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Molecular Characterization of the Interaction between Porins of Neisseria gonorrhoeae and C4b-Binding Protein

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Neisseria gonorrhoeae, the causative agent of gonorrhea, is a natural infection only in humans. The resistance of N. gonorrhoeae to normal human serum killing correlates with porin (Por)-mediated binding to the complement inhibitor, C4b-binding protein (C4BP). The entire binding site for both porin molecules resides within complement control protein domain 1 (CCP1) of C4BP. Only human and chimpanzee C4BPs bind to Por1B-bearing gonococci, whereas only human C4BP binds to Por1A strains. We have now used these species-specific differences in C4BP binding to gonococci to map the porin binding sites on CCP1 of C4BP. A comparison between human and chimpanzee or rhesus C4BP CCP1 revealed differences at 4 and 12 amino acid positions, respectively. These amino acids were targeted in the construction of 13 recombinant human mutant C4BPs. Overall, amino acids T43, T45, and K24 individually and A12, M14, R22, and L34 together were important for binding to Por1A strains. Altering D15 (found in man) to N15 (found in rhesus) introduced a glycosylation site that blocked binding to Por1A gonococci. C4BP binding to Por1B strains required K24 and was partially shielded by additional glycosylation in the D15N mutant. Only those recombinant mutant C4BPs that bound to bacteria rescued them from 100% killing by rhesus serum, thereby providing a functional correlate for the binding studies and highlighting C4BP function in gonococcal serum resistance. The Journal of Immunology, 2007, 179: 540–547.

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cause local infections. Despite the differences in the nature of the interactions, binding sites on C4BP for both Por1A and Por1B lie solely within CCP1 (5).

Natural infection with *N. gonorrhoeae* is restricted to humans. Several animal species have been tested as models for gonococcal infection with limited success. Prolonging the estrus cycle of mice using 17β-estradiol facilitates vaginal infection and has produced a useful model (12). Intrarethral inoculation of male chimpanzees with only Por1B- but not Por1A-bearing gonococcal strains enables an experimental infection that simulates human disease (13). We showed recently that the (mammalian) species specificity of gonococcal infection correlates with the ability of gonococci to bind host C4BP (14). Interestingly, chimpanzee C4BP seemingly binds exclusively to Por1B-bearing gonococci and only Por1B gonococcal infection correlates with the ability of gonococci to infect chimpanzees (12). Intraurethral inoculation of male chimpanzees with gonococci.

We exploited the binding of gonococci to select primate C4BP molecules to construct recombinant C4BP's to identify amino acids that are important for interaction with Por1A and Por1B. Mapping the sites of Por binding in C4BP CCP1 provides further insights into gonococcal pathogenesis and a deeper understanding of how this pathogen is uniquely adapted to infect humans exclusively.

**Materials and Methods**

**Sequence comparison**

The amino acid sequences of human, chimpanzee, and rhesus monkey CCP1 modules were aligned using Insight (Accelrys). Amino acid differences among the sequences were evaluated in the context of the three-dimensional nuclear magnetic resonance structure of human C4BP CCP1 (15) to better plan point mutations and facilitate the subsequent analysis of functional data.

**Proteins**

Human plasma C4BP (16), C4 (17), and factor I (18) were purified as described. C4b and C3b were purchased from Complement Technology. In some experiments that required large amounts of C3b or C4b, C3b- or C4b-like molecules (C3met or C4met) were used (19). C3met and C4met were prepared by the incubation of purified C3 or C4 with 100 mM methy-lylamine (pH 8.0 – 8.5) for 4 h at 37°C followed by dialysis against 50 mM Tris-HCl in 150 mM NaCl (pH 7.5). Peptides used in this study were at least 95% pure as judged by Coomassie staining of proteins resolved by SDS-PAGE. All proteins were stored at –20°C until use. Protein concentrations were determined from absorbance at 280 nm or from amino acid analysis following 24 h of hydrolysis in 6 M HCl. The C4b and C3b used in cofactor analysis experiments (see below) were labeled with [35S]methionine T method. The initial specific activity was 0.4 – 0.5 MBq/μg protein.

