"Streptococcus pneumoniae" recruits complement factor H through the amino terminus of CbpA*
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"Streptococcus pneumoniae", a human pathogen, is naturally capable of colonizing the upper airway and sometimes disseminating to remote tissue sites. Previous studies have shown that S. pneumoniae is able to evade complement-mediated innate immunity by recruiting complement factor H (FH), a complement alternative pathway inhibitor. Pneumococcal binding to FH has been attributed to choline-binding protein A (CbpA) of S. pneumoniae and its allelic variants, all of which are surface-exposed proteins. In this study, we sought to determine the molecular basis of CbpA-FH binding interaction. Initial deletional analysis of the CbpA protein in strain D39 (capsular serotype 2) revealed that the amino (N)-terminal region of 89 amino acids in the mature CbpA protein is required for FH binding. Immunofluorescence microscopy analysis showed that this region of CbpA is also necessary for FH deposition to the surface of the intact pneumococci. Moreover, recombinant proteins representing the 104 amino acids of the N-terminal CbpA alone was sufficient for high affinity binding to FH (K<sub>D</sub><1 nM). The FH-binding activity was finally localized to a 12-amino acid motif in the N-terminal CbpA by peptide mapping. Further kinetic analysis suggested that additional amino acids downstream of the 12-amino acid motif provide necessary structural or conformational support for the CbpA-FH interaction. The 12-amino acid motif and its adjacent regions contain highly conserved residues among various CbpA alleles, suggesting that this region may mediate FH binding in multiple pneumococcal strains.

"Streptococcus pneumoniae", a Gram-positive bacterium, is a natural resident of the human upper airway. It is also an important human pathogen that causes a wide spectrum of bacterial infections including community-acquired pneumonia, otitis media, meningitis, bacteremia, and sinusitis (1). In healthy human adults, pneumococcal colonization can last weeks or months without apparent illness (2). Epidemiological studies suggest that 20-50% of healthy individuals may carry pneumococci at any given time (2), demonstrating that S. pneumoniae is capable of withstanding host defense mechanisms. Both innate and adaptive immune responses are critical for limiting and clearing pneumococcal infections (1). Complement-mediated phagocytosis is a major innate immune mechanism against pneumococci (3,4). The products of activated complement bound on pneumococci enhances phagocytosis by binding to complement receptors on professional phagocytes (5). Internalized pneumococci within phagocytes are killed in phagolysosomes (6). Consistent with the key role of the complement system in bacterial clearance, recent studies have indicated that S. pneumoniae is able to evade complement activation. Pneumococcal surface protein A (PspA) has been shown to inhibit complement activation in mouse models, likely by blocking recruitment of the alternative pathway (4). Mice infected with PspA mutants showed significantly attenuated levels of colonization and bacteremia (7). Pneumolysin, a major pneumococcal toxin, is able to deplete complement by activating the classical complement pathway (8,9). Pneumolysin-deficient strains of S. pneumoniae showed reduced virulence in mice (10). Recent studies suggest that the pneumococci are also able to evade complement-mediated phagocytosis by recruiting complement factor H (FH) (11,12).
Choline binding protein A (CbpA) of *S. pneumoniae* has been demonstrated as the FH binding factor (13,14).

CbpA, also known as PspC (15), SpSA (16), Hic (13), or C3 binding protein (17), is expressed at the surface of *S. pneumoniae* via its C-terminal choline-binding domain (18). All virulent strains of *S. pneumoniae* tested thus far contain the *cbpA* locus (19,20). Genetic disruptions of CbpA expression in various pneumococcal strains led to significant loss of nasopharyngeal colonization, lung infection, and bacteremia in mice and rats (18,21-23). These observations indicate CbpA as an important virulence factor. The precise mechanisms of CbpA action in pneumococcal survival in vivo and pathogenesis are not completely understood. There are considerable sequence variations among *cbpA* allelic variants from various *S. pneumoniae* strains. Based on sequence diversity, Iannelli et al. have divided 43 *cbpA* variants into 11 major types (20). CbpA of strain D39 (capsular serotype 2) is composed of an N-terminal signal sequence for protein secretion (38 amino acids) and a mature portion of the protein (663 amino acids) (18). The C-terminal region of mature CbpA represents a choline-binding domain consisting of 10 tandem repeats of 20 amino acids. The N-terminal region is predicted to contain six alpha-helical structures, consisting of an N-terminal domain (96 amino acids), two direct repeats R1 and R2 (107 amino acids each), and a proline-rich linker (97 amino acids) (19,24). The R1 and R2 repeats are capable of independent binding to domains 3 and 4 of human polymeric immunoglobulin receptor (pIgR) (24-27). Binding interaction between CbpA and human pIgR has been shown to enhance pneumococcal adhesion to and invasion of cultured respiratory epithelial cells. Because the extracellular domains of pIgR are naturally present in mucosal secretions as free secretory component (SC) or as a component of secretory IgA (SIgA), the R1/R2 region of CbpA also binds to SC and SIgA (16,27).

FH is a 155-kDa plasma glycoprotein, which is composed of 20 short consensus repeats (SCR) (28). Each of the SCR shares high levels of sequence similarity. FH inhibits the alternative pathway of the complement system by preventing the binding of factor B to C3b, enhancing the decay of the C3-convertase (C3bBb) and acting as a cofactor for the cleavage of C3b by complement factor I (29). Deposition of FH on host tissue and cellular surfaces prevents non-specific damage and avoids the wasteful consumption of complement components (29). Interestingly, many microbial pathogens have been shown to bind to FH as a common mechanism for evasion of complement-mediated host immunity (28,30,31). This is exemplified by FH binding to *Borrelia burgdorferi* (32,33), *Neisseria gonorrhoeae* (34,35), *Streptococcus pyogenes* (36), and *Streptococcus agalactiae* (37,38). CbpA and its allelic variants have been identified to bind to SCR 8-11 (11), 13-15 (39), and 6-10 (40) of FH. Previous studies have located the FH-binding activity within the first 225 amino acids of CbpA (strain D39 of type 2) (41) and the first 223 amino acids of Hic (a CbpA variant in strain A66 of type 3) (13,38). To gain further understanding of the biology of pneumococcal interactions with the host innate immune factors, we attempted to identify the FH binding motif on CbpA in this work. Our data have localized the FH binding activity within a 12-amino acid region in the N-terminal domain of CbpA from *S. pneumoniae* strain D39.

### EXPERIMENTAL PROCEDURES

**Bacterial strains and chemical reagents** - All *S. pneumoniae* strains used in this work are listed in Supplement Table 2. Strains D39 (serotype 2) and TIGR 4 (serotype 4) were used as the parental strains from the American Type Culture Collection (ATCC) (Manassas, VA). Pneumococci were routinely grown in Todd-Hewitt broth containing 0.5% yeast extract (THY) or on tryptic soy agar plates containing 3% (v/v) sheep blood. When necessary, kanamycin (200 µg/ml) or streptomycin (150 µg/ml) was included in the broth and agar media for selection purposes. *Escherichia coli* strain DH5α was used for routine cloning; strains M15 and BL21 were used for expression of recombinant CbpA polypeptides. *E. coli* cultures were grown in Luria-Bertani (LB) broth or on LB agar plates. *E. coli* strains harboring the protein expression vectors were selected on LB medium containing ampicillin at a final concentration of 100 µg/ml. All ingredients for bacterial culture media and other chemicals used in this work were
obtained from Sigma (St. Louis, MO) unless otherwise stated.

