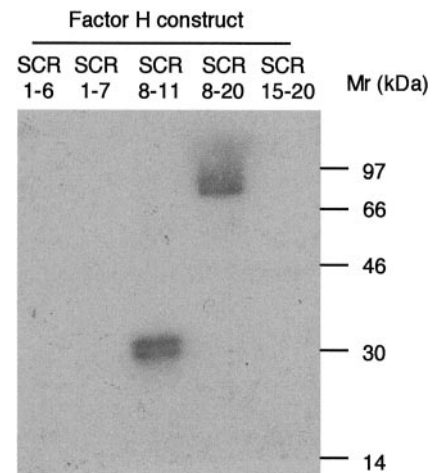


FIGURE 2. Radioligand blotting analysis binding of ¹²⁵I-Hic-GST to fH constructs. Recombinant fH constructs SCR1–6, 1–7, 8–11, 8–20, and 15–20 (390–580 ng protein/lane) were run on an SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was incubated with ¹²⁵I-Hic-GST (8 μ g) and washed, and binding was detected by exposure on an x-ray film. Binding of radioactive Hic-GST could be seen to fH fragments SCR8–11 and SCR8–20 but not to the other proteins tested.

Surface plasmon resonance analysis of Hic-fH interactions

The surface plasmon resonance technique provides direct and dynamic information on binding interactions between two proteins without a need to label either of them. The binding interactions between Hic and fH were analyzed by using the surface plasmon resonance assay and the different recombinant constructs of fH. First, we tested the binding of fH to flow cells coupled with Hic-GST, to GST alone, and to the control flow cell with no coupled protein. As shown in Fig. 4A, fH bound to Hic-GST but not to GST or to the control flow cell. When the recombinant N-terminal SCR1–7 and the C-terminal SCR8–20 constructs were tested, only SCR8–20 bound to Hic-GST (Fig. 4B). To further define the binding site, smaller fragments of fH were tested. As in the radioligand binding assay, SCR8–11 bound to Hic-GST (Fig. 4C), but SCR15–20 did not (data not shown). The same results were obtained when binding was tested to a flow cell coated with Hic without GST.

Competition experiments with the radioligand assay

Because of difficulties in expressing the SCR12–14 construct, we could not directly exclude a possible additional binding site in this region. To study whether there are Hic binding sites outside the SCR8–11 region, e.g., on SCRs 12–14, we tested whether the binding of Hic to SCR8–20 is inhibited by SCR8–11 and vice versa. As can be seen from a radioligand binding assay shown in Fig. 5, the addition of SCR8–11 inhibited the binding of Hic-GST to SCR8–20 by 95%. In the reverse experiment, SCR8–20 also inhibited the binding of Hic-GST to SCR8–11 by 89% (Fig. 5). These results, together with the lack of SCR1–7 binding to Hic, suggest that there are no major additional binding sites for Hic outside the SCR8–11 region on fH.

The effect of heparin on the interaction between Hic-GST and the SCR8–11 and 8–20 constructs was examined by incubating increasing amounts of heparin (0, 30, and 300 IU/ml) with ¹²⁵I-Hic-GST in the radioligand binding assay. Heparin had little (SCR8–20) or no (SCR8–11) inhibitory effect (data not shown) on the binding of Hic-GST to the two constructs, which is in accordance with the fact that there are no known binding sites for heparin in the SCR8–11 region.

