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Human C4b-Binding Protein Has Overlapping, But Not Identical, Binding Sites for C4b and Streptococcal M Proteins[1]

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Many strains of *Streptococcus pyogenes* bind C4b-binding protein (C4BP), an inhibitor of complement activation. The binding is mediated by surface M proteins in a fashion that has been suggested to mimic the binding of C4b. We have previously shown that a positively charged cluster at the interface between complement control protein domains 1 and 2 of C4BP α-chain is crucial for the C4b-C4BP interaction. To extend this observation, and to investigate the interaction with M proteins, we constructed and characterized a total of nine mutants of C4BP. We identified a key recognition surface for M proteins that overlaps with the C4b binding site because substitution of R64 and H67 by Gln dramatically reduces binding to both ligands. However, the analysis of all mutants indicates that the binding sites for C4b and M proteins are only overlapping, but not identical. Furthermore, M proteins were able to displace C4BP from immobilized C4b, whereas C4b only weakly affected binding of C4BP to immobilized M proteins. We found that the molecular mechanisms involved in these two interactions differ because the binding between M proteins and C4BP is relatively insensitive to salt in contrast to the C4BP-C4b binding. In addition, six mAbs directed against the α-chain interfered with C4b-C4BP interaction, whereas only two of them efficiently inhibited binding of C4BP to M proteins. Collectively, our results suggest that binding between C4b and C4BP is governed mostly by electrostatic interactions, while additional noncovalent forces cause tight binding of C4BP to streptococcal M proteins. ***The Journal of Immunology,*** **2000, 164: 5328–5336.**

The C4b-binding protein (C4BP)[3] is a plasma glycoprotein involved in the regulation of the classical pathway of complement activation both by accelerating the decay of C4bC2a (C3 convertase) (1) and by acting as a cofactor in the cleavage of C4b by factor I (2, 3). C4BP is a large protein (570 kDa) consisting of several polypeptides: seven identical α-chains and one β-chain (4, 5). As shown by electron microscopy and biochemical studies, C4BP consists of a ~160-kDa central core that can be released by chymotrypsin treatment and several extended tentacles formed by the α-chains (6, 7). The α- and β-chains are connected by disulfide bridges and consist of eight and three CCP (complement control protein) units, respectively (8). CCP units are often encountered in complement factors and consist of ~60 aa forming a compact hydrophobic core surrounded by five or more β-strands organized into β-sheets (9). The mechanism by which C4BP functions as a factor I cofactor in the degradation of C4b is still unclear. However, we have recently shown that a cluster of positively charged amino acids present on the interface between CCP1 and CCP2 of the C4BP α-chain is crucial for binding of C4b and for factor I cofactor function (10).

An interesting property of C4BP is its ability to bind to *Streptococcus pyogenes* (group A streptococcus) and *Bordetella pertussis*, two bacterial pathogens that are major causes of disease in humans (11, 12). The binding of C4BP to *S. pyogenes* is mediated by surface M proteins, which are important virulence factors (11, 13). The M proteins form fibrillar coiled-coil dimers on the streptococcal surface and have been studied extensively, due to their important ability to inhibit phagocytosis (14, 15).

Many strains of *S. pyogenes* bind C4BP (11), and the available evidence indicates that binding of C4BP to M proteins plays an important role in pathogenesis. In particular, studies of several different M proteins showed that the binding site for C4BP is localized to the hypervariable N-terminal region of the M proteins (16). This finding implies that the interaction with C4BP is of physiological importance, because the ability to bind C4BP has been retained in spite of extensive sequence variation in the hypervariable region. The role of bacteria-bound C4BP may be to down-regulate complement activation in the bacterial microenvironment, thereby protecting the bacterium against opsonization and phagocytosis (11).

In a study employing whole *S. pyogenes* bacteria, the binding site in C4BP was mapped to an area of C4BP encompassing CCP1-CCP3, and it was suggested that the binding site of surface M proteins mimics that of C4b (17). In this study, we report a comparison between the binding sites for C4b and M proteins in the C4BP α-chain, and we point out important differences in the molecular mechanisms governing these two interactions. Using a set of C4BP mutants, we first considerably extended our previous study on the binding site for C4b (10) and confirmed the conclusion that a cluster of positively charged amino acid residues at the CCP1-CCP2 interface is essential for the binding of C4b. All nine

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[3] Abbreviations used in this paper: C4BP, C4b-binding protein; 3D, three-dimensional; CCP, complement control protein domain; CR1, complement receptor 1; ^{125}I-C4BP, ^{125}I-labeled C4BP.

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C4BP mutants were then analyzed for ability to bind two purified streptococcal M proteins, Arp4 and Sir22 (11, 18), which have different N-terminal regions responsible for binding of C4BP (16). We concluded that a key binding site on C4BP for Arp4 and Sir22 lies on the interface between CPP1 and CCP2. However, the recognition sites in C4BP for C4b and M proteins are only overlapping, but are not identical. Furthermore, the intermolecular forces involved in these interactions are different. Collectively, these results extend our knowledge about the binding properties of C4BP and provide new insights into the molecular mechanisms by which *S. pyogenes* interacts with the immune system of the infected host. The present characterization of binding mechanisms may apply to other regulatory proteins of the complement system and to other pathogens that have evolved similar strategies to avoid complement attack.

Materials and Methods

Proteins

Human plasma C4BP, C4, and the streptococcal Arp4 and Sir22 proteins were purified, as described previously (18–20). Arp4 and Sir22 are equal to Emm4 and Emm22 in an alternative nomenclature (13). Protein concentrations were determined by measurement of absorbance at 280 nm, and the extinction coefficients (1%, 1 cm) used were 14.1, 8.3, and 19.4 for C4BP, C4, and Arp4, respectively. Due to a very low content of aromatic amino acids in Sir22, it is not possible to use absorbance measurement. Instead, concentration of Sir22 was determined from amino acid composition analysis after 24-h hydrolysis in 6 M HCl.

