Novel Purification Scheme and Functions for a C3-Binding Protein from *Streptococcus pneumoniae*†

Qi Cheng,‡ David Finkel,‡ and Margaret K. Hostetter*,§

Yale Child Health Research Center, Yale University School of Medicine, 464 Congress Avenue, New Haven, Connecticut 06520-8081

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ABSTRACT: To isolate microbial proteins capable of binding the third component of complement (C3), we coupled the free sulfhydryl group of methylamine-inactivated C3 to a thiolSepharose matrix. This simple technique facilitated the purification of the first C3-binding protein isolated from a bacterium (*Streptococcus pneumoniae*). Both metastable (native) and thioester-disrupted C3 were recognized by this protein; binding of C3 was noncovalent, independent of thioester conformation, and preferential for the C3 α-chain. Sequencing of amino-terminal and internal peptides from the C3-binding protein disclosed a proline-rich region spanning approximately 20 amino acids and a signal peptide that had not been previously reported. The gene was isolated from a library of genomic DNA from laboratory strain CP1200 by screening with a 1200 bp PCR product amplified from degenerate oligonucleotides encoding the amino terminal sequence and the internal proline-rich sequence. The open reading frame spanned 1692 bp; all peptide sequences were identified in the translated gene product, which also contained at least three choline-binding repeats at the carboxy-terminus. The gene was conserved, and the translated protein was functionally active in pneumococcal clinical isolates of serotypes 1, 3, 4, 14, and 19F. Serum from a patient recovering from acute pneumococcal infection contained IgG antibodies specific for this protein by immunoblot. Wide conservation among clinical isolates, saturable binding of C3, and the ability to stimulate the human immune response have not previously been reported for this choline-binding protein. A similar biochemical approach should enable the identification of other C3-binding proteins in microorganisms able to elude complement-mediated host defense.

The third component of complement, C3,[1] is a key component of innate immunity. In primitive hosts such as sharks, lampreys, sea urchins, or invertebrates such as ascidians, C3 serves as a surveillance protein and mediates phagocytosis of invading pathogens (*1*−*5*). In mammals, C3 links the classical, alternative, and lectin-mediated pathways of complement activation and targets microbes for removal. This process, termed opsonization, is greatly enhanced in the presence of specific antibodies directed against immunogenic epitopes of the pathogen.

C3-dependent opsonization requires the presence of an internal thioester bond, formed in the native protein by the propinquity of a sulfhydryl group (Cys^{1010}) and a glutamyl carbonyl (Gln^{1012}) on the C3 α-chain (*6*, *7*). Proteolytic cleavage of a 77-residue peptide from the amino terminus of the C3 α-chain leads to a conformational change in which a nucleophilic residue (His^{1126}) attacks the glutamyl carbonyl, forming an acyl-imidazole that binds covalently (*8*), either in amide linkage to proteins or in ester linkage to free hydroxyl residues found on circulating carbohydrates or on polysaccharide-coated microbial surfaces (*9*, *10*).

In infancy and early childhood, humans functionally or genetically deficient in C3 show increased susceptibility to a narrow range of pathogens, including *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Haemophilus influenzae* (*11*, *12*). Because these encapsulated bacteria display abundant surface polysaccharides that should otherwise serve as sites for opsonic deposition of C3b (*13*, *14*), we hypothesized that these organisms might well express heretofore uncharacterized surface proteins that interfere with opsonic deposition by binding or degrading circulating C3. Using a biochemical approach that relied upon exposure of the cysteinyl sulfhydryl group in the C3 thioester, we report here the successful purification of a C3-binding protein and the identification of the responsible gene in clinical and laboratory isolates of *S. pneumoniae*.

MATERIALS AND METHODS

Materials. Reagents for polyacrylamide gel electrophoresis, electrophoresis standards, and polyvinylidine difluoride membranes (PVDF) were obtained from Bio-Rad (Hercules, CA). Horseradish peroxidase (HRP)-avidin, HRP-goat anti-human IgG, HRP-goat anti-human C3, FITC-goat anti-rabbit Ig, and FITC-avidin were obtained from Cappel/ICN (Costa

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* To whom correspondence should be addressed. Phone: (203) 737-4489. Fax: (203) 737-5972. E-mail: margaret.hostetter@yale.edu.

‡ University of Minnesota.

§ Yale University School of Medicine.

[1] Abbreviations: C3, third component of complement; CH_3NH_2·C3, methylamine-treated C3; CNBr, cyanogen bromide; EDTA, ethylenediaminetetraacetic acid; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; PT22, 34, 42, internal peptides from the purified C3-binding protein; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SIgA, secretory IgA; TCA, trichloroacetic acid; THB, Todd Hewitt broth; TIGR, The Institute for Genome Research.

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Mesa, CA). Thiopropyl-Sepharose 6B, Protein A-Sepharose 4B, and CNBr activated Sepharose 4B were obtained from Pharmacia (Piscataway, NJ). Sulfo-NHS-LC Biotin, Supersignal chemiluminescent substrate, stable peroxide solution and luminol/enhancer were obtained from Pierce (Rockford, IL). Digoxigenin (DIG)-11-dUTP, and DIG luminescent detection kit were obtained from Boehringer Mannheim (Indianapolis, IN). SIgA was obtained from Sigma (St. Louis, MO). GeneAmp PCR kit with AmpliTaq DNA polymerase was obtained from Perkin-Elmer (Branchburg, NJ). T7QuickPrime kit was obtained from Pharmacia Biotech (Piscataway, NJ).

