Mutations in α-Chain of C4BP That Selectively Affect Its Factor I Cofactor Function*

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C4b-binding protein (C4BP) inhibits all pathways of complement activation, acting as a cofactor to the serine protease factor I (FI) in the degradation of activated complement factors C4b and C3b. C4BP is a disulfide-linked polymer of seven α-chains and a unique β-chain, the α- and β-chains being composed of eight and three complement control protein (CCP) domains, respectively. In previous studies we have localized cofactor activity and binding of C4b to α-chain CCP1–3 of C4BP, whereas the binding of C3b required additionally CCP4. Likewise, introduced point mutations that decreased binding of C4b/C3b caused a decrease in cofactor activity. In the present study, we describe two mutants of C4BP, K126Q/K128Q and F144S/F149S, clustered on α-chain CCP3, which selectively lost their ability to act as cofactors in the cleavage of both C4b and C3b. Both mutants show the same binding affinity for C4b/C3b as measured by surface plasmon resonance and have the same inhibitory effect on formation and decay of the classical pathway C3-convertase as the wild type C4BP. It appears that C4b and C3b do not undergo the same conformational changes upon binding to the C4BP mutants as during the interaction with the wild type C4BP, which then results in the observed loss of the cofactor activity.

The complement system is the key component of innate immunity, providing the first line of defense against invading pathogens. Limited proteolysis is central for both activation and inhibition of the complement system. Factor I (FI) is a serine protease responsible for down-regulation of classical, lectin, and alternative pathways of complement (1, 2). FI degrades activated forms of complement factor 3 (C3b) and 4 (C4b) only when they are bound to a cofactor protein such as C4b-binding protein (C4BP) or factor H (FH). C3 and C4 are homologous proteins processed intracellularly into α- and β-chains (C3) or α-, β-, and γ-chains (C4), which are linked with disulfide bridges. During degradation by FI, several peptide bonds in the α-chains of C3b and C4b are cleaved. Molecular events leading to the cleavage of C3b/C4b by FI are not well defined at present. It has been suggested that C3b undergoes a conformational change upon binding to FH, which results in the accessibility of peptide bonds in C3b that can be cleaved by FI (3). Simultaneously, it appears that FI is able to weakly interact with C3b even in the absence of cofactor protein and that this binding induces conformational change in FI, as diisopropyl fluorophosphate (DFP) can inhibit FI in complex with C3b (4). A similar phenomenon was observed, but to a lesser degree, when FH was also present. However, no inhibition could be observed when FI and FH were incubated with diisopropyl fluorophosphate either alone or together. Direct albeit low affinity (0.7 mM at physiological ionic strength) binding between FI and C3b was demonstrated to be ionic strength-dependent (5). In the presence of FH, the affinity between C3b and FI increased manifold.

C4BP is a large plasma protein consisting of seven identical α-chains and a unique β-chain, which are linked together by short amphipathic helices that are further stabilized by disulfide bridges (6). The α- and β-chains contain eight and three complement control protein (CCP) modules, respectively. CCP modules consist of ~60 amino acids forming a compact hydrophobic core that is surrounded by five or more β-strands organized into β-sheets (7). Electron microscopy of C4BP demonstrated a spider-like conformation with the seven α-chains forming extended tentacles (8, 9). C4BP interacts not only with C4b (10) but also with protein S (single binding site on the β-chain) (11), serum amyloid P component (SAP) (12), heparin (13), and a number of bacterial proteins (14–18).

C4BP controls C4b-mediated reactions, thereby inhibiting classical and lectin pathways of complement. Apart from acting as a cofactor to FI, C4BP also prevents formation of the C4bC2a complex, which is the classical pathway C3-convertase and accelerates its natural decay (19). Furthermore, C4BP acts as a FI cofactor in the cleavage of fluid phase C3b and therefore may contribute to regulation of the alternative pathway (20). Each α-chain of C4BP contains a C4b binding site, but most likely because of steric hindrance as high as four C4b can bind to one C4BP molecule (21). We have previously localized the binding site for C4b to CCP1–3 (22) and identified an important cluster of positively charged amino acids at the interface between CCP1 and CCP2 that is required for the recognition of C4b (23).