C4b-like molecules (C4 met) were prepared by incubation of purified C4 with 100 mM methylamine, pH 7.6, for 1 h at 37°C and subsequent dialysis against 100 mM Tris-HCl, 150 mM NaCl, pH 7.5. It has been demonstrated previously that C4 molecules treated this way are functionally equivalent to the C4b that results from cleavage by C1s component of the complement pathway (21). Throughout the study, the C4 met derivative was used, but will be referred to as C4b for reasons of clarity.

cDNA clones for the C4BP mutants R39Q, R64Q/R66Q, and R39Q/R64Q/R66Q and purification of the recombinant proteins were described previously (10). Briefly, the proteins were expressed from pcDNA3 vector (Invitrogen, San Diego, CA) in a human kidney cell line (293; American Type Culture Collection (Manassas, VA) catalog number 1573-CRL) and purified by affinity chromatography on a mAb 104 column. Mutations K63Q, R64Q, R66Q, H67Q, and K79Q were introduced according to QuikChange kit (Stratagene, La Jolla, CA); primers used were: K63Q (5′-ACC TTC TGT ATC TAC CAA CGA TGC AGA CAC-3′); R64Q (5′-TTC TGT ATC TAC AAA CAA TGC AGA CAC CCA-3′); R66Q (5′-ATC TAC AAA CGA TGC CAA CAC CCA GGA GAG-3′); H67Q (5′-AAA CGA TGC AGA CAA CCA GGA GAG TTA CGT-3′); K79Q (5′-GGG CAA GTA GAG ATT CAA ACA GAT TTA CTA TTT-3′); and their antisense counterparts. Nucleotides corresponding to mutated amino acids are underlined. All of the mutations were confirmed by DNA sequencing (Perkin-Elmer, Norwalk, CT). For construction of the R39Q/R64Q/R66Q/K79Q mutant, we used as a template cDNA coding for R39Q/R64Q/R66Q C4BP. Concentrations of rC4BP mutants were determined from amino acid composition analysis after 24-h hydrolysis in 6 M HCl. Proteins were labeled with ^{125}I using the chloramine T method. The sp. act. was 0.4–0.5 MBq/μg of protein.

Abs used in this study were: mAb 67 recognizing CCP4 of the α-chain; mAb 70, CCP1 of the α-chain; mAb 92, mAb 96, mAb 102, and mAb 104 binding to CCP1-CCP2 of the α-chain (22). In the previous study, it was shown that only mAb 70 could displace 70% of the binding of C4BP to mAb 96; all other Abs bound to different epitopes (22).

C4b/Arp4/Sir22 ligand-binding assay

Microtiter plates (Maxisorp; Nunc, Naperville, IL) were incubated overnight at 4°C with 50 μl of solution containing 10 μg/ml C4b, Arp4, or Sir22 in 75 mM sodium carbonate, pH 9.6. The wells were washed three times with 50 mM Tris-HCl, 0.15 M NaCl, and 0.1% Tween, pH 7.5 (washing buffer), and then incubated at room temperature with 200 μl of quench solution (washing buffer supplemented with 3% fish gelatin). After another three washes, increasing concentrations of plasma-purified C4BP or recombinant proteins diluted in 50 mM Tris-HCl, 150 mM NaCl, pH 8 (TBS), supplemented with 0.1% BSA and 0.1% Tween 20 were added, and the plates were incubated for 4 h at room temperature. The plates were then washed three times and incubated with biotinylated mAb mAb 67 diluted in quench solution. The Ab recognizes the middle part (CCP4) of the α-chain (22). After 1 h of incubation, the plates were washed and incubated for 1 h with streptavidin-conjugated HRP (Dakopatts, Glostrup, Denmark), washed again, and developed according to the manufacturer's instructions. The results are shown as a percentage of the maximal binding of wild-type rC4BP obtained in each set of experiments. Experiments were repeated three times, and means ± SD were calculated.

Competition assay

Microtiter plates were incubated overnight at 4°C with 50 μl of solution containing 10 μg/ml C4b, Arp4, or Sir22 in 75 mM sodium carbonate, pH 9.6. The wells were washed three times with washing buffer and then incubated at room temperature with 200 μl of quench solution. After another three washes, ^{125}I-labeled C4BP (^{125}I-C4BP) was added (20 kcpm/well) together with enough plasma-purified C4BP in 50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, and 0.1% BSA, pH 7.5, to achieve ~75% of the binding obtained with the tracer alone. Thus, 0.7 nM or 4.7 nM C4BP was added when C4b or Arp4/Sir22 was immobilized, respectively. Increasing concentrations of various mAbs were also added to the samples (mAb 67, 70, 92, 96, 102, and 104) or C4b, Arp4, and Sir22. In the experiments testing the influence of salt concentration on the binding, ^{125}I-C4BP was added to plates covered with C4b, Arp4, or Sir22 in buffer with NaCl content ranging from 25 mM NaCl to 1.5 M NaCl in 20 mM Tris-HCl, pH 7.4. The samples were incubated for 4 h at room temperature and washed five times, and the amount of radioactivity associated with each well was measured in a gamma counter. In experiments with varying salt concentrations, the plates were washed with buffer consisting of 50 mM Tris-HCl, pH 7.5, and 0.1% Tween 20. The results are shown as percentage of the maximal binding of ^{125}I-C4BP obtained in each experiment. Approximately 70–75% or 18–24% of applied ^{125}I-C4BP bound when Arp4/Sir22 or C4b was immobilized. Experiments were repeated three times, and means ± SD were calculated.

Binding of C4BP to S. pyogenes

S. pyogenes strain AL168, expressing Sir22 (18), was used for binding experiments, and a nonbinding mutant was used as a negative control. *S. pyogenes* strains were grown overnight in Todd-Hewitt broth (Difco, Detroit, MI) at 37°C. The streptococci were diluted in a suspension of the non-C4BP-binding *Escherichia coli* strain LE392, which was cultivated overnight at 37°C in LB medium. Binding assays were performed essentially as described (11). Briefly, the bacteria were washed twice in PBSAT (120 mM NaCl, 30 mM sodium phosphate, 0.02% NaN_3, 0.05% Tween 20, pH 7.2) and resuspended to 10^9 organisms/ml. Duplicate samples of bacteria (200 μl) were mixed with 20 μl of ^{125}I-C4BP variants (15 kcpm) and incubated at room temperature for 2 h. After addition of PBSAT (2 ml), the samples were centrifuged at 4200 × *g* for 12 min and the supernatants were discarded. This washing step was repeated once. Radioactivity in the pellets was determined, and binding was expressed as the percentage of radioactivity added that remained in the bacterial pellet. The mean of the duplicate samples was calculated, and nonspecific binding (<7%), recorded with strain LE392, was subtracted. The experiment was repeated three times, and means ± SD were calculated.