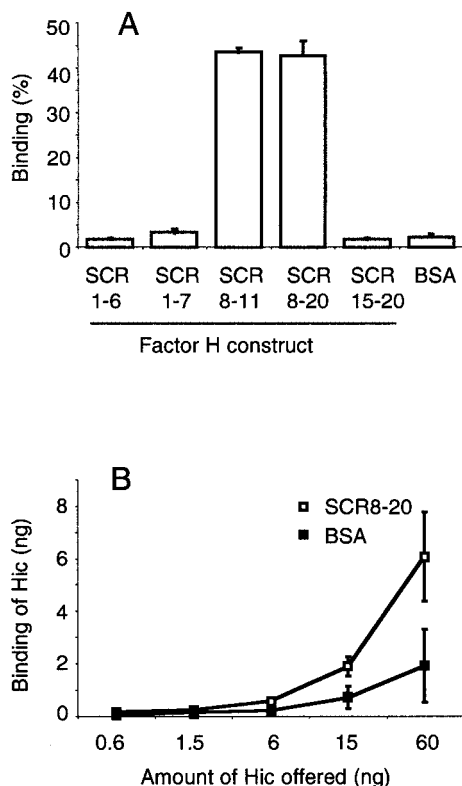


FIGURE 3. Binding of ¹²⁵I-Hic-GST to fH constructs coated on a microtiter plate. *A*, Factor H constructs SCR1–6, 1–7, 8–11, 8–20, and 15–20 or BSA (control) were coated (at 10 μg/ml) on a microtiter-well plate. After incubation with radiolabeled Hic-GST (75 ng/well) and washing, the binding of Hic-GST was determined as the percentage of well-bound radioactivity offered. The binding of Hic-GST is markedly stronger to SCR8–11 and 8–20 than to the other constructs. *B*, Dose-response effect of Hic binding to SCR8–20. SCR8–20 and BSA were coated (10 μg/ml) on a microtiter plate. Varying amounts of ¹²⁵I-Hic (0.6–60 ng/well) were added and binding was determined as described above.

To see whether the Hic binding site on fH would be recognized by any mAbs, we screened different mAbs against fH. We found that the mAb 131X binds to SCR8–11 but does not interfere with the binding of Hic-GST to SCR8–11 or 8–20 (data not shown). Also, no inhibitory effect was seen with the control mAb 196X, which recognizes SCR1.

Effect of CRP on the Hic-fH interaction

As CRP binds to several pneumococcal strains and to the SCR8–11 region of fH (35), we examined whether CRP interferes with the binding of Hic to fH. In a competition assay, CRP (0–300 μg/ml) was incubated with radiolabeled Hic-GST in wells coated with SCR8–11. CRP had no apparent effect on the binding of ¹²⁵I-Hic-GST to the fH construct (data not shown).

Because CRP bound directly to all the three pneumococcal strains studied (data not shown), we hypothesized that CRP might affect the binding of fH to the pneumococcal surfaces. We examined this by a direct binding experiment, in which varying amounts of CRP (0–100 μg/ml) were mixed with radiolabeled SCR8–20 (30 ng) in the presence of either calcium (1 mM) or EDTA (10 mM) and incubated with the unencapsulated PR218 (Hic⁺) and FP13 (Hic⁻) strains. No significant enhancing effect of CRP on the binding of SCR8–20 to the Hic-positive PR218 strain was seen (Fig. 6A). However, CRP dose dependently enhanced the binding of SCR8–20 to the Hic-negative FP13 pneumococcus (Fig. 6B).

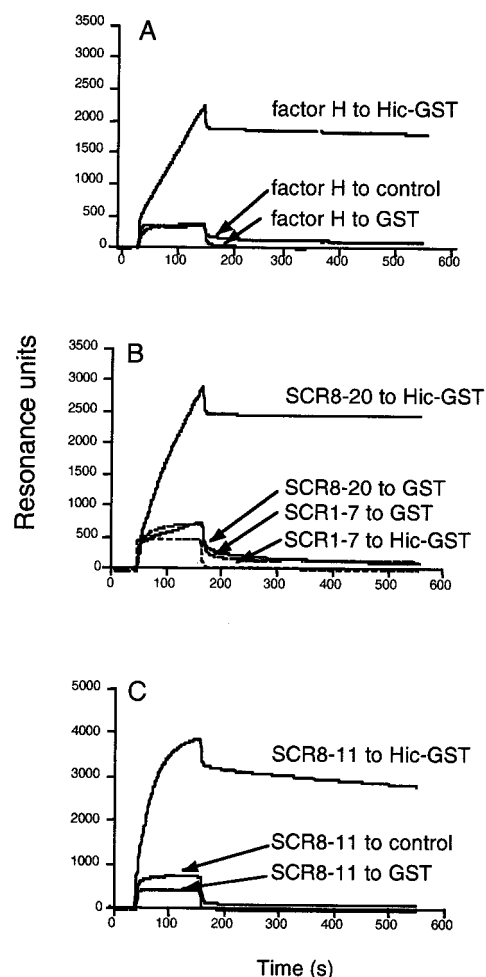


FIGURE 4. Surface plasmon resonance analysis of Hic-fH interactions. In the Biacore experiments, Hic-GST was coupled to the chip. GST coupled alone and a flow cell with no proteins were used as controls. *A*, Binding of fH to Hic-GST. fH showed a rapid association with and slow dissociation from Hic-GST on the chip. No or negligible binding to the GST-coupled or to the control flow cell was seen. *B*, Binding of SCR1–7 and 8–20 to Hic-GST. The binding curve of SCR8–20 resembled the fH binding curve. There is no binding when the SCR1–7 fragment is injected. Neither of the constructs bound to GST alone. Also, no binding to the control flow cell was observed (data not shown). *C*, Binding of SCR8–11 to Hic-GST. SCR8–11 represents the smallest construct of fH that bound to Hic. The association of SCR8–11 to Hic is faster than that of fH, whereas no dissociation is observed. Again, no binding is seen to GST or the control flow cell.

