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The Emerging Pathogen *Moraxella catarrhalis* Interacts with Complement Inhibitor C4b Binding Protein through Ubiquitous Surface Proteins A1 and A2

Therése Nordström,* Anna M. Blom,* Arne Forsgren,* and Kristian Riesbeck*1

*Moraxella catarrhalis* ubiquitous surface protein A2 (UspA2) mediates resistance to the bactericidal activity of normal human serum. In this study, an interaction between the complement fluid phase regulator of the classical pathway, C4b binding protein (C4BP), and *M. catarrhalis* mutants lacking UspA1 and/or UspA2 was analyzed by flow cytometry and a RIA. Two clinical isolates of *M. catarrhalis* expressed UspA2 at a higher density than UspA1. The UspA1 mutants showed a decreased C4BP binding (37.6% reduction), whereas the UspA2-deficient *Moraxella* mutants displayed a strongly reduced (94.6%) C4BP binding compared with the wild type. In addition, experiments with recombinantly expressed UspA150–770 and UspA230–530 showed that C4BP (range, 1–1000 nM) bound to the two proteins in a dose-dependent manner. The equilibrium constants (Kd) for the UspA150–770 and UspA230–530 interactions with a single subunit of C4BP were 13 μM and 1.1 μM, respectively. The main isoform of C4BP contains seven identical α-chains and one β-chain linked together with disulfide bridges, and the α-chains contain eight complement control protein (CCP) modules. The UspA1 and A2 bound to the α-chain of C4BP, and experiments with C4BP lacking CCP2, CCP5, or CCP7 showed that these three CCPs were important for the Usp binding. Importantly, C4BP bound to the surface of *M. catarrhalis* retained its cofactor activity as determined by analysis of C4b degradation. Taken together, *M. catarrhalis* interferes with the classical complement activation pathway by binding C4BP to UspA1 and UspA2.


*Moraxella catarrhalis* was earlier considered to be a harmless commensal in the respiratory tract, but is now acknowledged as an important mucosal pathogen. It is the third leading bacterial cause of acute otitis medium in children after *Streptococcus pneumoniae* and *Haemophilus influenzae* (1–3). *M. catarrhalis* is also a common cause of sinusitis and lower respiratory tract infections in adults with chronic obstructive pulmonary disease. In recent years, focus has been on both its outer membrane protein composition and on its interactions with the human host (4). The outer membrane proteins of *M. catarrhalis* that are suggested as virulence determinants include, among others, Moraxella IgD binding protein (MID)3 (3), *catarrhalis* outer membrane protein B (CopB), protein CD, *M. catarrhalis* adherence protein, and ubiquitous surface protein (Usp) (for reviews, see Refs. 3 and 4). The MID, also designated Hag (for hemagglutinin), has recently been demonstrated to function as an adhesin (5–11). Moreover, mice immunized with MID764–913 cleared *M. catarrhalis* much more efficiently as compared with mice immunized with BSA (12). The conserved 81-kDa CopB plays a role in iron acquisition, and the heat-modifiable CD protein functions as an adhesin to nasal and inner ear mucins (13–15). In addition to MID, immunization with CopB or CD proteins has shown to induce protective Abs in a mouse pulmonary clearance model (16, 17). *M. catarrhalis* adherence protein was recently discovered and shown to exhibit both lipolytic and adhesive properties (18).

The UspA family has been studied in detail and consists of UspA1, UspA2, and the hybrid protein, UspA2H (19, 20). UspA1 and UspA2 have molecular masses of 88 and 62 kDa, respectively. However, both proteins migrate as high molecular mass complexes in SDS-PAGE. The amino acid sequences of UspA1 and UspA2 are only 43% identical, but both proteins bear a common epitope of 140 aa residues with 93% identity. Abs against the common epitope have been found to be protective against *M. catarrhalis* infections in a mouse pulmonary clearance model (21). Both UspA1 and UspA2H are responsible for adhesion of *M. catarrhalis* to epithelial cells in vitro (10, 20).

The majority of clinical *M. catarrhalis* isolates are serum resistant (22, 23). Interestingly, *Moraxella* mutants deficient in UspA2 or both UspA1 and UspA2 are much more susceptible to killing by human serum as compared with the wild-type counterpart or a UspA1 mutant. Therefore, it has been suggested that *M. catarrhalis* UspA2 is an important outer membrane protein associated with serum resistance due to interaction with vitronectin (24, 25).