Bacterial Strains and Media. Laboratory isolates of *S. pneumoniae* included strains R6x, R6, and its *cbpa*$^-$ mutant (the kind gifts of Dr. Elaine Tuomanen, St. Jude Children's Research Hospital, Memphis, TN); CP1200 (the kind gift of Dr. Donald Morrison, University of Illinois, Chicago, IL); and WU, JY1119, JY183, and JY53 (the kind gifts of Dr. David Briles, University of Alabama, Birmingham, AL). Construction of the mutant CP1r has been previously described (*15*). Clinical isolates of *S. pneumoniae* included serotypes 1, 3, 4, 14, and 19F obtained from the Diagnostic Microbiology Laboratory of the University of Minnesota Hospital; two serotype 3 isolates obtained from Dr. Douglas Fine (University of Oklahoma); and a virulent serotype 23F (the kind gift of Dr. Steve Pelton, Boston City Hospital). The heavily encapsulated serotype 3 isolate 6303 was obtained from the American Type Culture Collection; its unencapsulated transposon mutant, DW3.8, was the kind gift of Dr. David Watson (Houston VA). These organisms were grown to exponential phase in Todd Hewitt broth (THB) or on blood agar at 37 °C and stored as frozen stocks at −80 °C in growth medium containing 10% glycerol. NZY media and Todd Hewitt broth were purchased from Gibco BRL (Grand Island, NY).

Purification of Human C3. C3 was purified from fresh plasma according to published methods (*16*). Methylamine-treated C3 (CH_3NH_2·C3) was generated by incubation of purified human C3 with 0.1 M methylamine in 0.1 M Tris/0.01 M EDTA, pH 8, for 90 min at 37° C. CH_3NH_2·C3 was then dialyzed overnight at 4° C against phosphate-buffered saline (PBS). For some experiments, CH_3NH_2·C3 was biotinylated by incubating 10 mg of CH_3NH_2·C3 with 0.2 mg of SulfoNHS-LC-biotin in 50 mM bicarbonate buffer, pH 8.5, for 2 h on ice. After biotinylation, CH_3NH_2·C3 was dialyzed against PBS overnight at 4 °C and stored at 4 °C.

Characterization of C3-Binding Activity. Laboratory and clinical isolates of *S. pneumoniae* were grown to mid-exponential phase in Todd Hewitt broth until OD_{600nm} reached ∼0.3. Pneumococcal cells were pelleted by centrifugation and lysed at room temperature for 30 min in the presence of 5% sodium dodecyl sulfate (SDS). Proteins in pneumococcal lysates and culture supernatants were electrophoresed on 15% SDS−PAGE under either nonreducing or reducing conditions (*17*) and transferred to nitrocellulose membranes for 60 min at 4 °C according to published methods (*18*). Membranes were incubated in 10% nonfat dry milk for 60 min at room temperature prior to a second 60 min incubation with 2 μg/mL of biotinylated native C3 or CH_3NH_2·C3 in Tris-buffered saline containing 0.1% Tween 20 and 3% BSA. After washing in Tris-buffered saline, C3 bound to pneumococcal proteins was detected by incubation with 1:20000 dilution of horseradish peroxidase (HRP)-avidin for 60 min at room temperature prior to development of luminol-enhanced chemiluminescence with the Supersignal system.

Table 1

PEPTIDE SEQUENCES	DEGENERATE OLIGONUCLEOTIDES
N-TERM TENEGSTQAATS [S] NMAKTE [H]	5' XCXGAAAACGAAGGXAGXACXCA 3' G T G TC
PT-22: EK/IPAEQPQPAPATQP	5' GGCTGXGGCTGCTCXGCXG 3' T T T
PT-34: SSDSSVGEETLPSS [S] L	
PT-42: S/VKEAELELVKEEAK	

Purification and Functional Analysis of the C3-Binding Protein. S. pneumoniae CP1200 were grown to mid-exponential phase in THB at 37 °C. Pneumococcal cells were removed by centrifugation at 10000*g* for 10 min. Proteins in the culture supernatant were precipitated with a final concentration of 10% TCA at 4 °C overnight. The precipitant was resuspended in 100 mM Tris and the final pH adjusted to 7.0 with 1 M NaOH. The resuspended proteins were chromatographed on a 1.2 mL column of Thiopropyl Sepharose 6B coupled to 4 mg of CH_3NH_2·C3. The column was then washed with 40 mL of 100 mM Tris-HCl, pH 7.0, containing 0.5 M NaCl. The C3-binding protein was eluted from the column with 20% ethanol in the Tris-HCl/NaCl wash buffer. Fractions of 1 mL were collected and analyzed spectrophotometrically at OD_{280}, by SDS−PAGE, and by C3-binding assay. Fractions containing the C3-binding protein were pooled, concentrated by precipitation in 90% ethanol, and stored at −20 °C. Approximately 80 pmol of purified protein immobilized on PVDF membrane was subjected to amino-terminal and internal peptide sequencing at Harvard Microchemistry Facility, Cambridge, MA. Four peptide sequences were obtained and used to derive degenerate oligonucleotides (Table 1).

To determine which chain(s) of C3 was bound, 1 mg of the purified pneumococcal protein was coupled to 1 mL of CNBr-activated Sepharose 4B according to manufacturer's instructions. A total of 2 mg of native C3 in PBS was passed through the affinity column. The column was washed with PBS, followed by 1 mM dithiothreitol (DTT) in PBS; 20% ethanol in PBS; and 1× electrophoresis sample buffer. C3 chain(s) in each wash were electrophoresed on 7.5% SDS−PAGE and detected by incubation with a 1:10000 dilution of HRP-conjugated goat anti-human C3. Since CH_3NH_2·C3 is more stable than native C3, CH_3NH_2·C3 was used for the remainder of the binding studies.

Release of the C3-binding protein from pneumococcal cells was assessed after growth of *S. pneumoniae* (5×10^8 cells) to mid-exponential phase in THB and repetitive washes in increasing concentrations of choline (0.5−10%). Choline washes were examined for the presence of the C3-binding protein by Western blot using IgG antibodies from rabbits injected with 500 μg of purified pneumococcal protein (Cocalico Biologicals, Inc. Reamstown, PA). Titers of rabbit antisera were determined by Western blot using 100 ng of

purified C3-binding protein and a 1:20000 dilution of HRP-conjugated goat anti-rabbit IgG as secondary antibody.