The binding was calcium dependent because it was inhibited by EDTA. No inhibition of the CRP-SCR8–20 interaction was seen with phosphorylcholine (data not shown). These results indicated that CRP can act as a link between Hic-negative pneumococci and fH but that it does not act synergistically with Hic.

Effect of Hic-fH interaction on C3b inactivation

The ability of pneumococci to bind fH from the fluid phase and use it as a cofactor for factor I in the cleavage of C3b was tested with a cofactor assay. Pneumococci were incubated with varying amounts (100 ng/ml–3 μg/ml) of fH, washed four times, and incubated with factor I (20 μg/ml) and radiolabeled C3b (200,000 cpm/tube). The samples were separated by SDS-PAGE gel and the cleavage of ¹²⁵I-C3b was visualized by autoradiography. Both Hic-positive strains, encapsulated HB565 (data not shown) and

The accumulation of C3 and its cleavage fragments expressing the C3c epitope (C3b plus iC3b plus C3c) on the pneumococci was detected by an ELISA using a polyclonal anti-C3c Ab. Generation of iC3b was monitored with a mouse mAb. From the results, the amount of C3b cleaved on the surface was calculated as a ratio of iC3b:C3. The ratio was higher (0.83) for the Hic-positive strain than for the Hic-negative strain (0.37) ($p < 0.05$; Student's *t* test) (Fig. 8). Thus, C3b was more efficiently cleaved to inactive iC3b on the Hic-positive than on the Hic-negative pneumococcal surface.

Effect of Hic on phagocytosis of pneumococci

Finally, we analyzed whether the expression of Hic and differences in C3b degradation were reflected in the efficiency with which the pneumococci became phagocytosed. FITC-labeled Hic-positive (PR218) and Hic-negative (FP13) unencapsulated pneumococci (5×10^6 /cells) were incubated with 0–50% NHS and MgEGTA for opsonization. Freshly isolated polymorphonuclear leukocytes (2.5×10^{10} cells) were added to allow phagocytosis. After washing, the amounts of phagocytosed pneumococci were determined by FACS analysis. As can be seen in Fig. 9, Hic-negative pneumococci became more readily phagocytosed at all three concentrations of NHS-MgEGTA compared with Hic-positive pneumococci.

Discussion

In the present study, we have shown that serotype 3 pneumococci use the Hic protein for binding to the middle region of the C inhibitor factor H. Thereby, pneumococci can protect themselves against C attack and opsonophagocytosis. The binding site of the pneumococcal PspC family member Hic in SCRs 8–11 of fH represents a novel binding region on fH for microbes. The fact that the acute phase protein CRP also binds to the same region establishes this part of factor H as a physiologically and pathophysiologically relevant site. For pneumococci, Hic and probably other PspC proteins (12) as well are important virulence factors that are expressed on the bacterial surface and extend through the cell wall.

Pneumococci are a significant cause of upper and lower respiratory tract infections and of systemic infections, including bacteremia and meningitis (1). Despite the development of antibiotics and capsular polysaccharide vaccines, pneumococci have re-

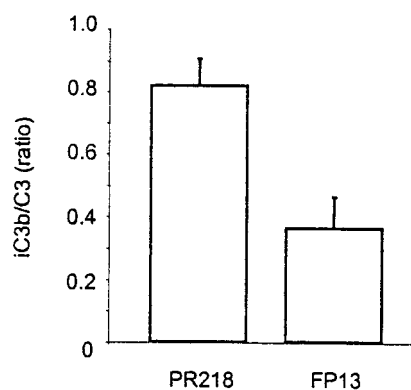


FIGURE 8. Analysis of C3b inactivation (iC3b:C3 ratio) in serum by ELISA. Hic-positive (PR218) and Hic-negative (FP13) pneumococci (9×10^8 cells/reaction) were incubated (10 min) with 10% NHS in the presence of MgEGTA to allow the activation of the AP. The relative amounts of iC3b and C3c were measured by a whole-cell ELISA. The ratio iC3b:C3c reflects the degree of C3b inactivation. The ratio (i.e., formation of iC3b) is higher with the Hic-positive strain PR218 than with the Hic-negative strain FP13 ($p < 0.05$; Student's *t* test).