Cofactor activity of C4BP in the cleavage of C3b in addition requires C4P (i.e. CCP1–4) (20). The elucidation of the regulatory mechanisms of complement.

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1 The abbreviations used are: FI, factor I; ANS, 8-anilino-1-naphthalenesulfonate; C3b, activated complement factor 3; C3met, C3b-like molecule prepared with methionine; C4b, activated complement factor 4; C4BP, C4b-binding protein; C4met, C4b-like molecule prepared with methionine; CCP, complement control protein repeat; CR1, complement receptor 1; FH, factor H; MCP, membrane cofactor protein.
is hindered by the lack of structural information. To overcome these limitations, we have used a combination of molecular modeling and site-directed mutagenesis to study the F1 cofactor activity of C4BP. In the present study we describe mutants K126Q/K128Q and F144S/F149S that are modified in residues clustered on CCP3 of the C4BP α-chain. These mutants were able to bind C4b/C3b and accelerate decay of classical C3 convertase compared to the wild type C4BP. However, they could not act as cofactors in cleavage of C4b/C3b, most probably due to the fact that they did not induce conformational changes in C3b/C4b.

**EXPERIMENTAL PROCEDURES**

_CDMA Clones for Recombinant Proteins—Full-length cDNA coding for the human C4BP α-chain was cloned to an eucaryotic expression vector, pcDNA3 (Invitrogen). It was then used as the template, and the following mutations were introduced using the QuickChange site-directed mutagenesis kit (Stratagene): Met14 → Gln (primers: 5'-TTT GCT GCC CCG CAA GAT ATT ACG TTC-3' and corresponding antisense primer); Lys109 → Gln/Lys110 → Gln (primers: 5'-CAA TGT CAA TCT CCA GAC ATC-3' and corresponding antisense primer); Phe144 → Ser (primers: 5'-GTC CAA TGT CTC TCT GGC TTC-3' and corresponding antisense primer); Lys126 → Gln (primers: 5'-AAA ACT GGA ACT CTA CTT TGA AAC TAC ACC-3' and corresponding antisense primers). Nucleotides corresponding to changed amino acid residues are underlined. All experiments were confirmed by automated DNA sequencing (PerkinElmer Life Sciences).

Proteins—Human plasma C4BP (24), C1 (25), C2 (26), C4 (27), and F1 (28) were purified as described in their respective references. C3, C3b, and C4b were purchased from Advanced Research Technologies. In some experiments requiring large amounts of protein, C4b- and C3b-like molecules (C4met/C3met) were used. C4met and C3met were prepared by incubation of purified C4 or C3 with 100 mM methylamine, pH 8.5 overnight at 3 °C, and subsequent dialysis against 50 mM Tris-HCl, pH 7.5, and 150 mM NaCl. C1 and C2 were only functionally pure, i.e., they were devoid of other complement factors, whereas all the other proteins used in the study were at least 95% pure as judged by Coo- nassie staining of proteins separated by PAGE performed in the presence of SDS. All proteins were stored at 80 °C. Protein concentrations were determined from absorbance at 280 nm or amino acid analysis following 24 h of hydrolysis in 6 M HCl. C4b and C3b were labeled with 125I using the chloramine T method. The specific activity was 0.4–0.5 MBq/µg of protein.

Purification of Recombinant Proteins—Human kidney 293 cells (American Type Culture Collection no. 1573-CBL) were transfected with the pLXSN C4BP expression construct according to the manufacturer's instructions (Invitrogen). The neomycin analogue G418 at a concentration of 400 µg/ml penicillin and streptomycin and 400 µM G418. Medium from 250 ml of solution containing 10 mM sodium phosphate and 100 mM NaF (pH 7.4) before analysis. Approximately 120 µg of each mutant were analyzed in the far UV region (185–250 nm). The resolution was 1 nm, the sensitivity 20 C with constant shaking, and 50-µl aliquots were removed at time intervals and added to 100 µl of guinea pig serum diluted 1:30 in GVB-EDTA (2.5 mM veronal buffer, pH 7.3, containing 100 mM NaCl, 0.1% gelatin, and 40 mM EDTA). After 1 h at 37 °C the samples were centrifuged, and the amount of erythrocyte lysis was determined spectrophotometrically (Abs350).