**Construction and expression of recombinant mutant C4BPs**

Full-length cDNA encoding the human C4BP a-chain that had been cloned into the eukaryotic expression vector pcDNA3 (Invitrogen Life Technol-

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Primers*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A12V, M14V</td>
<td>5'-TTATCATTTGCTGCCCAGTGGATATTACGTTG-3'</td>
</tr>
<tr>
<td>D15N</td>
<td>5'-GCTGGCCCGAGTATATTACGTTGACTGCT-3'</td>
</tr>
<tr>
<td>R22H</td>
<td>5'-TTGACTGAGACAACCTTAAACCTGA-3'</td>
</tr>
<tr>
<td>K24E</td>
<td>5'-GAGGACGCTTTGAACTGAAACTACT-3'</td>
</tr>
<tr>
<td>L34R</td>
<td>5'-AATACACTTCGTGCCGGCGCAGGTTCACTTC-3'</td>
</tr>
<tr>
<td>T43D</td>
<td>5'-AGATCCCATTCAACTTGAGCTTACCTGTAAT-3'</td>
</tr>
<tr>
<td>T45M</td>
<td>5'-AGATCCCATCCTCAACTTGAGCTTACCTGTAAT-3'</td>
</tr>
<tr>
<td>T43D, T45M</td>
<td>5'-AGA GCC CAT TCA GAT CAG AGC CTT ACC TGT ATT-3'</td>
</tr>
<tr>
<td>E53Q, V55T, N57T</td>
<td>5'-AATCTGATGCAATTGTAATTCCTCGTATC-3'</td>
</tr>
<tr>
<td>D15Q</td>
<td>5'-GAGACACGCTTCGAAACTGGAACTACT-3'</td>
</tr>
</tbody>
</table>

*Nucleotides corresponding to the mutated amino acids are underlined.

**Deglycosylation with N-glycosidase F (NFG)**

Purified wt C4BP and mutants that contained a potential N-glycosylation site at position 15 (D15N, D15N/K24E, and D15N/K24E/T43D/T45M) were dialyzed in PBS containing 50 mM EDTA, 1% SDS, and 1% 2-ME (pH 8.0) and incubated for 3 min at 95°C. NGF (1 U; Roche) and 0.5% *N*-octyl-glucoside were added to 3 µg of each protein and incubated for 24 h at 37°C. The reaction was terminated by the addition of a 20-µl sample buffer (0.5 M Tris-HCl (pH 6.8), 15% glycerol, 0.005% Coomassie G-250, 4% SDS, and 10 mM DTTO) and then the samples were heated at 95°C for 3 min and applied on a 7.5% SDS-polyacrylamide gel for electrophoresis. Separated proteins were transferred to a PVDF membrane and visualized by Western blotting.

**Circular dichroism spectroscopy**

Recombinant wt C4BP and the 12 mutant proteins were dialyzed extensively against 150 mM NaF before analysis. Approximately 60 µg of each protein was washed in the far UV region (200–250 nm) using a Jasco J-720 spectropolarimeter. Resolution was 1 nm, sensitivity was 20 millidegrees, and the speed was 20 nm/minute. Each protein was analyzed five times, an average was calculated, and buffer background values were subtracted.
To assess the binding of recombinant C4BP mutant proteins to C4b, C4BP (10 μg/ml) was coated onto microtiter plates overnight at 4°C. Plates were washed and 0.1–100 nM each recombinant mutant protein, diluted in TBS with 1% BSA and 0.05% Tween 20, was added at 0.01–10 nM and diluted in washing buffer. After incubation and washing, HRP-conjugated rabbit anti-mouse Ab (DakoCytomation), diluted 1/5000, was added and incubated for 1 h at room temperature. Wells were washed and substrate solution (8-mg o-phenylenediamine tablets (DakoCytomation) in 12 ml of H2O and 5 μl of H2O2) was added. Absorbance (492 nm) was read using a microtiter plate reader.

**Binding to heparin.** Binding to heparin was measured using surface plasmon resonance (BIAcore2000). Heparin was immobilized on an streptavidin chip (GE Healthcare) by the injection of 1.2 μg of biotinylated heparin in running buffer (10 mM HEPES, 75 mM NaCl, 5 mM EDTA and 0.002% Tween 20 (pH 7.4)). Increasing concentrations (2.5–20 nM) of each recombinant mutant protein were injected over an empty control flow cell without coupled heparin and then over the heparin-containing flow cell. In each determination, 40 μl of the protein solution was injected during the association phase at a constant flow rate of 20 μl/min. NaCl (2 M) was used to remove bound ligands between analyte injections.