Results

Design and expression of C4BP mutants

We have previously shown that a cluster of positively charged amino acids, present at the interface between CCP1 and CCP2 of the C4BP α-chain, is crucial for C4b binding and factor I cofactor function (10). In that study, we used three mutants of C4BP, in which either one, two, or three Arg residues were replaced by polar Gln, generating mutants R39Q, R64Q/R66Q, and R39Q/R64Q/R66Q. We found that all three variants of C4BP showed decreased binding ability for C4b and were poor cofactors for factor I. We decided to generate additional C4BP mutants to investigate in detail the interaction with C4b and other ligands that might bind to the same site. Fig. 1 shows the 3D model of the structure for CCP1 to CCP3 in human C4BP (8, 10) that we used to design these mutations. Five new single amino acid mutants and one quadruple mutant were constructed. Thus, a total of nine different C4BP mutants were used, including the three mutants described previously (see Figs. 1 and 2 for the designations of these nine mutants). In all nine cases, we exchanged positively charged, surface-exposed

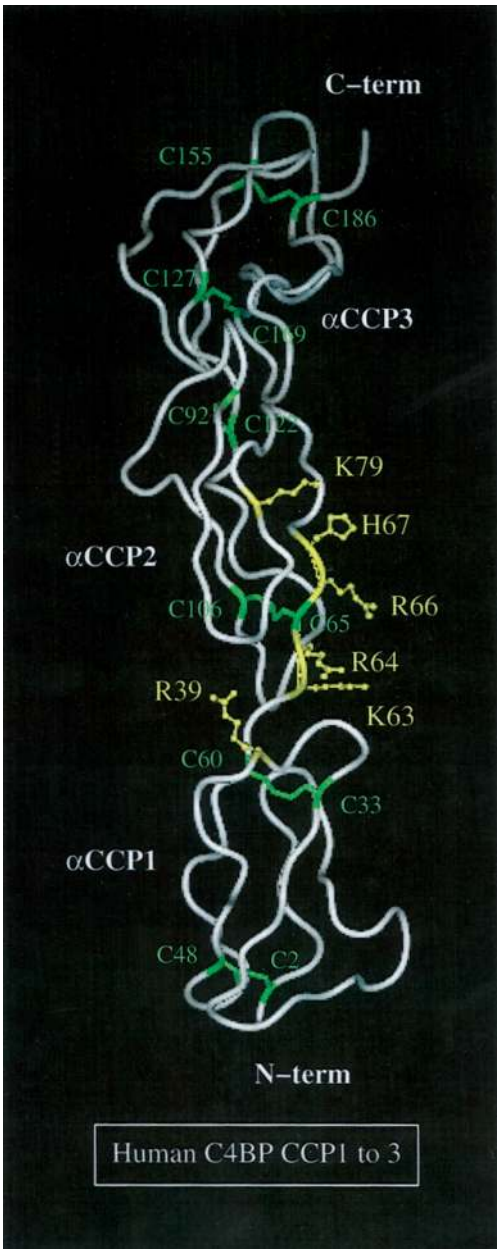

FIGURE 1. Model for the CCP1–3 modules of the C4BP α-chain. Solid ribbon representation of the 3D model for the CCP1, CCP2, and CCP3 modules of the human C4BP α-chain (8). The side chains of amino acids subjected to mutagenesis are shown in yellow, while disulfide bonds are marked in green for orientation.

amino acids to Gln residues. These changes were designed taking the 3D model structure into account and were thus expected to be structurally well tolerated. The rC4BP molecules were expressed in the human kidney cell line 293 and purified from culture media by affinity chromatography. Fig. 2*A* shows the recombinant proteins stained with Coomassie brilliant blue after separation on a 10% polyacrylamide gel under reducing conditions. Unreduced samples were applied on a 5% gel and stained with Coomassie brilliant blue (Fig. 2*B*) or transferred to a polyvinylidene difluoride membrane and detected with mAb 67, recognizing CCP4 of C4BP (Fig. 2*C*). All mutant proteins were expressed by the cells at similar levels and had similar mobilities upon SDS-PAGE. They were then probed with a panel of mAbs directed against α-chain of C4BP (mAb 67, 70, 92, 96, 102, and 104). Approximately 50% binding to immobilized wild-type C4BP was observed at 0.4–1 nM of each Ab (results not shown). Similar results were obtained for all variants of C4BP. This shows that the mutations did not affect the recognition of the C4BP by the mAbs, suggesting that the conformation of the C4BP variants was correct.

Binding of C4BP mutants to C4b

The interaction between C4b and rC4BP mutants was probed using a direct binding assay. Purified C4BP was added to immobilized C4b, and the amount of bound protein was detected using biotinylated mAb 67. Fig. 3*A* (*left panel*) shows binding curves for the six available single amino acid mutants of C4BP. Due to the multimeric nature of C4BP, this analysis only allowed a qualitative analysis of the effect of the mutations on interactions, but did not permit calculation of affinity constants. Maximal binding of wild-type rC4BP was set as 100%, and the apparent affinities for each mutant were estimated from the midpoint of the corresponding binding curve. The different apparent affinities are shown relative to that obtained for wild-type rC4BP (Fig. 3*B*, *left panel*). Plasma-purified and wild-type rC4BP bound to C4b with similar apparent affinities, confirming that 293 kidney cells are able to assemble correct C4BP molecules. Approximately 3 nM of each protein was required to reach half-maximal binding. All introduced mutations resulted in decreased binding ability of C4BP, in accordance with our hypothesis that positively charged residues at the CCP1-CCP2 interface are crucial for binding of C4b (10). In particular, substitution of Arg^{64}, His^{67}, Lys^{63}, and Arg^{39} caused a strong decrease in apparent affinity between C4b and C4BP.

Binding of C4BP mutants to streptococcal M proteins

To investigate whether the electropositive cluster of amino acids at the interface between CCP1 and CCP2 was also important for the interaction with streptococcal M proteins, we analyzed the ability of the different C4BP mutants to bind the two M proteins Arp4 and Sir22. This was of particular interest because it has been suggested that *S. pyogenes* binds to a site in C4BP indistinguishable from the C4b binding site (17). The interaction between C4BP mutants and streptococcal M proteins was evaluated in a direct binding assay (as described for the analysis of C4b binding), using purified preparations of M proteins (Fig. 3*A*). The apparent affinities of the different C4BP mutants for the M proteins were estimated from the midpoint of the binding curves and are shown in Fig. 3*B* as relative change when compared with wild-type rC4BP. Approximately 0.3 nM of wild-type rC4BP was needed to obtain half-maximal binding to both M proteins. Several of the C4BP mutants were strongly affected in their ability to bind the two M proteins.

We found that two of the tested single amino acid mutants, R64Q and H67Q, showed dramatically decreased apparent affinity to Arp4, by 180- and 40-fold, respectively. This result clearly indicates that these two amino acids constitute a vital part of the Arp4 recognition site on C4BP. R39Q and K63Q showed only a slight decrease in binding ability, whereas R66Q and K79Q bound to Arp4 better than to a wild-type rC4BP. Analysis of the double mutant R64Q/R66Q and the triple mutant R39Q/R64Q/R66Q was of particular interest, because the R66Q mutation alone increased binding. Both mutants showed strongly decreased binding, once again emphasizing the importance of R64 for efficient binding. However, the quadruple mutant R39Q/R64Q/R66Q/K79Q gave a surprising result, in that it bound to Arp4 almost as well as wild-type rC4BP.