The complement system is the first line of innate defense against pathogenic microorganisms, and activation of this system leads to a cascade of protein deposition on the bacterial surface, resulting in formation of the membrane attack complex and opsonization of the pathogen, followed by phagocytosis. C4b-binding protein (C4BP) is a fluid phase regulator of the classical pathway of complement activation. C4b inhibits the formation and accelerates the decay of the C3 convertase (C4bC2a), and it also serves as a cofactor to factor I in the proteolytic degradation of C4b (26–28).
C4BP is a large glycoprotein that is present in the plasma in several forms. The major form is composed of seven identical α-chains (70-kDa subunits) and one β-chain (45 kDa) (Fig. 1A) (29). The α- and β-chains consist of repeating domains of ~60 aa designated complement control protein (CCP) domains (30, 31). The unique β-chain of the major isof orm of C4BP binds to the vitamin K-dependent anticoagulant protein S. Consequently, in the majority of serum, C4BP circulates in a 1:1 high affinity noncovalent complex with protein S (29).

Several bacterial pathogens bind C4BP, and hence avoid deleterious effects of the complement system. C4BP binding has been demonstrated in many isolates of Streptococcus pyogenes, and the amino-terminal hypervariable regions of several members of the M family proteins are responsible for the binding (32). All clinical isolates of Bordetella pertussis expressing filamentous hemagglutinin, and also Escherichia coli (33), and also Streptococcus pyogenes, bind C4BP (33, 34). Furthermore, Neisseria gonorrhoeae has been reported to bind C4BP through porin protein 1A rhoeae protein A, bind C4BP (33, 34). Furthermore, Neisseria gonor-

Figure 1. Schematic illustration of plasma C4BP and recombinant C4BPSTOP8. A, The main C4BP isoform in serum consists of seven identical α-chains and one β-chain. The α-chains have eight CCP domains, whereas the β-chain has three CCP domains. All chains are held together by disulfide bridges involving the nonrepeat carboxy-terminal regions. B, recombinant C4BPSTOP8 has a single α-chain with a stop codon introduced after CCP8 and is devoid of the carboxy-terminal. recombinant C4BPSTOP8 was manufactured in kidney cells 293; ATCC CRL-1573.

Materials and Methods

Bacterial strains and culture conditions

The clinical M. catarrhalis isolates BBH18, RH4, and Bc5 have recently been described in detail (5, 8). The M. catarrhalis strains were routinely cultured in brain heart infusion (BHI) broth or on BHI agar plates at 37°C. The UspA1-deficient mutants were cultured in BHI supplemented with 1.5 µg/ml chloramphenicol (Sigma-Aldrich, St. Louis, MO), and UspA2-deficient mutants were incubated with 7 µg/ml zeocin (Invitrogen Life Technologies, Carlsbad, CA). Both chloramphenicol and zeocin were used for growth of the double mutants.

Antibodies

Rabbits were immunized i.m. with 200 µg of recombinant full-length UspA1 emulsified in CFA (Difco; Becton Dickinson, Heidelberg, Germany), and boosted on days 18 and 36 with the same dose of protein in IFA (9). Blood was drawn 3 wk later. To increase the specificity, the anti-UspA1 antisera was affinity-purified with Sepharose-conjugated recombinant UspA1. To ensure that the antiserum reacted with the same affinity to both recombinant UspA1100–770 and UspA230–539, the antiserum was examined with ELISA. UspA1100–770 or UspA230–539 (20 nM) were immobilized in microtiter plates and incubated with increasing concentrations of the antiserum, followed by HRP-conjugated goat anti-rabbit antisera diluted 1/1000 (Dakopatts, Glostrup, Denmark). The binding of anti-UspA1 antisera to both UspA1 and UspA2 was also confirmed by Western blots. Hence, the antiserum was designated anti-UspA1/A2 polyclonal Ab (pAb).

The UspA1–C4bp binding site was localized within the CCP2, CCP5, and CCP7 domains of the C4BP α-chain. By investigating the degradation of C4b, C4BP bound to the surface of M. catarrhalis was found to have preserved cofactor activity.

Construction and characterization of UspA1/A2-deficient M. catarrhalis

The UspA1-coding genes were amplified as two cassettes using DyNAzyme II DNA polymerase (Finzymes, Espoo, Finland), introducing the restriction enzyme sites, BamHI and HindIII, at the ends of the first cassette, and HindIII and XhoI at the ends of the second cassette. Resulting PCR fragments were digested with appropriate restriction enzymes and cloned into the pET28b (+) vector (Novagen, Madison, WI). A chloramphenicol resistance gene cassette from pLysS (Novagen) was amplified by PCR, using specific primers introducing the restriction enzyme site for HindIII. After digestion, the PCR product was ligated into the uaspA1 gene. A zeocin resistance gene cassette was amplified from the plasmid pEM7/Zeo (Invitrogen Life Technologies) with specific primers introducing HindIII, and the resulting PCR product was digested and ligated into the uaspA1 gene. M. catarrhalis strains RH4 and BHBI8 were transformed by electroporation using a Gene pulser apparatus (Bio-Rad, Hercules, CA) and the settings 2.5 kV, 25 µF, and 200 Ω. After transformation, bacteria were cultured in BHI broth without antibiotics for 6 h, and thereafter grown on BHI solid medium supplemented with chloramphenicol and/or zeocin. Resulting mutants were screened by PCR and the protein expression was analyzed by Western blot and flow cytometry.

