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# The Human Complement Regulator Factor H Binds Pneumococcal Surface Protein PspC via Short Consensus Repeats 13 to 15

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**The innate ability of *Streptococcus pneumoniae* to resist complement activation and complement-mediated phagocytosis may be a direct consequence of the ability of the bacteria to bind components of the complement regulatory system. One such component, factor H (fH), is a crucial fluid-phase negative regulator of the alternative pathway of complement and is utilized by a number of pathogenic organisms to resist complement attack. The pneumococcal surface protein C (PspC [also known as CbpA] and SpsA) has been shown to bind fH, although the exact binding site within one or more of the 20 short consensus repeats (SCRs) of the molecule is not known. The purpose of the current study was to map specific SCRs on fH responsible for this binding. Initial experiments utilizing type 2 pneumococcal strain D39 and its isogenic PspC-negative derivative (D39/*pspC* mutant) showed that fH binding was PspC dependent. A purified recombinant protein derivative of PspC that lacked the proline-rich region (PspCΔPro) had a reduced binding efficiency for fH, thereby directly showing the importance of this region for the fH interaction. We have specifically shown by inhibition experiments that SCRs responsible for heparin and C3b binding of fH are not involved in binding PspC and the interaction between fH and PspC is largely hydrophobic, since no inhibition was observed in the presence of high concentrations of NaCl. Construction of SCR proteins encompassing the whole fH molecule showed that SCRs 8 to 15 (SCR 8-15) mediated binding to PspC. Further localization experiments revealed that SCR 13 and SCR 15 were required for full binding, although partial binding was retained when either SCR was removed.**

*Streptococcus pneumoniae* (the pneumococcus) is a major cause of a range of human diseases including pneumonia, bacteremia, meningitis, otitis media, and sinusitis. Although the capsule has long been recognized as the major virulence determinant, several proteins, including pneumolysin (Ply) (28), pneumococcal surface adhesin A (PspA) (23, 24, 37), PspC (also known as CbpA, SpsA) (26, 31), Hic (18, 19), *S. pneumoniae* histidine protein A (PhpA) (40), and pneumococcal surface antigen A (PsaA) (36) have demonstrated importance in either virulence or evasion of host defense mechanisms.

PspC is structurally related to PspA and is a member of the choline-binding family of pneumococcal proteins, which are comprised of an N-terminal α-helical domain, a proline-rich domain, and choline-binding regions (7). PspC holds promise as a potential vaccine candidate against *S. pneumoniae*, given that the *pspC* gene is detectable in 75% of all isolates and immunization of mice with a purified PspC fragment comprising 1 to 455 amino acids from the type 2 strain D39 generated cross-reactive antibodies against PspA, and protects against sepsis from a heterologous type 3 strain expressing PspA but not PspC (7). More recently, immunization of mice with an identical PspC fragment was shown to protect against challenge with a thousand times the 50% lethal dose of *S. pneumoniae* D39, and the protection conferred was stronger than that observed for mice immunized with a recombinant derivative of Ply (26).

PspC has been shown to bind cytokine-activated lung cells (31), complement component C3 (34), the secretory component of human secretory immunoglobulin A (IgA) via a hexapeptide motif located in the N-terminal region (14, 15) and a human polymeric immunoglobulin receptor that facilitates mucosal invasion (39). More recently, PspC has been shown to bind the complement regulatory protein factor H (9).

Factor H (fH) is a 150-kDa serum protein that is critical for negatively regulating the alternative pathway of complement to prevent inappropriate complement activation on host cells, while allowing activation to proceed on the surfaces of invading microorganisms, resulting in opsonophagocytosis. fH is composed of 20 repetitive domains termed short consensus repeats (SCRs) composed of ~60 amino acids each, with distinct SCRs responsible for the differing functions attributable to fH. Sites on fH specific for cofactor activity and decay acceleration are located in SCRs 1 to 4 (SCR 1-4) (12, 21, 22) and those specific for mediating fH binding to C3b have been localized to three parts of the molecule, namely, SCR 1-5, SCR 6-10, or SCR 12-14 and SCR 19-20 (20, 33). Several sialic acid-binding sites on fH have been mapped by examining the binding of the polyanion heparin to SCR 7, SCR 20, and possibly in or around SCR 13 (4, 5, 27).

Several pathogenic microorganisms have exploited the properties of fH to prevent complement activation on their surfaces, thereby conferring serum resistance. Bacterial surface

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proteins known to bind fH include *S. pyogenes* M protein (3, 11), group B streptococcus β protein (1), Hic from type 3 *S. pneumoniae* (17, 18), YadA from *Yersinia enterocolitica* (8), OspE of *Borrelia burgdorferi* (16), and loop 5 of Por1A from *Neisseria gonorrhoeae* (29). Sialylated *N. gonorrhoeae* strains are also able to bind fH via a sialic acid-binding site between SCR 16-20 (30).

The purpose of the present study was to identify the key SCRs responsible for mediating the interaction between fH and purified recombinant PspC derivatives or D39 pneumococci and an isogenic mutant deficient in PspC. Our results now establish a new region on fH between SCR 13-15 that is utilized by a pathogenic bacterium to evade host immune responses.

## MATERIALS AND METHODS

**Bacterial strains, growth conditions, and plasmids.** The virulent *S. pneumoniae* type 2 strain D39 (NCTC 7466) and the erythromycin-resistant isogenic PspC insertion-duplication mutant strain D39/*pspC* mutant (2) were routinely grown on blood agar at 37°C in 5% $CO_2$ with 0.2 μg of erythromycin/ml (for the mutant strain). For assays, the strains were grown overnight on blood agar and then subcultured in Todd-Hewitt broth containing 0.5% yeast extract (THY) to the mid-log phase of growth (optical density at 600 nm [$OD_{600}$] = 0.3) at 37°C in 5% $CO_2$. *Escherichia coli* DH5α (Bethesda Research Laboratories, Gaithersburg, Md.) harboring the various plasmid constructs was propagated in low-salt Luria-Bertani broth containing 25 μg of Zeocin (Invitrogen, Carlsbad, Calif.)/ml, with aeration at 37°C.