**C4b and C3b degradation assay**

To assess the preservation of cofactor activity of mutant C4BPs, we examined for the degradation of C4b to C4c and C4d and that of C3b to C3c in the presence of factor I and the mutants of C4BP. One-hundred nM each mutant C4BP was mixed with 750 nM C3met (or 250 nM C4met), 60 nM factor I, and trace amounts of 125I-labeled C3b (or 125I-labeled C4b) in 50 μl of 50 mM Tris-HCl in 150 mM NaCl (pH 7.4). Samples were incubated for 1 h at 37°C and reactions were terminated by adding SDS-PAGE sample buffer with a reducing agent (DTT). Samples were then incubated at 95°C for 3 min and applied to 10–15% gradient SDS-polyacrylamide gels for electrophoresis to resolve degradation products of C4b or C3b. Separated proteins were visualized using a PhosphorImager device (Molecular Dynamics/GE Healthcare).

**Gonococcal strains**

Isogenic mutant strains of *N. gonorrhoeae*, FA6611 (Por1B strain MS11, where the native Por1A had been replaced with the FA19 Por1A molecule) and FA6616 (strain MS11, where the native Por1B had been reintroduced), have been described previously (21). For simplicity, we refer to strain FA6611 as the Por1A-bearing strain (or Por1A), and FA6616 as the Por1B-bearing strain (or Por1B). For binding and bactericidal assays, bacteria were grown on chocolate agar plates at 37°C with 5% CO2 and then suspended in HBSS2+ containing 2.5 μg of the recombinant mutant C4BP to be tested and 5 μl of hemolytically active *R. macaque* serum (Bioreclamation); the final concentration of bactericidal reaction mixtures was brought up to 150 μl with HBSS2+. Reaction mixtures were incubated at 37°C with shaking. Duplicate samples of 25 μl were plated on to chocolate agar at 0 and 30 min. Following overnight incubation at 37°C with 5% CO2, colonies were counted and survival was expressed as the percentage of the average of the number of colonies at 30 min divided by the number at 0 min.

**Results**

Sequence comparison

We compared the amino acid sequences of CCP1 of C4BP in three primate species: human, chimpanzee (*Pan troglodytes*), and the *R. macaque* monkey (Fig. 1). These species were chosen because human C4BP binds both gonococcal Por1A and Por1B, chimpanzee C4BP binds Por1B, and rhesus monkey C4BP binds neither Por1A nor Por1B (14). Furthermore, the binding site of human C4BP to *N. gonorrhoeae* resides entirely within CCP1 (5). Human and chimpanzee C4BP CCP1 differ by four amino acids only, suggesting that these amino acids are crucial for the binding of Por1A. Chimpanzee and rhesus monkey CCP1 differ by an additional 10 amino acids (an eleventh (aa no. 12) is shared between human and rhesus but not by chimpanzee), which presumably include the binding site for both Por1A and Por1B. In the mutagenesis experiments, we elected to change unique human amino acids to the corresponding chimpanzee or rhesus monkey counterparts (see Fig. 7). In total, 13 recombinant mutants of C4BP were constructed and expressed (Figs. 2 and 3).

