Interaction of human factor H with PspC of *Streptococcus pneumoniae*

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**Background & objectives:** *Streptococcus pneumoniae* has acquired virulence factors such as the polysaccharide capsule and various surface proteins, which prevent opsonization mediated by the complement system. PspC is one of the multi-functional pneumococcal surface proteins capable of eliciting an antibody response in mice. Our study further explores the role of pneumococcal surface proteins in resistance to complement mediated opsonophagocytosis by providing evidence that PspC binds human Factor H (FH), a regulatory protein of the alternative complement pathway. The present study was carried out to map the binding regions on PspC and FH, and to assess the functional activity of FH upon binding to PspC.

**Methods:** FH binding to D39 and other pneumococcal strains was observed by flow cytometry. A series of FH truncated and deletion mutants and PspC mutants were used to localize binding regions within these molecules. The functional activity of FH upon binding to PspC was measured by a haemolysis assay.

**Results:** FH binding to D39 and not to TRE108 (PspC-) cells was demonstrated by flow cytometry. Pneumococcal isolates of 14 different strains varied in their ability to bind FH. The binding region of FH within PspC to the first 225 amino acids of the α-helical domain was localized. The corresponding binding site for PspC is located within the SCR 6-10 region of FH. Haemolysis of rabbit red blood cells was inhibited by FH even in the presence of PspC.

**Interpretation & conclusion:** FH binding is specific to PspC on the pneumococcal cell surface. The binding region on PspC mapped to the non-conserved N-terminal region of the α-helical domain. The binding site on FH to PspC is different from the active site that functions in degradation of C3b. A haemolysis assay provided evidence that the functional activity of FH was maintained upon binding to PspC. Thus, binding of FH to PspC might be an important mechanism by which *S. pneumoniae* resist complement activation and opsonophagocytosis.

**Key words** Complement - factor H - PspC - *Streptococcus pneumoniae*

The alternative complement pathway functions as a nonspecific defense system against microbial pathogens.

The pathway is initiated by deposition of C3b on the surface of the invading organism resulting in either lysis, neutralization, or phagocytosis (Fig. 1). Host cells are protected from the complement cascade by cell surface molecules and complement regulatory proteins. One regulatory protein is Factor H (FH) which functions in rapid degradation of bound C3b on the host cell surface.

FH is a 150-kDa single glycoprotein that is found in the plasma at a concentration of about 400 mg/l. The protein is composed of 20 short consensus repeat domains (SCRs) each containing approximately 60 amino acid residues that fold into β-strands and sheets. The
The functional properties of FH depend on the interaction of individual SCR domains with each other. Microorganisms have evolved various mechanisms of escaping the alternative pathway by minimizing deposition of activated C3b on their surface. One such strategy involves expression of receptors that bind FH to the microbial surface. M6 protein of group A streptococci, YadA of Yersinia enterocolitica, Por1A of Neisseria gonorrhoeae, CRASP1 of Borrelia burgdorferi, and Hic of type 3 Streptococcus pneumoniae all bind FH. We recently demonstrated that PspC of S. pneumoniae also binds FH.

PspC, also known as CbpA and SpsA, is similar in structure to another pneumococcal surface protein, PspA. It consists of an N-terminal α-helical domain, a proline-rich domain, and a choline-binding domain. The α-helical domain of PspC is larger (50 to 100%) than the α-helical domain of most PspAs. The PspC choline binding and proline-rich domains are over 90% homologous to the corresponding domains of PspA. Due to structural similarity, it is possible that the antibodies directed to the proline-rich or choline binding domains cross-react with both PspA and PspC molecules.

PspC is a potential virulence factor of S. pneumoniae that functions by interacting with various components of the immune system. PspC binds to the secretory component of immunoglobulin A. It may also inhibit the complement system by binding to either the C3 protein or components on the surface of activated human epithelial and endothelial cells. The focus of our research is the interaction of FH with PspC.

In this study, we localized the binding sites on both FH and PspC. The PspC segment that binds FH was localized to amino acids 1 to 225 in the α-helical domain of PspC. The corresponding binding site for PspC was located within the SCR 6-10 region of FH. The functional activity of FH in the presence of PspC was also assessed using a haemolysis assay.