Isolation of the Gene. Degenerate oligonucleotides (2 μM) derived from the amino-terminal sequence and one internal peptide sequence (PT-22) were used as primers to amplify 75 ng of CP1200 chromosomal DNA by polymerase chain reaction with the GeneAmp PCR reagent kit with AmpliTaq DNA polymerase. The amplified product of 1200 bp was cleaned by Wizard PCR Preps DNA purification system (Promega, Madsion, WI), labeled with ^{32}P using T7 QuickPrime, and used to screen replica filters of ~15 000 plaques of randomly sheared CP1200 DNA in Lambda ZAPII (Stratagene) plated on NZY media. After denaturation (0.5 M NaOH and 1.5 M NaCl) and neutralization (1.5 M NaCl and 0.5 M Tris-HCI, pH 8.0) of the filters, genomic DNA was attached to the filters by baking at 80 °C for 2 h prior to hybridization with the 1200 bp probe in standard hybridization solution (6× SSC, 0.5% SDS, 5× Denhardt's reagent, 100 μg/mL denatured, fragmented salmon sperm DNA). Twelve clones identified in the initial screening were rehybridized with the 1200 bp probe in a secondary screening, and three clones with the strongest signal were chosen for in vivo excision of the pBluescript SK(−) phagemid by coinfection with helper phage according to manufacturer's procedure. The clone containing the largest insert (5.2 kb) was partially mapped by digestion with restriction enzymes and by hybridization with degenerate oligonucleotides encoding either the amino terminus or the internal peptide PT-22 (Table 1) after labeling with ^{32}P. A 2.0 kb *Hin*dIII fragment that failed to hybridize with the oligonucleotide probes was removed by restriction digestion, and the remaining 3.2 kb fragment containing the putative open reading frame was sent for automated sequencing (Interdisciplinary Center for Biotechnology Research, University of Florida, Gainesville). The Genbank accession number for the translated sequence is 3153898.

DNA Extraction and Analysis by Southern Blotting. Clinical and laboratory strains of *S. pneumoniae* were grown to mid-exponential phase (OD_{600nm} = 0.3−0.4) in THB. After rapid chilling of cells on ice, EDTA was added to a final concentration of 10 mM, and cells were centrifuged at 8000 rpm for 10 min at 4 °C. Cells were resuspended in 1/10 volume of ice-cold 50 mM Tris, pH 8.0/10 mM EDTA/0.1 M NaCl, centrifuged again, and resuspended in 1/100 of the original culture volume. Bacterial cells were lysed by sequential 5-min incubations with 1% Triton X-100 at 37 °C and 1% SDS at 50−60 °C and then cooled to 37 °C. Lysed cells were sequentially incubated with 100 μg/mL RNase at 37 °C for 2 h followed by 50 μg/mL proteinase K at 37 °C for 1 h. Chromosomal DNA was extracted twice with phenol:chloroform and once with chloroform and precipitated with 2 vol of cold ethanol overnight. DNA pellets were washed with 70% ethanol twice before drying and storage at 4 °C. A total of 5 μg of chromosomal DNA was digested with 10 units of *Eco*RI at 37 °C for 2 h and then analyzed by Southern blotting (*19*), using the 1200 bp fragment as a probe after digoxigenin labeling with DIG-11-dUTP (Boehringer Mannheim).

Immunodetection of the C3-Binding Protein in Strains CP1200, R6, and Their Respective Mutants. After growth of pneumococcal strains to exponential phase in THB at 37 °C, secreted pneumococcal proteins were precipitated from THB with 10% TCA overnight at 4 °C. The precipitant was resuspended in 100 mM Tris and the final pH adjusted to 7.0 with 1 M NaOH. Proteins were electrophoresed on 10% SDS−PAGE under reducing conditions and transferred to nitrocellulose membranes for 60 min at 4 °C as described above. After blocking with 10% nonfat dry milk for 60 min at room temperature, the membrane was incubated in Tris-buffered saline containing 0.1% Tween 20 and 3% BSA with a 1:500 dilution of rabbit IgG raised against purified C3-binding protein, washed three times in Tris-buffered saline, and then incubated with a 1:20000 dilution of HRP-conjugated goat anti-rabbit IgG for 60 min at room temperature, prior to development with the Supersignal system.

Conservation of the Protein in Clinical Isolates. Clinical specimens of *S. pneumoniae* serotypes 1, 3, 4, 14, and 19F were grown to mid-exponential phase in THB as described above. Lysates from 5×10^8 cells or supernatant proteins secreted by 2×10^8 cells were then electrophoresed on 15% SDS−PAGE under reducing conditions, transferred to nitrocellulose membranes, and subjected to Western blotting with 2 μg of biotinylated CH_3NH_2·C3 as described above.

Comparative Binding of C3 and SIgA. Wells of a 96-well ELISA plate were coated with 100 ng of C3-binding protein in carbonate buffer (pH 9.0) by overnight incubation at 4 °C. After blocking with 5% skim milk in PBS/Tween for 60 min at room temperature, varying amounts of biotinylated CH_3NH_2·C3 or biotinylated SIgA were added to the wells and incubated for 60 min at room temperature. The bound C3 or SIgA was detected by avidin-HRP. To determine background, wells coated with 100 ng of C3-binding protein were incubated with avidin-HRP in the absence of C3 or SIgA. Background readings were subtracted from those for wells with added C3 or SIgA. Concentrations of biotinylated C3 or SIgA ranging from 0 to 200 ng were used to generate a standard curve that was repeated with every experiment. All samples were assayed in triplicate; the mean of triplicate values was used for calculations.

In the competitive inhibition assay, wells were coated with 500 ng of the C3-binding protein overnight at 4 °C and then blocked with skim milk for 60 min. To test whether SIgA inhibited the binding of CH_3NH_2·C3 to the C3-binding protein, the binding of biotinylated CH_3NH_2·C3 was quantitated by avidin-HRP: (1) 10 μg of biotinylated CH_3NH_2·C3 without SIgA, incubated for 60 min at room temperature; (2) 10 μg of biotinylated CH_3NH_2·C3 added simultaneously with 20 μg of nonbiotinylated SIgA, incubated for 60 min at room temperature; (3) 200 μg of nonbiotinylated SIgA incubated for 60 min at room temperature, prior to a 60 min incubation of 10 μg of biotinylated CH_3NH_2·C3.