**Assessment of structural integrity of C4BP mutant proteins**

Amino acid residues that were targeted for mutagenesis were solvent exposed (as evidenced by the nuclear magnetic resonance three-dimensional structure of the human C4BP α-chain CCP1 (15)) and not involved in clearly stabilizing interactions with the remaining parts of the molecule (i.e., salt bridges). Therefore these amino acid substitutions were expected to be well tolerated structurally. The wt and mutant C4BP molecules were expressed in eukaryotic cells and purified from cell culture medium using affinity chromatography. We assessed the structural integrity of the mutant proteins by circular dichroism spectroscopy, which yielded similar spectra compared with wt C4BP, confirming that mutagenesis did not alter the three-dimensional structure of the recombinant molecules (not shown). The purified mutant proteins were...
separated by electrophoresis on 10% SDS-polyacrylamide reducing gels and validated both by staining with Coomassie brilliant blue (Fig. 2, right panel) and by immunoblotting of the similarly migrating proteins using a polyclonal Ab against C4BP (Fig. 2, left panel). Interestingly, the three mutants carrying the D15N substitution migrated as slightly larger proteins when compared with the wt. Because the alteration introduced a potential N-linked glycosylation site composed of asparagine at position 15 followed by isoleucine and threonine, we also expressed these mutants in the presence of tunicamycin to inhibit the N-linked glycosylation of secreted proteins. Indeed, we found that under these expression conditions the migration of D15N and the wt proteins were similar upon SDS/PAGE analysis (Fig. 3A). The experimental conditions used resulted in deglycosylation of ~50% of secreted C4BP (Fig. 3A, lower band marked with an arrow); attempts to use higher concentrations of tunicamycin to fully deglycosylate the proteins resulted in toxicity to the cells. The wt and the mutants containing the D15N mutation were also deglycosylated enzymatically using NGF. Consequently, all four proteins migrated with similar velocities in SDS-PAGE, indicating that the differences in migration detected among native (glycosylated) proteins were likely attributed to the additional glycan at N15 (Fig. 3B). To gain further evidence for additional glycosylation of the D15N mutant and to obtain a mutant that could determine the exact role of D15 in binding to heparin and gonococci, we expressed an additional mutant in which D15 was replaced by Q15 (Fig. 3C). The D15Q mutant showed a migration pattern in SDS-PAGE similar to that of the wt; this supports further the hypothesis that the D15N mutant carries an additional N-linked glycan.

Recombinant mutant C4BP proteins were also screened with a panel of conformation-dependent mAbs against the C4BP γ-chain. The recombinant proteins were coated onto microtiter plates and mAbs were used at different concentrations for detection. The two mutant proteins that contained an Asn residue at position 15 (instead of Asp) showed significantly decreased binding to mAb 102 (Fig. 4A). There were no differences in binding by other mAbs (67, 70, 92, 96 and 104) to the mutant C4BP molecules (not shown), providing further evidence of correct folding and stability. Evidence for the importance of D15 in the binding to heparin and gonococci, we expressed an additional mutant in which D15 was replaced by Q15 (Fig. 3C). The D15Q mutant showed a migration pattern in SDS-PAGE similar to that of the wt; this supports further the hypothesis that the D15N mutant carries an additional N-linked glycan.
binding, suggesting that the glycans possessed by D15N further compromised mAb102 binding, presumably by steric hindrance.

The heparin-binding site on C4BP resides in CCPs 1–3; CCP2 has been shown to be the most important domain for this interaction (22). We examined recombinant mutant protein binding to heparin by using surface plasmon resonance (Fig. 4B). The D15N, K24E, and T43D mutations resulted in decreased binding to heparin compared with wt C4BP. In contrast, both the L34R and the D15Q mutants showed increased binding to heparin.

The binding site of C4BP for C4b is located in CCPs 1–3 (22). A cluster of positively charged amino acids at the CCP1–2 interface are important in the C4BP-C4b interaction (23). We wished to test whether the mutations within CCP1 that we had constructed affected the binding of C4BP to C4b and also whether a corresponding change in function, i.e., cofactor activity of C4BP, might have resulted. Binding to C4b was tested by direct binding assay; no differences between any of the recombinant mutants and wt C4BP were seen (not shown). Cofactor activity that was tested by a C4b and C3b degradation assay that used factor H as the degrading enzyme showed quantitatively similar amounts of C4c, C4d, and iC3b generated by each of the recombinant proteins compared with wt C4BP (not shown).

**Binding of recombinant C4BP mutants to gonococci**

Each recombinant mutant C4BP was incubated with Por1A- and Por1B-bearing *N. gonorrhoeae*. After washing, binding was detected by flow cytometry using mAb 67 that is directed against CCP4, followed by FITC-labeled anti-mouse Ab (Fig. 5). Each mutant protein was tested using wt C4BP as a positive control. Neither Por1A- nor Por1B-bearing gonococci bound rhesus C4BP (14). We determined which of the human to rhesus mutations resulted in decreased C4BP binding to Por1A-bearing gonococci (Fig. 5A). The human-to-rhesus recombinant protein mutations D15N, K24E, T43D, or T45M, when altered individually or in combination (D15N/K24E or T43D/T45M), all had a strong negative impact on the binding of the corresponding mutant C4BP to Por1A. The effect of D15N upon the resulting mutant’s binding was due to the presence of the additional glycosylation site in D15N, because the D15Q mutant bound Por1A-bearing gonococci similarly as to the wt. In the case of Por1B-bearing gonococci among the mutant proteins that bore a single amino acid mutation, only the K24E mutation showed diminished binding of the resultant mutant C4BP to Por1B. Although mutating D15N alone had a minimal effect on the mutant’s binding to Por1B, the combined mutant protein that contained D15N/K24E resulted in complete loss of binding. This suggests that additional glycosylation on N15 sterically hinders the Por1B-C4BP interaction, especially when K24 is simultaneously mutated. The D15Q mutant bound Por1B gonococci similarly as to the wt, implying that D15 itself does not contribute to the interaction. As predicted (5), the C4BP mutant protein containing the E53Q/V55T/N57T rhesus substitutions located at the C terminus of CCP1 displayed full binding (Fig. 5).