The effects of mutations obtained for the two different M proteins were in most cases alike, but they were less pronounced for Sir22. Using direct binding assay, we found that H67Q, R64Q, and K63Q bound with a lower apparent affinity to Sir22, similar to

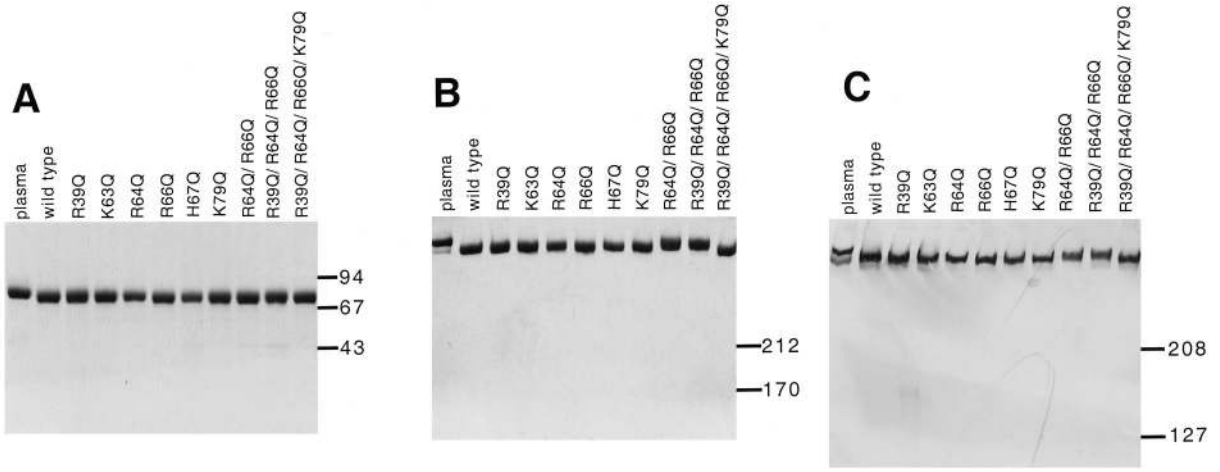

FIGURE 2. Analysis of purified C4BP and its mutants by SDS-PAGE. Plasma-purified C4BP and recombinant proteins (~2 μg/well for Coomassie staining and 0.04 μg/well for immunoblotting) were separated by SDS-PAGE. *A*, Coomassie-stained proteins separated on a 10% gel under reducing conditions. Unreduced proteins were separated on a 5% gel and Coomassie stained (*B*) or transferred to a polyvinylidene difluoride membrane and detected with mAb 67 against C4BP (*C*).

what we observed for Arp4. R39Q, R66Q, and K79Q bound with increased affinity to Sir22. Results obtained for double, triple, and quadruple mutants were comparable with those described for Arp4. The results acquired from the direct binding assay were confirmed by competition experiments, in which radiolabeled wild-type rC4BP was allowed to compete with various C4BP mutants for binding to C4b and Arp4 (results not shown).

Observations made for C4b and the two M proteins (Fig. 3) were similar for several of the C4BP mutants. For example, the H67Q mutant showed strongly decreased binding to all three ligands.

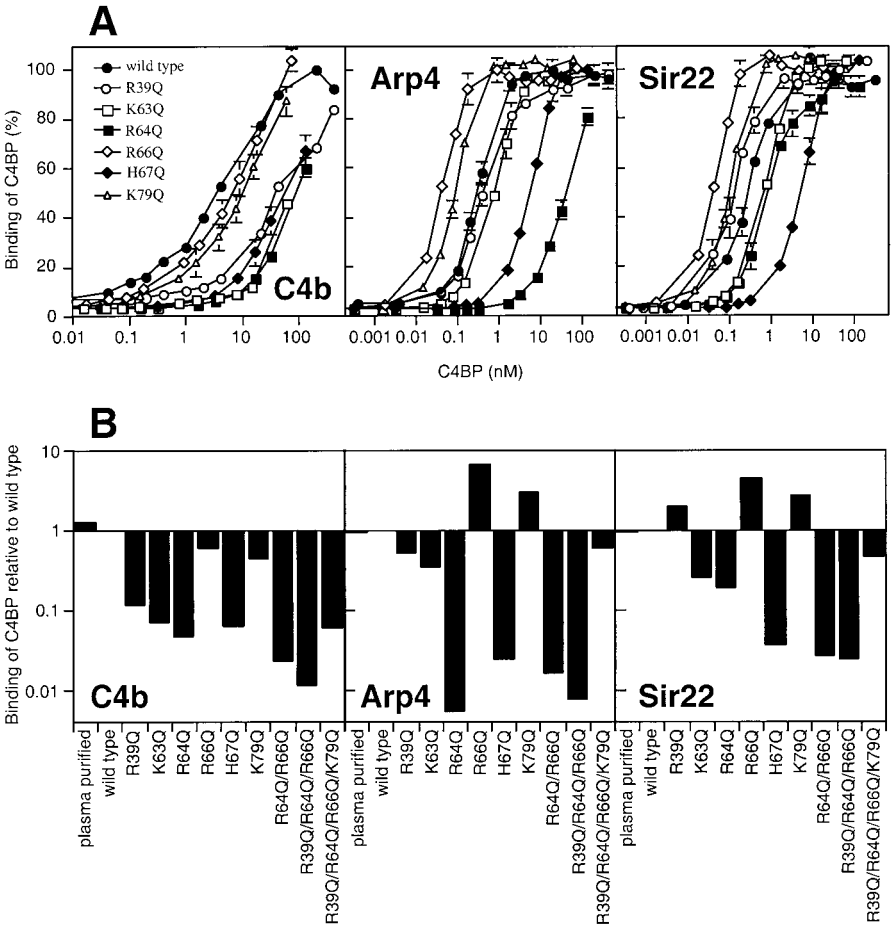

FIGURE 3. Binding of C4BP mutants to C4b and streptococcal M proteins Arp4 and Sir22. *A*, Microtiter plates were coated with C4b, Arp4, or Sir22, and allowed to react with increasing concentrations of plasma-purified C4BP or rC4BP molecules carrying various mutations. Amount of bound C4BP was detected with biotinylated mAb 67. Binding was expressed as percentage of the maximum binding of wild type observed in each experiment (mean value of three independent determinations performed in doublets ± SD). *B*, The apparent affinities for plasma-purified C4BP and each mutant were estimated from the midpoint of the corresponding binding curve, and are shown as relative to that of wild-type rC4BP.

