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The Host Immune Regulator Factor H Interacts via Two Contact Sites with the PspC Protein of *Streptococcus pneumoniae* and Mediates Adhesion to Host Epithelial Cells

Sven Hammerschmidt,* Vaibhav Agarwal,* Anja Kunert,† Steffi Haelbich,† Christine Skerka,† and Peter F. Zipfel‡‡

Pneumococcal surface protein C (PspC) of *Streptococcus pneumoniae* is a key virulence factor that mediates adhesion to host cells and immune evasion of the host complement. PspC binds the host immune and complement regulator factor H, which is composed of 20 short consensus repeats (SCR). This interaction contributes to pneumococcal virulence. In this study, we identified within the factor H protein two separate PspC binding regions, which were localized to SCR8–11 and SCR19–20, by using recombinant factor H deletion constructs for Western blotting assays and surface plasmon resonance studies. A detailed analysis of binding epitopes in these SCR by peptide spot arrays identified several linear binding regions within the sequences of SCR8–11 and SCR19–20. In addition, the factor H binding site was mapped within the pneumococcal PspC protein to a 121-aa-long stretch positioned in the N terminus (residues 38–158). Factor H attached to the surface of pneumococci via PspC significantly enhanced pneumococcal adherence to host epithelial and endothelial cells. This adhesion was specific and was blocked with a truncated N-terminal factor H-binding fragment of PspC. In conclusion, the acquisition of factor H by pneumococci via PspC occurs via two contact sites located in SCR8–11 and SCR19–20, and factor H attached to the surface of the pneumococcus promotes adhesion to both host epithelial and endothelial cells. The *Journal of Immunology*, 2007, 178: 5848–5858.

*Streptococcus pneumoniae* is a natural resident of the nasopharyngeal cavity. In addition to colonization, pneumococci cause severe local infections including otitis medium, sinusitis, and life-threatening invasive diseases such as lobar pneumonia, sepsis, and meningitis (1–4). The bacterium uses several strategies for colonization of the respiratory tract, transcytosis through host cells, and transmigration of the blood-brain-barrier. Several virulence factors of *S. pneumoniae* have been identified that are involved in the progression of pneumococcal diseases (5–7). Pneumococcal surface protein C (PspC; also named CbpA or SpsA) is a major pneumococcal virulence factor. To date, 11 different subtypes of PspC proteins have been identified and, based on their different anchorages in the bacterial cell wall, divided into two subgroups (8).

The classical PspC proteins (subtypes 1–6) are choline-binding proteins and constitute subgroup 1. The C-terminal choline-binding domain attaches the classical PspC proteins noncovalently to a cell wall via an interaction with the phosphorylcholine of lipoteichoic and teichoic acids. Members of the second subgroup, representing atypical or PspC-like proteins (subtypes 7–11) such as Hic (PspC11.4), are anchored in a sortase-dependent manner to the peptidoglycan of the cell wall by an LPXTG motif. The N-terminal regions of the first PspC subgroup show a common structure and organization. All proteins have a leader peptide and an N-terminal domain that is followed by either one or two single repeated domains (termed R1 and R2) and a proline-rich sequence (8–11).

PspC mediates pneumococcal adherence by binding the extracellular Ig-like domain, also known as the secretory component (SC), of the polymeric Ig receptor (pIgR) (12, 13). The specific binding to the human Ig-like ectodomains D3 and D4 of the SC occurs through hexapeptide motifs that are located in the direct repeats R1 and R2 of PspC (11, 13–15). One R domain is sufficient for binding to the SC of pIgR, to a free SC, or to a SC as part of secretory IgA (10, 11, 15).

In addition to its role as an adselin, PspC also mediates immune evasion by binding the host complement and the innate immune regulators, factor H and C3 (16, 17). Apparently PspC uses two different epitopes for binding the soluble host proteins factor H and SC (18). The factor H binding residues of the subgroup II PspC11.4 protein (Hic) were mapped to residues 29–269 (19). Interestingly, a region (38–149) of Hic shows considerable sequence homology with the N-terminal sequences of the subgroup I PspC proteins (8, 19).

Factor H is a fluid phase regulator of the alternative complement pathway and consists of 20 domains that are termed short consensus repeats (SCR), each consisting of ~60 aa. This plasma glycoprotein is the key fluid phase regulator of the alternative complement pathway.
An agent of the factor H pathway acts as a cofactor in the factor I-mediated proteolysis of C3b. Proteolytic cleavage of C3b results in the formation of the inactive iC3b fragment, which remains covalently linked to the surface (20).

