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

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Ubiquitous Surface Proteins A1 and A2

Therése Nordström,* Anna M. Blom,* Arne Forsgren,* and Kristian Riesbeck2*

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 UspA230–539 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 UspA230–539 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. The Journal of Immunology, 2004, 173: 4598–4606.

M. 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), 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 been recently 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. C4BP 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 isoform 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 K1 expressing the outer membrane protein A, bind C4BP (33, 34). Furthermore, Neisseria gonorrhoeae protein A, bind C4BP STOP8. Furthermore, Neisseria gonor- rhoeae protein A, bind C4BP STOP8. Furthermore, Neisseria gonor-

**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 liquid 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 antiserum was affinity-purified with Sepharose-conjugated recombinant UspA1. To ensure that the antiserum reacted with the same affinity to both recombinant UspA1 (10-70 and UspA2 (50-89), the antiserum was examined with ELISA. UspA1 (50-70) or UspA2 (50-89) (20 nM) were immobilized in microtiter plates and incubated with increasing concentrations of the antiserum, followed by HRP-conjugated goat anti-rabbit antiserum diluted 1/1000 (Dakopatts, Glostrup, Denmark). The binding of anti-UspA1 antiserum to both UspA1 and UspA2 was also confirmed by Western blots. Hence, the antiserum was designated anti-UspA1/A2 polyclonal Ab (pAb). The pAbs, mAb104 and mAb67, against C4BP were kindly provided by Dr. B. Dahlbäck (Department of Clinical Chemistry, Lund University, Malmö, Sweden). mAb104 was raised against CCP1, whereas mAb67 was against CCP4 as described by Blom et al. (37). The rabbit anti-C3b pAb was purchased from Dakopatts. The anti-C4c and anti-C4d mAbs were from Quidel (San Diego, CA).

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

The UspA-coding genes were amplified as two cassettes using DyNAzyme II DNA Polymerase (Finnzymes, Espoo, Finland), introducing the restriction enzyme sites, BamHI and HindIII, at the ends of the first cassette, and HindIII and Xhol 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 pLyS8 (Novagen) was amplified by PCR, using specific primers introducing the restriction enzyme site for HindIII. After digestion, the PCR product was ligated into the uspA1 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 uspA2 gene. M. catarrhalis strains RH4 and BBH18 were transformed by electroporation using a GenePulser 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 agar plates.

**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 DGVB (2.5 mM Veronal buffer, pH 7.3, containing 0.1% (w/v) gelatin, 1 mM MgCl2, and 0.15 mM CaCl2). Bacteria (107 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 Be5 using a DNeasy tissue kit (Qiagen, Hilden, Germany). The UspA-coding genes were amplified using DyNAzyme II DNA Polymerase with specific primers introducing BamHI and HindIII restriction enzyme sites. The UsPA1 and Uspa2 are considered to be autotransporters (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 \(^{50–70}\) and Uspa2 \(^{50–539}\). The PCR products were cloned into pET26b (+) vector and the resulting plasmids were transformed into the host E. coli DH5\(\alpha\). Thereafter, 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 the membrane proteins of the UsPA1/2-deficient M. catarrhalis mutants and the wild-type strains, vesicle formation was induced using 0.05 M \(\text{Na}_2\text{HPO}_4\), 0.15 M NaCl, 0.01 M EDTA (pH 7.4) at 56°C (39). After centrifugation, the supernatants were concentrated using Vivaspin columns (Vivascience, Hannover, Germany). Finally, vesicles were analyzed on SDS-PAGE and Western blot.