Material & Methods

Bacterial strains, growth conditions, and cell lysates: The pneumococcal strains used in this study for flow cytometry and Western blot analysis were cultured as previously described. The pneumococci were grown on blood agar at 37°C in 5% CO2 overnight and transferred to Todd-Hewitt broth with 0.5% yeast extract. The bacteria were harvested at late mid-log phase by centrifugation and suspended in phosphate-buffered saline (PBS; pH 7.2) for flow cytometry. The bacterial concentration (approximately 1 x 10^8 cfu/ml) was estimated by spectrophotometer (A600) and confirmed by viable counts on blood agar plates. For Western blot analysis, pneumococcal cell lysates were prepared as described previously and stored at -20°C until use. Escherichia coli Y1090 was grown in Luria-Bertani medium and used to prepare a control lysate.

Flow cytometry: FH purified from human serum was biotinylated as described previously using the EZ-Link sulfo-NHS-LC-biotinylation kit (Pierce, Rockford, IL). The bacteria in 100 µl of 1% bovine serum albumin-PBS were incubated with 100 µl of biotinylated FH (1/30) for 1 h at 37°C. The bacteria were washed with 1 ml PBS three times and suspended in 100 µl of fluorescein isothiocyanate-streptavidin (Southern Biotechnology Associates, Birmingham, Ala) for 30 min at room temperature. The cells were washed as before, suspended in 2 ml of PBS, and analyzed by FACScan cytometer (Beckton Dickinson, USA).

Cloning and expression: Chromosomal DNA was isolated from LM91 (PspA-), an isogenic strain of D39 by DNeasy Tissue Kit (Qiagen Inc., Valencia, CA). Oligonucleotide primers LSM370 (5’ C A C C C C G C G

Fig. 1. Activation of the alternative pathway of complement in response to a pathogen. The pathway is activated by spontaneous hydrolysis of C3 to C3b in the fluid-phase. Deposition of C3b on the target leads to the formation of C3bBb, also known as the C3 convertase. The C3 convertase functions to increase deposition of C3b on the activated-target surface resulting in opsonization and phagocytosis of the pathogen by macrophages and neutrophils.
A C A G A G A A C G A G G G A A G T A C3') and LSM371 (5'C T A G G A T G A G C T T G G A A G A G T 3') were used in a polymerase chain reaction (PCR) to amplify a 700 bp fragment, which encodes amino acids (aa) 1 to 255 of the α-helical region of PspC. The PCR fragment was cloned into TOPO TA cloning Version L (Invitrogen, Carlsbad, CA) and sub-cloned into ProEx C for expression (Life Technologies, Rockville, MD). The ProEx vector produces a fusion protein with a six-histidine tag at the N-terminus of the expressed protein. Expression was induced by the addition of 60 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to exponentially growing E. coli TOP10 cells. Two other fragments of PspC (aa 1 to 445 and aa 255 to 445) from D39 (kindly provided by A. Brooks-Walter) were also expressed as fusion proteins with a six-histidine tag in the pET20b expression vector (Novagen, Madison, Wis.) as described previously14. The His-tagged proteins were column purified, and the purity of the proteins was confirmed by SDS-polyacrylamide gel electrophoresis.

Western analysis: Western blot analyses were performed as previously described10. Purified PspC fragments and D39 lysate were separated on 4-20 per cent tris-glycine pre-cast gels (BioWhittaker Molecular Applications, Rockland, ME) and transferred to nitrocellulose membrane. The membranes were incubated with either biotinylated FH or polyclonal anti-PspC antibody14. Bound antibodies and FH were detected using streptavidin-conjugated horseradish peroxidase and a chemiluminescent substrate (Pierce, Rockford, Il).