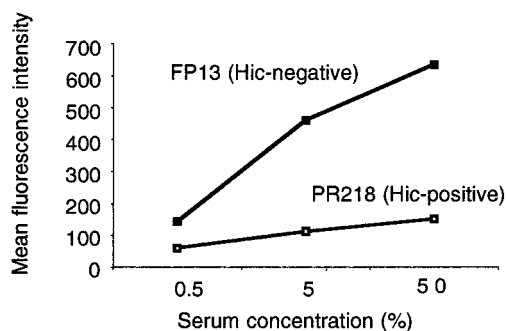


FIGURE 9. Phagocytosis of the Hic-positive and -negative pneumococci. FITC-labeled Hic-positive (PR218) and Hic-negative (FP13) pneumococci were incubated (10 min, 37°C) with fresh NHS (0.5, 5, and 50%) in the presence of MgEGTA to allow opsonization via the alternative pathway. Subsequently, the pneumococci (5×10^6 cells) were mixed with PMNs (2×10^5 cells) and incubated at 37°C for 12 min. After washing, the cells were analyzed by flow cytometry. The figure shows the mean fluorescence intensities of PMNs after phagocytosis of fluorescently labeled Hic-positive and Hic-negative pneumococci.

mained an important cause of disease and death. The factors that make one pneumococcal strain virulent and another less virulent have not all been clearly defined. The virulence mechanisms have been a target for vigorous research for several decades. It has been observed that different strains of pneumococci exploit different mechanisms to avoid host immune defense. The capsule is required for virulence (inhibition of phagocytosis and suppression of immune responses) (47, 48), but other factors, like pneumolysin (7) and PspA (5, 6), probably also play important roles.

We focused our study on serotype 3 pneumococcus, which is among the most frequently isolated strains in clinical samples (8, 9) and known to be resistant to phagocytosis (10). In accordance with an earlier study (11), we found that the PspC-related surface protein Hic bound C factor H. As a consequence, Hic promoted the cleavage of C3b by factor I. Accelerated breakdown of C3b on the pneumococcal surface impaired opsonization and suppressed phagocytosis by polymorphonuclear leukocytes.

The binding site for Hic was located to SCRs 8–11 of fH. Even though another binding site on SCRs 12–20 cannot be totally excluded, it seems unlikely according to the inhibition experiment in which the addition of SCR8–11 almost completely abolished the binding of Hic-GST to SCR8–20 (Fig. 5). Heparin did not influence binding of Hic to SCR8–11 but slightly inhibited binding of SCR8–20. This suggests that the polyanion binding sites on SCR13 or on SCR20 could have a minor contributing role in the binding of fH to Hic. Other microbial binding sites on fH have been located to SCR7 (streptococcal M-protein) (17), SCRs 19–20 (OspE of *Borrelia burgdorferi* sensu stricto) (19), and SCRs 16–20 for neisserial lipo-oligosaccharides (23). Because the co-factor and decay accelerating activities of fH in SCRs 1–4 are situated distant from SCRs 8–11, it was envisaged that the Hic-bound fH retained its functional activity when bound to the pneumococcal surface. This was directly proven in experiments in which fH bound to Hic on the pneumococcal surface promoted factor I-mediated C3b inactivation. The C3b-binding sites on fH are probably also outside the SCR8–11 region. One of the three C3b recognition sites has been located to the neighboring SCRs 12–14 (27), but yet an additional site in the region SCR6–10 (26) has not been excluded. Distinct binding sites for Hic and C3b on factor H thus allow an effective recruitment of fH by pneumococci when initial C3b deposition has occurred.

So far, the only identified function for SCRs 8–11 of fH has been the interaction with CRP (35). However, in our experiments CRP did not affect the binding of Hic to fH, suggesting that CRP and Hic have distinct binding sites within SCRs 8–11. Also, the fact that the binding of CRP to fH was calcium dependent, whereas the binding of Hic was not, suggests that the two interactions are of a different nature. Furthermore, because CRP also has a binding site in SCR7, the binding site on the SCR8–11 region may be a supplementary or secondary interaction site for CRP.