We next studied the effects of human-to-chimpanzee mutations on the binding of C4BP to intact gonococci. We have shown previously that only Por1B- but not Por1A-bearing strains bind chimpanzee C4BP. Only four amino acid changes (A12V, M14V, R22H, and L34R) differentiate human and chimpanzee CCP1 (Fig. 1), and alterations in one or more would be predicted to decrease the ability of C4BP to bind to the Por1A but not to the Por1B strain. Mutations of A12V and M14V simultaneously or of R22H and L34R individually showed only a slight decrease in binding to Por1A (Fig. 5B). However, binding to the Por1A-bearing strain was almost completely abrogated when these four amino acids were mutated simultaneously (Fig. 5B; rChimp). As expected rChimp bound to Por1B, but it is noteworthy that rChimp C4BP bound slightly better to the Por1B strain than human C4BP. When the concentration of rChimp was increased ~5-fold to 125 µg/ml, some binding to Por1A was also detected, although the fluorescence intensity was still lower than that observed with 25 µg/ml wt C4BP (Fig. 5C). This suggests that while the avidity of rChimp for Por1A is significantly decreased compared with that for Por1B, it is not absent.

**Bactericidal assay**

Rhesus serum is bactericidal against gonococci that otherwise resist killing by NHS. We have shown previously that adding wt C4BP rescues gonococci from killing by rhesus serum (14). We examined the ability of recombinant mutant C4BPs to rescue Por1A- and Por1B-bearing gonococci from complement-dependendent killing by rhesus serum (Fig. 6). Gonococci were mixed with buffer containing the appropriate C4BP mutant or wt C4BP, and rhesus serum was added. Samples were plated at 0 and 30 min and colonies of surviving bacteria were counted. In accordance with the flow cytometry binding assays, Por1A strains were not rescued by the mutants carrying the mutations D15N, K24E, T43D, or T45D alone or in combination (D15N/K24E and T43D/T45M) as shown in Fig. 6A. The D15Q-containing mutant C4BP protein, however, did rescue both Por1A- and Por1B-bearing gonococci in rhesus serum, suggesting that glycosylation at N15 in rhesus may be important in preventing rhesus C4BP from binding to gonococci and functioning as a complement regulator. As expected, the E53Q/V55T/N57T containing mutant protein rescued killing by
rhesus serum supplemented with increasing concentration of rChimp C4BP mutant surviving in the presence of sera at 30 min compared with the number at 0 min. C4BP correlates with serum resistance (5).

**FIGURE 6.** Bactericidal activity of rhesus serum supplemented with mutant C4BPs. A. Gonococci were incubated for 30 min at 37°C with 3.3% rhesus serum supplemented with 2.5 μg of each mutant C4BP in a final volume of 150 μl. Bacterial mixtures were plated on chocolate agar at times 0 and 30 min. Results (mean ± SD) are expressed as the number of gonococcal CFU surviving in the presence of sera at 30 min compared with the number at 0 min. B. The bacteria (Por1A and Por1B strains) were each incubated with rhesus serum supplemented with increasing concentration of rChimp C4BP mutant and the results expressed as in A.

The binding site for heparin, a negatively charged polymer, is responsible for resistance of these bacteria to phagocytosis (25). Other pathogens known to bind C4BP include *S. pyogenes* (26), *Moraxella catarrhalis* (27), *Bordetella pertussis* (28), *Escherichia coli* strain K1 (29, 30), *Borrelia recurrentis* (31), *Neisseria meningitidis* (32), *Candida albicans* (33), and *Haemophilus influenzae* (34).