Prevention of the Classical Pathway C3-convertase Assembly—The EAC14 cells were generated as described above and incubated in DGVB− containing C2 alone (~8 µg/ml) or together with an inhibitor. The mixtures were kept at 30 °C with constant shaking, and 50-µl aliquots were removed at time intervals and added to 100 µl of guinea pig serum diluted 1:30 in GVB-EDTA to measure residual C3-convertase activity. After 60 min of incubation at 37 °C, the samples were centrifuged, and the lysis of the erythrocytes was determined spectrophotometrically.

8-Aminolo-1-naphthalenesulfonate (ANS) Fluorescence Measurement—ANS fluorescence emission spectra were recorded on a Fluorolog-3 spectrometer (Jobin Yvon). C3met or C4met (0.7 µM each) in 20 mM sodium phosphate, pH 7.4, and 100 mM NaCl were incubated with 20 µM ANS in the presence of several concentrations of wild type C4BP or the mutants. Excitation was at 368 nm, and emission was recorded at 475 nm using 1.50-nm band pass excitation and emission slits. Measurements were performed at 25 °C. The fluorescence signal obtained from the buffer alone, −15% of the signal obtained for C3met, was subtracted. The signal obtained for each concentration of C4BP alone was also subtracted.

**RESULTS**

Selection of Mutants and Assessment of Their Structural Integrity—Our three-dimensional model of the C4BP α-chain (29) was used as the basis for the selection of mutations. Several mutants were produced, but only two of them (K126Q/K128Q and F144S/F149S), located in CCP3 (Fig. 1), provided new insights into the cofactor activity of C4BP. The residues that were targets for the mutagenesis were solvent-exposed and not involved in any clear stabilizing interactions with the remaining parts of the molecule (i.e. salt bridges), and the amino acid substitutions were therefore expected to be well tolerated. The C4BP mutants as well as the wild type molecule were expressed in eucaryotic cells and purified from cell culture media using affinity chromatography. The expression levels of the wild type and mutant C4BP were sim-
ilar, and −2 mg of pure protein was obtained from 1 liter of culture medium. Purified proteins migrated with similar mobilities upon electrophoresis (Fig. 2A) and polymerized correctly into hexamers (not shown). We also assessed the structural integrity of the mutants by circular dichroism analysis, and the results are presented as strength of the signal relative to the lowest point (millidegrees per second) measured (Fig. 2B). Both mutants yielded very similar spectra to the wild type C4BP, once more confirming that mutagenesis did not cause folding problems.

Mutagenesis of C4BP and Loss of FI Cofactor Function—To elucidate whether the FI cofactor activity of the C4BP variants was affected, they were incubated with C4b, FI, and trace amounts of 125I-labeled C4b. Proteins were then separated by SDS/PAGE, and C4b was visualized by autoradiography (Fig. 3, top panel). In the presence of FI and the wild type C4BP, the a-chain subunit of C4b disappeared, and a strong band corresponding to a degradation fragment (C4d) became apparent. Two C4BP variants, M14Q and T28Q/T47Q, were equally good cofactors as the wild type. However, both the K126Q/K128Q and the F144S/F149S mutants lost their ability to act as FI cofactors in the cleavage of C4b.

To assess the ability of the mutants to function as cofactors in the cleavage of C3b, a degradation assay similar to the one described previously for C4b was used. After incubation of 125I-C3b, FI and C4BP radioactively labeled fragments of C3b (68 kDa and 43/46 kDa, respectively) were visualized by autoradiography. Similarly to cleavage of C4b, we found that the two mutants, K126Q/K128Q and F144S/F149S, lost their ability to act as FI cofactors in the cleavage of C3b, whereas the M14Q and T28Q/T47Q variants were good cofactors (Fig. 3, bottom panel).