Biotinylated C3 bound in 1 was normalized to 100%.

To test whether CH_3NH_2·C3 inhibited the binding of SIgA to the C3-binding protein, bound SIgA was quantitated in three experimental mixtures: (4) 20 μg of biotinylated SIgA without CH_3NH_2·C3, incubated for 60 min at room temperature; (5) 20 μg of biotinylated SIgA added simultaneously with 10 μg of biotinylated CH_3NH_2·C3, incubated for 60 min at room temperature; (6) 100 μg of nonbiotinylated SIgA, incubated for 60 min at room temperature, prior to a 60 min incubation with 20 μg of biotinylated SIgA.

Biotinylated SIgA bound in 4 was normalized to 100%.

Immunogenicity. S. pneumoniae CP1200 was grown to exponential phase in 10 mL of THB. Proteins in the

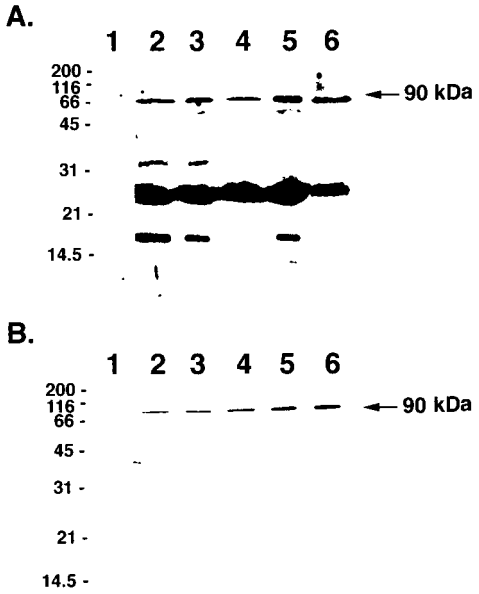

FIGURE 1: Identification of C3-binding protein in ligand affinity blots from lysates (A) and cell supernatants (B) of *S. pneumoniae* strain CP1200. Pneumococcal proteins were separated by 15% SDS−PAGE, transferred to nitrocellulose, and incubated with 2 μg/mL of biotinylated CH_3NH_2·C3; bound C3 was detected by avidin-horseradish peroxidase. Lane 1 is a media control. OD_{600nm} of lanes 2−6 were 0.127, 0.220, 0.325, 0.536, and 0.735, respectively. In panel A, each lane represents cell lysate from 5×10^8 cells. In panel B, each lane represents cell supernatant from 2×10^8 cells.

supernatant were precipitated with 10% TCA overnight at 4 °C, electrophoresed on 15% SDS−PAGE under nonreducing conditions, and transferred to nitrocellulose. The membrane was incubated with 10% nonfat dry milk, followed by a 1:10000 dilution of IgG antibodies isolated from the serum of an adult with acute pneumococcal pneumonia by affinity purification on Protein A Sepharose. The membrane was washed in Tris-buffered saline, incubated with a 1:50000 dilution of HRP-conjugated goat anti-human IgG for 60 min at room temperature, washed again, and developed with Supersignal according to manufacturer's instructions.

RESULTS

Cell-associated forms of C3-binding proteins in the *S. pneumoniae* strain CP1200 are synthesized from early logarithmic growth to stationary phase (Figure 1A, lanes 2−6) and include major bands of M_r 90 and 26 kDa. Minor bands of M_r ~32 and 16 kDa are not consistently present. Figure 1B shows that only the 90 kDa form of the C3-binding protein is secreted throughout pneumococcal growth. After biotinylation, both native C3 and methylamine-treated C3 (CH_3NH_2·C3) were bound in the absence of other complement proteins; thus, the binding of C3 by pneumococcal proteins is independent of the conformation of the thioester bond and does not represent the typical opsonic interaction, which requires the participation of multiple complement proteins (*10*).

Purification of the C3-binding protein was achieved by affinity chromatography on a CH_3NH_2·C3 matrix after TCA precipitation of secreted proteins in culture supernatants of exponentially growing *S. pneumoniae* strain CP1200. Because coupling of native C3 to a Sepharose matrix did not constitute an effective affinity matrix, presumably because