**Proteins.** The PCR amplification, cloning, expression, and purification of PspC comprising amino acids 1 to 445 of the mature PspC polypeptide, which lacks the choline-binding region but retains the α-helical and proline-rich regions, has been previously described (26). A second PspC derivative comprising amino acids 1 to 371 of the polypeptide, which is identical to PspC, except that it lacks the proline-rich region (denoted PspCΔPro), was similarly prepared except that construction utilized the downstream primer AD8 (5′-TGGTTTTTCTTAAGCTTATCTTCTTCTGC-3′), which incorporates a *Hin*dIII site (underlined) (26).

fH was purified from plasma as previously described (13) and then subjected to an immunoaffinity chromatography step with goat anti-fH as the coupling ligand. To confirm the fH remained functionally active, factor I-mediated cofactor assays were performed with C3b.

**Construction of recombinant fH proteins.** The recombinant SCR constructs comprising SCR 1-7, SCR 8-15, SCR 16-20, SCR 8-10, SCR 8-12, SCR 8-13, SCR 8-14, and SCR 8-15Δ13 of fH were constructed by PCR and cloned into the yeast expression vector pPICZαA (Invitrogen), downstream of the AOX1 methanol-inducible promoter. PCRs, cloning, and transformation into *E. coli* were carried out according to standard protocols (32). Construction of SCR 1-7 utilized the primers H1For (5′-GTCCGAATTCGAAGATTGCAATGAACTTCCTC-3′) and H7Rev (5′-GGTCTAGACA CGGATGCATCTGGGA-3′), which incorporates *Eco*RI and *Xba*I sites, respectively (underlined). SCR 8-15 was PCR amplified with H8For 5′-GTCGGTACCAAAACATG TTCCAAATCAAG-3′ and H15Rev 5′-GGTCTAGAGCCTTCACACTGAGGTGGAG-3′ incorporating *Kpn*I and *Xba*I sites, respectively (underlined). SCR 16 to 20 utilized H16For (5′-G GGAATTCGGCCTTCCTTGTAAATCTCC-3′) and H20Rev (5′-GGTCTAGATCTTTTTGCACAAGTTGGAAC-3′) primers incorporating *Eco*RI and *Xba*I sites, respectively (underlined). SCR 8-12, SCR 8-13, and SCR 8-14 were constructed by using H8For and H12Rev (5′-GGTCTAGATCACACACAC ACT GGGGAAGTTG-3′), H13Rev (5′-GGTCTAGATCATGAGCAGTTCA CTTCTGGATC-3′), and H14Rev (5′-GGTCTAGATCAAACACAGAGTGGT ATTGACTG-3′), all of which contain *Xba*I sites (underlined). SCR 8-15Δ13 was constructed by utilizing an SCR 1-20Δ13 template DNA (5) with amplification carried out by using H8For and H15Rev primers. All PCR-amplified SCR constructs were digested with the appropriate restriction enzymes, cloned into the multiple cloning site of pPICZαA, and transformed into *E. coli* DH5α.

**Expression in *Pichia pastoris*.** A sample of up to 10 μg of *Sac*I digested pPICZαA DNA containing the various SCR constructs was electroporated into *P. pastoris* strain X33, with transformants selected on 100 μg of Zeocin/ml. Expression of recombinant proteins was induced for 2 to 4 days in the presence of 2% methanol according to the manufacturer's instructions (Invitrogen).

**Purification of recombinant proteins.** SCR proteins expressed by using the *P. pastoris* system were purified by immunoaffinity chromatography on CNBr-activated Sepharose (Amersham Pharmacia Biotech, Amersham, United Kingdom) coupled with sheep anti-human fH IgG prepared by caprylic acid precipitation (35). Briefly, 100 to 200 ml of *P. pastoris* supernatant was passed over 5-ml affinity columns several times prior to extensive washing in 50 mM phosphate buffer and elution with 3 M potassium thiocyanate. Eluates were immediately dialyzed against phosphate buffer, and the concentration of proteins was determined against a fH standard curve generated by enzyme-linked immunosorbent assay (ELISA).

**PspC-fH binding ELISA.** A PspC-binding ELISA was performed by coating 0.5 μg of purified PspC or PspCΔPro in the presence of 100 mM bicarbonate buffer (pH 9.5) overnight at 4°C on Maxisorb ELISA trays (Nunc, Roskilde, Denmark). The samples were blocked with 5% (wt/vol) skim milk in phosphate-buffered saline (PBS) for 1 h at room temperature (RT) and washed three times in phosphate buffer. Samples (100 μl) of either fH or the recombinant fH constructs (5 μg/ml in phosphate buffer) were added and incubated at RT for 1 h. Samples were washed then incubated with goat anti-fH (Calbiochem, San Diego, Calif.; 1:2,000 [vol/vol]) in 1% skim milk-PBS for 1 h at RT. Samples were washed and incubated with horseradish peroxidase (HRP)-conjugated donkey anti-goat IgG (Silenus, Melbourne, Australia) added at 1:2,000 (vol/vol) in 1% skim milk-PBS for 1 h at RT. After a washing step, Sigma Fast OPD substrate (Sigma, St. Louis, Mo.) was added, and the $OD_{490}$ was determined. Experiments were repeated three times in triplicate and mean and standard deviations were determined.