DNA electrophoresis and Southern hybridization - DNA cloning and manipulations were performed according to standard methods (42). *S. pneumoniae* genomic DNA was prepared by the phenol-chloroform extraction method as described previously (43). Restriction enzyme-digested DNA fragments were separated by agarose gel electrophoresis. All restriction enzymes and DNA standards were purchased from New England Biolabs (Beverly, MA). DNA bands were visualized by staining agarose gels with ethidium bromide (2 µg/ml). For Southern hybridization, DNA was blotted to Hybond-N+ nylon membranes by the alkaline transfer method (42). Briefly, agarose gels containing DNA fragments were soaked in 0.25 M HCl for 10 min to facilitate DNA transfer. The DNA fragments were transferred overnight to a Hybond-N+ nylon membrane (Amersham, Arlington Heights, IL) with 0.4 N NaOH. Following transfer, the membranes were neutralized with 0.5 M Tris-HCl (pH 7.2) for 5 min, rinsed in 2X SSC (1X SSC: 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), and air dried. DNA blots were reacted with digoxigenin (DIG)-dUTP labeled probes according to the supplier’s instructions. DNA probes were prepared using a PCR DIG Probe Synthesis Kit as described by the supplier (Roche, Indianapolis, IN). Pre-hybridization was performed for 1 h at 50 °C in a DNA Hybridization Buffer (Roche). Labeled PCR products (25 to 50 ng) were denatured by boiling for 5 min and added to the DNA Hybridization Buffer. After hybridization overnight at 50 °C, the membranes were sequentially washed (15 min per wash) with 1X SSC containing 0.1% SDS at 50 °C and 0.5X SSC containing 0.1% SDS at 60 °C. Hybridization was detected with a DIG luminescent detection kit (Roche) at room temperature as follows. The membranes were washed with the washing buffer (0.1 M maleic acid, 0.15 M NaCl, and 0.3% Tween-20; pH 7.5) for 2 min, incubated with 1% blocking reagent in 0.1 M maleic acid and 0.15 M NaCl (pH 7.5) for 30 min, and reacted with a 1:5,000 dilution of the anti-DIG antibody for 1 hr. After washing three times with the washing buffer (10 min per wash), the membranes were treated with the CSPD substrate and hybridized DNA bands were visualized using a Futura 2000K Automatic X-ray Film Developer (Fischer Industries, Geneva, IL). Sizes of DNA fragments were estimated based on DIG-labeled DNA molecular weight standards (Roche).

Construction of *S. pneumoniae* mutants - The *cbpA*-null and truncated mutants of *S. pneumoniae* were generated in the streptomycin-resistant derivatives of strains D39 and TIGR4 by allelic replacement with the counter selectable Janus cassette as described previously (44). Streptomycin-resistant pneumococcal strains were prepared by transformation with the recessive *rpsL* allele *rpsL1* (or *str1*). The *rpsL1* allele (45), conferring streptomycin resistance in the absence of the wild type *rpsL* + allele, was amplified by PCR from chromosomal DNA of strain CP1200 (46) (a gift from Donald A. Morrison) using primers Pr387/Pr388. The Janus cassette consists of a kanamycin-resistance gene and a dominant wild type *rpsL* + allele encoding protein S12 of the small ribosomal subunit, a target for streptomycin (44). To construct *cbpA*-null mutants in strains D39 and TIGR4, the upstream and downstream sequences flanking the *cbpA* coding region were used to target the precise deletion sites by double-crossover homologous recombination.

The upstream (1,314 base pairs, bp) and downstream (1,145 bp) *cbpA* flanking regions in strain D39 were separately amplified from genomic DNA preparations by polymerase chain reaction (PCR) using primer pairs Pr252/Pr313 and Pr314/257, respectively (see Supplement Table 1 for primer sequences). Despite sequence diversity in the *cbpA* coding regions of various pneumococcal strains, the flanking sequences of the *cbpA* locus are highly conserved including the 5’ (78 bp) and 3’ (229 bp) intergenic regions (20). We thus amplified the *cbpA* flanking sequences of strains D39 and TIGR4 by using the same primer pairs. PCR amplifications were performed using conditions as described previously (47). To minimize the possibility of introducing sequence errors during PCR amplifications, a high-fidelity DyNAzyme EXT DNA polymerase (MJ Research, Waltham, MA) was used for all PCR amplifications in this study. All of the primers were commercially synthesized by Invitrogen (Carlsbad, CA). The Janus cassette was amplified by PCR from chromosomal DNA of *S.
pneumoniae strain CP1296 using primers Pr311/Pr312. The primers were designed based on GenBank accession AF411920. Strain CP1296 contains a chromosomal insertion of the Janus cassette (44), and was kindly provided by Donald A. Morrison. The PCR products of the Janus cassette and the cbpA flanking sequences were digested by appropriate restriction enzymes as determined by the nested restriction sites (Asc I and Fse I) at the 5′ end of the primers, purified from agarose gels using a DNA Gel Purification Kit (Qiagen, Valencia, CA), and ligated using a Quick Ligation Kit (New England Biolabs). The ligation mixtures were used to transform streptomycin-resistant D39 (strain ST594) or streptomycin-resistant TIGR4 (strain ST630) by natural transformation as described previously (48). The transformants were selected for resistance to kanamycin (150 µg/ml) on blood agar plates. Kanamycin-resistant colonies were chosen to detect the loss of the entire cbpA coding region and the presence of the Janus cassette in the cbpA locus by PCR amplification, DNA sequencing, and Southern hybridization.

In-frame deletions in the cbpA gene of strain D39 were generated by allelic exchange at the background of the cbpA-null mutant strain ST588 as described previously (44). DNA segments flanking the deleted sequences of the cbpA gene were initially amplified by PCR using primers listed in Supplement Table 1 and illustrated in Fig 1A. Primers were designed to create desirable in-frame deletions according to the complete genome sequence of strain R6, an unencapsulated derivative of strain D39 (accession AE008564) (49). PCR products of the initial PCR amplifications were subsequently joined together by overlap extension PCR (50). The final PCR products were purified from agarose gels and directly used to transform strain ST588; transformants were selected for streptomycin-resistant clones (200 µg/ml). The resulting strains became resistant to streptomycin and sensitive to kanamycin due to the loss of the Janus cassette (44). The sequences of the resultant cbpA alleles were further characterized by PCR amplification, Southern hybridization, and DNA sequencing using chromosomal DNA preparations of S. pneumoniae strains. The DNA sequence analysis was performed using the DNASTAR Lasergene v6.1 (Madison, WI).

Recombinant protein expression - The recombinant proteins CbpA1, CbpA2 and CbpA4 were expressed in the pQE30 vector and purified as six-histidine (His)-tagged proteins in E. coli as described previously (24). A similar strategy was initially used to express CbpA7 as a His-tagged protein, but the construct with a correct sequence configuration did not yield detectable CbpA7 after appropriate induction with IPTG of the E. coli strain M15 containing the plasmid construct. CbpA7 was subsequently expressed as a fusion protein with the glutathione S transferase (GST). Specifically, the 1089-bp DNA segment encoding the CbpA7 region was amplified from genomic DNA of strain D39 by PCR using primers Pr569/Pr604. The PCR product was cloned into the Bam HI/Eco RI sites of the pGEX-2T expression vector (Pharmacia, Piscataway, NJ) to produce a GST fusion protein (designated GST-CbpA7) in E. coli strain BL-21 (DE3) according to the supplier’s instructions. The sequence of the insert was verified prior to use for protein expression. The full CbpA4 polypeptide and its smaller truncates (CbpA8-10) were also expressed as the GST fusion constructs in the pGEX-2T vector in the same manner. The coding sequences of CbpA4, CbpA8, and Cbp9 were amplified by PCR using genomic DNA of strain D39 and primer pairs Pr105/Pr644, Pr105/Pr645, and Pr643/Pr645, respectively. The coding DNA sequence for CbpA10 was constructed to delete the FH binding motif by a two-step PCR amplification method. The upstream and downstream coding regions flanking the FH binding motif were first separately amplified from the genomic DNA of strain D39 with primer pairs Pr105/Pr641 and Pr642/Pr644, respectively. The two PCR products were subsequently joined together by overlap extension PCR using primers Pr105 and Pr644 (50).

Western blot and antibodies - Western blot was performed essentially as described previously (25). Briefly, cell lysates and purified proteins were boiled for 5 min in standard SDS-PAGE gel loading buffer in the presence of reducing agent β-mercaptoethanol and subjected to electrophoresis in 10-20% Tris-Tricine SDS-PAGE gels. Proteins were electro-transferred to polyvinylidene difluoride (PVDF) membranes (Millipore,
Bedford, MA). CbpA expression was detected with a rabbit antiserum for the full-length CbpA as described previously (24). The blots were blocked with 5% milk (w/v), reacted with the anti-CbpA antibody (1:5,000 dilution), washed three times in phosphate-buffered saline (PBS), and reacted with peroxidase-conjugated goat anti-rabbit IgG antibody (1:5,000 dilution) (BioRad, Hercules, CA). For analysis of FH binding, the protein blots were incubated with purified human FH at a final concentration of 0.4 µg/ml (Sigma) overnight at 4 °C, followed by extensive washing with PBS and reaction with goat anti-human FH antibody (1:2,500 dilution) (Calbiochem, San Diego, CA). The blots were finally incubated with peroxidase-conjugated rabbit anti-goat IgG antibody (1:5,000) (BioRad). S IgA binding of pneumococci was measured in a similar manner with the exception of using purified human S IgA from Sigma (0.4 µg/ml), rabbit anti-human SC (Biomed, Foster City, CA) (1:2,000 dilution), and peroxidase-conjugated goat anti-rabbit IgG antibody (1:5,000). Reactive protein bands were visualized by an enhanced chemiluminescence (ECL) Western Blot Kit (Pierce, Rockford, IL) according to the supplier’s instruction.