The binding of factor H to the surface of pathogens has been observed for other streptococci such as Streptococcus agalactiae and Streptococcus pyogenes and several other pathogenic microbes including Borrelia burgdorferi, Candida albicans, Neisseria gonorrhoeae, and Neisseria meningitidis (21–28). Host-derived factor H attached to the surface of a pathogen inhibits complement activation and thus prevents complement-mediated killing. The lack of PspC in the pneumococci of serotype 2 strain D39 and the lack of Hic in serotype 3 strain A66 attenuated pneumococcal virulence during sepsis. Apparently this defect results in decreased uptake by polymorphonuclear leukocytes (29, 30). In addition, pneumococci deficient in PspC, but not wild-type pneumococci, are efficiently killed by microglia cells, which represent the resident phagocytes in the brain (31). Thus, PspC expression seems to be important for colonization and for pneumococcal survival in an immune-competent host (6).

Recently, it was shown that factor H binds to the cell surfaces of host cells via polyanionic cell surfaces such as proteoglycans, sialic acids, heparan sulfate chains, or glycosaminoglycans (32–34). Moreover, factor H and its splicing variant factor H-like protein 1 (FHL-1), which consists of the first seven SCR, bind via an RGD sequence of SCR4 to host cells. Factor H binding interferes with fibronectin binding, suggesting that both molecules use identical cellular receptors (35). Similarly, human polymorphonuclear leukocytes bind to immobilized factor H via integrin CD11b/CD18, i.e., CR3 (36). In contrast, binding to human endothelial cells is mediated via the heparin/glycosaminoglycan-binding site within SCR20 of factor H (37).

In part, inconsistent data were reported for the interaction of PspC and Hic with factor H. The pneumococcal protein Hic, which is preferentially produced by serotype 3 strains, binds to SCR8–11 and SCR12–14 of factor H. In contrast, for the PspC of serotype 2 strain D39, binding to SCR6–10 and SCR13–15 of factor H has been reported (16, 38). The goal of this study was to evaluate the interaction of complement regulator factor H with the bacterial PspC protein by localizing the binding sites within the host protein as well as within the bacterial protein. In addition, the impact of bacterial cell surface-bound factor H on pneumococcal adhesion to host cells was demonstrated for the first time.

### Materials and Methods

**Bacterial strains, culture conditions, and PspC protein purification**

*S. pneumoniae* was cultured on blood agar plates (Oxoid) at 37°C and 5% CO₂ or in Todd-Hewitt broth (Roth) supplemented with 0.5% yeast extract to a density of 5 × 10⁸ CFU ml⁻¹ (OD₆₅₀ of ~0.5). The wild-type pneumococcal strains and isogenic mutants that do not produce PspC or strains that are deficient for the capsular polysaccharide (CPS) are listed in Table I. The PspC protein has a modular organization and is divided into different subtypes; therefore, the PspC nomenclature is also included in Table I. Isogenic mutants that do not express PspC were constructed for the nonencapsulated R6x (PspC3.1) and *S. pneumoniae* NCTC 10319 (PspC3.3), which is a low encapsulated strain and perfectly suitable for cell culture infection experiments as described earlier (13, 39). PspC-deficient mutants of R6x and NCTC 10319 were generated by replacement of the pspC sequence with the erythromycin gene cassette. Briefly, the full-length pspC gene was amplified by PCR from the chromosomal DNA of *S. pneumoniae* NCTC 10319 with the primers 5'-GGACCTTGTGTAAGCAGAAATG-3' and 5'-AAGGCTGTTTAGTTATCCACATTCAGCTGG-3', which incorporated flanking BamHI and HindIII (underlined) restriction sites. The amplified DNA was cloned into similarly digested pQ30E0 (Qiagen). A SpeI digest of the inserted pspC fragment (nt 550-1080) was deleted and the erythromycin gene cassette was blunt-end ligated with the plasmid. The integrity of the antibiotic gene cassette was verified by sequence analysis using ABI Prism dye terminator cycle sequencing (Applied Biosystems). Erythromycin (5 μg ml⁻¹) was added to the growth medium for the mutants. The transformation of pneumococci was performed as previously described (15).

<table>
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<tr>
<th>Strain</th>
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**SDS-PAGE and binding of ¹²⁵I-radiolabeled factor H**

Whole cell lysates of pneumococci or His₅-tagged PspC derivatives were subjected to SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore) using a semidry blotting system (Bio-Rad). Factor H was radiolabeled with ¹²⁵I by a standard chloramine-T method and binding assays with pneumococci were performed as described (10) and quantitated in a counter (1600 TR; Packard Instrument).

For blot overlay assays with ¹²⁵I-radiolabeled factor H, whole protein lysates of pneumococci were separated by SDS-PAGE and transferred to a PVDF membrane. After blocking with 10% skim milk (Oxoid), the membrane was washed and incubated with ¹²⁵I-radiolabeled factor H (300,000 cpm ml⁻¹) in 5 ml of PBS-Tween 20 (0.05%) for 4 h at room temperature. After extensive washing, bound factor H was detected by autoradiography.