A whole-cell ELISA utilizing D39 and D39/*pspC* mutant was performed essentially as previously described (40). Mid-log-phase pneumococcal strains were washed in PBS, resuspended to an $OD_{550}$ of 1.0, and 50 μl was added per well to a Maxisorb ELISA plate. The bacteria were dried onto the plate overnight at RT, and the ELISA was performed as described above, except that SCR proteins were used at a concentration of 10 μg/ml. Experiments were repeated three times in triplicate, and the mean and standard deviations were determined.

**Western blot analysis.** Recombinant fH proteins or whole-cell pneumococcal lysates prepared by growing the bacteria in THY to an $OD_{600}$ of 0.3 (i.e., ~$10^9$ CFU/ml) and 1 ml of pelleted bacteria resuspended in 40 μl of lysis buffer were separated by sodium dodecyl sulfate–12% polyacylamide gel electrophoresis (SDS–12% PAGE) and transferred onto Hybond C+ nitrocellulose (Amersham Pharmacia Biotech). For detection of PspC, mouse anti-PspC polyclonal antibody was used (1:3,000 [vol/vol] dilution), whereas the fH constructs were detected with goat anti-fH antiserum. Antigen-antibody complexes were reacted with the appropriate secondary antibody at a 1:2,000 dilution and visualized by chemiluminescence according to the manufacturer's instructions (Amersham Pharmacia Biotech). Blots were exposed to HyperFilm ECL (Amersham Pharmacia Biotech).

## RESULTS

**Expression of PspC on the pneumococcal cell surface and the interaction with fH.** To initially confirm that the PspC-negative derivative of the virulent type 2 D39 strain (D39/*pspC* mutant) does not express PspC, a Western immunoblot of whole-cell lysates was carried out with polyclonal mouse anti-PspC antiserum. The results clearly demonstrate that the D39/*pspC* mutant strain does not produce detectable amounts of PspC, whereas PspC and PspA were readily detected in the parent strain D39 (Fig. 1A), which is in agreement with previous experiments (2, 26). Since cross-reactivity between PspC antibodies to PspA has been reported previously (7) the antiserum was absorbed extensively against D39/*pspC* mutant to remove such antibodies, which may hinder experiments designed to detect PspC only. Figure 1B shows that no PspA was detected in either D39 or D39/*pspC* mutant after absorption, whereas PspC was only detected in the D39 strain.

The interaction of fH with PspC was examined by ELISA. Previous experiments have established that fH can bind PspC from D39 cell extracts but not PspA (9). Two PspC protein constructs were utilized. The first construct consisted of amino acids 1 to 445, comprising the α-helical region and proline-rich region but lacking the C-terminal choline-binding region and

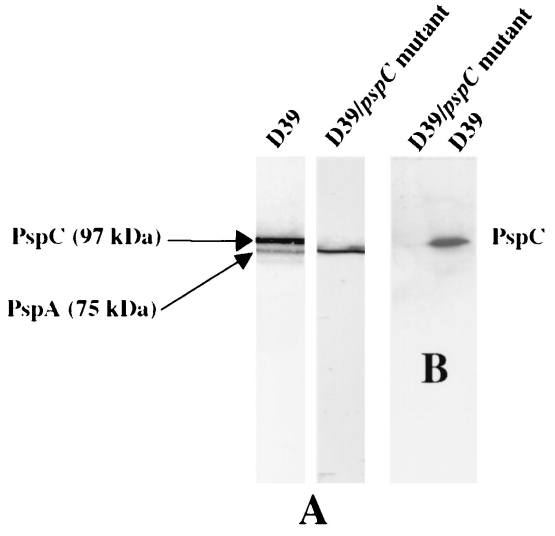


FIG. 1. Western immunoblot analysis of whole-cell lysates from pneumococcal strains D39 and the isogenic insertion-duplication mutant D39/*pspC* mutant. Samples (10 μl) were electrophoresed by SDS–12% PAGE, transferred to nitrocellulose, and blotted with a 1:3,000 dilution of either mouse anti-PspC antiserum (A) or mouse anti-PspC antiserum absorbed extensively across D39/PspC mutant to remove cross-reacting antibodies against PspA (B). Proteins were detected with HRP-conjugated goat anti-mouse IgG (1:2,000) prior to visualization by enhanced chemiluminescence. Protein bands corresponding to PspC (97 kDa) or PspA (75 kDa) are indicated.

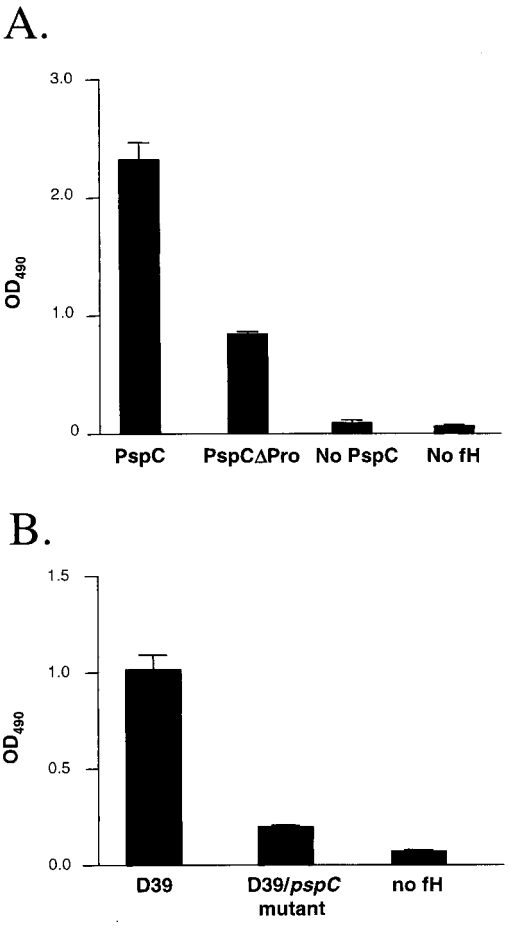


FIG. 2. Binding of fH to purified PspC and PspCΔPro (A) or pneumococcal strains D39 or D39/*pspC* mutant (B) as determined by ELISA. fH (5 μg/ml) was incubated with 0.5 μg of bound PspC, PspCΔPro, D39, or D39/*pspC* mutant dried onto microtiter plates overnight. Binding of fH was detected by the sequential addition of goat anti-fH (1:2,000), HRP-conjugated donkey anti-goat IgG (1:2,000) and substrate prior to reading the $OD_{490}$. PspC and fH controls are also indicated on both the graphs. Experiments were repeated three times in triplicate, with the means and standard deviations indicated.