Ligand dot blot: For ligand dot blotting, a procedure similar to the one described for interaction of FH with M protein20 was used. Briefly, 5 µg of purified PspC protein (aa 1 to 445), albumin, and E. coli TOP10 cells were dried onto a nitrocellulose membrane (Millipore Corporation, Bedford, MA ) at 37°C for 30 min. After blocking in 1 per cent BSA-PBS overnight at 4°C, the membranes were incubated with 0.3 µg of full-length FH or recombinant FH representing specific deletions of the twenty SCR domains that were cloned and expressed as previously described21 for 1 h at room temperature. The membrane was further incubated with goat anti-FH antibody (Quidel, Mountain View, CA) followed by rabbit anti-goat-biotinylated antibody (Southern Biotechnology Associates, Birmingham, Ala) before detection with streptavidin-conjugated horseradish peroxidase. Bound FH was visualized using a chemiluminescent substrate (Pierce, Rockford, Il).

Haemolysis assay: The inhibition of the alternative pathway mediated by FH was examined by a haemolysis assay9,22. C2-deficient serum (1/12 dilution) (Advanced Research Technology, San Diego, California) was used as a source of complement and rabbit red blood cells (RBCs) (5 x 109) (Advanced Research Technology, San Diego, California) were used as target cells. A dilution of serum was used that resulted in 80 per cent lysis of target cells following incubation at 37°C for 40 min. Subsequently, C2-deficient serum with or without recombinant purified PspC (30 mM), FH (15 µM), or a pre-incubation mixture of PspC and FH (2:1 molar ratio) in 200 µl of veronal-buffered saline (VBS) with 13.5 mM EGTA, Mg2+ and 0.1 per cent gelatine was incubated for 5 min at 37°C. The rabbit RBCs (50 µl) were added to the incubation mixture. The reaction was stopped with 750 µl of cold VBS, 10 mM EDTA at 5, 10, 20, and 40 min intervals. After centrifugation at 3000 rpm for 5 min, the supernatants were removed to measure the amount of haemoglobin released by spectrophotometer at 412 nm.

Fig. 2. Binding of FH to exponentially growing capsular type 2 S. pneumoniae (a) TRE108 (PspC-) compared to (b) D39 (PspC+) by flow cytometry. The bacteria were incubated with or without biotinylated FH and stained with fluorescein isothiocyanate-streptavidin. FH binding was measured by FACScan cytometer as change in fluorescent intensity in comparison to the cells incubated without FH.
Results

Binding of human FH to exponentially growing pneumococci: S. pneumoniae cells of various isolates were incubated with human FH, suspended in FITC-conjugated streptavidin, and examined by FACScan cytometer. Change in mean fluorescence intensity was observed upon incubation of wild-type D39 cells with FH (Fig. 2b). TRE 108 cells, an isogenic mutant of D39 that does not express PspC, did not bind FH (Fig. 2a). Variations in FH binding among the different pneumococcal isolates were also observed by flow cytometry (Fig. 3). These results were consistent with previous data obtained by Western analysis and immunofluorescence microscopy (unpublished data).

Mapping of the FH binding region on PspC: Western blotting was used to determine the region of PspC, which binds FH. Purified recombinant PspC proteins expressing either full-length or portions of the α-helix domain from D39 or its isogenic strain LM91 were incubated with biotinylated FH. Cell lysates from strain D39 and E. coli were used as positive and negative controls, respectively. FH bound to the PspC (aa 1 to 445), and PspC (aa 1 to 225) fragments from D39 and LM91, respectively (Fig. 4). PspC (aa 255 to 445) fragment from D39 lacked this binding activity. These results indicated that the FH binding region was located between amino acids 1 to 225 of PspC.
Localization of the FH binding region to PspC:
The localization of PspC binding site on FH was determined by a ligand dot blotting assay. PspC immobilized on a nitrocellulose membrane was incubated with either full-length FH or recombinant truncated proteins with specific deletions in the 20 SCR domains. BSA and E. coli lysate were used as negative controls. The membranes were incubated with either full-length FH or FH with specific deletions in the 20 SCR domains and detected with anti-FH antibody.