**Table I. Streptococcus pneumoniae strains used in this study**

*Escherichia coli* BL21 (DE3) (Stratagene), cultivated at 30°C on Luria-Bertani agar or broth containing 100 μg/ml of ampicillin, was used as the host strain for expression of a His₅-tagged fusion protein. The His₅-tagged fusion proteins were purified by Ni²⁺ affinity chromatography with the Protino Ni prepacked column kit according to the manufacturer’s instructions (Macherey-Nagel). The His₅-tagged PspC proteins used in this study have been described earlier (13, 15). Briefly, the recombinant His₅-tagged PspC proteins represent PspC group 2.1, which is expressed by strain ATCC 33400 (serotype 1), or PspC group 3.3, which is expressed by strain NCTC 10319 (see Fig. 34).

**Factor H, Abs, and human sera**

Human factor H and the polyclonal anti-factor H Ab were purchased from Calbiochem. Recombinant factor H deletion mutants representing SCR1–7, SCR8–20, SCR8–11, SCR11–15, and SCR15–20 were expressed in the baculovirus expression system as described (40, 41). Purification of anti-PspC IgG, which was generated by immunization of a rabbit with PspC2.1, and polyclonal anti-pneumococcal IgG (13) was performed by protein A-Sepharose 4B affinity chromatography. The inhibitory role was assayed for the mAbs L20, E14, and C18, whose binding sites in the factor H protein have been mapped to SCR19 (13, 39). Nonimmune human sera or plasma proteins were obtained from healthy individuals upon informed consent.
Immunoblot and Western blot analysis

Identical amounts of His6-tagged PspC deletion mutants were spotted on a PVDF membrane using a Bio-Dot SF microfiltration apparatus (Bio-Rad). Immobilization of the proteins was confirmed by immunodetection using purified anti-PspC IgG together with a HRP-conjugated rabbit Ab. The binding of factor H (2 μg ml⁻¹) to the various PspC deletion mutants was visualized using factor H antiserum (diluted 1/800; Calbiochem) in combination with a HRP-conjugated goat antiserum and detected by ECL chemiluminescence (GE Health Care).

PepSpot analysis

A library of 115 peptides representing SCR8–11 and a library of 64 peptides representing SCR19–20 of factor H were synthesized and spotted on a cellulose membrane (Intavis MultiPep System). Each peptide was 12 aa in length and differed from the next peptide in two amino acid residues; thus the peptides have an overlap of 10 residues. Membranes were incubated with recombinant PspC protein (PspC SH2; 10 μg ml⁻¹) as described (13, 37) and bound PspC protein was detected using polyclonal PspC IgG in combination with HRP-conjugated rabbit IgG and ECL.

Surface plasmon resonance assays

Protein-protein interactions were analyzed by a surface plasmon resonance technique using a Biacore 3000 instrument as described (13, 37). Briefly, factor H, factor H SCR8–20, and the recombinant PspC derivative SH2 were dialyzed against 10 mM sodium acetate buffer (pH 4.0). All proteins were coupled via a standard amine-coupling procedure to the flow cells of a sensor chip (CM5, Biacore). Control flow cells were prepared in the same way but without injecting the protein. The analytes including PspC proteins, factor H, or the recombinant truncated factor H derivatives SCR1–7, SCR8–20, SCR8–11, SCR11–15, and SCR15–20 were dialyzed against running buffer (i.e., PBS (pH 7.4)). Binding of the analytes was analyzed after separate injection into the flow cells coupled with the binding partners and into a control cell using a flow rate of 10 μl min⁻¹ at 25°C in all experiments. Each interaction was measured at least three times.

Flow cytometric analysis of factor H binding to pneumococci

Binding of factor H to viable pneumococci in competitive inhibition experiments was tested using flow cytometry. Bacteria were cultured in Todd-Hewitt broth supplemented with 0.5% yeast extract, and 5 × 10⁷ bacteria in 100 μl of PBS were incubated in the absence or presence of PspC proteins that were used as competitors. The suspensions were incubated for 30 min at 37°C and thereafter bacteria were washed three times. The binding of factor H to pneumococci was detected after incubation with the factor H antiserum for 30 min at 37°C followed by FITC-conjugated anti-goat Ig Ab (MoBiTec). Bacteria were washed and fluorescence was analyzed by flow cytometry using a FACSScalibur apparatus (BD Biosciences). The pneumococci were detected using log forward and log side scatter dot plot, and a gating region was set to exclude debris and larger aggregates of bacteria. Ten thousand bacteria were analyzed for fluorescence using log scale amplification. The geometric mean fluorescence intensity (GMFI) × percentage of labeled bacteria was recorded as a measure for binding activity.