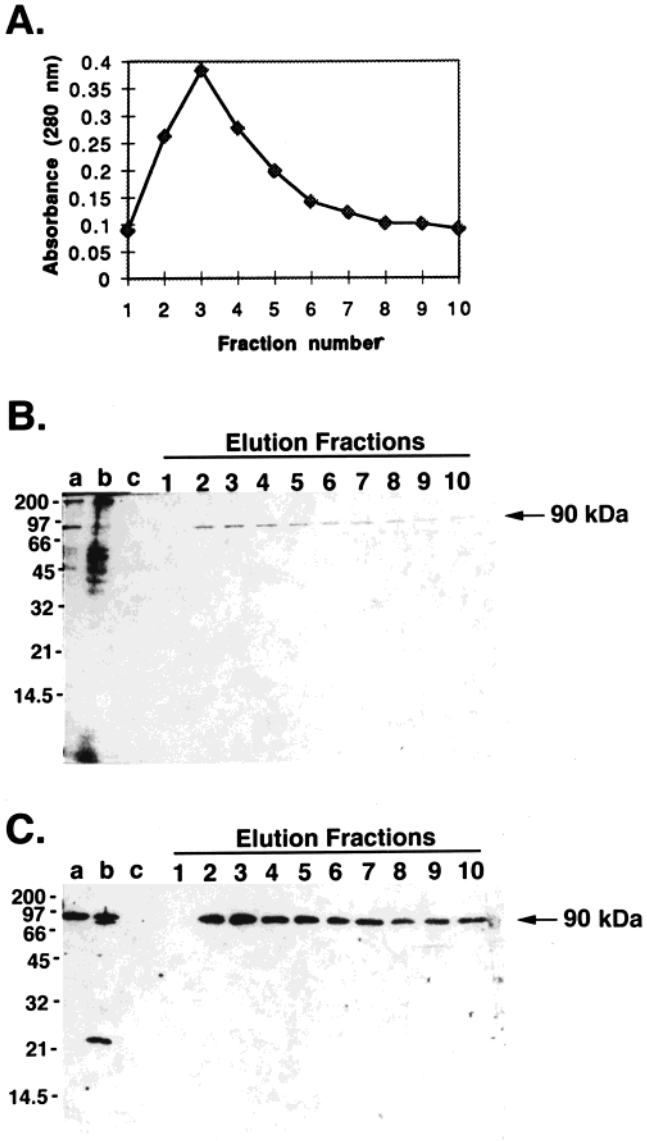

FIGURE 2: Purification of the C3-binding protein. (A) Elution profile from CH_3NH_2·C3-thiopropyl Sepharose 4B column. (B) Coomassie blue staining of 15% SDS−PAGE. (C) Detection of the C3-binding protein with biotinylated CH_3NH_2·C3. Lane a, supernatant; b, void volume; c, 0.5 M NaCl wash; and 1−10, 20% ethanol elution fractions. The yield was 1 mg from 4 L of pneumococcal supernatant.

of multiple orientations of C3 when bound to Sepharose, the single sulfhydryl group expressed by CH_3NH_2·C3 was linked by disulfide bond to Thiopropyl Sepharose 6B, thereby orienting each molecule of CH_3NH_2·C3 identically. Elution with 20% ethanol yielded a homogeneous product of 90 ± 5 kDa that was functionally active (Figure 2). The mass of the eluted protein corresponded to that identified in pneumococcal lysates and supernatants (Figure 1).

Native C3 is composed of an α-chain of M_r 115 kDa and a β-chain of M_r 75 kDa. The α-chain contains the thioester bond which mediates opsonic binding; linkage by interchain disulfide bonds to the β-chain is thought to stabilize this otherwise labile-binding site (*20*). To determine which chains of C3 were recognized by the C3-binding protein, 500 μg of the purified pneumococcal protein was coupled to CNBr-activated Sepharose 4B as an affinity matrix for purified human C3. The C3 β-chain was readily eluted by 1 mM

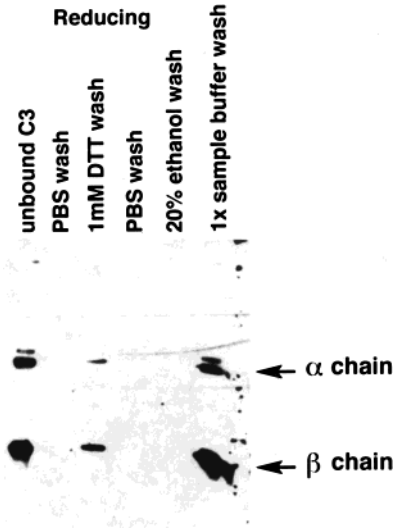

FIGURE 3: Western blot analyzing the binding of C3 α- and β-chains to 1 mg of purified C3-binding protein coupled to CNBr-activated Sepharose 4B. C3 polypeptides eluted under the conditions indicated were electrophoresed on 7.5% SDS−PAGE under reducing conditions and detected with HRP-conjugated goat anti-human C3.

DTT or 20% ethanol (Figure 3). Elution of the β-chain appears consistent with preferential binding of the C3 α-chain to the pneumococcal protein. However, elution of the α-chain, albeit in smaller quantities, after treatment with DTT emphasizes that the interaction of the pneumococcal C3-binding protein with C3 is noncovalent and therefore distinct from the covalent deposition of C3b on the pneumococcal surface during opsonization.

An amino-terminal sequence (N-term) and three internal peptides, PT-22 (proline rich), PT-34, and PT-42, were obtained from tryptic digestion of the protein (Table 1). Because the TIGR sequence of the pneumococcal genome was not available at the time of protein purification, the sequences of the isolated peptides were compared to the NCBI protein database and were unlike any published sequence. Degenerate oligonucleotides derived from the N-term peptide and PT-22 (Table 1) amplified a 1200 bp fragment from CP1200 DNA; this fragment was used to probe a library of genomic DNA from the homologous strain. The largest clone (pDF143) isolated from the genomic library spanned 2520 bp and contained a large open reading frame of 1692 bp (nucleotides 383−2074) encoding a deduced polypeptide of 564 amino acids with a molecular mass of 67 280 Da and a pI of 9.52 (Genbank accession no. 3153898). The open reading frame was preceded by a typical ribosome-binding site (Shine-Delgarno sequence), with TATAGA at −10 and TTGAAG at −35 of the putative promoter region. All internal peptides were identified in the translated amino acid sequence (Figure 4).

Common motifs encountered in pneumococcal surface proteins include a proline-rich region of 27 amino acids (aa 413−439), which has 88% identity (23/27) with the proline-rich region of several cell wall proteins from *S. pneumoniae*, including PspA (aa 335−360) (*21*). The carboxy-terminus is preceded by three tandem repeats of 20 amino acids, each of which has 90% identity to the choline-binding repeats of PspA (*21*). However, in contrast to PspA, which is released from the pneumococcal cell wall by incubation in

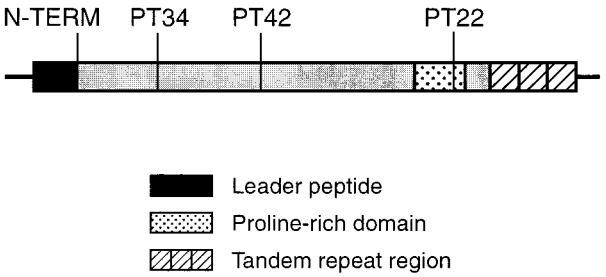

FIGURE 4: Structure of the protein, as isolated from strain CP1200. The region of the signal sequence is shaded. The four peptide sequences derived from the purified protein are indicated as N-term, PT34, PT42, and PT22.