**Epi-immunofluorescence microscopy** - Fluorescence staining of live pneumococci was carried out essentially as described previously (51). Bacterial cultures were grown in THY broth to exponential phase (OD600=0.3-0.4) and centrifuged at 1,800 x g for 10 min. The bacterial pellets were washed three times in PBS and resuspended to a final density of approximately 5x10⁸ CFU/ml in PBS. To detect CbpA expression, aliquots (100 µl each) of the bacterial suspension were mixed with the rabbit anti-CbpA serum (1:100 dilution) for 30 min at room temperature, followed by incubating with FITC-conjugated goat anti-rabbit IgG antibody (1:100 dilution in PBS) (Pierce). Each incubation step was followed by three washes in PBS (centrifugation at 1,500 x g for 5 min and resuspension of bacterial pellets in the same volumes of PBS). Similar procedures were used to detect FH binding to live pneumococci. Aliquots (100 µl each) of the above bacterial suspension (~5x10⁸ CFU/ml) were mixed in subsequent order with purified human FH (10 µg/ml), goat anti-rabbit IgG antibody (1:100 dilution) (Pierce), and FITC-conjugated rabbit anti-goat IgG antibody (1:100 dilution) (Pierce). The amounts of C3 and iC3b on pneumococcal surfaces were also determined in a similar manner. Pneumococci were incubated with 10% fresh normal human serum for 20 min at 37 °C. After three washes with PBS, the bacteria were reacted with mouse monoclonal antibody against human C3 (1:100 dilution) (GeneTex, San Antonio, TX) or human iC3b (Quidel, San Diego, CA) for 1 hour at room temperature. The pneumococci were subsequently treated with a FITC-conjugated rabbit anti-mouse IgG antibody (1:100 dilution) (Pierce). At the end of the final wash, the pneumococci were inspected by standard phase-contrast and fluorescence microscopy using an Olympus BX51 upright fluorescence microscope. To quantify pneumococcus-bound C3 or iC3b, total or fluorescent pneumococci in five random fields at 400X were counted by phase contrast or fluorescent microscopy.

**Isothermal Titration Calorimetry (ITC).** All ITC measurements were performed at 30 °C as described previously (52). Before titration, the purified human FH, CbpA4, and 12-AA FH binding peptide (ALNIKLASAIKTK) were dialyzed in PBS (pH 7.42) and adjusted with PBS to final concentrations of 4.2 µM, 126.7 µM, and 1.8 mM, respectively. The FH binding peptide was synthesized (Sigma-Genosys, Woodland, TX). To perform the titrations, a stock solution of the CbpA4 protein or the peptide was injected in 10 µL increments to the solution containing the purified human FH. To extract the enthalpy of binding (ΔH), dissociation constant (K_D), and binding stoichiometry (N), data were plotted and analyzed with Origin version 5.0.

**Detection of FH binding by spot synthesized CbpA peptides** - The N-terminal FH-binding domain (amino acids 37-140 in CbpA of strain D39) represented by CbpA4 was divided into 31 overlapping synthetic peptides. Each peptide consisted of 15 amino acids except for the last peptide (only 14 amino acids) with an offset of three amino acids. The peptides were commercially synthesized and covalently coated onto the cellulose membrane (Sigma-Genosys). Each spot on the membrane carried approximately
5 nmol of distinct peptide. The spot membrane was used to detect FH binding according to the manufacturer’s instruction and a previous study (27). Briefly, non-specific binding of the peptides to the antibodies was initially evaluated. The membrane was incubated with the goat anti-human FH antibody (1:3,300 dilution) and the peroxidase-coupled secondary antibody (1:5,000 dilution) each for 2 hr at room temperature. Reactivity was assessed by the ECL Western Blot Kit as described above. The membrane was subsequently regenerated according to the supplier’s manual. Specific binding of human FH to the spot membrane was assessed in a similar manner with the exception of incubating the membrane with 5 µg/ml human FH overnight at the first step. Only the “new” spots detected in the presence of FH were considered to represent FH-specific binding.

Enzyme-linked immunosorbent assay (ELISA) - The FH binding activity of CbpA was quantified using an ELISA method as described previously (25,53). Six-histidine-tagged recombinant protein CbpA4 (4 µg/well) was coated to the wells of 96-well plates (Nalge Nunc International) by incubating overnight at 4°C. BSA was coated as a negative control. After washing and blocking, purified human FH (1 µg/well) was added to the CbpA4-coated wells for 2 hr at room temperature. The bound FH was reacted with a goat anti-human FH antibody (1:200 dilution) and detected using a peroxidase-conjugated rabbit anti-goat IgG antibody (1:200 dilution). Absorbance was read on a microtiter plate reader at a wavelength of 500 nm (BioRad). The ELISA results are presented as the absorbance units. To determine the blocking effects of recombinant CbpA polypeptides, various dilutions of these reagents were thoroughly mixed with FH and immediately added to the CbpA4-coated wells. Purified GST was treated in the same manner as a negative control.

RESULTS

Deletional mutagenesis in the cbpA coding region - To identify the FH binding domain of CbpA, we constructed a series of deletions in the CbpA protein of S. pneumoniae strain D39. D39 is a type 2 virulent isolate commonly used to study pneumococcal biology and pathogenesis. Dave et al. have demonstrated that CbpA of strain D39 is able to bind to human FH (14,40). In addition, the DNA sequences of the cbpA gene and flanking regions are available in the complete genome sequence of R6 (an avirulent derivative of D39) as represented in accession AE008564 (49). We first replaced the entire cbpA-coding region of a streptomycin-resistant derivative ST594 of strain D39 with the Janus cassette by allelic exchange, resulting in cbpA-null strain ST588. ST588 was used to generate a series of unmarked in-frame deletions in the very N-terminal domain (in mutant ST650), R1 repeat (in mutant ST660), both R1 and R2 (R1/R2) repeats (in mutant ST592), proline-rich linker (in mutant ST665), and C-terminal choline binding domain (ST656) of the cbpA coding region (Fig 1A). The resultant cbpA mutants did not show detectable differences from D39 in growth rate in THY broth (data not shown). Restriction digestion and Southern hybridization of the genomic DNA preparations from these cbpA mutant strains revealed loss of the expected DNA fragments in the cbpA locus (Fig 1B). As expected, only the cbpA-null mutant ST588 carried the Janus cassette as determined by Southern hybridization using the Janus-specific probe (Fig 1C). This result thus confirmed the loss of the original Janus cassette in the strains with unmarked deletions in the cbpA coding region. Furthermore, the Southern blot results also showed that the Janus cassette was inserted only in the cbpA locus of strain D39 instead of non-specific insertions in other loci of the pneumococcal chromosome. The DNA sequences in the cbpA coding region of all the cbpA mutant strains were amplified by PCR and used to determine the accuracies of the mutations by DNA sequencing. All six mutants represented in Fig 1B were confirmed to carry designed deletions in the cbpA locus.

The N-terminal domain of CbpA is necessary for FH binding - We characterized the CbpA expression of the cbpA deletional mutants by Western blot using cellular lysates of log-phase cultures. The CbpA proteins were detected in strain D39 and type-4 strain TIGR4 (Fig 2A). There is extensive sequence diversity between the CbpA proteins of strains D39 and TIGR4 (20). We thus reasoned that determining FH binding activity of strain TIGR4 could be informative to
identify the important amino acid residues in CbpA that are involved in FH binding. TIGR4 is another important virulent strain that has been recently used to study pneumococcal pathogenesis because the complete genome sequence of this strain is available as well (54). As expected, the cbpA-null mutants in both D39 (strain ST588) and TIGR4 (strain ST640) backgrounds did not show detectable CbpA, whereas all the truncational mutants expressed mutant forms of the CbpA protein with decreased molecular sizes (Fig 2A). Strain ST656 consistently showed a much weaker signal in the Western blot experiments, which is likely due to the lack of the C-terminal choline-binding domain and thereby the release of the mature protein into the culture medium.