Serum bactericidal assay

Normal human serum ( NHS) was obtained from five healthy volunteers. The blood was clotted for 30 min at room temperature (RT), and thereafter incubated on ice for 60 min. After centrifugation, the sera were pooled, aliquoted, and stored at ~70°C. Serum that was inactivated at 56°C for 30 min was used as a control. The M. catarrhalis strains and mutants were diluted in DGBV2+ (2.5 mM Veronal buffer, pH 7.3, containing 0.1% (w/v) gelatin, 1 mM MgCl2, and 0.15 mM CaCl2). Bacteria (106 CFU) were incubated together with 10% of NHS or heat-inactivated NHS in a final volume of 100 µl. This mixture was incubated at 37°C, and at time 0, 5, 10, 15, 20, and 30 min, 10-µl aliquots were removed and spread onto BHI agar plates.
DNA cloning and protein expression

Genomic DNA was extracted from _M. catarrhalis_ Bc5 using a DNeasy tissue kit (Qiagen, Hilden, Germany). The UspA-coding genes were amplified using DsNaAzyme II DNA Polymerase with specific primers introducing BumIII and HindIII restriction enzyme sites. The UspA1 and UspA2 are considered to be Pneumotransporters (38). Thus, the signal peptides and the C-terminal sequences are most likely not involved in the function of the proteins. Therefore, to avoid presumptive toxicity and to increase solubility of the signal peptides and the hydrophobic C-terminal regions were not included. The resulting PCR products corresponded to the truncated proteins designated UspA1<sup>1-70</sup> and UspA2<sup>30-539</sup>. The PCR products were cloned into pET26b<sup>*/H11001</sup> vector and the resulting plasmids were transformed into _E. coli_ DH5α. The plasmids encoding for the Usp-proteins were transformed into the expressing host BL21(DE3) (Novagen). All constructs were sequenced using the BigDye Terminator Cycle Sequencing version 3.1 Ready reaction sequencing kit (Applied Biosystems, Foster City, CA). The expression and purification of the recombinant proteins was done as previously described (7).

Outer membrane protein preparations

_**M. catarrhalis**_ forms vesicles and secretes outer membrane components into the surrounding medium (39). EDTA and heat induce vesicle formation, and this has proved to be a convenient and reliable method for extracellular _M. catarrhalis_ outer membrane proteins. To analyze membrane proteins of the UspA1/A2-deficient _M. catarrhalis_ mutants and the wild-type strains, vesicle formation was induced using 0.05 M Na<sub>2</sub>HPO<sub>4</sub>, 0.15 M NaCl, 0.01 M EDTA (pH 7.4) at 56°C. The bacteria were washed and incubated for 30 min at 37°C. After thorough washings in the same buffer, the bacteria were mixed with E.coli DH5α. Thereafter, the supernatants containing the BigDye Terminator Cycle Sequencing version 3.1 Ready reaction sequencing kit (Applied Biosystems, Foster City, CA). The expression and purification of the recombinant proteins was done as previously described (7).

**SDS-PAGE and detection of proteins on membranes (Western blots)**

SDS-PAGE was run as described before (5). Gels were stained with Coomassie brilliant blue R-250 (Bio-Rad). Electrophoretical transfer of protein bands from the gel to an Immobilon-P membrane (Millipore, Bedford, MA) was done at 20 V overnight to transfer the high molecular mass complexes. After transfer, the Immobilon-P membrane was blocked in PBS with 0.1% Tween 20 (PBS-Tween) containing 5% milk powder. After several washings in PBS-Tween, the membrane was incubated with rabbit anti-UspA1/A2 antisera diluted 1/500 in PBS-Tween, including 2% milk powder, for 1 h at RT. HRP-conjugated goat anti-rabbit antisera diluted 1/1000 was added after washings in PBS-Tween. After incubation for 1 h at RT and additional washings in PBS-Tween, development was performed with ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Uppsala, Sweden).