signal peptide (PspC), and has been described elsewhere (26). The second construct comprised amino acids 1 to 371, which lacks the proline-rich region, the C-terminal choline-binding region, and signal sequence, thus only containing the variable N-terminal α-helical portion of the protein (denoted PspCΔPro). Both the PspC and PspCΔPro proteins readily bound purified fH compared to control wells (Fig. 2A), although the PspC construct, which contains the proline-rich region, bound two to three times more fH than PspCΔPro that lacks this region.

The interaction between fH and PspC on the pneumococcal cell surface was also examined by ELISA. Wells were coated with either D39 or the isogenic D39/*pspC* mutant strain and binding of fH determined. FH bound the D39 parent strain and not the mutant (Fig. 2B), further confirming that the interaction with fH on the D39 cell surface is PspC dependent (9).

**Heparin, C3b, and sodium chloride do not inhibit the fH-PspC interaction.** The effect of heparin on the ability of fH to interact with PspC was examined to determine whether the characterized heparin binding sites on fH within SCR 7, SCR 20, and possibly SCR 13 (3, 4, 27) overlap with the PspC binding site. Purified fH (5 μg/ml) was preincubated with either 100 or 10,000 U of heparin/ml for 30 min at RT, prior to incubation with PspC precoated on an ELISA tray. Even in the presence of 10,000 U of heparin/ml, which has been shown to completely inhibit streptococcal M protein binding to fH (3), the fH-PspC binding interaction was not affected (data not shown). Therefore, the heparin binding sites on fH do not overlap with the PspC binding site.

It was also determined by ELISA whether the C3b binding sites on fH (20, 33) were overlapping with PspC. Samples of fH (5 μg/ml) and purified C3b (a gift from T. S. Jokiranta, Haartman Institute, Helsinki, Finland) were preincubated for 1 h at 37°C at molar ratios of 1:1, 1:10, and 1:50, respectively, and the percent inhibition was calculated versus fH binding to PspC without C3b. Even in the presence of a 50-fold molar excess of C3b, fH retained full binding ability to PspC (Fig. 3). C3b did not interact with PspC directly by ELISA (data not shown). Therefore, the PspC binding site is also distinct from the C3b binding site on fH.

Finally, we also determined the effects of increasing salt concentrations on the fH-PspC interaction to ascertain whether the interactions involve electrostatic or hydrophobic forces. fH (5 μg/ml) was preincubated with increasing concentrations of NaCl of from 50 mM to 1 M at RT for 1 h, and the effect on binding to coated PspC on ELISA trays was examined. The fH-PspC interaction is insensitive to salt concentra-

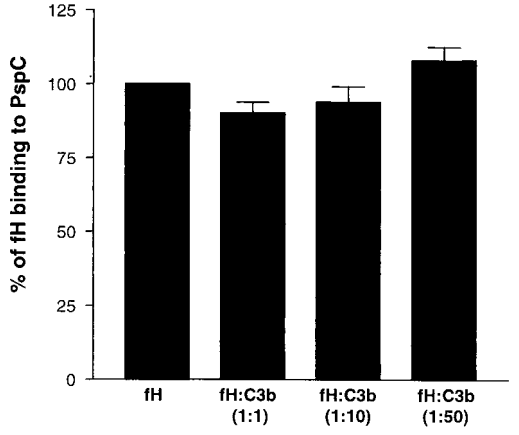


FIG. 3. The C3b and PspC binding sites on fH do not overlap as determined by ELISA. Wells of a microtiter tray were coated with 0.5 μg of PspC and incubated with either fH (5 μg/ml) or fH preincubated at 37°C for 1 h with increasing molar amounts of C3b at a ratio of 1:1, 1:10, or 1:50. Sample $OD_{490}$ readings were expressed as a percentage relative to the fH-PspC interaction without C3b present. fH binding was detected by the sequential addition of goat anti-fH (1:2,000), HRP-conjugated donkey anti-goat IgG (1:2,000) and substrate. Experiments were repeated three times in triplicate, with the means and standard deviations indicated.

tions of up to 1 M, which indicates that fH binding to PspC is likely to be hydrophobic, but not electrostatic, in nature (Fig. 4).