To further verify the CbpA mutant strains represented in Fig 2A, we characterized the binding activities of these strains to human SlgA by Western blot. Our previous study has revealed that each of the R1 and R2 repeats of the CbpA protein contains an independent binding site for human SlgA/SC/plgR (24). Consistently, strain ST592 lacking the R1/R2 repeat region had no detectable binding capacity to human SlgA (Fig 2B). In contrast, SlgA binding was observed in the mutants lacking the N-terminal domain (strain ST650), R1 repeat (ST660), or proline-rich linker (ST665), confirming that these CbpA segments are not essential for SlgA binding. The positive SlgA binding activity in the absence of the R1 repeat was due to the presence of the second SlgA binding site on the R2 repeat in strain ST660. The lack of SlgA binding in strain ST588 (CbpA-null) and the weak reactivity of ST656 with SlgA are consistent with the CbpA expression patterns as determined by the CbpA (Fig 2A). These results demonstrate that the truncated forms of the CbpA protein were properly expressed in these mutants of strain D39.

A similar approach was used to screen these CbpA mutants for FH binding activities. In line with the results obtained with the CbpA antibody (Fig 2A) and SlgA (Fig 2B), the wild type strains D39 and TIGR4 showed strong binding to human FH, but the isogenic CbpA-null mutants ST588 and ST640 had undetectable binding to FH (Fig 2C). Interestingly, there was no detectable FH binding with strain ST650 lacking the N-terminal domain of 89 amino acids, although this strain was capable of binding to the CbpA antibody and human SlgA (Fig 2A, 2B). This N-terminal region has not been assigned to any known function. This result suggested that the N-terminal 89 amino acid region of mature CbpA is necessary for FH binding. In contrast, deletions in the R1 repeat (ST660), the R1/R2 repeats (ST592), or the proline-rich linker (ST665), did not affect CbpA binding to FH, indicating that these regions are not essential for the FH binding activity. To rule out potential non-specific binding, the protein blots of the above pneumococcal strains were used to perform Western blotting in the absence of human FH. This control experiment did not yield any detectable protein bands (data not shown), indicating that the anti-FH and secondary antibodies did not react with pneumococcal proteins. Additional experiments showed that the anti-FH antibody recognizes only the human FH protein but not various irrelevant proteins including human SlgA, complement C3, serum albumin and bovine serum albumin (Fig 2D-E). The CbpA antibody, SlgA, and FH sometimes also reacted with multiple protein bands below the intact CbpA polypeptides (Fig 2A-C). This phenomenon was frequently observed when overexposure of the X-ray films was necessary to show the weaker signals of certain samples on the same blots. These lower bands appeared to represent degraded CbpA during the sample preparation because they entirely disappeared in the CbpA-null strains. These results strongly suggest that CbpA is the only FH binding protein in multiple pneumococcal strains. Together, these data strongly imply that the N-terminal region of CbpA contains a binding motif(s) for human FH.

The N-terminal domain of CbpA is necessary for FH binding to live pneumococci -The above Western blot result in Fig 2C demonstrated the significance of the N-terminal domain of CbpA in FH binding, but the experiment was conducted with denatured and reduced CbpA. To verify whether the FH binding activity of this region operates in the cellular context of pneumococci under natural conditions, we evaluated FH binding of the intact pneumococci by epi-immunofluorescence microscopy. The pneumococci from mid-log phase cultures were resuspended in PBS and reacted with the CbpA antiserum (middle panel, Fig 3A) or purified
human FH (right panel, Fig 3A). Under these conditions, the bacteria did not show apparent autolysis and maintained viability based on the actual counts of colony-forming units (data not shown). Phase-contrast microscopy showed that the parent strain D39 exhibited similar cellular morphology and arrangements as the isogenic mutants ST588 (CbpA null), ST650 (N-terminal domain deletion), and ST592 (R1/R2 region deletion) (left panel, Fig 3A). Immunofluorescence staining detected CbpA in strain D39 but not in the CbpA-null mutant ST588. CbpA was also readily detectable in the CbpA mutants lacking the N-terminal domain (ST650) or the entire SlgA binding region (ST592). Thus, this result confirmed the data obtained with these pneumococcal strains by Western blot as represented in Fig 2A. Human FH was able to deposit on the surfaces of the pneumococci in a CbpA-dependent fashion, since strong FH binding was detected in the parent strain D39 but not in the CbpA-null strain ST588. The isogenic strain ST592 lacking the entire SlgA/SC/plgR-binding R1/R2 repeats retained FH binding activity, confirming the R1/R2 repeats are not necessary for this activity. In contrast, the deletion in the N-terminal domain of CbpA (strain ST650) completely abolished the FH-binding activity.

We also tested whether the N-terminal CbpA is necessary for FH-mediated inhibition of complement activation by assessing the levels of pneumococcus-bound iC3b. iC3b has been used as an indicator of FH activity because FH is able to degrade C3b to iC3b by acting as a cofactor for complement protein factor I, another negative regulator of the alternative complement pathway (38). Consistent with the FH binding result (Fig 3A), deleting the N-terminal CbpA (strain ST650) or the entire CbpA (ST588) resulted in substantial reduction of iC3b bound to pneumococci (Fig 3B). In contrast, deletion of the R1/R2 region (strain ST592) did not affect the level of iC3b. The reduced iC3b in strain ST650 were not due to the effect of the CbpA deletion on C3 binding to pneumococci because this strain and the CbpA-null mutant showed comparable levels of C3 deposition with the parent strain D39. On the basis of enumerating the C3- and iC3b-positive pneumococci in five random microscope fields at 400X, the ratios of C3- and iC3b-stained bacteria were estimated to be 1:0.52, 1:0.22, 1:0.49, and 1:0.31 for strains D39, ST558, ST592, and ST650, respectively. Together with the Western blot data (Fig 2), these results demonstrated that the N-terminal domain of CbpA is essential for FH binding and activity under natural conditions.

The N terminus of CbpA is sufficient for binding to human FH - The above results indicated the necessity of the N-terminal CbpA for pneumococci to bind to FH. We wanted to determine whether this region alone is able to confer binding activity to human FH. Four recombinant forms of CbpA were expressed in E. coli and purified by affinity chromatography (see Experimental Procedures). As illustrated in Fig 4A, these recombinant proteins individually represent the region of the N-terminal domain and the R1/R2 repeats (CbpA1), the N-terminal domain and the R1 repeat (CbpA2), the N-terminal domain alone (CbpA4), or the region of the R1/R2 repeats and proline-rich linker (CbpA7). The C-terminal choline-binding domain was excluded from all of the recombinant proteins to improve protein solubility. These CbpA polypeptides were visualized by Western blot using the CbpA antibody (Fig 4B). As noted in our previous study (24), the recombinant CbpA polypeptides migrated more slowly than the predicted positions based on their amino acid sequences. The slower migration of CbpA7 was due to the N-terminal addition of the 26-kDa GST protein. It was also noticed that two protein bands were consistently present in the CbpA2 lane. The lower band may represent a degradation product. Alternatively, the upper band could be a dimer that was not readily separated under these conditions.

The CbpA recombinant proteins were first tested for the binding capacity to human SlgA. Strong SlgA binding activity was detected with CbpA1, CbpA2, and CbpA7 but not with CbpA4 (Fig 4C), thus confirming our previous finding that the R1/R2 repeats are required for CbpA binding to human SlgA/SC/plgR (24). While CbpA1 and CbpA2 also bound to purified human FH, CbpA7 missing the N-terminal domain was not able to bind to FH (Fig 4D), further confirming the importance of the N-terminal domain of pneumococcal CbpA in interacting with FH. Finally, CbpA4 representing the N-terminal domain alone showed strong binding activity to
FH as determined by isothermal titration calorimetry (ITC) (Fig 5). The ITC results indicated that CbpA4 binds to FH with a very high affinity (dissociation constant or \(K_D<1\) nM). A precise binding constant could not be unequivocally predicted because the sample titration data only exhibited one point in the binding site saturation region. The binding enthalpy (\(\Delta H\)) is exothermic and estimated to be \(-22.5\) kcal/mole. The stoichiometry data indicated the formation of a 1:1 complex between the two proteins. These results demonstrated that the N-terminal domain of CbpA is necessary and sufficient to confer binding capability to human FH.