**Complement proteins**

Human C4BP was purified from human plasma (40). Recombinant wild-type C4BP (rC4BP) was expressed in human kidney cells 293 (ATCC CRL-1573; American Type Culture Collection, Manassas, VA), and purified using affinity chromatography with mAbs against the a-chains of C4BP (41). rC4BP<sup>41-73</sup>, containing only one a-chain (Fig. 1B), was prepared in the same way, with the exception of a deletion of the nonrepeat carboxy-terminal region involved in the polymerization of the chains. rC4BP lacking single CCP domains (C4BP mutants) were constructed by overlapping extension PCR, and were expressed in kidney cells 293, followed by purification with affinity chromatography (37). All the mutants were extensively characterized to show that no folding problem was introduced by mutagenesis (37) and were previously used to define binding sites for several ligands (34–36, 42, 43). C4b, C3b, and factor I were from Advanced Research Technologies (San Diego, CA).

**Flow cytometry analysis**

The UspA1/A2-protein expression and the capacity for _M. catarrhalis_ to bind C4BP were analyzed by flow cytometry. The _**M. catarrhalis**_ wild-type strains and UspA1/A2-deficient mutants were grown on solid medium overnight and washed twice in PBS containing 3% fish gelatin (Sigma, Aldrich) (PBS-gelatin). To estimate which CCP domain of C4BP is involved in the binding of UspA1 and UspA2, microtiter plates were immobilized with 40 nM UspA1<sup>1-70</sup> or UspA2<sup>30-539</sup> in 75 mM sodium carbonate (pH 9.6) at 4°C overnight. Unbound proteins were measured to ensure that C4BP bound equally well to the plastic. Plates were washed four times with washing buffer (50 mM Tris-HCl, 0.15 M NaCl, and 0.1% Tween 20, pH 7.5), and blocked for 2 h with washing buffer supplied with 3% fish gelatin (blocking buffer). After four washings, the wells were incubated for 1 h at 37°C with C4BP diluted in 2-fold steps in blocking buffer, with the highest concentration at 1000 nM. Thereafter, the wells were washed and incubated with mAb104 (1/5000) in blocking buffer for 1 h in RT. After additional washings, HRP-conjugated rabbit anti-mouse pAbs (Dokapotts) diluted 1/1000 was added for 1 h at RT. The wells were washed four times and the plates were exposed and measured as previously described below.

**Determination of K<sub>D</sub>**

Equilibrium affinity constants were obtained from plots of C4BP binding of UspA1<sup>1-70</sup> or UspA2<sup>30-539</sup> as a function of the C4BP concentration (45). The data were fit to the following equation (Equation 1) for a single-site binding isotherm using nonlinear least squares regression analysis: B =
PBS-Tween. Thereafter, 30 kcpm 125I-labeled UspA1 was mixed with PBS-Tween containing 5% milk powder in RT and washed four times with a blot device. After saturation, the membranes were incubated for 2 h with increasing concentrations with UspA1. The binding was analyzed using surface plasmon resonance (Biacore 2000; Biacore). The recombinant UspA1 50–770 or UspA2 30–539 were manually applied to nitrocellulose membranes (Schleicher & Schuell). 0.1 M Tris-HCl (pH 9.0) were manually applied to nitrocellulose membranes (Schleicher & Schuell) for 100 s to achieve saturation during the association phase at a constant flow rate of 30 μl/min, and the dissociation phase was analyzed for 200 s at the same flow rate. Signals were then normalized by subtracting the nonspecific signal measured in flow cell 1. Between each different concentration, flow cell surfaces were regenerated with a 30-μl injection of 2 M NaCl to remove bound ligand. All sensograms were analyzed using the BioEvaluation 3.0 software (Biacore) to calculated equilibrium affinity constants.

Dot blot assays

Purified c4BP variants diluted in 3-fold steps (0.0014–3 μg/ml) in 100 μl of 0.1 M Tris-HCl (pH 9.0) were manually applied to nitrocellulose membranes (Schleicher & Schuell Microscience, Dassel, Germany) using a dot blot device. After saturation, the membranes were incubated for 2 h with PBS-Tween containing 5% milk powder in RT and washed four times with PBS-Tween. Thereafter, 30 kcpm 125I-labeled UspA1 50–770 or UspA2 30–539 in PBS-Tween with 2% milk powder was added for 3 h in RT. The bound protein was visualized with a Personal FX (Bio-Rad) using intensifying screens.