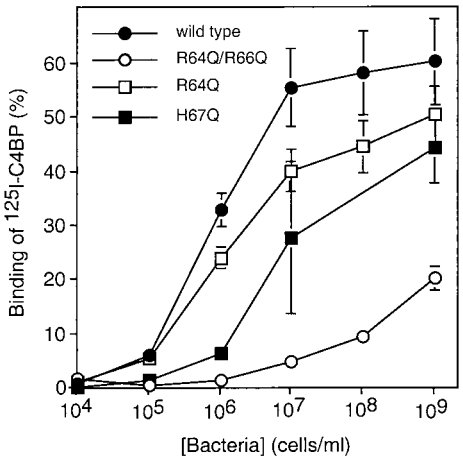

FIGURE 4. Binding of C4BP to *S. pyogenes* AL168, expressing Sir22. Increasing amounts of bacteria were suspended in solution containing wild-type ^{125}I-rC4BP, or various mutants, and incubated at room temperature for 1 h. After washing, the amount of C4BP associated with bacteria was measured in a gamma counter. Binding is shown as a function of bacterial concentration. Shown are mean results ± SD of three different experiments performed in doublets.

However, some differences are noteworthy. In particular, none of the C4BP mutants showed increased binding to C4b, while R66Q and K79Q showed considerably increased binding to the two M proteins. Moreover, the quadruple mutant, which was almost unaffected in its ability to bind M proteins, showed strongly reduced binding to C4b (but also in the case of C4b the quadruple mutant bound better than the triple mutant). Taken together, these data suggest that C4BP has overlapping, but not identical, binding sites for C4b and M proteins.

We also tested whether the point mutations introduced in C4BP had similar effect on the binding to whole *S. pyogenes* bacteria as to purified M proteins. In this experiment, bacteria in suspension were analyzed for ability to bind wild-type ^{125}I-rC4BP or various mutants. Binding was analyzed for the Sir22-expressing strain AL168, and the results were in agreement with those obtained with purified Sir22. As shown in Fig. 4, up to 60% of wild-type rC4BP bound to this strain. The binding of R64Q, H67Q, and R64Q/R66Q was decreased as compared with recombinant wild type. Results obtained for remaining mutants were also similar to those obtained with purified Sir22 (not shown). The fact that comparable results were obtained with purified streptococcal proteins and proteins expressed on the bacterial surface shows that physiologically relevant conclusions can be drawn from our experiments.

Cross-competition assay for C4BP binding to immobilized ligands

Results obtained with mutants of C4BP prompted us to perform competition assays to assess whether or not C4b and M proteins utilize the same binding site on C4BP. C4b, Arp4, or Sir22 was immobilized in wells of microtiter plates, and ^{125}I-C4BP was added together with various fluid-phase competitors: C4BP, C4b, Arp4, or Sir22. Both Arp4 and Sir22 were able to displace ^{125}I-C4BP from the immobilized C4b (Fig. 5, *upper panel*). In a reciprocal experiment, C4b had only a weak effect on binding of ^{125}I-C4BP to immobilized Arp4 or Sir22 (Fig. 5, *middle* and *lower panels*). These results suggested that either the affinities between the C4b-C4BP and M protein-C4BP interactions are very different and/or that these proteins bind to nonequivalent sites on C4BP.

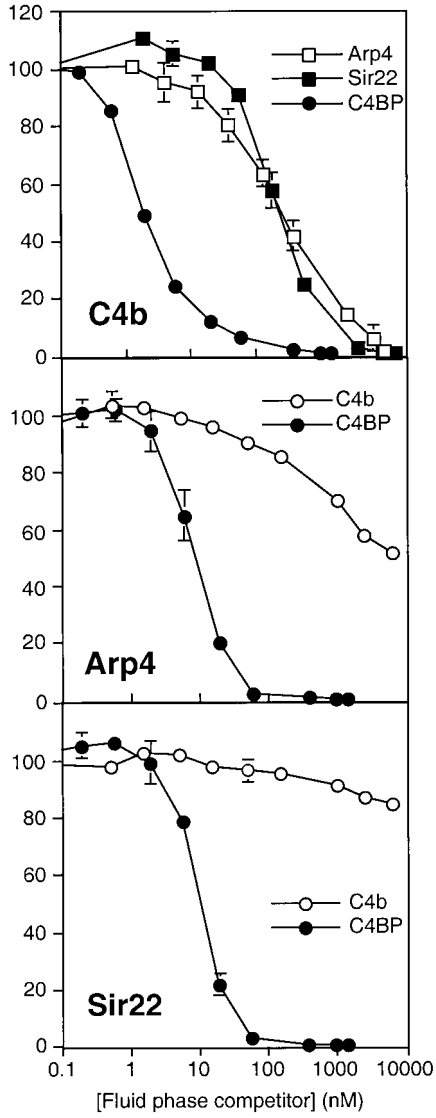

FIGURE 5. Cross-competition assay for C4BP binding to immobilized ligands. Increasing concentrations of fluid-phase proteins competed with trace amounts of ^{125}I-C4BP for binding to immobilized C4b (*upper panel*), Arp4 (*middle panel*), and Sir22 (*lower panel*). The 100% binding was estimated in the absence of fluid-phase competitor. Results of at least three different experiments performed in doublets are shown; bars represent SD values.

Similar results have been obtained previously in a competition assay in which C4b was allowed to compete with Arp4 for binding to immobilized C4BP (11). In such a set up, Arp4 displaced ^{125}I -labeled C4b from C4BP, whereas the addition of C4b did not prohibit binding between ^{125}I-labeled Arp4 and immobilized C4BP.

The effect of salt on the C4b and Arp4/Sir22 binding to C4BP

To further characterize the binding sites for C4b and *S. pyogenes*, we tested whether these interactions were inhibited in the presence of increasing salt concentration. To this end, plasma-purified ^{125}I-C4BP was incubated with C4b, Arp4, or Sir22 immobilized in wells of a microtiter plate. The buffer (20 mM Tris-HCl, pH 7.4) was supplemented with 0.1% BSA, 0.1% Tween 20, and NaCl concentrations ranging from 25 mM to 1.5 M. After 4-h incubation and washing with 50 mM Tris-HCl, pH 7.5, supplemented with 0.1% Tween 20, bound C4BP was measured in a gamma counter. Fig. 6 shows that binding between C4BP and C4b (black bars) was