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-UspA1/2 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 \(\alpha\)-chains of C4BP (41). rC4BP \(^{539}\), containing only one \(\alpha\)-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 UspA1/2-A2 protein expression and the capacity for M. catarrhalis to bind C4BP were analyzed by flow cytometry. The M. catarrhalis wild-type strains and UsPA1/2-deficient mutants were grown on solid medium overnight and washed twice in PBS containing 3% fish gelatin (Sigma Aldrich) (PBS-gelatin). To analyze UsPA1 expression, the bacteria (10\(^8\)) were incubated with the anti-UsPA1/2 antisera in 100 \(\mu\)l of PBS-gelatin for 1 h at 37°C. The bacteria were washed and incubated for 30 min at RT with FITC-conjugated goat anti-rabbit pAb (Dakopatts) diluted according to the manufacturer’s instructions. After three additional washes, the bacteria were analyzed in a flow cytometer (EPICS, XL-MCL; Coulter, Hialeah, FL). C4BP binding to whole bacteria was analyzed by incubation of the bacteria (10\(^8\)) with 2.5 \(\mu\)g of C4BP in PBS-gelatin for 1 h at 37°C. After washings, the bacteria were incubated with 0.5 \(\mu\)g of anti-C4BP pAb per milliliter for 30 min at RT. Thereafter, the bacteria were washed and incubated for 30 min at RT with FITC-conjugated goat anti-rabbit pAb. After three additional washes, bacteria were analyzed by flow cytometry. All incubations were kept in a final volume of 100 \(\mu\)l of PBS-gelatin, and the washings were done with the same buffer. The anti-C4BP pAb and FITC-conjugated anti-rabbit pAb were added separately as a negative control for each strain analyzed.

To analyze C4BP cofactor activity, M. catarrhalis BBH18 wild-type (10\(^8\)) was incubated with 5 \(\mu\)g of C4BP in PBS-gelatin for 1 h at 37°C, followed by washings and addition of 10% of C4BP depleted normal human serum. After 30 min at 37°C, the bacteria were washed and 1 \(\mu\)g/ml anti-C4c or anti-C4d mAbs (Quidel) were added. After another 30 min, the bacteria were washed and FITC-conjugated goat anti-mouse pAb (Dakopatts) was added. In another set of experiments, the bacteria were incubated with anti-C3d pAb (Dakopatts) (1/20) after incubation with serum, followed by a FITC-conjugated anti-rabbit pAb. The bacteria were washed and analyzed by flow cytometry. M. catarrhalis BBH18 wild type without preincubation with C4BP was used as negative control.

C4b-degradation assay

M. catarrhalis RH4 wild type (5 \(\times\) 10\(^8\)) was incubated with 15 \(\mu\)g of C4BP in 50 nM Tris-HCl (pH 7.4) supplemented with 150 mM NaCl for 1 h at 37°C. After thorough washings in the same buffer, the bacteria were mixed with 80 nM C4b, 60 nM factor I, and trace amounts of 125 I-labeled C4b in 50 \(\mu\)l of buffer. As a positive control, 100 nM C4BP was used in fluid phase instead of the preincubated bacteria. As a negative control, the RH4 wild-type Moraxella without preincubation of C4BP was used. The samples were incubated for 2 h at 37°C and the reaction was terminated by the addition of SDS-PAGE sample buffer. The reduced SDS-PAGE was run as described above. Thereafter, the gel was dried and the proteins were visualized with a Personal FX (Bio-Rad) using intensifying screens.

Protein labeling and RIA

Purified recombinant UsPA1 \(^{50–70}\), Uspa2 \(^{50–359}\), or C4BP were labeled with 0.05 mol iodine (Amersham Biosciences, Buckinghamshire, U.K.) per mol protein, using the chloramine-T method (44). The specific activity of 125I-labeled C4BP was 5.7 kcpmng. M. catarrhalis strains BBH18 and RH4 wild types and corresponding mutants were grown overnight on solid medium and were washed in PBS with 2% BSA. Bacteria (10\(^7\)) were incubated for 1 h at 37°C with 125I-labeled C4BP (1600 kcpm/sample) in PBS, 2% BSA. After three washings with PBS containing 2% BSA, radiolabeled C4BP bound to bacteria was measured in a gamma counter (Wallac, Espoo, Finland).

Direct ligand binding

Microtiter plates (Nunc-Immuno Module; Nunc, Roskilde, Denmark) were coated with 20 nM of purified recombinant UsPA1 \(^{50–70}\)/Uspa2 \(^{50–359}\) in 75 mM sodium carbonate (pH 9.6) at 4°C overnight. Unbound proteins were measured to ensure that UsPA1 \(^{50–70}\)/Uspa2 \(^{50–359}\) 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 at RT 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 plates 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 (Dakopatts) diluted 1/1000 was added for 1 h at RT. The wells were washed four times and the plates were developed and measured using ECL substrate to detect bound 125I-labeled C4BP for each triplet of rC4BP mutants (each lacking one CCP) in a concentration of 90 nM, respectively. Thereafter, the wells were washed and incubated with mAb104 and/or mAb67 (1/5000) in blocking buffer for 1 h at RT. After additional washings, HRP-conjugated rabbit anti-mouse pAbs (Dakopatts) diluted 1/1000 was added for 1 h at RT. The wells were washed and developed as above.