**The PspC binding site on fH resides within SCR 8-15.** To initially localize the PspC binding site on fH, three SCR constructs consisting of SCR 1-7, SCR 8-15, and SCR 16-20 were analyzed for the ability to bind to PspC and PspCΔPro by ELISA. The SCR proteins used in the present study were expressed in *P. pastoris* and immunoaffinity purified with goat

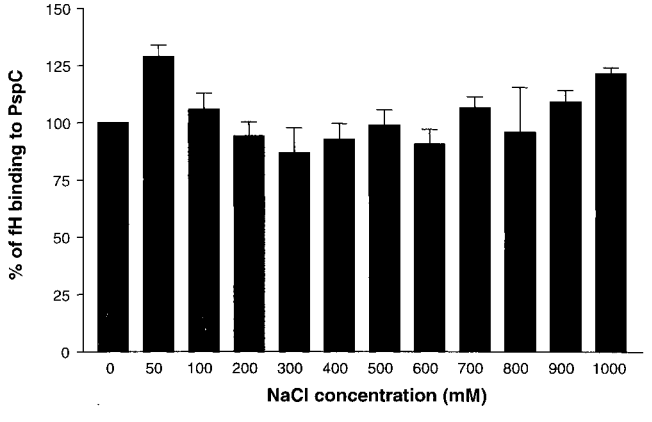


FIG. 4. Effect of increasing NaCl concentrations on the fH-PspC interaction. Microtiter tray wells were coated were coated with 0.5 μg of PspC and incubated with fH (5 μg/ml) in the presence of increasing molar amounts of NaCl from 50 to 1,000 mM. Sample $OD_{490}$ readings were expressed as a percentage of the fH-PspC interaction without NaCl present. fH binding was detected by the sequential addition of goat anti-fH (1:2,000), HRP-conjugated donkey anti-goat IgG (1:2,000), and substrate prior to determining the $OD_{490}$. Experiments were repeated three times in triplicate, with the means and standard deviations indicated.

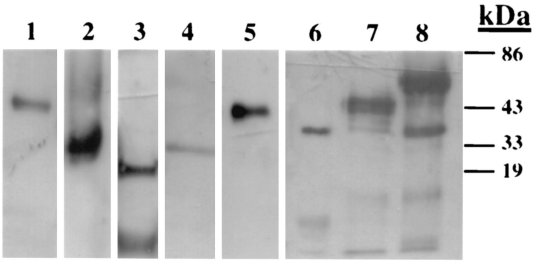


FIG. 5. Western blot analysis of the various fH SCR protein constructs used in the present study. SCR 1-7 (lane 1), SCR 16-20 (lane 2), SCR 8-10 (lane 3), SCR 8-12 (lane 4), SCR 8-15Δ13 (lane 5), SCR 8-13 (lane 6), SCR 8-14 (lane 7), and SCR 8-15 (lane 8) proteins were expressed in *P. pastoris* with secreted proteins immunoaffinity purified across CNBr-activated Sepharose with sheep anti-human fH used as the coupling ligand. A sample of each protein was subjected to SDS–10% PAGE prior to transfer to nitrocellulose and specific detection by using goat anti-human fH (1:2,000), followed by HRP-conjugated donkey anti-goat IgG (1:2,000) and visualization by enhanced chemiluminescence. Molecular mass standards are shown to indicate the relative sizes of each SCR construct.

anti-fH as the coupling ligand. A Western blot of the different proteins used in the present study is shown in Fig. 5. The ELISA data showed that SCR 8-15 bound PspC and to a lesser extent PspCΔPro (Fig. 6), whereas SCR 1-7 and SCR 16-20 did not bind to either of the constructs (Fig. 6). Furthermore, a 10-fold increase in the protein concentration over SCR 8-15 of either SCR 1-7 or SCR 16-20 still resulted in no PspC binding (data not shown). SCR 8-15 bound PspC to a similar degree compared to fH binding, although to a lesser extent to PspCΔPro. Therefore, the PspC binding site resides solely within SCR 8-15 of fH.

**The SCR 13 domain of fH is required for PspC binding.** Construction of distinct SCR proteins within the SCR 8-15 region of fH was utilized to further localize the PspC binding

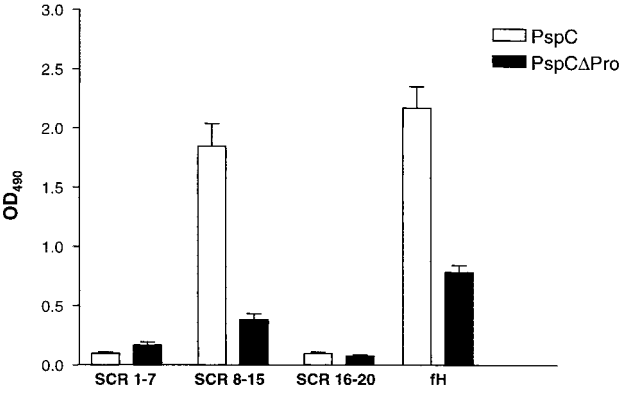


FIG. 6. Determination by ELISA of the region of fH responsible for mediating binding to PspC. fH (5 μg/ml) and three SCR constructs encompassing the entire fH molecule, namely, SCR 1-7, SCR 8-15, and SCR 16-20 (5 μg/ml) were assessed for the ability to bind 0.5 μg of PspC or PspCΔPro immobilized onto a microtiter tray. Binding of SCR containing proteins was detected by using goat anti-fH (1:2,000) then HRP-conjugated donkey anti-goat IgG (1:2,000), followed by the addition of substrate and a reading at $OD_{490}$. Experiments were repeated three times in triplicate, with the means and standard deviations indicated.

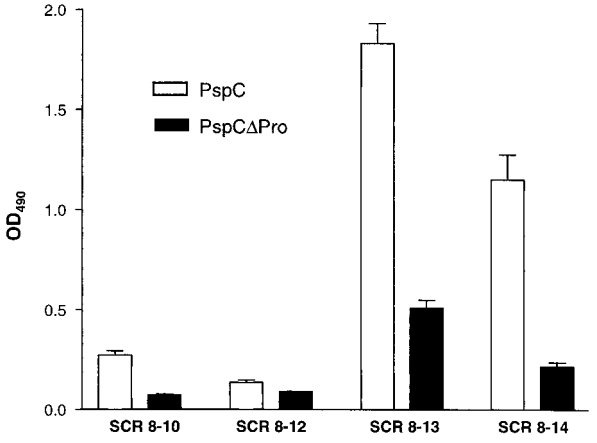


FIG. 7. Localization by ELISA of the PspC binding site within SCR 8-15. SCR 8-10, SCR 8-12, SCR 8-13, and SCR 8-14 proteins (5 μg/ml) were assessed for the ability to bind 0.5 μg of PspC or PspCΔPro immobilized onto a microtiter tray. The binding of SCR-containing proteins was detected by using goat anti-fH (1:2,000) and then HRP-conjugated donkey anti-goat IgG (1:2,000), followed by the addition of substrate and a reading at $OD_{490}$. Experiments were repeated three times in triplicate, with the means and standard deviations indicated.