Using recombinant C4BP mutants that lack individual domains, we have shown previously that human C4BP binds to gonococci exclusively via the N-terminal CCP1 of the α-chain (5). We compared the amino acid sequences of human C4BP α-chain CCP1 with the CCP1 sequence of chimpanzee and the *R. macaque* monkey and noted that human and chimpanzee sequences and human and *Rhesus* sequences varied at four or 12 amino acid positions, respectively. This suggested that the four differing amino acids between human and chimpanzee C4BP CCP1 were crucial for the interaction between Por1A (does not bind chimpanzee C4BP at lower concentrations) and C4BP. Similarly, the 10 amino acids that differentiate chimpanzee from rhesus monkey C4BP were likely to be important for the binding to Por1B. Based on these differences in CCP1 amino acid sequences among the three species, we constructed and expressed 13 recombinant mutants of C4BP using site-directed mutagenesis. The mutations were all targeted at the CCP1 of the α-chain, whereas the amino acid sequences of CCPs 2–8 were maintained entirely as found in the human sequence. The resulting recombinant proteins did not differ in structural integrity as evidenced by circular dichroism spectroscopy, which yielded similar spectra as the wt recombinant C4BP. This indicates the presence of similar contents of β-strands and α-helices to ensure proper folding and stability, which are independent attributes that would be important in binding. Functional integrity was also preserved as evidenced by the maintenance of serine protease cofactor activity to factor I-mediated cleavage of C4b and C3b. Decreased binding of mAb102 to all recombinant proteins containing the D15N mutation (D15N, D15N/K24E, and D15N/K24E/T43D/T45M) as well as the D15Q mutant suggested that D15 is a critical component of the C4BP epitope that binds this mAb. The remaining mAbs against CCP1 bound equally to all the recombinant mutant proteins, independently confirming that the effect of the D15 mutation on mAb102 binding was specific and not attributable to misfolding.

The binding site for heparin, a negatively charged polymer, is located in CCPs 1–3 of the α-chain; CCP2 is the most important domain (22). In a previous study, the mutagenesis of R39, R64, or R66 strongly decreased the binding of C4BP to heparin (23). The three amino acids are localized in a positively charged patch on the interface of CCP1 and 2. In the heparin-binding assay that was performed here, mutagenesis of the individual amino acid residues K24 and T43 also resulted in decreased binding to heparin compared with the recombinant wt C4BP. T43 is close to the principal...
positively charged heparin-binding region located at the CCP1-CCP2 interface (15, 35), while K24 is located more distant. However, it is likely that heparin molecule (the heparin polymer can be 50-A long compared with the ~40-A length of a single CCP domain) wraps around the domain and contacts other residues in addition to the key positively charged amino acids that constitute the "hot-spots" for heparin binding. Therefore, the present finding that amino acids away from the key heparin-binding region can contribute directly or indirectly to the binding of this negatively charged polymer is compatible with the known structural data. Interestingly, the change of the hydrophobic leucine residue at position 34 to the positively charged arginine as well as the change of the negatively charged aspartic acid at position 15 to neutral glutamine both resulted in increased binding of heparin, which is consistent with our previous finding because these residues are located near the heparin binding site. This suggests that these newly formed positive charges could facilitate the approach and/or the interaction with a negatively charged polymer. In most instances heparin uses its negatively charged groups to bind via electrostatic forces, although other types of contact are clearly important for high affinity and specificity (36). An increase in positive surface charge of the protein near the site of heparin interaction tends to increase the binding, whereas a loss of positive charge(s) or replacement with negative charge(s) usually lead to decreased avidity and binding. The D15N mutation impaired the binding of C4BP to heparin because of the introduction of the additional glycosylation site, probably due to steric hindrance because the D15Q mutation did not decrease the interaction.