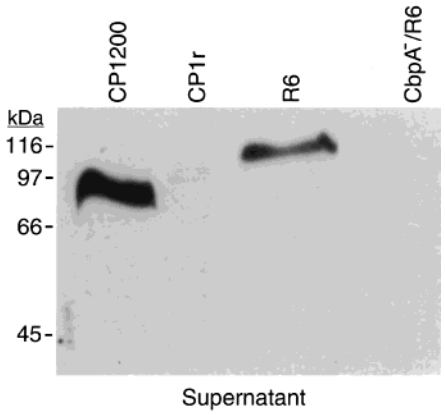

FIGURE 5: Structural similarities between secreted forms of the 90 kDa C3-binding protein isolated from CP1200 and CbpA from R6, as analyzed by the binding of polyclonal antibodies raised against the 90 kDa protein. CP1200 (lane 1) and its isogenic mutant Cp1r (lane 2); R6 (lane 3) and its isogenic (*cbpa*$^-$) mutant (lane 4). Molecular mass markers are indicated in the left margin.

0.5% choline, approximately 90% of the C3-binding protein remained bound to pneumococcal cells in choline concentrations up to 10% (data not shown). In contrast to surface proteins of other Gram-positive bacteria, no LPXTGX motif (*22*) was found at the carboxy-terminus or elsewhere in the amino acid sequence of the C3-binding protein.

The deduced amino acid sequence of the C3-binding protein (Genbank accession no. 3153898) has two close homologues in the choline-binding proteins CbpA (Genbank accession no. 2425109) and SpsA (Genbank accession no. 2511705) (*23*, *24*). The C3-binding protein is most closely related to CbpA, a choline-binding protein isolated by choline affinity matrix chromatography (*23*). The C3-binding protein reported here contains a signal peptide not present in CbpA and a C-terminus with only three choline-binding motifs, as opposed to 10 in CbpA; the remainder of the molecule is identical. The C3-binding protein is also highly homologous to SpsA; both the C3-binding protein and SpsA have an identical leader peptide of 38 amino acids, but the former contains a 153 amino acid insertion (aa 212−264) that is absent in SpsA (*24*). On the basis of structural analysis made possible after the publication of the TIGR database, we propose that the C3-binding protein and CbpA are one and the same and that the designation CbpA may now be broadened to include C3-binding protein as well as choline-binding protein.

The functional similarity of the C3-binding protein and CbpA was confirmed by the fact that polyclonal antibodies to the 90 kDa C3-binding protein from strain CP1200

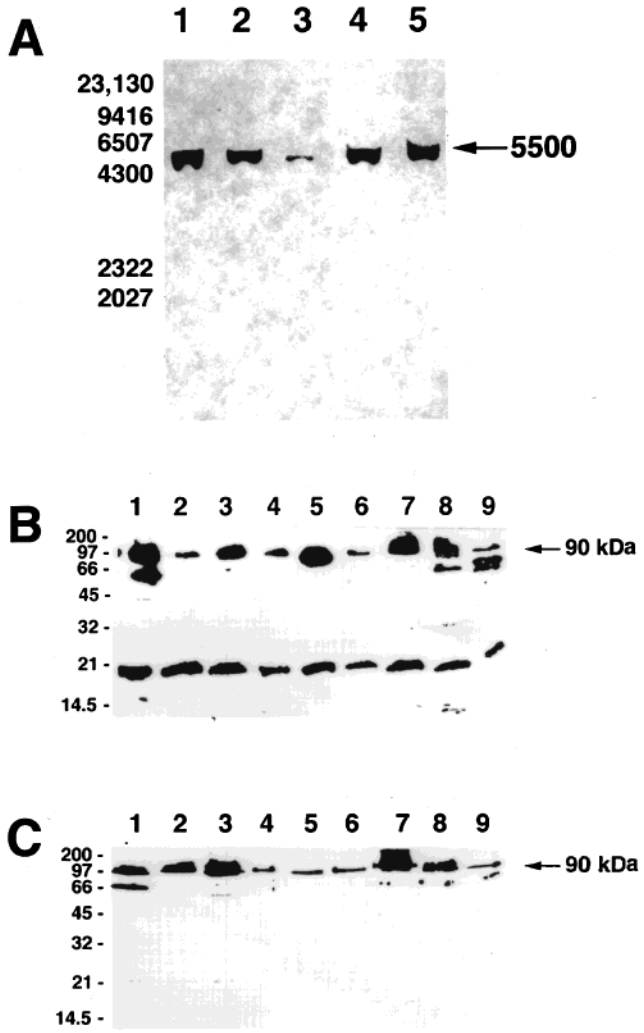

FIGURE 6: Structural and functional conservation of the C3-binding protein in clinical isolates of *S. pneumoniae*. (A) Southern blot illustrating conservation of the gene in chromosomal DNA from *S. pneumoniae* serotypes 1 (lane 1), 4 (lane 2), 14 (lane 3), and 23F (lane 4). Laboratory strain CP1200 is shown in lane 5. (B and C) Western blot analysis of the binding of biotinylated CH_3NH_2·C3 to the 90 kDa C3-binding protein in lysates (B) and supernatants (C) of *S. pneumoniae* clinical isolates, as follows: serotype 1 (lane 1); serotype 3 (lanes 2, 4−6); unencapsulated serotype 3 mutant DW3.8 (lane 3); serotype 4 (lane 7); serotype 14 (lane 8); serotype 19F (lane 9).

recognized as well the 120 kDa protein CbpA in strain R6 (*23*). No proteins in the supernatant of exponentially growing CP1r, in which the gene encoding the C3-binding protein was disrupted by insertional mutagenesis (*15*), or in the *cbpa*$^-$ mutant of strain R6 were recognized by polyclonal antibodies to the 90 kDa C3-binding protein (Figure 5). These studies suggest structural homology between the C3-binding protein identified in strain CP1200 and the previously described choline-binding protein CbpA (*23*).