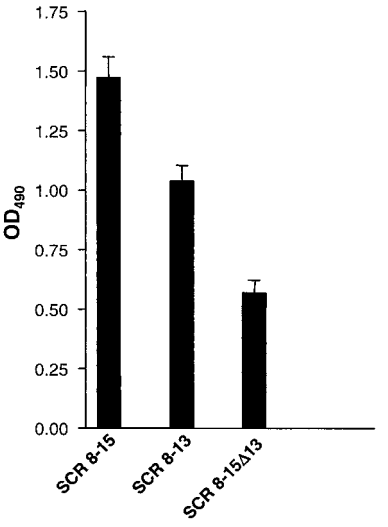


FIG. 8. ELISA analysis of SCR 8-15Δ13 binding to PspC versus SCR 8-15 and SCR 8-13. The various SCR proteins (5 μg/ml) were assessed for the ability to bind 0.5 μg of PspC immobilized on a microtiter tray. The binding of the SCR-containing proteins was detected by using goat anti-fH (1:2,000) and then HRP-conjugated donkey anti-goat IgG (1:2,000), followed by the addition of substrate and a reading at $OD_{490}$. Experiments were repeated three times in triplicate, with the means and standard deviations indicated.

site on fH. The constructs consisted of SCR 8-10, SCR 8-12, SCR 8-13, and SCR 8-14 (Fig. 5) and the purified proteins (5 μg/ml) analyzed for binding to PspC or PspCΔPro by ELISA. SCR 8-10 and SCR 8-12 were incapable of mediating binding to either of the PspC constructs (Fig. 7), even after their concentrations were increased 10-fold in the ELISA (data not shown). In contrast, binding to both PspC and PspCΔPro was observed for SCR 8-13, suggesting that SCR 13 is responsible for this interaction (Fig. 7).

SCR 8-14 also bound PspC and PspCΔPro. However, the binding was approximately two-thirds that of SCR 8-13, which may indicate that the presence of the additional SCR may constrain the PspC binding site within SCR 13 (Fig. 7). Some evidence for this observation was the fact that a 5- or 10-fold increase in the concentration of SCR 8-14 did not result in any significant increase in binding to PspC (data not shown). Conversely, it appears as though two adjacent SCRs, with the addition of SCR 14-15 removes this inhibition, resulting in comparable PspC binding compared to fH in the PspC ELISA (see Fig. 6). This indicates that a second binding site may exist in SCR 15 or alternatively SCR 15 contributes to the overall binding of fH.

**Effect of an SCR 8-15Δ13 construct on PspC binding and inhibition experiments.** To further analyze the role of SCR 13 and SCR 15 in mediating binding of fH to PspC, we deleted SCR 13 and examined the ability of this SCR 8-15Δ13 protein compared to SCR 8-15 and SCR 8-13 to bind PspC. The ELISA data in Fig. 8 indicate that, although SCR 8-15Δ13 retains the ability to bind PspC, there is an approximately 60% reduction in binding in comparison to SCR 8-15. Furthermore, SCR 8-15 showed an approximately 25% increase in binding compared to SCR 8-13, indicating that SCR 15 is also required for maximal binding to PspC; however, SCR 13 or SCR 15 alone is capable of mediating an interaction with PspC (Fig. 8).

We also examined the ability of SCR 8-15 and SCR 8-14 to competitively inhibit each other in binding to PspC. Wells of an ELISA tray were coated with either 0.1 μg of SCR 8-15 or SCR 8-14, and the ability of PspC (200 ng/ml) preincubated with either of the two proteins (10 μg/ml) for 30 min at RT to bind the coated proteins was determined by using mouse anti-PspC antiserum. Figure 9 indicates that SCR 8-15 almost totally

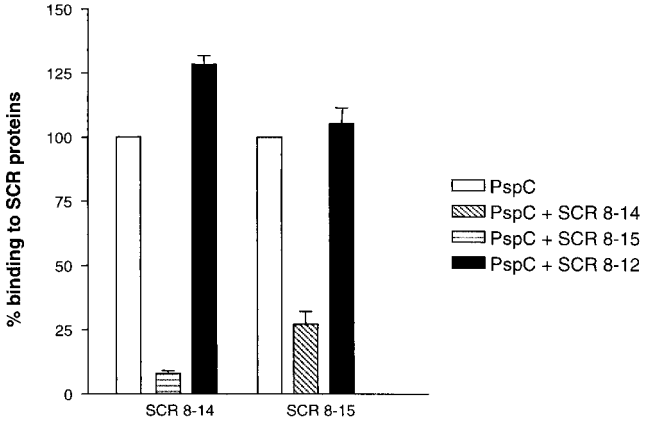


FIG. 9. Competition experiments between fH SCR 8-14 and SCR 8-15 binding to PspC. SCR 8-14 and SCR 8-15 proteins (0.1 μg) were coupled to wells of an ELISA tray prior to the addition of a mixture of PspC (200 ng/ml) and SCR 8-14 or 8-15 (10 μg/ml) preincubated for 30 min at RT. Specific binding of PspC to the immobilized proteins was determined by the addition of polyclonal mouse anti-PspC antiserum (1:1,000) and then HRP-conjugated goat anti-mouse IgG (1:2,000), followed by the addition of substrate and a reading at $OD_{490}$. The readings were expressed as a percentage versus of SCR-PspC interaction alone in the absence of competitor. Experiments were repeated three times in triplicate, with the means and standard deviations indicated.