Determination of \(K_D\)

Equilibrium affinity constants were obtained from plots of C4BP binding of UsPA1 \(^{50–70}\)/Uspa2 \(^{50–359}\) 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:

\[
B = (B_\text{sat}} \times C \times \frac{1}{K_D + C}
\]
PBS-Tween. Thereafter, 30 kcpm $^{125}$I-labeled UspA1 was incubated 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 recombinant C4BP STOP concentration, the specificity of which was confirmed elsewhere (36). Briefly, microtiter plates were incubated with 20 nM recombinant UspA1 $^{50-770}$ or UspA2 $^{30-539}$ overnight at 4°C in 75 mM Na$_2$CO$_3$ (pH 9.6). Thereafter, the wells were washed and blocked as described above. After four washings, $^{125}$I-labeled C4BP was added (50 kcpm/well), together with various concentrations of unlabeled proteins diluted in blocking buffer, and followed by an overnight incubation at 4°C. After four additional washings, the radioactivity was measured in a gamma counter.

Surface plasmon resonance (Biacore, Uppsala, Sweden)

The interaction between UspA1 $^{50-770}$ or UspA2 $^{30-539}$ and C4BP was further analyzed using surface plasmon resonance (Biacore 2000; Biacore). Three flow cells on a CM5 sensor chip were activated using 20 µl of 0.2 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide with 0.05 M N-hydroxy-sulfosuccinimide at a flow rate of 30 µl/min. The recombinant UspA1 $^{50-770}$ and UspA2 $^{30-539}$ were then injected at a concentration of 20 µg/ml in 10 mM sodium-acetate buffer (pH 4) over flow cell 2 and 3, respectively, to reach 1700 resonance units. The unreacted groups were washed out with the standard flow buffer (10 mM HEPES-KOH, pH 7.4, 70 mM NaCl, 0.005% Tween 20, 5 mM EDTA). Protein solutions were injected 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 C4BP concentration, the 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 calculate equilibrium affinity constants.

Dot blot assays

Purified C4BP variants diluted in 3-fold steps (0.0014–3 µg) 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 buffer (10 mM sodium-acetate buffer containing 0.005% Tween 20, 5 mM EDTA). Thereafter, 30 kcpm $^{125}$I-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 intensified 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 antisera. The BBH18ΔuspA1 mutant was deficient in a 115-kDa protein corresponding to UspA1, whereas the BBH18ΔuspA2 mutant lacked both the high molecular mass complex (>250 kDa) and a 100-kDa protein, both corresponding to UspA2 (Fig. 2). The double mutant did not express any proteins that could be recognized by the anti-UspA1/A2 pAb.

To further examine the M. catarrhalis UspA1 and UspA2 mutants, bacteria were analyzed by flow cytometry using anti-UspA1/A2 pAb. As can be seen in Fig. 3B, only a minor decrease in mean fluorescence intensity (mfi) (from 63.2 to 51.2) was observed with the BBH18ΔuspA1 mutant when it was compared with the wild-type counterpart (Fig. 3A). In contrast, M. catarrhalis BBH18ΔuspA2 displayed a decreased number of UspA molecules (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.
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 observed with the M. catarrhalis RH4 isolate and the corresponding RHΔuspA1/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 RNA 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 C4d, 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 found 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.
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., Biacore) 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. 1) 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 isofrom 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. 10 A), 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.
attack in two ways. Firstly, C4BP bound to the surface of \( M. \) \textit{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 (C4b2a) will most likely be inhibited and its decay accelerated. This may help \( M. \) \textit{catarrhalis} to avoid membrane attack complex-mediated lysis.

Taken together, we have presented several lines of evidence on \( M. \) \textit{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 \( M. \) \textit{catarrhalis} binding to C4BP and, therefore, contributes to \( M. \) \textit{catarrhalis} serum resistance and, consequently, virulence.

References

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