The gene encoding CbpA is conserved in a variety of patient isolates of *S. pneumoniae* (serotypes 1, 4, 14, and 23F) from several geographic locations (Figure 6A). CbpA is expressed in cell lysates and supernatants from exponentially growing cultures of these and other clinical isolates, including the heavily encapsulated strain 6303 and its transposon mutant DW3.8 (*25*) (Figure 6, panels B and C, lanes 6 and 3, respectively). Interestingly, all of the clinical isolates express a smaller species of C3-binding protein at

Table 2: Inhibition of C3 Binding by SIgA[a]

experimental mixture	bound C3 (ng)	% of C3 binding
10 μg of **CH_3NH_2·C3**	3.488	100
10 μg of **CH_3NH_2·C3** and 20 μg SIgA	2.549	73
200 μg of SIgA first, then 10 μg of **CH_3NH_2·C3**	1.382	44

[a] The biotinylated component is in bold type.

Table 3: Inhibition of SIgA Binding by C3[a]

experimental mixture	bound SIgA (ng)	% of SIgA binding
20 μg of **SIgA**	12.039	100
20 μg of **SIgA** and 10 μg of CH_3NH_2·C3	10.101	84
100 μg of CH_3NH_2·C3, then 20 μg of **SIgA**	9.462	78

[a] The biotinylated component is in bold type.

M_r 20 kDa in cell lysates; this species is not seen in supernatants. CbpA was also detected in cell lysates of PspA mutants JY119, JY118, and JY53 and their parent strain WU (*21*), thereby confirming that the proteins encoded by *pspA* and *cbpa* are distinct (data not shown). Such wide conservation of CbpA has not been recognized previously.

Because of the structural homology with SpsA, a protein thought to bind secretory IgA (*24*), the ability of SIgA to bind to CbpA and to compete with binding of CH_3NH_2·C3 was tested by ELISA. SIgA readily bound to CbpA and inhibited C3 binding by ~50% (Table 2). Scatchard analysis of C3 binding was linear with a K_d of 200 nM; Scatchard analysis of the binding of SIgA was nonlinear with a K_d of 1 μM (Figure 7). These results suggested that CbpA may have multiple sites for the binding of SIgA, one or more of which may overlap with the binding site for C3.

In a previous report, the binding of SIgA to a serotype 1 pneumococcal isolate was reported as linear, with a K_d of 9.3×10^{-9} M (*24*). The substantive differences with our results may relate to the fact that there are several SIgA-binding proteins on pneumococci with widely varying masses (~50−90 kDa) (*24*) and that the authors of the earlier report performed their binding studies with intact pneumococci, while we measured the binding of purified SIgA to purified CbpA.

Evidence supporting the immunogenicity of CbpA in humans is presented in Figure 8. A serum sample from an adult recovering from pneumococcal pneumonia did not contain IgG antibodies to any secreted pneumococcal proteins within the first week of illness; however, convalescent serum obtained approximately 30 days after discharge from the hospital contained IgG antibodies that readily recognized CbpA. Thus, CbpA elicits an IgG antibody response in humans recovering from pneumococcal infection. That CbpA is immunogenic for humans has not previously been reported.

DISCUSSION

In organisms too primitive for antibody-based immune systems, C3 stands as the primary means of host defense. In higher vertebrates and mammals, genetic or functional deficiency of C3 predisposes these hosts to a variety of

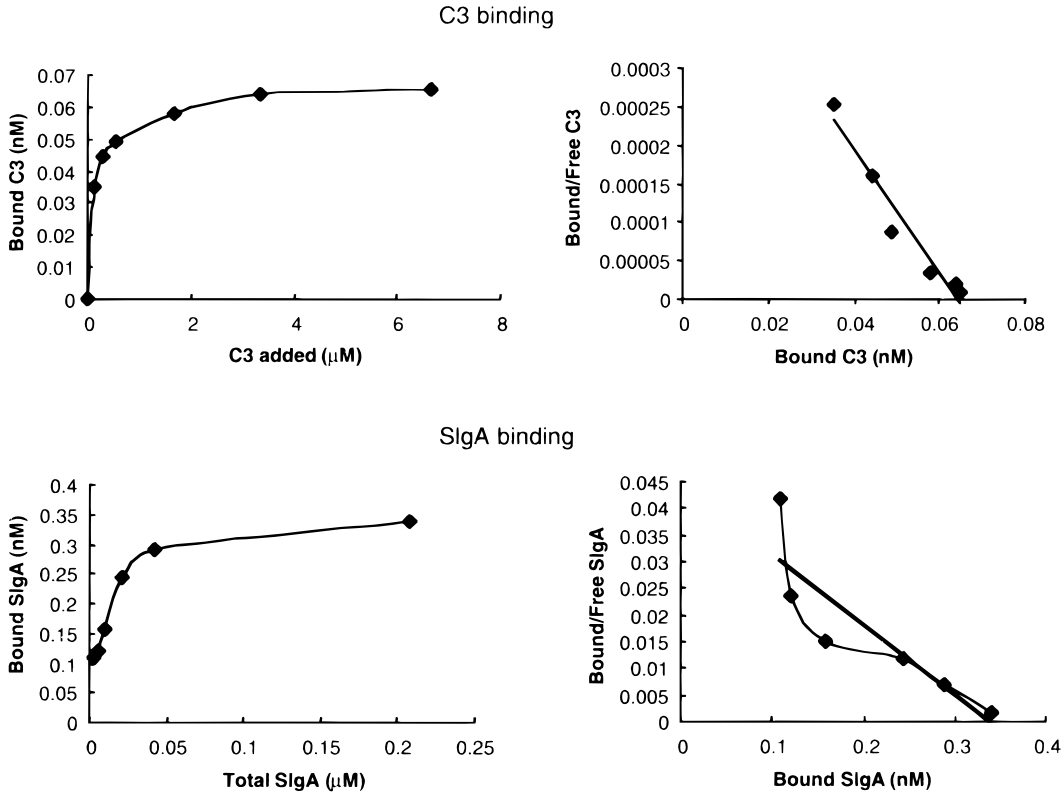

FIGURE 7: Equilibrium binding of C3 (top, left panel) and SIgA (bottom, left panel) to the pneumococcal C3-binding protein CbpA. Scatchard analysis of the respective data points is shown in the right-hand panels. Each value represents the mean of three separate experiments performed in triplicate.