Cell lines and culture conditions

The cultivation of cell lines was performed as described (39, 43). Briefly, human A549 cells (lung alveolar epithelial cells type II pneumocytes; ATCC catalog no. CCL-185) were cultivated in DMEM supplemented with 10% FCS, 5 mM glutamine, 100 U/ml penicillin, and streptomycin (all from PAA Laboratories) at 37°C in 5% CO₂. Detroit 562, human nasopharyngeal epithelial cells (ATCC catalog no. CCL 138) that express the polymeric Ig receptor, were cultivated in RPMI 1640 (PAA Laboratories) and ECL chemiluminescence. The samples were separated by SDS-PAGE, transferred to a PVDF membrane, and analyzed with factor H antiserum. Lane 1, Plasma as control (Ctrl); lanes 2 and 6, whole cell lysate of pneumococci (W) incubated in PBS; lane 3, proteins eluted from the pneumococcal cell surface (E) by treatment with 2 M NaCl after incubation in human plasma; lanes 4 and 8, whole cell lysate (W) after incubation of pneumococci with human plasma or serum; lane 5, serum as control (Ctrl); lane 7, proteins eluted (E) from the pneumococcal cell surface after incubation in human serum; M, protein marker (New England Biolabs).

Binding of 125I-radiolabeled factor H to wild-type (WT) NCTC10319 and isogenic ΔpspC mutant. Bacterial lysates were separated by SDS-PAGE, transferred to a PVDF membrane, and used for an overlay assay with 125I-labeled factor H. Binding of soluble 125I-radiolabeled factor H to viable pneumococcal wild-type (WT) strains NCTC10319 (Cps⁻) and R6x (Cps⁻) and their isogenic ΔpspC mutants. Both wild-type strains bound strongly factor H and binding was reduced in the mutants that are deficient in PspC. D. Recruitment of factor H to encapsulated and nonencapsulated pneumococci producing different PspC subtypes. Binding of factor H (2 μg) was determined by flow cytometry and results were expressed as GMFI × percentage of FITC-labeled and gated bacteria. Representative data from independent experiments are shown.

Pneumococcal adhesion assay

Epithelial cells and HBMEC were seeded at a density of 5 × 10⁴ cells in 100 μl of DMEM-HEPES (PAA laboratories) supplemented with 1% FCS at 37°C using a multiplicity of infection of 40. The role of factor H for adherence was analyzed by incubating pneumococci for 10 min with factor H in a total volume of 100 μl DMEM-HEPES at 37°C before infection and the infection assay was conducted in a total volume of 500 μl after adding the preincubated bacteria. After infection, cells were washed three times with PBS to remove unbound bacteria. Adherent bacteria were visualized following the fixation of cells with 3.7% paraformaldehyde by immunofluorescence or by plating the bacteria on blood agar after the detachment and lysis of cells with

![Image](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org/)

FIGURE 1. Binding of factor H to pneumococci. A, Immunoblot analysis of the binding of factor H to S. pneumoniae serotype 35A (NCTC10319), which were incubated in human serum and plasma, respectively. The samples were separated by SDS-PAGE, transferred to a PVDF membrane, and analyzed with factor H antiserum. Lane 1, Plasma as control (Ctrl); lanes 2 and 6, whole cell lysate of pneumococci (W) incubated in PBS; lane 3, proteins eluted from the pneumococcal cell surface (E) by treatment with 2 M NaCl after incubation in human plasma; lanes 4 and 8, whole cell lysate (W) after incubation of pneumococci with human plasma or serum; lane 5, serum as control (Ctrl); lane 7, proteins eluted (E) from the pneumococcal cell surface after incubation in human serum; M, protein marker (New England Biolabs); B, Binding of 125I-radiolabeled factor H to wild-type (WT) NCTC10319 and isogenic ΔpspC mutant. Bacterial lysates were separated by SDS-PAGE, transferred to a PVDF membrane, and used for an overlay assay with 125I-labeled factor H. C, Binding of soluble 125I-radiolabeled factor H to viable pneumococcal wild-type (WT) strains NCTC10319 (Cps⁻) and R6x (Cps⁻) and their isogenic ΔpspC mutants. Both wild-type strains bound strongly factor H and binding was reduced in the mutants that are deficient in PspC. D, Recruitment of factor H to encapsulated and nonencapsulated pneumococci producing different PspC subtypes. Binding of factor H (2 μg) was determined by flow cytometry and results were expressed as GMFI × percentage of FITC-labeled and gated bacteria. Representative data from independent experiments are shown.
saponin (1%; w/v). The number of viable intracellular bacteria was quantitated by an antibiotic assay as described (43).

Determination of adherent pneumococci by immunofluorescence
Pneumococci attached to the cells were stained with a polyclonal pneumococcal antiserum in combination with a secondary goat anti-rabbit IgG coupled with Alexa Fluor 488 (green) or Alexa Fluor 568 (red) (MoBiTec) (43). After blocking nonspecific binding sites with 10% FCS, cells were thoroughly washed with PBS and then incubated for 30 min with the pneumococcal antiserum (1/100). Bound Abs were detected with an Alexa Fluor 488-labeled goat anti-rabbit Ig conjugate (MoBiTec). The glass cover slips were embedded “upside down” in Moviol, sealed with nail polish, and stored at 4°C. At least 50 cells were counted using a fluorescence microscope (Zeiss Axioskop) and a confocal laser scanning microscope and software (Zeiss) were used for image acquisition. Each experiment was repeated at least three times and results were expressed as mean ± SD.