A 12-amino acid region in the N-terminal domain of CbpA is required for FH binding - We further mapped the FH-binding motif on CbpA using spot-synthesized peptides. An array of 31 overlapping peptides (15 amino acids each with an offset of three amino acids) was synthesized to encompass the N-terminal FH-binding region of CbpA from strain D39 (37-140 amino acids). Equal amounts (5 nmol) of these peptides were covalently spotted onto a cellulose membrane and assayed for the ability to bind to purified human FH. To determine the background binding to the CbpA peptides, the peptides were first probed with the FH antibody followed by the peroxidase-conjugated secondary antibody in the absence of di-

Many pneumococcal isolates in multiple serotypes have been reported to be able to bind to human FH (13,41), although the sequence of the \(cbpA\) locus is highly variable among pneumococcal strains (19,20). The FH-binding activity has been attributed to the CbpA allelic variants of strains TIGR4 (type 4) (Fig 2C), G54 (type 19F) (13), A66 (type 3) (13), 3496 (type 3) (13), and ATCC33400 (type 1) (41). As represented in Fig 6B, sequence comparison showed that the amino acid sequence of the FH-binding motif in strain D39 is partially conserved among the FH-binding CbpA alleles of strains TIGR4, A66, 3496, and ATCC33400. Several amino acid residues are highly conserved including those at positions 2 (leucine), 6 (leucine), 9 (isoleucine), and 10 (lysine), whereas the other residues vary to different extents. This pattern of sequence conservation is also apparent in many other CbpA allelic variants based on sequence analysis of additional 51 CbpA sequences deposited in the databases (data not shown). Interestingly, the entire FH-binding domain is absent in the CbpA locus of strain G396 (type 3) (Fig 6B). This strain did not show detectable FH binding in our preliminary analysis (data not shown). The sequence similarity suggests that the FH-binding motif identified in strain D39 may be operative in other pneumococcal strains. Similar comparisons did not identify significant sequence similarity between the FH binding domain of CbpA and the FH-binding proteins of other pathogenic bacteria including the C beta antigen of \(S. agalactiae\) (37,38), M protein of \(S. pyogenes\) (36), and OspE protein of \(B. burgdorferi\) (32,33). This agrees with the divergent target sites for the CbpA, M, and OspE proteins on FH. CbpA and its allelic variant Hic appear to bind the short consensus repeats (SCR) 8-15 of factor H (11,39,40), whereas the M and OspE proteins interact with the SCR 7 (55) and SCR15-20 (56), respectively.

We sought to verify the results obtained by the peptide mapping. The recombinant protein CbpA4 (Fig 4) representing the FH binding domain was further modified by deleting the 12-AA FH binding motif (CbpA10) (Fig 6C). The entire CbpA4 region and deletion construct CbpA10 were expressed as GST-fusion proteins and tested for their abilities to block the binding between CbpA and FH by ELISA (Fig 6D). While the intact CbpA4 was able to block FH binding of immobilized CbpA4 in a dose-dependent manner, CbpA10 did not show significant inhibition even at the highest concentration (25 \(\mu g/ml\). As a
negative control, GST alone did not affect the CbpA-FH binding. The ELISA result further demonstrated that the 12-AA motif is essential for the FH binding. However, a synthetic peptide of the 12-AA motif failed to block the CbpA-FH binding at molar concentrations that were 10-fold higher than the blocking concentrations of CbpA4 (Fig 6D). The free peptide also showed negligible binding to FH as determined by ITC (data not shown). The lack of factor H binding by the free peptide suggested that the affinity of CbpA for FH also depends on adjacent sequences since there are multiple conserved amino acid residues surrounding the 12-AA motif (Fig 6B). To test the contribution of other amino acid residues to the interaction between CbpA and FH, two additional GST-CbpA fusion proteins lacking the highly conserved segments of the CbpA4 region were constructed and used to block the CbpA-FH binding by ELISA. CbpA9 with a deletion of the N-terminal 27 amino acids in the CbpA4 region showed similar blocking effect as the intact CbpA4 polypeptide, whereas CbpA8 lacking the 27 residues of the C-terminal CbpA4 did not show significant inhibition (Fig 6D). The result indicated that the CbpA segment downstream of the 12-AA motif amino acids is also necessary for the FH binding activity of the pneumococcal CbpA protein. However, the peptides representing the deleted sequence did not show detectable FH binding activity in the peptide mapping analysis (Fig 6A). These results thus suggest that the amino acids deleted in CbpA8 may provide structural or conformational support for the 12-AA binding motif rather than form a separate FH binding site.

**DISCUSSION**

Activated complement system is highly effective in mediating phagocytosis and pneumococcal clearance (4,57-59). On the other hand, S. pneumoniae can be carried in healthy individuals for long periods of time (2). It is thus intriguing how the pneumococci overcome host immunity that is mediated by the complement system. Recent studies have indicated that S. pneumoniae is capable of evading complement attack by multiple mechanisms (4,8,9,11,12). One of the mechanisms is the active recruitment of complement FH to the surface of the pneumococci (11,12). CbpA and its allelic variants of S. pneumoniae have been identified to confer molecular binding capacity to FH (13,14). The FH-binding activity has been mapped within the first 225 amino acids of CbpA (strain D39 of type 2) (41) and the first 223 amino acids of Hic (a CbpA variant in strain A66 of type 3) (13,38).

Our initial deletional analyses in the cbpA coding sequence of strain D39 showed that the 89-amino-acid region in the very N-terminus of mature CbpA is necessary for pneumococcal binding to purified human FH. This N-terminal region is a part of the N-terminal α-helical structure of CbpA (18,60). We further demonstrated that the very N-terminal domain of CbpA is required for the intact pneumococci to bind to FH by epi-immunofluorescence microscopy. Additional experiments with recombinant forms of CbpA further demonstrated this N-terminal region is sufficient for CbpA binding to FH. A 12-amino acid sequence in the N-terminal CbpA was identified to contain the FH binding activity by peptide mapping analysis. The importance of this sequence motif in the CbpA-FH binding was verified by deletional analysis, but the lack of FH binding by this motif alone indicates that addition CbpA sequence beyond the 12-AA motif is required for the full FH-binding capacity.

The lack of FH binding by the free CbpA peptide is reminiscent of a previous finding by Hammerschmidt et al. (27). A hexapeptide (YRNYPT) sequence based on the R1/R2 region of CbpA was found to bind human SlgA by a similar peptide mapping approach. However, the free synthetic peptide failed to inhibit the CbpA-SlgA binding in solution. Additional data in that study suggested that the hexapeptide motif represents only the minimal SlgA-binding sequence, but the binding affinity of CbpA to SlgA is dependent on the length and adjacent amino acids flanking the hexapeptide (27). It is thus possible that the surrounding amino acids of the 12-AA sequence also allow this motif to adopt a specific structure or conformation for FH binding. Consistent with this notion, deletion of the 27-amino acids downstream of the 12-AA motif (CbpA8) abolished the factor H binding in the ELISA experiments, although the peptides representing this region did not show detectable binding to FH in the peptide mapping analysis. Immobilization of the peptide on solid surfaces
might provide necessary structural support, which could be absent for the free peptide in solution. Alternatively, certain amino acids deleted in the CbpA8 construct may directly participate in the high-affinity reaction between CbpA and FH by binding to a different site on FH with a low binding affinity.

Surface-bound FH is able to inhibit the activation of the alternative pathway by at least two mechanisms (29). FH promotes the cleavage of C3b to iC3b by factor I and thus prevents the formation of the C3b-Bb complex (the alternative pathway C3 convertase). Jarva et al. have shown that Hic, a CbpA allelic variant of the type-3 pneumococci, is able to enhance the degradation of pneumococcal surface-bound C3b into iC3b by recruiting FH (11). Our analysis suggests that the FH binding domain of CbpA contributes to the cleavage of C3b into iC3b at the surfaces of encapsulated pneumococci. Factor H also decreases the deposition of C3b onto the bacterial surfaces by displacing the factor H from the C3b-Bb complex and thus inactivating the C3b amplification loop. We were not able to demonstrate a significant decrease of C3b deposition with the FH-binding deficient mutant strain. The most likely explanation is that C3b deposition can be mediated by the classic and lectin pathways, which are not controlled by factor H. It is well recognized that natural antibody against pneumococci and C-reactive protein are present in the bloodstream of healthy adults, and these factors can enhance C3b deposition by activating the classic pathway (1). Thus, the FH-mediated reduction in C3b deposition might be masked by other factors present in the human serum samples.