Functional activity of FH in the presence of PspC:
We examined the functional activity of FH in the presence of PspC using a haemolysis assay with C2 deficient serum and rabbit RBCs. The concentration of C2-deficient serum needed for approximately 80 per cent haemolysis of rabbit RBCs after 40 min was determined by preliminary experiments. Following incubation, 75.0 ± 7.64 per cent of rabbit RBCs were lysed by the C2-deficient serum. We observed 69.3 ± 8.9 per cent haemolysis of rabbit RBCs by the C2-deficient serum in the presence of PspC. As expected, the lysis was greatly inhibited (only 15 ± 0.58%) upon addition of purified FH to the C2-deficient serum (Table). Moreover, the inhibition of haemolysis of rabbit RBCs was maintained upon addition of a pre-incubated mixture of purified FH and PspC to the C2-deficient serum (12.0 ± 0.58%) suggesting that PspC bound FH is functional.

Discussion

PspC is a potential virulence factor of S. pneumoniae that functions by interacting with various components of the immune system. Recently, we showed that PspC binds to the major regulatory protein of the alternative pathway of complement, FH. In this investigation, we further confirmed the binding of FH to exponentially growing D39 and other pneumococcal strains by flow cytometry. We also mapped the binding sites on both PspC and FH with recombinant truncated mutant proteins. Moreover, we provided evidence that FH activity is maintained in the presence of PspC in a haemolysis assay. The interaction of PspC with FH might play an important role in prevention of opsonization by minimizing C3b deposition on the pneumococcal surface.

FH bound to the surface of D39 cells whereas the isogenic mutant strain of D39, TRE108 (PspC-) did not

Table. Haemolysis of rabbit red blood cells by alternative pathway of complement in the presence of FH and PspC

<table>
<thead>
<tr>
<th>rRBC</th>
<th>Time (min)</th>
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<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Serum</td>
<td>14.00 ± 0.00</td>
</tr>
<tr>
<td>Serum and PspC</td>
<td>13.67 ± 0.88</td>
</tr>
<tr>
<td>Serum and FH</td>
<td>12.50 ± 0.76</td>
</tr>
<tr>
<td>Serum and PspC-FH</td>
<td>12.53 ± 0.78</td>
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* Data expressed as geometric mean of per cent haemolysis of rabbit red blood cells ± standard error of the mean from three experiments
bind FH. This suggests that FH binding is specific to PspC on the surface of live D39 cells. As mentioned before, PspC shares over 90 per cent homology with PspA in the proline and choline binding domains. Since TRE108 expresses PspA but not PspC, FH failing to bind to this strain further implies that the binding region is present in the α-helical domain of PspC that does not share homology with PspA. It has been reported that the interaction between FH and PspC maybe hydrophobic since binding was not inhibited in the presence of increasing concentrations of salt.

PspC is found on approximately 75 per cent of pneumococcal strains. Recently, the polymorphic pspC locus of 43 different pneumococcal strains was characterized by DNA sequencing of PCR fragments. The majority (approximately 86%) of strains tested contained single copy while some strains (14%) had two tandem copies of the PspC gene. The DNA sequence of PspC varied in each of the 43 strains of pneumococci. The PspC proteins were placed in 11 different groups according to variations in the amino acid sequence. The C-terminal end of the PspC variants contained either the choline-binding domain or the LPXTG motif common to many Gram positive bacteria. This ability to express and use two different anchorage domains may be unique to PspC of S. pneumoniae. The LPXTG motif was present in PspC of type 3 pneumococcus and in the second protein of strains with two alleles of PspC.

The binding of FH to exponentially growing pneumococci varied among the 14 different isolates tested by flow cytometry. These observations were similar to previous studies in that there was no correlation between the intensity of FH binding and clade type of PspC or pneumococcal capsular type. It is well established that FH binds to a PspC variant, Hic, that contains an LPXTG motif in type 3 pneumococcus. However, it is not clear whether differences in FH binding are due to the highly variable nature of PspC among the different strains or presence of other FH binding molecules on the pneumococcal surface.