Statistical analysis
Adherence data were expressed as the mean ± SD. Differences in adherence were analyzed by the two-tailed unpaired Student’s t test. In all analyzes, \( p < 0.05 \) was considered statistically significant.

Results
Binding of factor H to pneumococcal PspC protein
PspC3.3 expressing pneumococci (NCTC10319, serotype 35A) were analyzed for factor H binding. Pneumococci were incubated either in human plasma or serum. After incubation in human plasma, the elute fraction and the extract prepared from plasma-treated pneumococci were separated by SDS-PAGE, and analyzed by Western blotting using a factor H antiserum. Factor H was detected in samples of eluted proteins and whole cell lysates (Fig. 1A, lanes 3 and 4), thus demonstrating that factor H derived from human plasma binds to the surface of pneumococci. The same results were observed when pneumococci were incubated in human serum (Fig. 1A, lanes 7 and 8). FHL-1, which is also present in serum, did not bind to pneumococci (data not shown).

Similarly, the binding of radiolabeled factor H was assayed. \(^{125}\)I-radiolabeled factor H bound to the wild-type pneumococci of strain NCTC10319 but not to the pneumococci of the isogenic pspC knockout strain (Fig. 1B). In addition \(^{125}\)I-radiolabeled factor H bound to the wild-type strain but not to the isogenic pspC mutant strains representing the encapsulated strain NCTC10319 or the nonencapsulated R6x. (Fig. 1C). These results indicate that PspC is the major and most likely the only surface protein of pneumococci that binds the host regulator factor H.

PspC is a highly variable surface protein with a modular organization (8, 10). To assess whether PspC variability affects the binding of factor H, pneumococci producing different PspC subtypes, including the serotype 3 strain A66 that expresses Hic (PspC11.4), were used in binding experiments. As demonstrated by flow cytometric analysis (Fig. 1D), all of the pneumococcal

![FIGURE 2. Binding of factor H to PspC protein analyzed by surface plasmon resonance. Binding of factor H to the PspC protein SH2 (PspC2.1) was analyzed by surface plasmon resonance. PspC derivative SH2 was coated on the surface of the sensor chip and human factor H was applied as an analyte with the indicated concentrations. Sensorgrams show concentration-dependent rates of factor H binding to PspC. Co, Injection of PBS.](http://www.jimmunol.org/)

![FIGURE 3. Schematic models of PspC deletion constructs and factor H. A, Structures of PspC2.1 and PspC3.3 deletion constructs. The binding region identified for factor H derivatives SCR8–20 and SCR8–11 is shown in black and the hexapeptide binding epitope (Y/R)RNYPT of the SC is indicated in the R domains of PspC variants. PspC2.1 is the PspC protein produced by S. pneumoniae ATCC 33400 (serotype 1) and PspC3.3 is produced by S. pneumoniae NCTC10319 (serotype 35A). The latter is identical to the PspC of pneumococcal strains D39 and R6x which have been used in previous studies to investigate the PspC-factor H interaction (16). LP, Leader peptide; CBD, choline-binding domain; P, proline-rich sequence; R, R domain. B, Factor H and factor H deletion constructs SCR8–20 and SCR8–11.](http://www.jimmunol.org/)
strains analyzed recruited factor H to the bacterial cell surface and binding was independent of the PspC subtype. Similarly as in binding studies with secretory IgA and SC (10), factor H binding efficiency increased significantly when nonencapsulated pneumococcal strains were used (Fig. 1D), indicating that the CPS interferes with factor H binding.

**Binding of factor H to PspC analyzed by surface plasmon resonance**

Surface plasmon resonance was used to characterize the binding of factor H to the SH2 domain of PspC2.1. Factor H binding to the immobilized PspC was dose dependent and increased with increasing concentrations of factor H (Fig. 2). The PspC derivative SH2^198–203, in which the critical amino acids for the interaction with the secretory component were exchanged (Fig. 3A), showed comparable binding (data not shown). Thus, factor H-PspC complex formation occurs independently of the SC-binding epitope of the PspC protein.

**Mapping of the factor H-binding domain in PspC**

To map the binding site within the bacterial PspC protein, deletion mutants of PspC2.1 and one of PspC3.3 were used (Fig. 3A). The factor H deletion mutants used in this study are shown in Fig. 3A. Both factor H mutants did bind to the PspC2.1 derivatives SH2, SH2^198–203, and SH3 and to the PspC3.3 fragment SH12. No binding was detected to PspC2.1 deletion mutants SM1, SM2, SM5, or SM6 (Fig. 4A). These results indicate that the factor H binding site of PspC is located within the N terminus, i.e., amino acid residues 38–158.