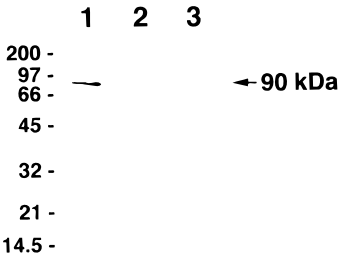

FIGURE 8: Immunogenicity of C3-binding protein by Western blot. Culture supernatant from 2×10^8 exponentially growing pneumococcal cells was electrophoresed on 15% SDS−PAGE and transferred to nitrocellulose. Membrane was blocked by 10% skim milk and incubated with the following sera: (1) convalescent serum, (2) acute serum, and (3) control serum depleted of IgG by absorption on protein A. Human IgG was detected by HRP-conjugated rabbit anti-human IgG.

bacterial or viral infections, even in the presence of antibodies (*11*, *12*, *26*, *27*). Thus, the ability of circulating C3 to recognize an invading pathogen and to deposit opsonically and covalently on its surface represents a highly conserved system of surveillance, targeting, and removal.

Hypothesizing that microorganisms would have developed strategies to elude C3-mediated host defense, we took advantage of the single sulfhydryl group of the C3 thioester in order to isolate the first C3-binding protein reported in bacteria. The simplicity of this biochemical approach should enable the identification of similar molecules among other streptococci and perhaps even among other genera.

Our studies of the C3-binding protein CbpA, one of eight or more choline-binding proteins in *S. pneumoniae*, have expanded upon the initial report of its characterization and that of its homologue SpsA (*23*, *24*) by identifying two novel functions: binding of C3 and stimulation of the human immune response. Moreover, we have demonstrated that CbpA is secreted in a functionally active form and is widely conserved among clinical and laboratory isolates of *S. pneumoniae*. Previously, choline-binding proteins were thought to remain bound to the rigid pneumococcal cell wall by virtue of a noncovalent interaction with the phosphorylcholine moiety of teichoic acid (*28*). This placement positions them optimally to participate in such processes as adhesion to epithelial or endothelial cells (*23*), to bind molecules synthesized at the mucosal barrier, such as secretory IgA (*24*), or to interfere with complement activation (*29*).

As isolated from the laboratory strain CP1200, the C3-binding protein CbpA has a signal sequence that was not identified in the original report, which utilized the strain LM91 (*23*). Our studies demonstrate secretion of CbpA in the absence of choline. Second, in the original descriptions of this choline-binding protein, a *cbpA*$^-$ mutant was substantially deficient in its ability to colonize the nasopharynx of infant rats but did not exhibit reduced virulence in a murine model of intravenous infection (*23*). However, in our studies, the ability of the C3-binding protein to elicit antibodies in humans recovering from pneumococcal disease suggests participation in the response to systemic pneumococcal disease in humans and not simply in colonization. The wide conservation of CbpA among both laboratory and clinical strains of *S. pneumoniae* (Figure 6, panels B and C) makes it one of the most prevalent pneumococcal surface proteins yet described; interestingly, the wide variation in molecular mass that is ascribed to differences in the number of choline binding repeats in related proteins such as PspA seems not to be associated with secreted forms of CbpA, at

least in the several serotypes analyzed in this report. Thus, the ability to interfere with C3-mediated attack, whether as a secreted protein or as bound to cell wall phosphorylcholine, appears to be widely conserved among pneumococci.

Prior to this report, the predominant microbial strategy to evade C3-mediated host defense involved secreted proteinases that cleave or degrade C3 (*30*, *31*). For example, *Pseudomonas aeruginosa* expresses an elastase-like molecule that cleaves the α-chain of C3, but leaves the *β*-chain intact (*32*, *33*). *Entamoeba histolytica* secretes a 56 kDa neutral cysteine proteinase that cleaves C3 between residues 78 and 79, only one amino acid residue distal to the natural site acted on by the C3 convertases (*34*). The binding of native C3, in contrast to its degradation, has not been previously reported, although glycoprotein 1C of herpes simplex is able to bind C3b, the activated form of the molecule (*35*). Deletion of the arp1 gene of *Aspergillus fumigatus* greatly increases C3b deposition on fungal conidia, suggesting that the gene product modulates C3 binding in some as yet uncharacterized fashion (*36*).

To our knowledge, streptococcal proteins that bind C3 have not previously been identified, although interference with opsonic deposition of C3b (*37*), a C5a peptidase (*38*), and inhibition of the membrane attack complex by the SIC protein (streptococcal inhibitor of complement-mediated lysis) (*39*) have been described in *S. pyogenes*. Within this context, the C3-binding properties of CbpA in *S. pneumoniae*, therefore, represent a novel contribution to the microbial arsenal because of its ability to engage either immobilized or fluid-phase C3. The conservation of CbpA among several encapsulated clinical isolates of *S. pneumoniae*, its production throughout pneumococcal growth, and its presence in both cell-bound and secreted forms suggest a potential role in combating opsonization and phagocytosis. Further study will be required to understand whether CbpA may amplify other known mechanisms of complement resistance by *S.pneumoniae*—the presence of polysaccharide capsule; the site, number, and structure of deposited C3 fragments; and their avidity for phagocytic receptors (*40*−*42*)—to augment the virulence of *S. pneumoniae*.

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