Mutagenesis of C4BP Is Not Associated with Loss of Binding to C4b and C3b—To assess whether the lost cofactor activity of C4BP mutants was due to decreased binding of C4b, we first used a competition assay in which the wild type and the mutants were allowed to compete with wild type 125I-C4BP for binding to C4b immobilized in wells of a microtiter plate. None of the mutants studied showed decreased binding to C4b, whereas the F144S/F149S mutant bound C4b 3-fold better than the wild type (Fig. 4). Furthermore, we determined the affinity of binding between C4BP mutants and C4b using surface plasmon resonance (BIAcore). Wild type C4BP and the mutants were immobilized via amino coupling in separate flow cells of CM5 chip, and C4met at various concentrations was injected over the surface. Binding was recorded in arbitrary resonance units, and the signal obtained from the empty control flow cell was subtracted. Obtained sensograms were evaluated using a steady state affinity model (BIAevaluation 3.0). In agreement with results obtained in competition assay, the F144S/F149S mutant bound C4met with 3-fold higher affinity than the wild type (Table I). The addition of 20 μM ZnCl2 increased the affinity of the wild type and the mutants up to KD = 0.1 μM, and the difference in affinity was no longer apparent. We also analyzed binding of C3met to C4BP under the same conditions; this interaction is weaker than C4met-C4BP and requires the presence of zinc for detection. Both mutants and wild type C4BP bound C3met with the same

![Figure 1](image1.png) **Model for the CCP1-CCP3 modules of the C4BP a-chain.** Residues shown to bind C4b are indicated in blue and amino acids investigated in the present study are in red. R39, Arg; K63, Lys; R64, Arg; R66, Arg; H67, His; K126, Lys; K128, Lys; F144, Phe; and F149, Phe.

![Figure 2](image2.png) **Analysis of purified C4BP mutants by SDS/PAGE and circular dichroism.** A, wild type recombinant C4BP and the mutants (−2 μg/well) were separated by SDS/PAGE on 10% gel under reducing conditions. Proteins were then visualized by Coomassie staining. B, CD spectra obtained for the wild type C4BP and the mutants are shown.
The C4BP mutants retained this ability. EAC14 cells were incubated with C2 alone or together with C4BP variants. At intervals, aliquots were drawn and incubated with guinea pig serum, a source of C3 and the terminal complement components. The degree of erythrocyte lysis was determined spectrophotometrically, and the results were expressed in terms of Z, which represents the number of EAC142 sites formed and equals the natural negative logarithm of (1 - percentage lysis). Z obtained at $T_{\text{max}}$ in the absence of inhibitor (−1) was set as 100%. C4BP inhibited the assembly of the C3-convertase by 50% at the used concentration (Fig. 5A). None of the C4BP mutants used in this study showed decreased ability to inhibit assembly of C3-convertase when compared with the wild type. In agreement with its increased affinity for C4b, the F144S/F149S mutant was ~2.5-fold more efficient than the wild type in preventing assembly of the convertase.

A hemolytic assay was also used to test whether the decay-accelerating property of C4BP variants was affected. EAC142 cells were generated by incubating EAC14 with C2 for 5 min (equal to the $T_{\text{max}}$ obtained in the previous experiment). The cells were then centrifuged and resuspended in DGV B2 (control) or DGV B2 containing varying concentrations of C4BP variants. After 5 min of incubation, guinea pig serum was added to determine the remaining convertase activity. Z obtained in DGV B2 without inhibitor was set as 100% in each experiment. Wild type C4BP accelerated decay of the C3-convertase in dose-dependent manner, and so did the mutants (Fig. 5B). Again, the F144S/F149S mutant was significantly more efficient than the wild type, but none of the mutants lost the ability to accelerate decay of the convertase.

**Mutagenesis of C4BP Is Associated with Loss of Ability to Induce Conformational Change in C3b/C4b—**Both C3b and C4b are able to bind a hydrophobic probe (ANS), and the binding can be measured as a fluorescence signal. We have found that ANS signal for both C3b and C4b decreased in the presence of the wild type C4BP but was not affected by the K126Q/K128Q and F144S/F149S mutants (Fig. 6). The significant difference between the wild type and the mutants suggests that the latter ones are not able to induce conformational change in C3b and C4b.