Because the factor H SCR8–20 mutant did not bind to N-terminal PspC mutants that contain only the R domain, a truncated R domain, or a C-terminal extended R domain (Figs. 3A and 4A), it is concluded that the SC-binding epitope within the R domain(s) of PspC2.1 is dispensable for factor H binding. These results are consistent with a previous study showing that factor H binding is independent of the SC binding epitope.
binding motif YRNYPT and that factor H binding does not compete with binding of SC (18). These results were confirmed by using a complementary approach. In surface plasmon resonance studies the PspC derivatives SH12, SH2, SH2198–203, SH3, and SM1 were used as analytes to measure the binding to immobilized factor H and factor H SCR8–20. Similarly as in the ligand blot overlay assays, the binding to factor H or SCR8–20 was observed with PspC proteins that contained the 121-aa-long (positions 38–158) N-terminal domain (Fig. 4, B and C).

**Factor H has two contact sites for pneumococcal PspC**

Given that the factor H SCR8–11 deletion mutant binds to PspC, we were interested in identifying the essential and minimal domains of factor H that are required for this interaction. First, surface plasmon resonance was used to verify and compare the binding of the factor H deletion mutants SCR1–7, SCR8–11, SCR11–15, SCR8–20, and SCR15–20 to immobilized PspC. The factor H mutant SCR8–11 bound to PspC2.1 as indicated by the
association and dissociation profile. Factor H construct SCR8–20 showed a more pronounced binding and construct SCR15–20 showed a rather low level of binding (Fig. 5A). In contrast, SCR1–7 and SCR11–15 showed no binding (Fig. 5A). These results are indicative of a second PspC-binding domain in factor H. To confirm the existence of a second binding site within the C terminus of factor H, the binding of factor H SCR8–20 to PspC was analyzed in the presence of three domain-mapped mAbs that bind to the C-terminal SCR19 (mAb L20) or to overlapping domains within the SCR20 of factor H (mAbs E14 and C18). The two mAbs that bind to SCR20, but not mAb L20, blocked the interaction with SCR19–20. In the presence of the two mAbs E14 and C18, which bind to SCR20, the binding of the SCR8–20 construct was reduced, whereas the mAb L20 had no effect on binding (Fig. 5B and data not shown). This effect of the blocking Abs is in agreement with presence of a second binding site in the C terminus of factor H. Factor H contains heparin binding sites in the SCR9 and the SCR20 of factor H (44). Heparin blocked the binding of SCR8–20 to PspC in a dose-dependent manner (Fig. 5C). Apparently the SCR20 binding region acts in concert with the binding domain in SCR8–11. These data suggest that factor H uses two binding domains for interaction with PspC2.1 that are located within SCR8–11 and SCR19–20.

Mapping of the linear PspC binding sites within SCR8–11 and SCR19–20 of factor H

Peptide spot analyses were used to identify linear PspC-binding sequences within two factor H regions. Peptides with a length of 12 aa spanning the region of the SCR8–11 and the SCR19–20 of factor H were generated and probed with the recombinant SH2 fragment of PspC2.1. Several linear binding regions were identified (Fig. 6). PspC binding was indicated to linear sequences in SCR8–11 (A) and SCR19–20 (B) were divided into 12-aa peptides with a 10-aa transition with the next peptide. The spot membrane of SCR8–11, which comprises aa 446–685 of factor H, contains 115 synthetic peptides. SCR19–20 was divided into 64 peptides and comprises aa 1104–1231 of factor H. Membranes were incubated with PspC derivative SH2 (PspC2.1) and binding was detected with an anti-PspC antiserum.

Factor H facilitates adhesion of pneumococci to host cells

Factor H mediates complement control at the surface of pneumococci. To identify additional functions for the attached host regulator, we assayed the role of factor H in the adhesion of pneumococci to human cells. The adhesion of pneumococci (NCTC10319) preincubated with factor H to human epithelial cells was studied. Factor H increased the attachment of pneumococci to host cells in a dose-dependent manner. Apparently factor H mediated adherence is a general mechanism, as this effect was observed for several human cell lines including epithelial and endothelial cells (Fig. 7, A and B). These data further confirm that factor H and the SC of pIgR do not share the binding sites in the bacterial protein PspC, because the factor H effect was also demonstrated for Detroit 562 cells, which produce the pIgR.

In addition, the internalization of S. pneumoniae (NCTC10319) to human cells was quantitated. The attached bacteria were killed by antibiotics and internalized bacteria were recovered. Factor H treatment of the bacteria significantly increased the number of internalized bacteria (Fig. 7C). However, the increase in uptake was lower as compared with the increase in adherence. These results suggest that bacteria-bound factor H plays a pivotal role in adhesion and influences internalization.