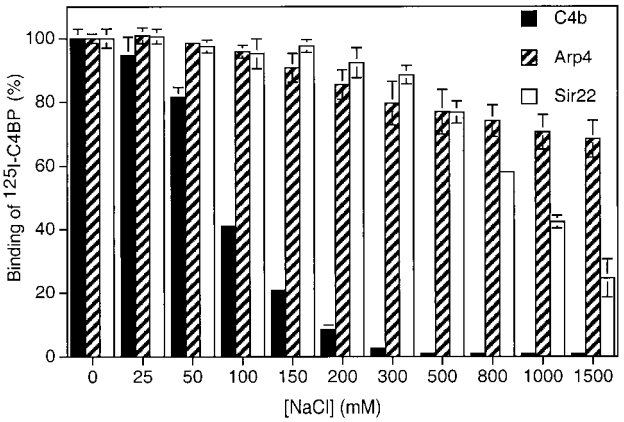

FIGURE 6. Effect of salt concentration on the binding of C4BP to C4b, Arp4, and Sir22. Plasma-purified C4BP was added to microtiter plates covered with C4b (filled bars), Arp4 (gray bars), or Sir22 (open bars) in buffer supplemented with increasing NaCl concentrations. After incubation for 4 h at room temperature, the plates were washed and the amount of bound C4BP was measured in a gamma counter. Results are shown as a mean value of three separate experiments ± SD.

sensitive to salt concentration and that binding was abolished already at 0.3 M NaCl. In contrast, binding between Arp4 and C4BP (gray bars) was relatively insensitive to salt concentration and only decreased by 30% at 1.5 M NaCl. The interaction between Sir22 and C4BP (white bars) was slightly more sensitive to NaCl concentration as compared with Arp4 and decreased by 50% at 1 M NaCl. These data support the conclusion that C4b and M proteins use different mechanisms to interact with C4BP.

Inhibition study with mAbs

A competition assay was used to study the effects of several mAbs against C4BP on the binding of C4b, Arp4, and Sir22. Trace amounts of plasma-purified ^{125}I-C4BP were added together with increasing amounts of various mAbs: mAb 67, 70, 92, 96, 102, and 104. In agreement with results reported previously, we found that all of these Abs, which have similar affinities for the α-chain of C4BP, inhibited the interaction with C4b (22). As shown in Fig. 7 (*upper panel*), mAb 104 was most efficient in inhibiting interaction with C4b: about 0.8 nM Ab was required to reach 50% inhibition of the binding, while other Abs were ~5- to 10-fold less efficient. The same experiment was performed with immobilized Arp4 (Fig. 7, *middle panel*). In this system, mAb 104 and also mAb 102 had a strong inhibitory effect on the interaction. However, higher concentration had to be used to obtain similar inhibition as for the C4b-C4BP interaction. Several Abs influenced the interaction with Arp4 weakly or had no effect. In particular, mAbs 67 and 92 had little or no influence on the binding between C4BP and Arp4, in contrast to their effect on binding between C4BP and C4b. Results obtained for Sir22 were similar to Arp4 (Fig. 7, *lower panel*). However, when Sir22 was immobilized, only mAb 102 and mAb 104 significantly inhibited the interaction with C4BP at the concentrations tested.

Discussion

The aim of this study was to characterize the mechanisms by which C4b and streptococcal M proteins interact with C4BP. Furthermore, we wanted to identify a key recognition area in the C4BP α-chain involved in the binding of these ligands. To address this question, we used nine C4BP mutants and compared the ability of these molecules to interact with C4b and with the M proteins

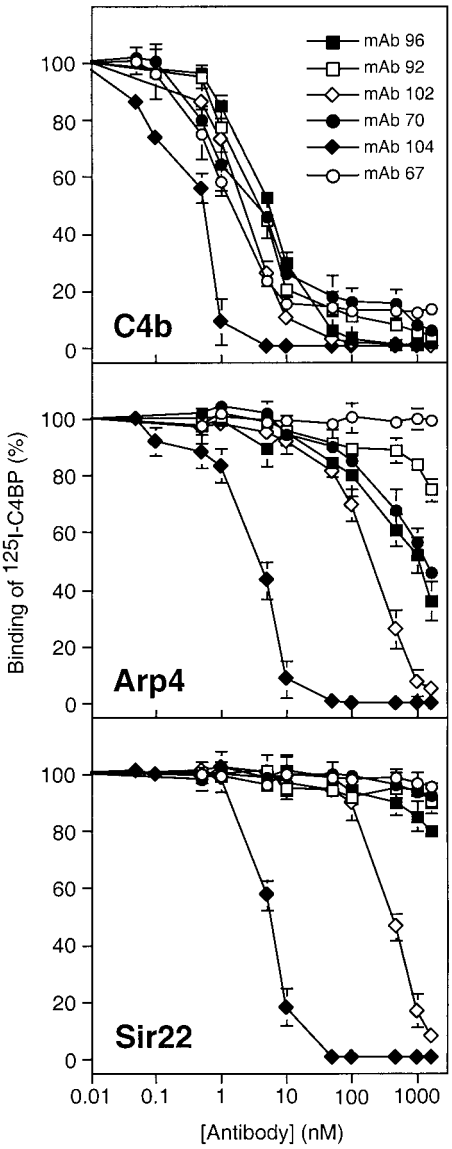

FIGURE 7. Effect of anti-C4BP mAbs on the binding of C4b, Arp4, and Sir22 to C4BP. ^{125}I-C4BP together with increasing concentrations of various Abs was added to microtiter plates covered with C4b (*upper panel*), Arp4 (*middle panel*), or Sir22 (*lower panel*). After 3 h of incubation at room temperature, the plates were washed and the amount of radioactivity bound was measured in a gamma counter. Results are shown as a mean value of three separate experiments ± SD.

Arp4 and Sir22. Effects of NaCl and mAbs were also tested, and the experimental results were then evaluated in conjunction with structural analysis of a recently reported 3D model structure of human (8) and mouse C4BP. Taken together, our data show that the key binding region for C4b overlaps with the surface interacting with Arp4/Sir22 and is located on CCP1 and CCP2. However, the recognition areas are not identical and the molecular mechanisms involved in these two processes differ.

Previously published data from our group (10) together with the present results show that Arg^{39}, Lys^{63}, Arg^{64}, and His^{67} are crucial for C4b binding. This observation indicates that an essential interaction site is located at the interface between the CCP1-CCP2 modules and on CCP2. These results are in good agreement with other published observations showing that the key binding site for C4b lies within the CCP1-CCP3 region (6, 17, 22, 23). We suggest that C4b displays a negative surface that could complement the

electropositive region in C4BP. This hypothesis is supported by a recent report showing that negatively charged residues in C3b (homologous to C4b) are essential for the binding to factor H and to complement receptor 1 (CR1; similar in structure and function to C4BP) (24). Thus, it is possible that C4BP, factor H, and CR1 all display a positively charged binding region, while the binding area at the surface of C3b or C4b is electronegative. Indeed, Krych and colleagues observed that altered binding of C3b or C4b to CR1 always correlated with mutations that removed a positive charge or added a negative charge in CR1 (25). Accordingly, it has been observed that binding affinity of cofactors to C3b and C4b increases concomitantly with decrease in ionic strength of the buffer (26). Long-range electrostatic forces together with favorable charge-charge interactions could be crucial for the formation of many short-lived macromolecular complexes within the complement cascade.