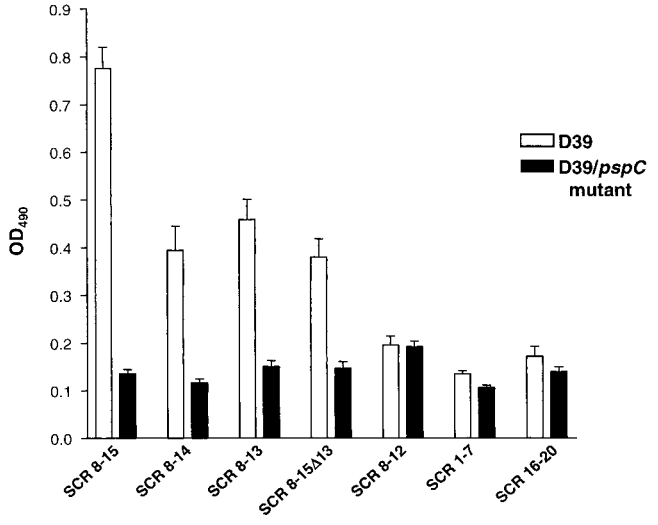


FIG. 10. Binding of fH SCR proteins to D39 and D39/*pspC* mutant pneumococci by ELISA analysis. Bacterial strains were dried overnight on microtiter trays and incubated with 10 μg of SCR 8-15, SCR 8-14, SCR 8-13, SCR 8-15Δ13, SCR 8-12, SCR 1-7, and SCR 16-20/ml. Binding of SCR containing proteins to the pneumococci was detected by using goat anti-fH (1:2,000) and then HRP-conjugated donkey anti-goat IgG (1:2,000), followed by the addition of substrate and a reading at $OD_{490}$. Experiments were repeated three times in triplicate, with the means and standard deviations indicated.

inhibits the binding of PspC to SCR 8-14; however, SCR 8-14 showed only ~75% inhibition of SCR 8-15 binding to PspC. These data are consistent with previous binding data and verify the importance of SCR 15 in mediating an interaction with PspC. The negative control protein, SCR 8-12, did not inhibit the binding of PspC to either SCR 8-14 or SCR 8-15 (Fig. 9), further confirming that SCR 8-12 is not involved in PspC binding. What remains to be determined is whether SCR 15 is able to act synergistically with SCR 13 to form the complete PspC binding site and hence partially displace the weaker-binding SCR 8-14 from PspC or, alternatively, whether a second binding site exists on PspC for SCR 15.

**Binding of SCR proteins to pneumococcus by ELISA.** The whole-cell ELISA by using D39 or D39/*pspC* mutant was utilized to further verify that the SCR constructs that bound purified PspC or PspCΔPro were also able to recognize PspC presented on the surface of the bacteria. This approach provides for a more physiologically relevant interaction. As expected, based on the purified PspC ELISA results, SCR 1-7, SCR 16-20, and SCR 8-12 did not show any discernible binding to the PspC-expressing strain D39 (Fig. 10). SCR 8-15 showed a six- to sevenfold increase in binding to the D39 strain versus the otherwise D39/*pspC* mutant, confirming that binding occurs via a PspC-dependent mechanism. SCR 8-15 bound to a greater extent than SCR 8-13, SCR 8-14, or SCR 8-15Δ13, which showed a three- to fourfold increase in binding to D39 over background levels observed in the mutant strain (Fig. 10). Interestingly, SCR 8-13 did not show a significant difference in binding to D39 compared to SCR 8-14 or SCR 8-15Δ13, which differ from the superior binding displayed by SCR 8-13 in the PspC ELISA (see Fig. 7 and 8). Therefore, the presence of both SCR 13 and SCR 15 is required for optimal binding to D39 pneumococci versus either SCR alone.

## DISCUSSION

PspC from the pneumococcal type 2 strain D39 binds to the human complement regulator fH, and in this report we have elucidated the PspC binding site on fH. We specifically showed that fH bound two purified PspC protein constructs, one consisting of amino acids 1 to 445 of the mature PspC polypeptide, which lacks the choline-binding region but retains the α-helical and proline-rich regions, and a second PspC derivative that lacks the proline-rich region (PspCΔPro). Interestingly, the absence of the proline-rich region significantly decreased the binding ability of fH, which underlies the importance of this region in recognition and/or binding. The binding of fH to the surface of *S. pneumoniae* D39 was shown to be PspC dependent, given that an otherwise isogenic PspC-negative mutant (D39/*pspC* mutant) did not show any discernible binding of fH by ELISA.

The contribution of electrostatic and hydrophobic forces to the fH-PspC interaction, namely, was determined by examining the effects of increasing NaCl concentrations. The binding of fH to PspC was insensitive to NaCl between 0.05 to 1.0 M, which is indicative of a short-range hydrophobic interaction, given that concentrations of NaCl up to 1.0 M are known to reduce electrostatic forces and salt bridge formation, while not interfering with hydrophobic forces (10). The binding of C4b-binding protein (C4BP) *to S. pyogenes* M4 and M22 proteins is also relatively insensitive to salt and occurs via hydrophobic forces between SCR 1 and 2 of C4BP (6). In contrast, 200 mM NaCl is sufficient to severely abrogate the fH-M6 protein interaction, mediated via a cluster of positively charged Lys and Arg residues between SCR 6 and SCR 7 and within SCR 7 itself (E. Giannakis, E., T. S. Jokiranta, D. A. Male, S. Ranganathan, R. J. Ormsby, V. A. Fischetti, C. Mold, and D. L. Gordon, unpublished data).