Our data cannot rule out possible contributions of other CbpA domains to the affinity or avidity of the FH binding activity, although the very N-terminal domain is necessary and sufficient for this function. Duthy et al. reported that the proline-rich linker of CbpA from strain D39 is involved in FH binding based on the observation that a recombinant CbpA containing the proline-rich linker (CbpA7) did not show detectable FH binding in the absence of the N-terminal FH binding domain. The R1/R2 repeats of CbpA do not appear to participate in FH binding, because the recombinant CbpA protein with (CbpA1 and CbpA2) or without the R1/R2 repeats (CbpA4) showed similar levels of FH binding.

Sequence comparison of CbpA allelic variants suggests that the FH binding domain identified in strain D39 operates in other pneumococcal strains. FH binding activities have been reported in many pneumococcal strains (13,14,41). The FH-binding motif identified in CbpA of strain D39 contains multiple amino acid residues that are shared by the CbpA allelic variants of other pneumococcal strains in various serotypes. The sequence conservation in this motif is particularly striking in the Hic protein (a CbpA allelic variant in strains A66 and 3496 of type 3). The 64% sequence identity in the FH binding motif is remarkably higher than the overall 29% sequence identity between CbpA and Hic. Hic is distantly related to CbpA of strain D39; most of the sequence homology is limited to the N-terminal regions of the two proteins. Furthermore, Hic is anchored to the cell surface of S. pneumoniae through the Gram-positive cell wall-anchoring LPSTG motif at the C terminus (13), whereas most CbpA allelic variants attach to the cell wall by binding to the cell wall choline (18,61). However, it is still possible that Hic binds to FH in a different manner. Three putative FH binding sites on Hic have been identified by peptide scanning of the first 223 amino acids (38). One of these FH binding sites includes the amino acids 90-101 of Hic corresponding to the FH binding motif of CbpA. Consistently, the binding affinity of CbpA (K_D < 1 nM) is at least 23-fold stronger than that of Hic (K_D = 23 nM) (13).

It is clear that pneumococci interact with human FH and SlgA/SC/plgR via two separate binding motifs in the N-terminal α-helical structure of CbpA. However, the binding mechanisms appear to be similar. Two identical hexapeptide motifs in the R1/R2 repeats have been identified to bind to human SlgA (27). The SlgA/SC/plgR-binding motif has been identified
in more than 70% of all pneumococcal isolates characterized thus far (16,20). Our limited sequence analysis showed that the N-terminal FH-binding domain of CbpA is present in all examined pneumococcal strains except for the strains harboring CbpA alleles of the PspC8 group (20). These lines of evidence suggest that most pneumococcal strains possess the binding motifs for both immune factors. Dave et al. have demonstrated that CbpA can bind simultaneously to FH and SlgA (40). Furthermore, denatured CbpA is able to bind to both human FH and SlgA/SC/plgR (16,24). In contrast, our recent study has shown that human SlgA/SC/plgR binds to pneumococcal CbpA via a conformational binding site(s) through the D3/D4 region of plgR (25). The intra-domain disulfide bonding of plgR is essential to maintain binding to pneumococci SlgA/SC/plgR, because either denatured or reduced SlgA/SC/plgR failed to bind to CbpA (25). It is unclear whether denaturation and reduction of FH have any effect on the binding efficiency of pneumococci. Lastly, CbpA binding to both FH (39) and SlgA/SC/plgR (J.-R. Z., unpublished data) are not affected by the presence of NaCl up to 1 M, indicating that both types of the CbpA-host binding occur via hydrophobic forces instead of electrostatic interactions. High concentrations of NaCl are known to diminish electrostatic forces mediated by clusters of positively charged lysine and arginine residues but not alter short-range hydrophobic interactions (62). For instance, the binding interaction between the \textit{S. pyogenes} M protein and C4b-binding protein (C4BP) is similarly resistant to salt (63), whereas the binding of the \textit{S. pyogenes} M protein to FH is electrostatic force-dependent and can be abolished by 200 mM NaCl (39,64,65).

A variety of pathogenic bacteria bind to complement FH and FH-like proteins to promote immune evasion, adherence, or intracellular localization (28,30,31). The biochemical mechanisms for these binding interactions are largely unclear. \textit{S. pyogenes} binds to FH through amino acids 256-292 of the M protein (64). It is unclear if this binding requires a conformational binding structure on the M protein. The BbCRASP-1 and OspE proteins of the Lyme disease agent \textit{Borrelia burgdorferi} interact with FH through conformational binding determinants (66-68). Truncations in either the N- or C-terminal region of OspE result in loss of FH binding. The different binding modes are also reflected in the variable receptor sites on FH for bacterial ligands. SCR 7, the heparin-binding region of FH, has been localized as the binding region for the M protein of \textit{S. pyogenes} (69) and the BbCRASP-1 protein of \textit{Borrelia burgdorferi} (66). The pneumococcal binding site(s) on FH remains to be fully characterized. Two separate studies have mapped the CbpA binding activity to SCR 6-10 (41) and SCR 13-15 (39). Since both studies used the CbpA protein from the same \textit{S. pneumoniae} D39 strain, it will be important to further determine whether CbpA is able to bind to distinct regions on FH under natural conditions. Jarva et al. have mapped the Hic binding site to the 8-11 SCR regions of FH (11). Additional studies are warranted to understand whether various pneumococcal strains interact with complement FH through totally different mechanisms.

In summary, the identified FH binding motif of pneumococcal CbpA is unique from all other known mechanisms of bacterium-host interactions. Thus, this study represents a significant advancement towards more fully understanding the pneumococcal survival mechanisms during persistent colonization in the upper respiratory tract and disease stages in remote tissues.

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**FOOTNOTES**
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1 The abbreviations used are: ATCC, American Type Culture Collection; CbpA, choline-binding protein A; FH, complement factor H; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetic acid; EGTa, ethylene-bis(oxyethylenenitrilo)tetraacetic acid; ELISA enzyme-linked immunosorbent assay; ITC, isothermal titration calorimetry; plgR, polymeric immunoglobulin receptor; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PspA, pneumococcal surface protein A; PspC, pneumococcal surface protein C; R1/R2, R1 and R2 repeats of CbpA; SC, secretory component; SCR, short consensus repeats; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SIgA, secretory immunoglobulin A; SpsA, *Streptococcus pneumoniae* SC/SIgA binding protein A.

**FIGURE LEGENDS**

Fig. 1. Domain deletions in the *cbpA* locus. A, Schematic map to illustrate in-frame deletions in the *cbpA* locus of strain D39. The domain structures of CbpA are indicated for the N-terminal signal sequence (SS), R1/R2 repeat region, proline-rich linker (PRL), and C-terminal choline-binding domain (CBD). The promoter of the *cbpA* gene is represented by an arrow. The *cbpA* mutant strains and deleted amino acids are labeled at the left and right ends of the constructs, respectively. The deleted regions are indicated by dashed lines. Primers for PCR amplifying individual regions of the *cbpA* locus and the flanking sequences are indicated at the top of each PCR product. The 5'-3'direction of each primer is marked with an arrow. The locations of the DNA probes used for the Southern blot in (B) and (C) are indicated with asterisks (*). The constructs are not drawn to scale. B, Southern hybridization of a DNA blot representing the wild type strain (D39) and various isogenic CbpA mutants. Approximately 10 µg chromosomal DNA of each strain was digested with AccI and separated in a 1% agarose gel. A 258-bp probe representing the immediate upstream sequence of the *cbpA* coding region was labeled by using primers Pr119/Pr313 and chromosomal DNA of strain D39 as a template. C, Same as (B) with exception of using a Janus cassette probe labeled by using primers Pr311/Pr425. The molecular sizes of DNA markers are marked in bases.