Previously, Accardo and co-workers have shown that the entire binding site for *S. pyogenes* lies within CCP1–3 of the C4BP α-chain and suggested that this site is indistinguishable from the C4b binding site (17). These authors have also shown that replacing Arg^{64} and His^{67} by uncharged amino acids decreased the ability of C4BP to bind whole *S. pyogenes* bacteria and C4b-Sepharose. Analysis of our results clearly shows that the cluster of positively charged amino acids between CCP1 and CCP2, which is crucial for binding of C4b, is also part of the site of interaction for the streptococcal M proteins Arp4 and Sir22. However, the mechanism of the latter binding appears to be different, and other forces than electrostatic most likely play an important role. This conclusion is based on the differential effects of increasing NaCl concentration on the binding of C4b and of M proteins and on analysis of the C4BP mutants.

Interactions between proteins involve complex mechanisms and are predominantly dictated by electrostatic forces, hydrogen bonds, and van der Waals contacts (27). Furthermore, binding surfaces are more hydrophilic than the protein core, but they also tend to be more hydrophobic than the average nonbinding surface areas (28, 29). Characterization of binding mechanisms by interpretation of mutagenesis results can be complicated because the side chains of some amino acids, like these of Arg or Lys, have dual, hydrophobic, and charged/polar characteristics. In this study, we attempted to differentiate between two binding mechanisms, one which seems to be highly dependent on electrostatic forces and the other being favored by hydrogen bonding and hydrophobic contacts. It is generally accepted that NaCl in the 0.1–1 M range reduces long-range electrostatic interactions and limits the formation of salt bridges (30), without significantly affecting their stability once they are formed at the interface of a specific protein-protein recognition site. In contrast, hydrophobic interactions between hydrophobic side chains present at protein interfaces together with hydrogen bonds are short ranged, and NaCl concentrations within the range used in this study should not significantly disturb such contact. The relatively minor effect of salt on the C4BP-Arp4/Sir22 interactions stands in sharp contrast to the C4b-C4BP binding that was entirely abolished already at 300 mM NaCl (Fig. 6). The most likely hypothesis that could account for these effects is that long-range electrostatic forces and ion pairings are essential for the attraction and binding of C4b to C4BP, while the binding of M proteins to C4BP also involves numerous hydrophobic interactions and hydrogen bonds. Possibly, the C4b-C4BP complex requires only short $t_{1/2}$ for physiological reasons (i.e., C4b interacts with C4BP, is cleaved by factor I, and then released), while the *S. pyogenes*-C4BP interaction probably has evolved to escape complement attack requiring different types of forces to further stabilize the binding with C4BP. These two different mechanisms could then result in the observed 10-fold difference in apparent affinity constants. It seems that although the electropositive region on C4BP CCP1 and CCP2 lies within the binding site for Arp4, the interaction is essentially depending on hydrogen bonds and hydrophobic contact. Interestingly, similar hydrophobic interaction seems to be used by measles virus, as it interacts with a hydrophobic loop present on the surface of CD46 (31).

Residues Lys^{63}, Arg^{64}, and His^{67} in C4BP are directly involved in the binding of Arp4 and Sir22 because changes of these residues resulted in greatly decreased binding of both streptococcal proteins. It seems that the binding involves the hydrophobic character of Lys^{63} and Arg^{64}, but not their ability to form salt bridges, in contrast to what is expected for the C4b-C4BP interaction, because the Arp4/Sir22-C4BP binding is rather insensitive to salt. There are slight differences between Arp4 and Sir22 with respect to their interaction with C4BP mutants, which is consistent with the fact that, although these two proteins share an overall sequence identity of ~78%, there is only ~25% identity within the hypervariable region binding C4BP (estimated with Lasergene; DNAstar, Madison, WI). The hypervariable region in Arp4 and Sir22 contains many negatively charged amino acids that could attract (in a nonspecific manner) the electropositive region on C4BP. However, negatively charged amino acids are also present in the hypervariable region of protein M5 that does not interact with C4BP (16). These findings agree with our results and imply that other forces than electrostatic play an important role in the interaction between C4BP and M proteins.

The conclusion that C4b and Arp4/Sir22 have overlapping but not identical binding sites on the surface of C4BP was confirmed by using a set of mAbs, directed against the α-chain of C4BP. Inhibition of the C4b-C4BP binding by all six Abs was reported previously, and our present results are in agreement with these data (22). Only two of these mAbs (no 104 and 102) could completely inhibit the binding of both Arp4 and Sir22 to C4BP. In particular, mAb 104 was the most efficient inhibitor both for C4b and for Arp4/Sir22. However, it should be noted that mAbs 67 and 92 had little or no effect on the binding of Arp4/Sir22, but could completely block binding of C4b, confirming that the two different ligands do not have identical binding sites. There are three additional lines of evidence for the conclusion that C4b and M proteins bind in different ways to C4BP. First, we have found that the pH dependencies of the C4b-C4BP and Arp4/Sir22-C4BP interaction differ. Binding between C4b and C4BP showed two maxima around pH 6 and 8, whereas Sir22-C4BP interaction did not change in the interval of pH 6–9 (results not shown). Second, the positively charged cluster on C4BP binds the electronegative polysaccharide heparin, which therefore competes with C4b for binding to C4BP (8), but influences the binding between C4BP and Arp4 to a much smaller extent (8). Third, both Arp4 and Sir22 block binding of C4b to C4BP, while C4b is not able to efficiently compete with M proteins for binding to C4BP even at a concentration of 10 μM (Fig. 5) (11). This observation suggests that the interaction between C4BP and M proteins on the surface on the bacteria is physiologically relevant even during a massive activation of the complement system when high concentrations of C4b are generated locally.