Results

Characterization of UspA1- and A2-deficient M. catarrhalis mutants

Two clinical isolates (BBH18 and RH4) were mutated by introduction of chloramphenicol and zeocin resistance cassettes in the genes encoding for UspA1 and UspA2, respectively. Resulting mutants were confirmed by PCR. Moreover, absence of UspA1 and/or UspA2 expression was proven by analysis of outer membrane vesicles (i.e., EDTA heat-induced vesicles) in Western blots using an anti-UspA1/A2 antiserum. The BBH18ΔuspA1 mutant lacked the 115-kDa band (filled arrowhead), whereas BBH18ΔuspA2 expressed neither the high molecular mass protein nor the 100-kDa band (open arrowheads). The BBH18ΔuspA1/A2 double mutant was deficient in all three bands.

FIGURE 2. Western blot analysis of M. catarrhalis BBH18ΔuspA1, ΔuspA2, and ΔuspA1/A2 mutants compared with the wild-type counterpart. To induce vesicle formation, the outer membrane proteins were extracted using an EDTA-containing buffer at 56°C. Resulting proteins were analyzed by Western blots using a rabbit anti-UspA1/A2 antiserum and HRP-conjugated goat anti-rabbit pAb. The BBH18ΔuspA1 mutant lacked the 115-kDa band (filled arrowhead), whereas BBH18ΔuspA2 expressed neither the high molecular mass protein nor the 100-kDa band (open arrowheads). The BBH18ΔuspA1/A2 double mutant was deficient in all three bands.

mfi, 10.3) (Fig. 3C) as compared with the UspA1-deficient mutant. When both UspA1 and UspA2 were knocked out, the resulting mutant was completely deficient in both proteins (Fig. 3D). Similar results were obtained with the M. catarrhalis RH4 wild-type isolate and corresponding mutants (data not shown). Thus, the phenotypes of all the M. catarrhalis mutants were defined and the Western blot results were correlated with the flow cytometry data. In addition, an interesting observation was that our two selected M.

FIGURE 3. Flow cytometry profiles of M. catarrhalis BBH18 wild-type and UspA1/A2-deficient mutants proving a UspA1/A2-dependent c4BP binding. The BBH18 wild-type clinical isolate (A and E), BBH18ΔuspA1 (B and F), BBH18ΔuspA2 (C and G), or BBH18ΔuspA1/A2 (D and H) were incubated with a rabbit anti-UspA1/A2 antiserum (A–D) or c4BP (E–H) followed by anti-c4BP pAb (E–H). Finally, a FITC-conjugated anti-rabbit antiserum was added. The mfi for each profile is also shown. A typical experiment of three is demonstrated.
M. catarrhalis isolates (BBH18 and RH4) displayed a higher density of UspA2 as compared with UspA1.

The M. catarrhalis ΔuspA2 mutants are serum sensitive

The wild-type strains and the mutant strains were tested in a serum bactericidal assay. The wild-type strains BBH18 and RH4 were completely resistant to NHS, whereas their derived UspA1 mutants were only partially resistant (Fig. 4). However, the M. catarrhalis ΔuspA2 mutants and the double mutants were killed by NHS after 5 min. Both strains and all derived mutants were resistant to heat-inactivated NHS.

M. catarrhalis devoid of UspA1 and UspA2 does not bind C4BP

Binding of C4BP to M. catarrhalis was analyzed by flow cytometry using a polyclonal anti-C4BP antiserum. Interestingly, the M. catarrhalis BBH18 isolate strongly bound C4BP (mfi, 99.4) (Fig. 3E). In contrast, BBH18ΔuspA1 showed a decreased C4BP binding (mfi, 62.0) compared with the wild-type counterpart (Fig. 3F). Furthermore, BBH18Δuspa2 attracted C4BP to a much lower degree (mfi, 5.4) compared with the BBH18ΔuspA1 mutant (Fig. 3G). Consequently, C4BP binding to the BBH18ΔuspA1/A2 double mutant was lower (mfi, 2.7) as compared with the single mutants (Fig. 3H). A similar pattern was obtained with the M. catarrhalis RH4 isolate and the corresponding RH4ΔuspA1/A2 mutants.

To further analyze the interaction between C4BP and M. catarrhalis, 125I-labeled C4BP was added to the two clinical isolates BBH18 and RH4. Both M. catarrhalis strains strongly bound 125I-labeled C4BP (Fig. 5), i.e., 45–55.7% of the added 125I-labeled C4BP bound. However, no significant difference was observed between the M. catarrhalis wild-type strains and the corresponding ΔuspA1 mutants. In contrast, the M. catarrhalis ΔuspA2 and double mutants did not bind 125I-labeled C4BP above background levels (1.2–5.0% of maximal binding). We also included M. catarrhalis RH4 and BBH18 devoid of the outer membrane protein MID (9) as positive controls. Experiments with these two M. catarrhalis Δmid mutants showed the same C4BP binding as the wild-type counterparts (Fig. 5). Taken together, M. catarrhalis ΔuspA1 mutants displayed a 38% decrease in C4BP binding when analyzed by flow cytometry (Fig. 3B), whereas the less sensitive RIA did not demonstrate any significant decrease in 125I-labeled C4BP binding to M. catarrhalis ΔuspA1 mutants. Furthermore, M. catarrhalis strongly bound C4BP, and a strict correlation existed between UspA1/A2 expression and C4BP binding.