Fig. 2. Localization of FH-binding domain. A, Western blot of *S. pneumoniae* strain D39 and isogenic mutants with the deletion in CbpA as depicted in Fig.1A. Strain TIGR4 and isogenic CbpA-null mutant ST640 were also included. The total cell lysates of pneumococcal strains with equivalent amounts of proteins were separated in an SDS-PAGE gel and reacted with the rabbit anti-CbpA antiserum. The sizes of protein standards are marked in kDa. B, Same as (A) except for using purified human SIgA. C, Same as (A) except for using purified human. D, Coomassie blue staining of an SDS-PAGE gel with multiple purified proteins. The same amount of human SIgA, human complement C3, human serum albumin (HSA), and bovine serum albumin (BSA) were loaded. A smaller amount of human factor H (FH) was loaded to minimize smearing in the subsequent Western blotting as shown in (E) with anti-FH antibody.

Fig. 3. Binding of complement proteins to the intact pneumococci. A, FH staining of *S. pneumoniae* strain D39 and isogenic mutants with the deletion of the entire CbpA (*ST588*), N-terminal CbpA (*ST650*), or R1/R2 repeats (*ST592*). After sequential staining steps with rabbit anti-CbpA serum and FITC-labeled secondary antibody, pneumococci in the same fields were visualized with a phase contrast (column 1) or a FITC filter (column 2). The pneumococci stained with FH were visualized with a FITC filter (column 3). B, pneumococcus-bound C3 and iC3b. The same strain panel was stained with fresh human serum and antibody against human C3 (columns 1 and 2) or iC3b (columns 3 and 4). The same fields of each strain were visualized with a phase contrast (columns 1 and 3) or a FITC filter (columns 2 and 4).
Fig. 4. Binding of recombinant CbpA polypeptides to human FH.  
A, Schematic representation of recombinant CbpA proteins. The amino acid positions of CbpA represented by each recombinant protein are indicated at the right side of each construct. The right panels summarize detectable (+) and undetectable (-) binding activities of each CbpA construct to human SlgA and FH as shown in (C) and (D).  
B, Detection of the recombinant CbpA proteins with the CbpA antiserum as in (2A).  
C, Detection of the recombinant CbpA proteins with human SlgA as in (2B).  
D, Detection of the recombinant CbpA proteins with FH as in (2C).

Fig. 5. ITC analysis of the CbpA-FH interaction.  
A, Raw ITC data for the interaction between CbpA and FH. The titration consists of 16 injections of the purified recombinant CbpA4 (126.7 μM) into the calorimeter cell containing ~1.4 ml of the purified human FH (5.5 μM).  
B, Data after baseline integration and concentration normalization. The binding enthalpy (ΔH), dissociation constant (K_D), and stoichiometry (N) are also indicated.

Fig. 6. Localization of the FH-binding motif on CbpA.  
A, Peptide spot array analysis by Western blot. Spot synthesized overlapping peptides were first reacted sequentially with a goat anti-human FH and peroxidase-conjugated secondary antibody to detect the background binding (left panel). The blot was stripped as recommended by the manufacturer and re-probed with human FH (right panel). Spots with specific binding to FH are indicated with arrows and the represented amino acid sequences.  
B, Sequence alignment of the N-terminal CbpA of strain D39 with the CbpA allelic variants of strains TIGR4 (accession NP_346601), G54 (accession AAF73804), ATCC33400 (accession CAA71783), A66 (accession AAG16729), 3496 (accession AAL90447), and G396 (accession AAF73793). The deleted regions in various protein constructs represented in (C) are indicated at the tops of the sequences. Identical amino acids are shaded. Gaps were introduced for optimal alignment as indicated by dashed lines. Amino acids positions of each allele are marked on the right side according to the relative distance from the first amino acid following the signal sequence.  
C, Schematic illustration of deletions (dashed lines) in the FH-binding domain of CbpA as represented in the recombinant CbpA4 protein.  
D, Inhibition of the CbpA-FH interaction by CbpA polypeptides. The CbpA4-coated wells of microtiter plates were incubated with purified human FH in the presence or absence of various concentrations of the CbpA truncates as illustrated in (C) or a synthetic peptide of the 12 amino acids representing the FH binding motif as identified from (A). Bound FH was detected with anti-FH antibody and peroxidase-conjugated secondary antibody by ELISA. The values represent the absorbance readings from one of the 4 separate tests.
Figure 1

A

D39

ST588

ST650

ST660

ST592

ST665

ST656

Wild type CbpA

ΔCbpA (1-701)

ΔCbpA (40-128)

ΔCbpA (136-271)

ΔCbpA (130-503)

ΔCbpA (406-502)

ΔCbpA (503-700)

SS → R1 → R2 → PRL → CBD

Pr252 Pr313 → Pr119

Pr252 Pr431 → Pr430

Pr433 Pr432

Pr390 → Pr389

Pr435 → Pr434

Pr429 → Pr428

 Kanf → Pr425 → rpsL+

B

cbpA probe

C

Janus cassette probe
Figure 2

A. Reaction with anti-CbpA

B. Binding to human SlgA

C. Binding to human factor H

D

E
Figure 3

A

D39
ST588
ST650
ST592

Phase contrast
Anti-CPbA staining
Factor H staining

B

D39
ST588
ST650
ST592

Phase contrast
C3 staining
Phase contrast
iC3b staining
Figure 4

A

| Protein | Domain | SlgA binding | Factor H binding |
|---------|--------|---------------|------------------|
| CbpA1   | His 36-414 | +             | +                |
| CbpA2   | His 36-292  | +             | +                |
| CbpA4   | His 36-140  | -             | +                |
| CbpA7   | GST 136-502 | +             | -                |

B

Anti-CbpA staining

C

SlgA binding

D

Factor H binding
Figure 5

A

Time (min)

\( \text{μcal/sec} \)

B

kcal/mole of injectant

\( \Delta H = -22.5 \text{ kcal/mole} \)

\( N = 0.824 \)

\( K_0 < 1 \times 10^{-9} \text{ M} \)
Figure 6

A) Without human factor H

B) Deletion in CbpA9

| Deletion in CbpA9 | Deletion in CbpA10 | Deletion in CbpA8 |
|------------------|-------------------|------------------|
| CbpA4-D39        | ATENEGSTQATSSMKTEH | YRENLMLXKSRKDELPS | YRENLMLXKSRKDELPS |
| D39              | ATENEGSTQATSSMKTEH | YRENLMLXKSRKDELPS | YRENLMLXKSRKDELPS |
| TIGR4            | ATENEGSTQATSSMKTEH | YRENLMLXKSRKDELPS | YRENLMLXKSRKDELPS |
| G54              | ATENEGSTQATSSMKTEH | YRENLMLXKSRKDELPS | YRENLMLXKSRKDELPS |
| ATCC33400        | ATENEGSTQATSSMKTEH | YRENLMLXKSRKDELPS | YRENLMLXKSRKDELPS |

CbpA4-D39: 63
D39: 63
TIGR4: 78
G54: 75
ATCC33400: 74
A66: 65
3496: 65
G396: 25

C) FH binding motif

D) Absorption at OD500 vs Concentration (μg)

- GST
- CbpA4
- CbpA8
- CbpA9
- CbpA10
- Peptide
Supplement Table 1. The DNA sequences and locations of the primers used in this study