The fH-PspC interaction is not inhibited by heparin (data not shown), suggesting that the cluster of positively charged amino acids residing in SCR 7, SCR 13, and SCR 20, which are postulated to play a role in heparin binding (4, 5, 27) are not involved in PspC binding. More recently, we have shown that the positive residues residing in SCR 7 are essential for heparin binding; this site overlaps with the M6 protein binding site and the fH-M6 protein interaction was reduced by ca. 50% in as little as 100 U of heparin/ml (Giannakis et al., unpublished). The group B streptococcal β protein also binds fH, and this interaction was also inhibited by heparin (1), indicating that both group A streptococcal M protein and group B streptococcal β protein, although structurally unrelated, may bind similar sites on fH.

The recently identified Hic protein isolated from a type 3 pneumococcal strain, which is similar to PspC, binds fH within SCR 8-11 (17, 18). This indicates that, although Hic and PspC are homologous in their N-terminal region, each is sufficiently divergent from the other to bind a unique site on fH. Furthermore, C3b has no effect on the fH-PspC interaction, since fH preincubated with up to a 50-fold molar excess of purified C3b did not affect PspC binding. C3b is assumed to interact with fH in the fluid phase in the assay, given that previous studies have

shown the affinity between fH and C3b in the fluid phase is the same as that between fH and non-activator-bound C3b (25). Presumably, once fH is bound by PspC on the surface of the pneumococci, fH is then able to interact with C3b directly via one or more of the three C3b binding sites, thereby limiting amplification of C3b deposition on its surface and subsequent phagocytosis.

Initial experiments with a series of fH constructs spanning the entire molecule (SCR 1-7, SCR 8-15, and SCR 16-20) indicated that only the middle construct bound PspC and PspCΔPro. We then constructed a series of SCR proteins within the SCR 8-15 region and specifically localized the binding site of PspC to SCR 13-15. The data indicate that the presence of either SCR 13 or SCR 15 is sufficient to mediate an interaction with PspC; however, the presence of both SCRs significantly increased binding to both purified PspC and PspC on the pneumococcal cell surface. Although SCR 814 showed a decrease in binding to pure PspC relative to SCR 8-13, on the bacterial surface there was no difference in binding between the two proteins versus SCR 8-12, which did not bind PspC in either assay. This finding indicates that SCR 14 is not directly involved in the interaction and, at least in the PspC ELISA, that the additional SCR may somewhat constrain the binding site in SCR 13. The involvement of SCR 15 in PspC binding was shown by an SCR 8-15Δ13 construct in which the entire SCR 13 was deleted. This protein retained the ability to bind purified PspC, although to a lesser extent than SCR 8-13 and SCR 8-15; however, on the bacterial surface SCR 8-15Δ13, binding was similar to that of SCR 8-13 but significantly less than SCR 8-15. What is clear from these studies is that the SCR 13-15 region involved in PspC binding lies well downstream from the regions required for both cofactor and decay acceleration activity within SCR 1-4. Therefore, the SCR 1-4 region is free to interact with complement proteins, whereas SCR 13-15 remains bound to the pneumococcal surface.

Given the importance of SCR 15 in enhancing the binding interaction with PspC in conjunction with SCR 13, we examined whether SCR 8-15 binding to PspC could be inhibited by SCR 8-14 and vice versa, in an ELISA system. When SCR 8-15 was coated onto an ELISA tray, a mixture containing SCR 8-14 and PspC still resulted in partial binding of PspC to SCR 8-15 (~25% versus control). In contrast, SCR 8-15 incubated with PspC almost completed inhibited PspC binding to immobilized SCR 8-14. SCR 8-12 acted as a negative control and was shown to play no role in binding PspC. Therefore, essentially two possibilities exist for SCR 8-15 binding to PspC. (i) SCR 13 and SCR 15 may come together to form the strongest PspC binding site, and this protein may partially displace the weaker binding SCR 8-14 (containing only SCR 13) from PspC. (ii) There may be an additional binding site for SCR 15 on PspC, which is distinct from the SCR 13 binding site, explaining why SCR 8-14 is only partially inhibitory of SCR 8-15/PspC binding. We are currently conducting experiments to distinguish between these two possibilities, although there is no doubt that both SCR 13 and SCR 15 are required for optimal binding to PspC.

The fH binding site on PspC has yet to be definitively mapped, although previous evidence suggests the site is located within the N terminus of the protein. The fH binding site on Hic has been localized to the N-terminal amino acids 39 to 261, a region that shares significant identity with that of PspC (17). Furthermore, Janulczyk et al. (17) showed that a D39 mutant with a 405-amino-acid N-terminal deletion of PspC did not absorb fH from plasma, although part of the deletion encompassed a region of PspC not homologous to Hic, including part of the proline-rich region. Therefore, the observation in our experiments that PspC was a far better binder of fH versus PspCΔPro may indicate that the proline-rich region functions either in presenting the N-terminal α-helical region in the correct conformation for binding fH or provides alternative binding sites for fH. The proline-rich region of PspA has a proposed role as a flexible tether to anchor PspA to the cell wall via the choline-binding region (38) and, given the high degree of amino acid identity between this region of PspC and PspA, such a role is probable for the PspC proline-rich region. Clearly, the proline-rich domain is important for host immunity, since mice immunized with PspC show cross-reactive and protective antibodies to PspA, and these antibodies were directed to the proline-rich region in both molecules (7).

In conclusion, the newly identified region between SCR 13 and SCR 15, responsible for binding PspC from type 2 pneumococci, is distinct from all other characterized bacterial protein-binding sites on fH and is therefore yet another important region of fH utilized by a pathogenic microorganism to evade host complement attack.

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