C4BP exhibits cofactor activity at the M. catarrhalis cell surface

C4BP serves as a cofactor to Factor I in the degradation of C4b, which results in appearance of the two fragments C4d and C4c. Upon cleavage, it has been demonstrated that C4d remains bound to the surface of the bacteria, but that C4c is released to the surrounding medium resulting in an increased C4d/C4c ratio at the bacterial surface (35). To investigate whether C4BP was active at the M. catarrhalis cell membrane, the BBH18 wild type was coated with C4BP and thereafter incubated with normal human serum depleted of C4BP. Surface-bound C4c and C4d was analyzed by flow cytometry using specific mAbs directed against C4c or C4d. Cofactor activity of C4BP will not alter the amount of C4b measured by mAb against C4b, but will decrease the amount of C4b detected by the mAb against C4c. A higher C4d/C4c ratio was detected at the bacterial surface when M. catarrhalis BBH18 that was preincubated with C4BP (Fig. 6B) was compared with M. catarrhalis that were not preincubated with C4BP (Fig. 6A). Thus, C4BP bound to M. catarrhalis retained its cofactor function because C4b was degraded to C4d. The activity of C4BP was confirmed by analysis of C3b deposition on M. catarrhalis. Because C4BP inhibits C3 convertase, a decreased C3b deposition will be detected if C4BP is functionally active. Bacteria were coated with C4BP, followed by incubation with C4BP-deficient serum. C3b deposition was then determined with anti-C3d pAb and FITC-conjugated anti-rabbit pAb. Interestingly, when bacteria were preincubated with C4BP, the C3b deposition significantly decreased, i.e., the mfi were 30–41% lower with C4BP-coated bacteria compared with bacteria only.

To further confirm the cofactor activity of C4BP bound to M. catarrhalis, the RH4 wild-type strain with or without addition of C4BP was incubated with factor I and 125I-labeled C4b. When 125I-labeled C4b was incubated in the presence of C4BP-coated M. catarrhalis RH4, the degradation product, C4d, strongly appeared (Fig. 6C). However, no C4b degradation (i.e., C4d was not formed) was seen with bacteria only. Thus, C4BP retained its cofactor capacity to degrade C4b when it was bound to M. catarrhalis.

FIGURE 4. The M. catarrhalis BBH18ΔuspA2 and ΔuspA1/A2 mutants were serum sensitive, whereas M. catarrhalis BBH18 wild type was serum resistant and the BBH18ΔuspA1 mutant was partially serum resistant. The wild-type ( ), ΔuspA1 mutant ( ), ΔuspA2 mutant ( ), and ΔuspA1/A2 mutant ( ) strains were incubated in the presence of 10% NHS. The double mutant was also incubated with 10% heat-inactivated NHS ( ). Numbers of bacteria (CFU) before addition of NHS was defined as 100%. Error bars indicate SD.

FIGURE 5. M. catarrhalis UspA2-deficient mutants do not bind C4BP. M. catarrhalis BBH18 and RH4 wild-type isolates were compared with mutants devoid of UspA1, UspA2, or both UspA1 and A2. In addition, E. coli BL21 were included as negative control and two M. catarrhalis Δmid strains as positive controls. Bacteria were incubated with 125I-labeled C4BP, followed by several washes and analysis in a gamma counter. The mean values of three experiments are shown. Error bars indicate SD.
Recombinant UspA1\(^{50-770}\) and UspA2\(^{30-539}\) bind C4BP in a dose-dependent manner

To further analyze the interaction between C4BP and the UspA1 and A2, the truncated proteins UspA1\(^{50-770}\) and UspA2\(^{30-539}\) were recombinantly produced in E. coli. To evaluate whether the C4BP/UspA interaction was dose dependent, microtiter plates coated with UspA1\(^{50-770}\) or UspA2\(^{30-539}\) were incubated with C4BP at increasing concentrations. Bound C4BP was detected by a specific anti-C4BP mAb. As can be seen in Fig. 7, UspA1\(^{50-770}\) and UspA2\(^{30-539}\) bound to C4BP in a dose-dependent manner; however, UspA1\(^{50-770}\) required higher concentrations of C4BP for binding as compared with UspA2\(^{30-539}\). The results from these experiments allowed calculation of apparent \(K_D\) values of the interactions. \(K_D\) values were obtained by fitting the data in Fig. 7 to Equation 1. The calculated \(K_D\) for UspA2\(^{30-539}\)/C4BP was 26.5 nM, whereas it was 57 nM for UspA1\(^{50-770}\)/C4BP.