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**Fig. 6.** Model of the mechanisms of inactivation of the alternative pathway by FH. (A) A diagramme of FH showing the 20 SCR domains along with the three C3b (shaded regions) and the PspC binding sites. The active site on FH involved in degrading C3b or the C3b convertase is contained within the first four SCR domains. Note that the binding region for PspC is different than the active site for complement regulatory function. (B) Three different mechanisms by which FH has been shown to decrease the amount of C3b deposited on host cell surface. B1: FH binds to C3b on host cell and serves as cofactor for cleavage of C3b by FI. B2: FH functions in dissociation of Bb from C3bBb (C3 convertase) upon binding to C3b. B3: The presence of molecules such as sialic acid can result in preferential binding of FH than B to C3b on host cell surface. The effect on the activation of the alternative pathway may be similar upon binding of FH to PspC on the pneumococcal cell surface.
To further study this interaction, we mapped the binding sites on FH and PspC. The FH binding was localized to amino acids 1 to 225 of PspC. This region was located within the large α-helical domain of the molecule. According to sequence analysis, variations in PspC have been attributed to the α-helical domain. The first 100 to 150 amino acids of α-helical domain are hypervariable in both size and sequence. The hypervariable region is followed by the first set of direct repeats ranging from 101 to 205 amino acids depending on the pneumococcal strain. Smaller amino acid repeats found in some strains are due to the lack of a sequence at the N-terminal end found in many strains with larger amino acid repeats. This region of the molecule is highly charged consisting of either lysine and glutamic acid residues. The binding of FH is also localized within the first 261 amino acids of Hic in type 3 pneumococci. However, Hic did not inhibit binding of FH to PspC suggesting that both molecules are binding to unique sites on FH. The binding site for the secretory component of IgA is within the hexapeptide YRNYPT between amino acids 198 and 203 on PspC. It will be important to determine if FH or secretory IgA interferes with the binding of either molecule to PspC.

We localized PspC binding site on FH to SCR 6-10 domains. Other studies mapped the binding site on FH to be within SCR 8-15 for PspC and SCR 8-11 for Hic of *S. pneumoniae*. These findings are similar to studies involving interactions of FH and FHL-1/reconnectin with M protein of the group A streptococcus. The M protein binding site in FH was first mapped within SCR 6-10. The binding of M protein since then is precisely localized to SCR 7 of human FH. The complement resistant strains of *Borreliae* also consist of two surface proteins, CRASP-1 and CRASP-2, that bind to SCR 5-7 of human FH and FHL-1/reconnectin, respectively.

There are three sites on FH that bind C3b (Fig. 6A). Complement regulatory activity due to C3b binding is directly attributed to only the first four SCR domains of FH. Thus, the binding of FH to PspC within SCR 6-10 implies that the active site is still available to bind C3b and inhibit complement activation.

Our data from the haemolysis assay provided evidence that FH was active upon binding to PspC. The regulatory activity of FH was also maintained after binding to Hic of type 3 pneumococci, Yad A of *Y. enterocolitica*, and CRASP-1 of *B. burgdorferi*. These proteins can control C3b degradation on or near the bacterial surface upon attachment since the binding site to the bacteria (SCR 5-7) and active site for C3b inhibition (SCR 1-4) are in separate regions within the molecule.

The spontaneous hydrolysis of C3b in the fluid-phase of serum can also result in deposition of C3b on host cell surfaces. However, progression of the complement cascade is prevented on host cells at early stages of activation by complement regulatory molecules on the cell or in the serum. The three mechanisms of complement inactivation on host surface by FH are presented in Fig. 6B. FH regulates the alternative complement pathway by serving as a cofactor for cleavage of C3b by factor I, dissociating the C3bBb complex, or competing with factor B for binding to C3b. The decrease in deposition of C3b on the cell surface leads to inactivation of the alternative pathway.

The binding of FH to PspC may be one of the mechanisms by which C3b deposition is also controlled by pneumococcus to prevent complement attack. The mechanisms for C3b degradation on the pneumococcal surface may be similar to the host upon binding of FH to PspC (Fig. 6B). Thus, the interaction of FH with PspC may be an important mechanism by which pneumococci escape opsonophagocytosis. It may also further explain the variations in virulence among the different pneumococcal strains.

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References


13. McDaniel LS, McDaniel DO, Hollingshead SK, Briles DE. Comparison of the PspA sequence from *Streptococcus pneumoniae* EF5668 to the previously identified PspA sequence from strain Rx1 and ability of PspA from EF5668 to elicit protection against pneumococci of different capsular types. *Infect Immun* 1998; 66: 4748-54.


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