Because human and mouse C4b interact with C4BP CCP1 to CCP3 (23), but only human and higher primate C4BP molecules interact with *S. pyogenes* (17), we decided to compare the molecular surface of 3D models of human and murine C4BP aiming at finding regions that present important amino acid differences (Fig. 8). Positively charged residues at the interface between CCP1-CCP2 are conserved in the two species, which is consistent with the hypothesis that they are crucial for the attraction and transient

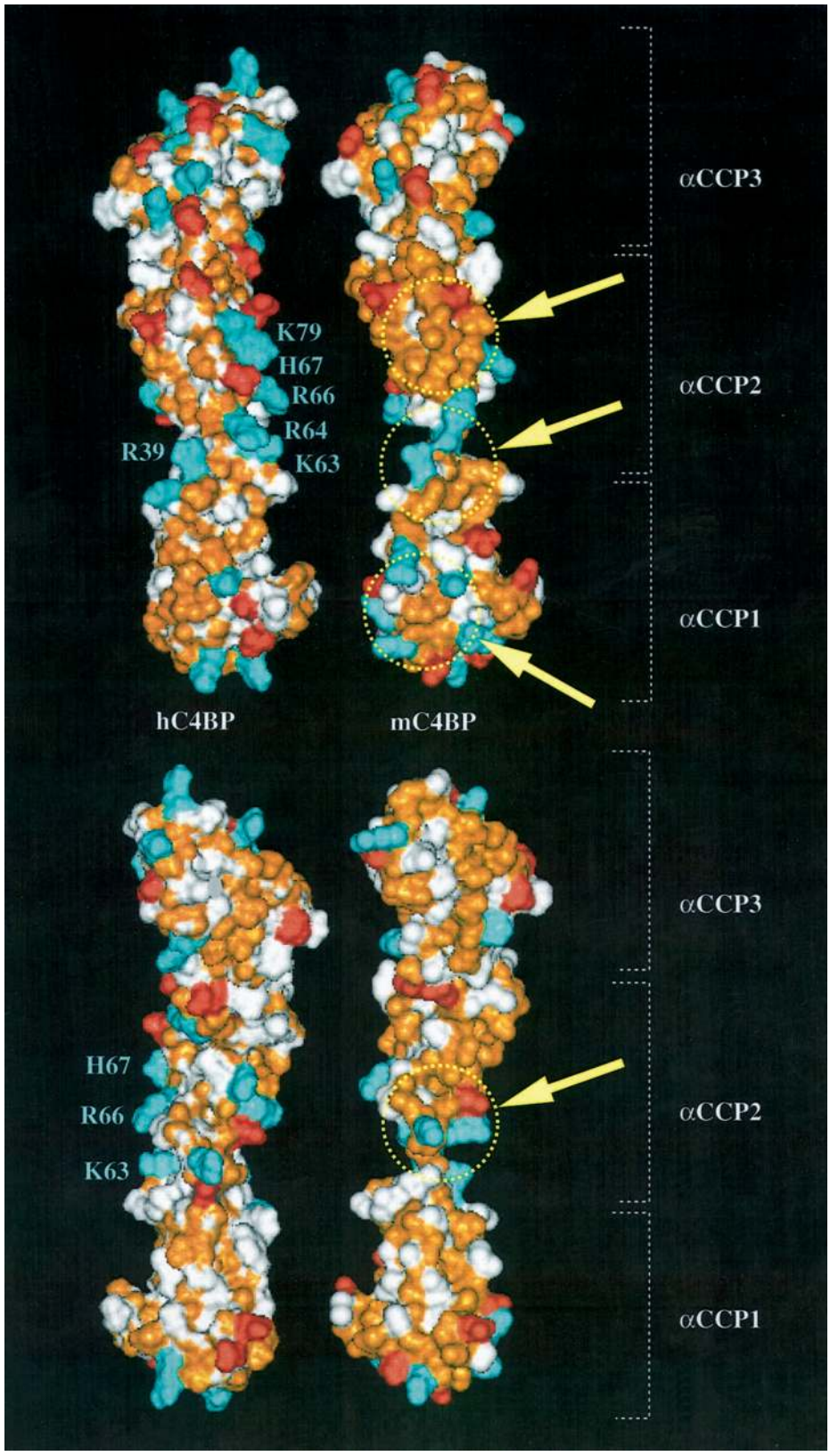

FIGURE 8. Comparison of 3D models for human and mouse C4BP CCP1–3. A solvent-exposed surface of the two models is presented. Further refinement of the intermodular angle requires additional experimental data. Negatively charged amino acid residues are displayed in red, positively charged in blue, polar regions are in orange, while the hydrophobic/aromatic side chains are white. In the *top panel* of the figure, the orientation of the models is similar to the one used in Fig. 1. The other face of the molecules is shown in the *lower panel*. Regions that are clearly different between the two proteins are indicated by arrows.

binding of C4b (via electrostatic forces). During evolution, the molecular surfaces of M proteins may have evolved in such a way that they not only occupy the area overlapping with the C4b binding site on the surface of C4BP, but also allow for M proteins to remain tightly bound. The surfaces of CCP3 in the two species are relatively similar, and the fact that *S. pyogenes* binds only to human C4BP but not to murine C4BP molecule suggests that CCP3 is not a key binding site for M proteins. There are striking differences within the linker region between CCP1 and CCP2, caused by the fact that it is one residue longer in mouse C4BP as compared with human C4BP (Fig. 8). This region is therefore a good candidate for constituting an epitope recognized by some of the tested

mAbs. The differences in length and amino acid distribution in this region could also account for the lack of interaction between mouse C4BP and streptococcal M proteins. Some differences are also seen at the surface of CCP2, which in human C4BP displays more polar surfaces (hydrogen bonds forming) that together with hydrophobic-aromatic regions at the surface of CCP1 could be crucial for M proteins binding. The latter forces may be individually weak, but since many such contacts can be established when C4BP binds to Arp4 or Sir22, the overall result is the formation of a tight, stable, and specific complex. It is possible that the molecular surface required for binding of C4b involves at least CCP1–3, and that it is larger than the binding area for M proteins, which appears to mainly involve CCP1–2. This could in part be explained by the six times larger molecular size of the C4b molecule as compared with the M proteins.

Studies of the interaction between streptococcal M proteins and C4BP are interesting not only from a structural point of view, but also for understanding of the host-parasite relationship during bacterial infection. In this respect, it is noteworthy that the C4BP-binding M proteins Arp4 and Sir22 have separate binding sites for C4BP and Ig-Fc (11, 16). The interactions with Ig-Fc and with C4BP appear to be restricted to proteins from primates (17, 32), a finding that probably is related to the fact that *S. pyogenes* normally causes disease only in humans. This adaptation to a human environment implies that the binding of Ig-Fc and C4BP to streptococcal M proteins plays an important role during the establishment of an infection. Evidence for an important role of C4BP in bacterial pathogenesis also comes from the finding that C4BP binds to all clinical isolates of *B. pertussis*, the etiologic agent of whooping cough (12). Interestingly, inhibition studies have suggested that *B. pertussis* binds to a region of C4BP overlapping with the C4b binding site (12). These interactions of C4BP with unrelated human pathogens encourage further studies of the molecular mechanisms involved and could help in the design of new therapeutic agents.

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