On the basis of the current and previous results, pneumococci could use either CRP or Hic to evade opsonophagocytosis. As can be seen in Fig. 6, CRP dose dependently increased the binding of fH SCR8–20 to Hic-negative but not to the Hic-positive pneumococci. It has been described earlier that the presence of CRP leads to an increased binding of fH to C3b on the pneumococcal surface (38, 49). The binding of CRP to the pneumococcal C-polysaccharide leads to classical pathway activation (37) and to the binding of fH directly to CRP. This leads, first, to C component deposition on pneumococci and, subsequently, to accelerated inactivation of C3b. Although the host can thus use CRP for defense against pneumococci, the pneumococci could also, by limiting the number of deposited C3 activation products, use CRP for its own evasion. However, although CRP and Hic both independently increased fH binding, no synergistic effect of the two factors on binding of fH to pneumococcus was observed. This may be due to spatial constraints that restrict the binding of the SCR8–11 region of fH simultaneously to Hic and CRP. On the basis of the current results, it thus appears that pneumococci primarily use Hic (or PspC) for their protection against C, but in cases where Hic is absent, CRP may enhance fH binding to the pneumococcal surface.

Hic is structurally closely related to the PspC family (also called choline binding protein A and secretory IgA binding protein). There is significant homology between Hic and other PspCs within N-terminal amino acids 1–60 and the C-terminal aa 170–612. The fH-binding region is located within aa 39–261 (11). At the C terminus, Hic is anchored to the pneumococcal cell wall by an LPSTG motif, which is similar to the streptococcal M protein cell wall binding motif. Because of the homology and previously observed binding of fH to PspC (12), it appears likely that fH also binds to other members of the PspC family.

Factor H is principally produced by liver hepatocytes, and the average concentration of fH in human plasma is ~500 µg/ml. In tissues, like in the alveolar spaces in lungs, the amount is presumably lower. However, the Hic-positive pneumococci bound fH even at a concentration of 10 ng/ml. This was sufficient for cofactor activity in factor I-mediated C3b cleavage (Fig. 7). The degradation pattern of C3b bound to serotype 3 pneumococci was described already 15 years ago (10). Serotype 3 pneumococci were found to promote C3b cleavage more efficiently than, for example, serotype 6 and 14 pneumococci. When type 3 pneumococci were incubated in nonimmune agammaglobulinemic serum, C3b became degraded in a pattern that typically occurs by factors I and H. This suggests that serotype 3 pneumococci can use fH for protection also at sites where the fH levels are low, like within the lungs or on meningeal membranes.

Surface-bound fH inhibits the formation of the AP C3 convertase C3bBb and thereby the net amount of C3b produced. By binding fH, pneumococci have less C3b deposited on their surface and, furthermore, most of this C3b quickly becomes cleaved to inactive iC3b. Our results show that the expression of Hic increases the ratio of iC3b:C3b on the pneumococcal surface (Fig. 8). This leads to somewhat reduced phagocytosis by PMNs (Fig. 9). Because of their C resistance, pneumococci can persist for prolonged periods in the body. Thereby, pneumococci remain immunogenic in their

hosts. The C3d end product of C3b cleavage may remain covalently bound on the pneumococcal Ags and promote the development of B and T cell-mediated immune responses. It is known that C3d is a powerful endogenous adjuvant recognized by the CR2 receptor on B lymphocytes (50). The importance of C3b opsonization in the defense against pneumococci can be observed in C3-deficient patients (51). These patients are susceptible to pneumococcal infections and are incapable of producing an effective Ab response.

The properties of Hic and other PspC group surface proteins suggest that they could be good protein vaccine candidates against pneumococcal infections. As proteins, the members of the PspC family would be more efficient in triggering Ab and cell-mediated immunity than the capsular polysaccharide. This group of proteins is expressed by several pneumococcal serotypes that are significant in causing disease. The fundamental basis of a PspC vaccine would be that Abs against PspC proteins could directly neutralize a virulence mechanism. The combination of the PspC-proteins to the pneumococcal polysaccharides would have the additional benefit that both components originate from the same bacterial species.

In conclusion, our findings locate the binding site of the pneumococcal surface protein Hic to SCR domains 8–11 in the central part of the C inhibitor fH. This is a novel microbial binding site on fH. The SCR8–11 region of fH has earlier been found to also bind the acute phase protein CRP. However, the binding sites on fH for CRP and Hic are not identical, because CRP and Hic do not compete for binding to fH and CRP requires calcium for binding. As a functional consequence, the binding of fH to Hic leads to reduced C activation on the pneumococcal surface and diminished opsonophagocytosis. The data suggest that Hic is an important virulence factor for type 3 pneumococci.

Acknowledgments

We thank Monica Schoultz for the FACS analysis, Marjatta Ahonen for skillful technical assistance, and Dr. Ilkka Seppälä (Haartman Institute) for providing the GST. The pneumococcal strains used in this study were kindly provided by Dr. Gianni Pozzi (University of Siena, Italy).

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