Our current data show that amino acids K24, T43, and T45 are important for the binding of C4BP to Por1A. The recombinant proteins that carried these mutations showed decreased binding to a Por1A-bearing gonococcal strain as measured by flow cytometry. Furthermore, these mutant C4BPs were unable to rescue Por1A gonococci from the bactericidal activity of rhesus serum. The D15N mutant showed similar impairment, but this was likely not due to a direct involvement of D15, but instead to the additional glycosylation site that is present in rhesus C4BP at N15. The replacement of Asn with a conserved (but unglycosylated) Gln residue preserved binding to C4BP and its complement ability to regulate on the Por1A surface. For Por1B, similar results were obtained for K24 because it was involved in binding and its alteration to E24 (rhesus-like) abolished the ability of the mutant protein to confer serum resistance. In contrast to Por1A, the additional glycosylation of the D15N mutant had only a weak negative impact on the interaction of C4BP with Por1B, and the complement-regulating function on the Por1B surface was retained. Interestingly, mutating the four amino acids that differ between human and chimp C4BP either singly (R22H or L34R) or in combination (A12 and M14) did not strongly impact binding to the Por1A strain. However, when the four amino acids (A12, M14, R22, and L34) were altered simultaneously to yield the equivalent of chimpanzee CCP1, the binding to Por1A was abolished at lower concentrations of the mutant protein used in binding assay. In the model of C4BP CCP1 (23), these four amino acids that are important for binding to Por1A are located on the same face of the molecule (Fig. 7). The binding of chimp C4BP to Por1A was partially restored when higher protein concentrations were used. This is consistent with prior observations of weak C4BP binding to and partial survival of certain Por1A strains (14) and our current observations that addition of 8-fold excess rChimp C4BP to rhesus serum could rescue gonococci from complement-mediated killing. To further evaluate the impact of our findings, we tested the binding of C4BP mutants used in this study to two additional Por1A (339063 and 401082) and two Por1B (FA1090 and 1291) strains known to interact with C4BP (5). We found that the effects of mutations on binding to these strains were consistent with those seen with the two strains used throughout this study. This implies that the structural requirements for porin-C4BP interactions are conserved between strains.

We have shown previously that the interaction between porin and C4BP differs in nature depending on the por allele expressed (5). Hydrophobic forces are mainly responsible for the Por1A-C4BP interaction because it is not dissociated at high NaCl concentrations. Neither C4b nor heparin influences the binding of C4BP to Por1A, suggesting that these two binding sites do not overlap. The binding of C4BP to Por1B in contrast was influenced by salt concentration, which points to ionic interactions between the two molecules. Both C4b and heparin can compete out the binding of C4BP to Por1B. In the present study we have identified the following amino acids that are involved to various degrees in binding to Por1A: K24, T43, T45, A12, M14, R22, and L34. Some of these amino acids are fully hydrophobic (A12 and M14) and the side chains of other residues are capable of making hydrophobic
contacts (e.g., the methyl group of Thr and the carbon atoms of Lys and Arg) and do not contribute exclusively via hydrogen bonding or ionic interactions. In accordance with the ionic nature of the Por1B-C4BP interaction, the K24 amino acid that is involved in this interaction is charged and acts via the formation of salt bridges and/or hydrogen bonds with Por1B. Furthermore, heparin binding to the K24E mutant was decreased, which correlates with the previous data and points to overlapping binding sites for heparin and Por1B. In contrast, heparin binding was also decreased for the T43D mutant, although the binding of C4BP to Por1A was not affected by heparin. This suggests that a region of the binding site for heparin and por1A also overlaps but to a lesser extent.

The recent finding that only human and chimpanzee C4BPs bind to Por1B gonococci and only human C4BP binds to Por1A gonococci provides an explanation for the species specificity of gonococcal infection. The capacity to bind host C4BP may enable the gonococcus to persist and cause disease. Gonococci are rapidly killed by nonhuman sera such as rat and rhesus sera unless human C4BP is added to the serum, and this suggests that transgenic mice expressing human C4BP could further improve the existing in vivo model (12) for studying N. gonorrhoeae infection. Our observations are also important in the evaluation of vaccines against pathogenic Neisseria and may explain why nonhuman complement sources are more bactericidal than human complement when vaccine-induced human Abs are tested for their complement-dependent killing activity.

In conclusion, we have mapped the binding sites on the CCP1 domain of the C4BP α-chain for gonococcal Por1A (T43, T45, K24, A12, M14, R22, and L34) and Por1B (K24). We found that an additional glycosylation of D15 as in rhesus C4BP sterically hinders interactions with both porins. Of course, the amino acids identified in the current study are unlikely to be the only amino acids involved in interactions with porins, which may be stabilized further by other neighboring amino acids. In addition, the decreased binding of mutant C4BPs correlated in every instance with the decreased ability of the proteins to rescue gonococci from the bactericidal action of rat serum. This further underlines the importance of C4BP binding in gonococcal pathogenesis.

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Disclosures

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References