| Primer | Sequence | Location* |
|--------|----------|-----------|
| Pr105  | 5'-CGGATCCGCGACAGAGAACGAGGGAAGTAC-3' | 4157-4179 |
| Pr119  | 5'-CGGATCCTGGGAGATATTTGGACAGTGAA-3' | 4602-4625 |
| Pr252  | 5'-CTTTGTCCCGGAGCATCAGT-3' | 5581-5603 |
| Pr257  | 5'-CTCTATGGGCTGGGAGCATCAGT-3' | 1038-1060 |
| Pr311  | 5'-ATTGGCCGCGCCCTTCTTCTTCTTCTTCTTCTTCT-3' | 773-796 |
| Pr312  | 5'-ATTGGCCGCGCCCTTCTTCTTCTTCTTCTTCTTCTTCT-3' | 2080-2100 |
| Pr313  | 5'-ATTGGCCGCGCCCTTCTTCTTCTTCTTCTTCTTCTTCTTCT-3' | 4277-4311 |
| Pr314  | 5'-ATTGGCCGCGCCCTTCTTCTTCTTCTTCTTCTTCTTCTTCT-3' | 2158-2182 |
| Pr387  | 5'-ATTGGCCGCGCCCTTCTTCTTCTTCTTCTTCTTCTTCTTCT-3' | 5197-5220 |
| Pr388  | 5'-ATTGGCCGCGCCCTTCTTCTTCTTCTTCTTCTTCTTCTTCT-3' | 5834-5857 |
| Pr389  | 5'-ATTGGCCGCGCCCTTCTTCTTCTTCTTCTTCTTCTTCTTCT-3' | 3049-3073 |
| Pr390  | 5'-ATTGGCCGCGCCCTTCTTCTTCTTCTTCTTCTTCTTCTTCT-3' | 3901-3924 |
| Pr401  | 5'-ATTGGCCGCGCCCTTCTTCTTCTTCTTCTTCTTCTTCTTCT-3' | 1905-1931 |
| Pr425  | 5'-ATTGGCCGCGCCCTTCTTCTTCTTCTTCTTCTTCTTCTTCT-3' | 1073-1093 |
| Pr428  | 5'-ATTGGCCGCGCCCTTCTTCTTCTTCTTCTTCTTCTTCTTCT-3' | 2170-2186 & 2841-2862 |
| Pr429  | 5'-ATTGGCCGCGCCCTTCTTCTTCTTCTTCTTCTTCTTCTTCT-3' | 2170-2186 & 2841-2864 |
| Pr430  | 5'-ATTGGCCGCGCCCTTCTTCTTCTTCTTCTTCTTCTTCTTCT-3' | 3883-3903 & 4168-4184 |
| Pr431  | 5'-ATTGGCCGCGCCCTTCTTCTTCTTCTTCTTCTTCTTCTTCT-3' | 3883-3903 & 4168-4184 |
| Pr432  | 5'-ATTGGCCGCGCCCTTCTTCTTCTTCTTCTTCTTCTTCTTCT-3' | 3454-3475 & 3881-3897 |
| Pr433  | 5'-ATTGGCCGCGCCCTTCTTCTTCTTCTTCTTCTTCTTCTTCT-3' | 3454-3475 & 3880-3897 |
| Pr434  | 5'-ATTGGCCGCGCCCTTCTTCTTCTTCTTCTTCTTCTTCTTCT-3' | 2824-2845 & 3074-3092 |
| Pr435  | 5'-ATTGGCCGCGCCCTTCTTCTTCTTCTTCTTCTTCTTCTTCT-3' | 2845-2822 & 3092-3073 |
| Pr436  | 5'-ATTGGCCGCGCCCTTCTTCTTCTTCTTCTTCTTCTTCTTCT-3' | 3862-3882 |
| Pr437  | 5'-ATTGGCCGCGCCCTTCTTCTTCTTCTTCTTCTTCTTCTTCT-3' | 2842-2859 |
| Pr438  | 5'-ATTGGCCGCGCCCTTCTTCTTCTTCTTCTTCTTCTTCTTCT-3' | 4027-4049 & 3966-3990 |
| Pr439  | 5'-ATTGGCCGCGCCCTTCTTCTTCTTCTTCTTCTTCTTCTTCT-3' | 3966-3990 & 4027-4049 |
| Pr440  | 5'-ATTGGCCGCGCCCTTCTTCTTCTTCTTCTTCTTCTTCTTCT-3' | 4107-4128 |
| Pr441  | 5'-ATTGGCCGCGCCCTTCTTCTTCTTCTTCTTCTTCTTCTTCT-3' | 867-3890 |
| Pr442  | 5'-ATTGGCCGCGCCCTTCTTCTTCTTCTTCTTCTTCTTCTTCT-3' | 3947-3970 |

*All the numbers indicate the primer positions in GenBank accession AE008564 except for those primers that were based on accession AF411920 (Pr311, Pr312, and Pr425) or AE008406 (Pr387 and Pr388). Primers with two locations were used to create targeted deletions by overlap extension PCR.*
Supplement Table 2. *S. pneumoniae* strains used in this study

| Strain  | Description                                                                 | Source or reference |
|---------|-----------------------------------------------------------------------------|---------------------|
| CP1200  | Rx derivative; *hex* mal, *rpsL1*; Hex<sup>+</sup>, Kan<sup>s</sup>, RecA<sup>+</sup> | (46)                |
| CP1296  | CP1200 derivative; *bgl-1*, *cbp3*::*kan-rpsL*<sup>+</sup>; Strep<sup>r</sup>, Kan<sup>s</sup> | (44)                |
| D39     | Clinical isolate, serotype 2; *rpsL*<sup>+</sup>, *cbpA*<sup>+</sup>; Strep<sup>r</sup>, Kan<sup>s</sup> | (70)                |
| ST588   | ST594 derivative; *rpsL1*, *cbpA* null, Δ*cbpA*::*kan-rpsL*<sup>+</sup>; the entire coding sequence of the *cbpA* gene was replaced by transformation with the Janus (*kan-rpsL*) amplified from CP1296 chromosomal DNA using primers Pr311/Pr312; ΔCbpA<sup>1-701</sup>, Strept<sup>r</sup>, Kan<sup>r</sup> | This study          |
| ST592   | ST594 derivative; *rpsL1*, *cbpA*; ΔCbpA<sup>130-406</sup>, the 130<sup>th</sup>-406<sup>th</sup> amino acids of CbpA (the R1 and R2 repeats) were deleted by allelic exchange in strain ST594; Strep<sup>r</sup>, Kan<sup>s</sup> | This study          |
| ST594   | D39 derivative; *rpsL1* (by transformation with the *rpsL1* allele, which was amplified by PCR from CP1200 chromosomal DNA using primers Pr387/PR388 and gel purified), *cbpA*<sup>+</sup>; Strep<sup>r</sup>, Kan<sup>s</sup> | This study          |
| ST650   | ST594 derivative; *rpsL1*, *cbpA*; ΔCbpA<sup>40-128</sup>, the 40<sup>th</sup>-128<sup>th</sup> amino acids of CbpA (the N-terminal domain) were deleted by allelic exchange in strain ST594; Strep<sup>r</sup>, Kan<sup>s</sup> | This study          |
| ST660   | ST594 derivative; *rpsL1*, *cbpA*; ΔCbpA<sup>136-271</sup>, the 136<sup>th</sup>-271<sup>th</sup> amino acids of CbpA (the R1 repeat) were deleted by allelic exchange in strain ST594; Strep<sup>r</sup>, Kan<sup>s</sup> | This study          |
| ST656   | ST594 derivative; *rpsL1*, *cbpA*; ΔCbpA<sup>503-700</sup>, the 503<sup>th</sup>-700<sup>th</sup> amino acids of CbpA (the choline-binding domain) were deleted by allelic exchange in strain ST594; Strep<sup>r</sup>, Kan<sup>s</sup> | This study          |
| ST665   | ST594 derivative; *rpsL1*, *cbpA*; ΔCbpA<sup>406-502</sup>, the 406<sup>th</sup>-502<sup>th</sup> amino acids of CbpA (the proline-rich linker) were deleted by allelic exchange in strain ST594; Strep<sup>r</sup>, Kan<sup>s</sup> | This study          |
| TIGR4   | Clinical isolate, serotype 4; *rpsL*<sup>+</sup>, *cbpA*<sup>+</sup>; Strep<sup>r</sup>, Kan<sup>s</sup> | (54)                |
| ST630   | TIGR4 derivative; *rpsL1* (by transformation with the *rpsL1* allele, which was amplified by PCR from CP1200 chromosomal DNA using primers Pr387/PR388 and gel purified), *cbpA*<sup>+</sup>; Strep<sup>r</sup>, Kan<sup>s</sup> | This study          |
| ST640   | ST630 derivative; *rpsL1*, *cbpA* null, Δ*cbpA*::*kan-rpsL*<sup>+</sup>; the entire coding sequence of the *cbpA* gene was replaced by transformation with the Janus (*kan-rpsL*) amplified from CP1296 chromosomal DNA using primers Pr311/Pr312; ΔCbpA<sup>1-663</sup>, Strept<sup>r</sup>, Kan<sup>r</sup> | This study          |

Kan<sup>r</sup>: kanamycin resistant, Kan<sup>s</sup>: kanamycin sensitive, Strep<sup>r</sup>: streptomycin resistant, Strep<sup>s</sup>: streptomycin sensitive
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