The interaction between C4BP and UspA1 or A2 was further confirmed using a competition assay after saturated conditions of UspA1\(^{50-770}\) or UspA2\(^{30-539}\) and \(^{125}\)I-labeled C4BP were defined (Fig. 8A), were incubated with \(^{125}\)I-labeled C4BP in the presence of increasing C4BP concentrations. Unlabeled C4BP specifically inhibited the binding between \(^{125}\)I-labeled C4BP and UspA1\(^{50-770}\) or UspA2\(^{30-539}\) (Fig. 8B). A total of 22 nM C4BP was required to block the UspA2\(^{30-539}\)/\(^{125}\)I-labeled C4BP interaction by 50% (IC\(_{50}\)), whereas 70 nM was required to block 50% of the UspA1\(^{50-770}\) binding to \(^{125}\)I-labeled C4BP.

To establish the affinity constant of the UspA/C4BP interaction, binding experiments using surface plasmon resonance (i.e., Bia-core) were performed. UspA1\(^{50-770}\) and UspA2\(^{30-539}\) were immobilized on the surface of a CM5 chip using amino coupling. A single \(\alpha\)-chain of recombinant C4BP (rC4BP\(^{STOP8}\), Fig. 1B) was injected until saturation was reached (Fig. 9). Due to the low affinity, the association or the dissociation rate affinity could not be calculated. However, the \(K_D\) (the equilibrium dissociation constant) was calculated from a binding curve showing response at equilibrium plotted against the concentration using steady-state affinity model supplied by BiaEvaluation software (Biacore). The \(K_D\) for the binding between rC4BP\(^{STOP8}\) and UspA1\(^{50-770}\) or UspA2\(^{30-539}\) was 13 \(\mu\)M and 1.1 \(\mu\)M, respectively.

CCP2, -5, and -7 subunits contribute to the binding of rC4BP to UspA1/A2

The main isoform of human C4BP circulating in plasma consists of two types of subunits, i.e., seven identical \(\alpha\)-chains and one \(\beta\)-chain. To identify the subunit that was responsible for the interaction of C4BP with UspA1/A2, we analyzed whether recombinantly produced C4BP containing polymerized \(\alpha\)-chains (but no \(\beta\)-chain) bound to M. catarrhalis. Flow cytometry analyses revealed that C4BP and plasma-derived C4BP equally bound to M. catarrhalis. This suggested that the \(\beta\)-chain was not involved in the interaction, but that the UspA-dependent binding was localized within the \(\alpha\)-chain.

To evaluate which \(\alpha\)-chain CCP subunit was involved in the C4BP/UspA interaction, eight mutant rC4BP proteins, each lacking one of the eight CCP subunits, were analyzed for binding. Immobilized UspA1\(^{50-770}\) or UspA2\(^{30-539}\) was incubated with equal amounts of the rC4BP CCP subunit mutants. Interestingly,
binding of UspA2 30–539 to rC4BP lacking either CCP2 or CCP7 strongly decreased (Fig. 10A), and the binding to the /H9004 CCP5 mutant decreased with two-thirds. Similar results were obtained with UspA1 50–770 (results not shown). The results were also confirmed by dot blots using iodine-labeled UspA1 50–770 or UspA2 30–539. Interestingly, we did not observe any inhibition of the interaction between M. catarrhalis and C4BP using NaCl, heparin, or C4b, all of which disrupt bindings based on ionic interactions with CCP1–2 of C4BP.

Discussion
In the present study, we demonstrate a novel interaction between the respiratory pathogen M. catarrhalis and the important complement regulator C4BP. Complement resistance is crucial for bacterial virulence. A large number of clinical Moraxella catarrhalis isolates (n = 1350) have been analyzed for serum resistance (23, 46). Interestingly, 89% of the Moraxella isolates from patients with lower respiratory tract infections were strongly or intermediate resistant to complement-mediated killing. In contrast, the percentage of serum-resistant M. catarrhalis harbored by healthy carriers was as low as 41.5%. Binding of complement inhibitors such as C4BP or factor H is a widely spread strategy of serum-resistant pathogens (47).