The CPS of pneumococci interferes with bacterial adherence to host cells (45). To elucidate whether the CPS also affects the factor H-mediated adherence of pneumococci to host cells, the adherence of the wild-type TIGR4 was compared with that of the CPS-deficient mutant TIGR4Δcps. As observed earlier for other pneumococcal strains (45), removal of the CPS, in this case TIGR4Δcps, significantly increased the number of host cell-attached bacteria as...
compared with the encapsulated wild-type TIGR4 (Fig. 7D). Similar to our results with NCTC10319, pretreatment of the nonencapsulated TIGR4Δcps with factor H increased adherence significantly (Fig. 7D). In contrast, pretreatment of the wild-type TIGR4 with factor H was less efficient. Adherence to host cells was only slightly enhanced and the absolute values of host cell-bound pneumococci remained relatively low (Fig. 7D). These data demonstrate that the factor H-binding protein PspC is at least partially concealed below the CPS. In conclusion, the cell culture infection assays demonstrated a significant role of bacteria-bound factor H in pneumococcal adherence independent of the cell type.

Blocking of factor H-mediated pneumococcal adherence

To confirm a role of surface-attached factor H in pneumococcal adhesion via binding to the very N-terminal part of PspC, blocking experiments were performed. First, the binding of factor H to pneumococci was measured by flow cytometry in the presence of the SH3 domain of PspC or PspC SM1. Flow cytometric analysis indicated a dose-dependent binding of factor H to pneumococci (data not shown) and a competitive inhibition of factor H binding to pneumococci by PspC protein SH3, which contains the factor H-binding epitope (Fig. 8A). In contrast, the PspC derivative SM1, which represents the SC-binding R domain of PspC and lacks the factor H binding region, showed no inhibitory effect (Fig. 8A).

In cell culture infections the PspC derivative SH3 was assessed for its ability to inhibit factor H-mediated adherence of pneumococci. Host cells were infected with pneumococci that had been pretreated with a mixture of factor H and the PspC derivative SH3. The results showed that the factor H-binding domain SH3 of PspC inhibited factor H-mediated pneumococcal adhesion to host cells (Fig. 8, B and C). In conclusion, inhibition experiments confirmed the specific interaction of amino acid residues 38–158 of PspC for factor H-mediated pneumococcal adherence, which is mediated by the interaction of PspC with the SCR8–11 and the SCR19–20 of factor H.

Discussion

In this study we characterize the interaction of the host complement inhibitor factor H and the microbial PspC surface protein and demonstrate that this interaction is relevant for the adherence of the human pathogenic *S. pneumoniae* to host cells. The microbial PspC protein has one major binding site for factor H. The binding site was localized to a 121-aa-long stretch in the N-terminal region of PspC comprising amino aa 38–158 of PspC, which is oriented to the outside of the pathogen. The host regulator factor H interacts with the pneumococcal PspC protein via two regions that were localized to SCR8–11 and SCR19–20. In our peptide spot arrays, PspC epitopes were mapped to three linear binding regions within SCR8–11 and to two linear binding regions within SCR19–20. The recruited factor H controls complement activation at the bacterial surface and we demonstrate that pneumococci exploit surface-attached host regulators for adhesion to host cells. In conclusion, the PspC interacts with factor H via two separate contacts sites in the host factor H and this interaction is essential for immune evasion and host cell attachment of *S. pneumoniae*.

The PspC protein is a multifunctional bacterial protein of *S. pneumoniae* and binds at least the SC of pIgR and the immune relative to untreated pneumococci. *D*. Factor H mediated adherence of wild-type pneumococcal strain TIGR4 and its nonencapsulated mutant TIGR4Δcps to Detroit 562 nasopharyngeal epithelial cells as determined by immunofluorescence microscopy. *, *p* < 0.005; **, *p* < 0.02; relative to infections conducted in the absence of factor H.
regulator factor H (5, 6). To date, 11 subtypes of the PspC protein family have been identified that are attached via two different ways to the bacterial surface (8). Subgroup I PspC proteins have a choline-binding domain and subgroup II proteins, including Hic, are anchored in a sortase-dependent mechanism in the microbial peptidoglycan backbone via the C-terminal LPXTG motif. All identified members of the PspC protein family show sequence polymorphism and sequence variability. The identified 121-aa-long factor H binding region the PspC of subgroup I proteins shows sequence identity percentages to the subgroup II protein Hic (PspC11.4) of 34% (PspC3.3), 62% (PspC2.1), and 100% (PspC3.4), respectively. Within the N-terminal region of Hic three putative factor H-binding epitopes were identified (19, 46). The identified sequences are conserved in all sequenced variants of PspC. Recently, a 12-aa factor H binding motif was identified in the first 104 amino acids of PspC3.1 (47). However, a synthetic peptide representing this 12-aa-long stretch did not inhibit the binding of factor H to PspC3.1 (47). Similarly, a synthetic hexapeptide that was identified as the minimal SC-binding epitope did not inhibit the binding of SC to PspC (15). Thus, amino acids adjacent to the binding epitope and also the structure of the binding region seem to be pivotal for the complex formations. The identification of factor H- and SC-binding epitopes in different N-terminal domains of PspC is furthermore in accordance with data from Dave et al. (18), who demonstrated that factor H and secretory IgA bind independently to PspC. This suggests that factor H and SC bind different regions in PspC. Taken together, experimental evidence shows that the binding epitope(s) for factor H are located in the very N-terminal region of PspC, whereas the hexapeptide SC-binding site is located in the R domains of PspC.