Several studies have indicated that complement proteins and regulators are present in the human respiratory tract (48, 49). In addition, complement activity can be detected in the extracellular matrix during inflammation and increased vascular permeability.
Because C4BP in complex with protein S binds to phospholipid membranes (51, 52), another possibility would be that C4BP promotes adhesion by binding simultaneously to *M. catarrhalis* and the epithelial cell surface.

Flow cytometry analysis and RIAs of UspA1- and UspA2-deficient mutants revealed that UspA1/A2 was crucial for binding of C4BP. Furthermore, the Western blot and flow cytometry showed that UspA2 was expressed at a much higher density compared with UspA1 (Figs. 2 and 3). Thus, the UspA2-deficient mutants displayed a much stronger decrease in C4BP binding than the UspA1-deficient mutants. This was supported by the results from the serum bactericidal assay (Fig. 4). Interestingly, the BBH18AuspA1 mutant was only partly serum resistant. This differed from a previous study showing that *M. catarrhalis* O35E devoid of UspA1 was as resistant to complement-mediated killing as the wild-type counterpart (22).

Recombinantly produced UspA150–770 and UspA250–599 bound C4BP to a similar extent, and both interactions were dose dependent and specific (Figs. 7 and 8). UspA1 and A2 are in part strongly related, but comparison of the amino acid sequences from four *M. catarrhalis* strains shows only 41.1–46.0% identity and 55.3–63.5% similarity (53). Interestingly, the two outer membrane proteins share a common epitope consisting of 140 aa residues with 93% identity (19), suggesting that the C4BP binding site may be, at least in part, located within this sequence.

We used a single rC4BP α-chain (rC4BP STOP8) as binding partner in our Biacore analyses. The *Kₐ* values determined from the interactions where C4BP wild type was used (Fig. 7) were in nanomolar range. Thus, UspA1 and UspA2 most likely interact simultaneously with more than one α-chain in polymeric C4BP molecule. It has been shown previously for other C4BP ligands that up to four interactions are possible at the same time (31, 54).

The interactions between UspA1 or UspA2 and C4BP appeared to be nonionic because they were not disrupted in presence of high salt concentration or heparin. Moreover, experiments with rC4BP mutants, each deficient in one CCP module, revealed that CCP2 and CCP7 on the α-chain are the main binding sites for both recombinant UspA50–599 (Fig. 10A) and UspA50–270 (51, 77–79). Furthermore, the ΔCCP5 mutant had lost two-thirds of its binding capacity. Several pathogens interact with CCP2, whereas *M. catarrhalis* is the first bacterium described that uses CCP7 as a recognition site for C4BP binding. *Streptococcus pyogenes* M proteins bind C4BP in a nonionic manner and recognize CCP1 and CCP2, and their binding sites overlap to some extent with the binding site for C4b (32, 41). Filamentous hemagglutinin from *Bordetella pertussis* interacts with C4BP in a very similar way as C4b; anionic binding with a cluster of charged amino acids on the interface of CCP1 and CCP2 (33, 43). In parallel, the interaction between porins PorIA and PorIB from *N. gonorrhoeae* and C4BP also requires CCP1. The PorIA-C4BP and PorIB-C4BP interactions are based on hydrophilic and ionic bindings, respectively (35). Furthermore, isolated type IV pili from *N. gonorrhoeae* display an ionic binding to C4BP that involves CCP1 and CCP2 (36). Finally, outer membrane protein A from *E. coli* K1 interacts with CCP3 on C4BP, and the binding is based on a hydrophobic interaction (34). Thus, with the exception of *M. catarrhalis*, CCP1–3 are the major modules of the C4BP α-chain that are used by several bacterial species.

The ability of *M. catarrhalis* to bind C4BP suggests that the species uses C4BP’s capacity to inhibit the complement-mediated attack in two ways. Firstly, C4BP bound to the surface of *M. catarrhalis* maintains its activity to degrade C4b to C4c and C4d (Fig. 6). Consequently, such degradation prevents C4b from participating in the opsonization of the pathogen. Secondly, because surface-bound C4BP binds C4b, the formation of C3 convertase (C4bC2a) will most likely be inhibited and its decay accelerated. This may help *M. catarrhalis* to avoid membrane attack complex-mediated lysis.

Taken together, we have presented several lines of evidence on *M. catarrhalis* UspA1 and UspA2 binding to C4BP, a factor that inhibits the classical pathway of the complement system. Because UspA2 is expressed at a higher density as compared with UspA1, UspA2 most likely has a stronger impact on *M. catarrhalis* binding to C4BP and, therefore, contributes to *M. catarrhalis* serum resistance and, consequently, virulence.

**References**