In this study we have localized the binding region of PspC to SCR8–11 in the middle region of factor H. In addition, we have identified a second contact site for PspC in the SCR19–20 of factor H. The sensorgrams of the surface plasmon resonance studies show a stronger PspC binding of SCR8–20, which includes the two binding regions, as compared with SCR8–11, which has only one binding region. In a previous study PspC binding has been mapped to SCR6–10 and it has been suggested that the SCR13–15 contributes to the complex formation (18, 38). As for PspC, the SCR8–11 of factor H has been shown to be involved in the interaction of factor H with the Hic protein of serotype 3 pneumococci and the Bac (β protein) of S. agalactiae (46). Hic has further been shown to bind to a region outside of the middle region of factor H. This second binding site of Hic, which has a lower affinity compared with the SCR8–11 binding region, has been localized to the SCR12–14 of factor H (46). In conclusion, the strength of complex formation seems to be determined by an interaction of the different PspC variants with two contact sites in factor H. The simultaneous interactions with discontinuous peptide sequences in these two contact sites might finally result in a high avidity binding of factor H to PspC. Independent of the exact attachment points, host factor H is attached to the bacterial surface in a way such that the complement regulatory region SCR1–4 is oriented toward the outside.

Peptide mapping analysis showed the binding of factor H to discontinuous and partially homologous sequences of pneumococcal Hic and Bac of S. agalactiae. Therefore, it was suggested that the bacterial adherence molecules Hic and Bac are related (30). Similarly, the Hic and PspC proteins of subgroup I are structurally related and the localized factor H binding regions of the two proteins show sequence homology. The homology of PspC, Hic, and Bac and their recognition of peptide sequences in SCR8–11 implies a general and probably conserved strategy for factor H acquisition to the streptococcal surface.

PspC and the other related bacterial proteins use a unique region for the attachment of the host immune regulator factor H. All three proteins bind to the middle region of factor H i.e., SCR8–11. Previously, a heparin-binding domain has been localized to the SCR9 of factor H (44). As shown here, the interaction of PspC and factor H SCR8–20 is blocked by heparin, suggesting that the heparin...
binding regions in SCR9 and SCR20 of factor H mediate binding. The peptide spot assays identified three linear regions in SCR9, SCR10, and CR11 that mediate binding and include a total of 10 positively charged amino acid residues. In addition, two linear binding regions with a total of eight positively charged residues were identified in SCR20 (Fig. 6). The presence of positively charged residues in the identified linear regions of factor H is in agreement with the observed inhibitory effect of heparin.

The identified type of attachment of factor H to PspC of *S. pneumoniae* differs from that of other pathogens such as *Candida albicans*, *Aspergillus fumigatus*, *S. pyogenes*, and *Borrelia* species (20, 48). Theses pathways use SCR6–7 and SCR19–20 or a combination of both domains for the surface attachment of factor H. In contrast to these pathogenic microorganisms, pneumococci do not bind FHL-1 from human plasma and none of the PspC variants interact with FHL-1.

Pneumococci are versatile pathogens that use multiple surface proteins or the capsular polysaccharide to control complement activation at the surface. A central role for complement, and particularly for the alternative pathway of complement, has been shown for clearance of pneumococci in mouse models. Several pneumococcal proteins were identified that mediate complement control. These include PbpA (also named PhbB and BVH-11), the toxin pneumolysin, which mediates complement-mediated clearance (49), and the PspA protein, which, similarly as PspC, is a member of the choline-binding protein family. The deletion of PspA attenuates virulence and increases the complement receptor-mediated clearance of pneumococci (50). It has been suggested that PspA functions as an inhibitor of C3b deposition by controlling factor B-mediated alternative complement pathway activation (51, 52). The recruitment of factor H to the surface of pneumococci efficiently prevents the activation of C3b and the complement-mediated opsonophagocytosis of pneumococci (46). The improved survival of pneumococci expressing PspC or Hic in a systemic mouse infection model and in microglial cells provides further evidence for the importance and versatility of PspC in different host niches (29, 31). A recent study indicated that carriage isolates, which produce lesser amounts of CPS than the invasive isolates, recruit significantly more factor H than the systemic isolates (53). Simultaneously, these isolates are less able to interact with FHL-1.

In conclusion, factor H binding to pneumococci occurs via an interaction of the N-terminal part of PspC with two contact sites in factor H. This complex formation on the pneumococcal cell surface plays dual roles in pneumococcal infections. On mucosal surfaces, bacteria-bound factor H promotes adherence to host cells. Moreover, in invasive infections factor H binding to pneumococci improves survival by inhibiting complement-mediated lysis of the bacteria.

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**Disclosures**

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**References**