**DISCUSSION**

Complement inhibitors act on key components of the converging pathways of complement to prevent pathological consequences of unwanted complement activation. The inhibitors bind C4b and C3b, and these interactions have two consequences, i.e. decay acceleration of C3-convertases that contain C4b/C3b and the degradation of these factors by proteinase F1. In recent years, significant efforts have been made to elucidate the binding sites for C3b and C4b on various cofactor proteins such as C4BP, complement receptor 1 (CR1), membrane cofactor protein (MCP), and FI. However, the mechanism by which the cofactors assist in the cleavage of F1 is not clear. FI is only able to cleave C3b/C4b when they are bound to a cofactor molecule and there is no doubt about the direct interaction of C4b/C3b with cofactor molecules. But it is not clear whether FI interacts only with bound C4b/C3b or if it is also able to form...
contacts with the cofactor proteins (e.g. C4BP). It has been suggested that there is a direct molecular interaction between FI and C3b leading to conformational change in FI (4). This, however, is not enough to result in the cleavage of C3b in the absence of a cofactor.

For several complement regulators, CR1, MCP, decay accelerating factor (DAF), and FH, the involvement of individual CCP domains in the complement regulatory function and binding of C4b/C3b has been investigated. In the case of the decay accelerating factor (four CCPs), it was shown that the classical pathway C3-convertase regulatory function resides within CCP2 and CCP3, whereas regulation of the alternative pathway requires CCP1, CCP2, and CCP3 (31). In MCP (four CCPs), sites for C4b/C3b interaction have been mapped primarily to CCP2, CCP3, and CCP4 (32, 33). In FH (20 CCPs), there are three C3b binding sites localized to CCP1–4 (34–37), CCP12–14, and CCP19–20 (38). CR1 (28 CCPs) is organized into four repeats, each consisting of seven CCP units. Full ligand binding (C4b and C3b) and functional activity requires the first four CCPs in each repeat (39–42). Taken together, analysis of several complement regulators suggests that a basic C3b/C4b binding unit consists of 3–4 CCP domains. Both CR1 and MCP were subjected to extensive mutagenesis, and several important observations concerning binding and cofactor activity of these proteins were made. For both proteins, the reduction of positive charge decreased binding of both C4b and C3b, whereas the introduction of additional positive charge increased binding (41–43). These observations are consistent with the hypothesis that the binding is based on ionic interactions between positively charged amino acids on cofactor protein and the negatively charged amino termini of the α’ chains of C3b and C4b (44). A reduction in binding of C4b/C3b always produced a proportional decrease in cofactor activity. However, the reverse was not always true, because for both CR1 and MCP, mutants were observed that bound ligands similar to wild type, whereas their cofactor activities were reduced (41–43). The authors suggested that cofactor activity is a two-step process requiring ligand binding and an interaction with the protease.

We have shown previously that CCP 1–3 of C4BP α-chain are required for the binding of C4b and the cofactor activity (22). Binding is based to a large extent on ionic interactions and requires a cluster of positively charged amino acids at the interface between CCP1 and CCP2 (23). C4BP is also a cofactor in the cleavage of C3b and, in that case, CCP1–4 are required (20). In the present study, we have identified a patch of amino acids on CCP3 that selectively affect cofactor activity of C4BP in the cleavage of both C3b and C4b. Mutagenesis did not affect affinity for C4b or the ability of C4BP to prevent assembly or accelerate decay of the classical C3-convertase. We believe that there are two possible explanations for these observations. Either the C4b/C3b molecules do not undergo conformational change upon binding to these mutants, or the mutated amino acids form an FI-binding site. We were not able to set up any assay that could measure binding of FI to C4b/C4BP complex, but we have used fluorescence spectroscopy to determine whether C4b/C3b undergo conformational change upon binding to C4BP and its mutants. It was shown previously that conversion of C3 to C3b is associated with an increase in surface hydrophobicity, which can be measured as an increase in ANS fluorescence (30). The increase is abrogated upon binding of C3b to a cofactor and even more so after cleavage by FI (3). We have found that C4BP had a similar effect on the binding of ANS to C3b as reported previously for FH. However, the mutants did not affect ANS fluorescence in a significant way. We have also found that ANS binding to C4b decreased in the presence of wild type C4BP but that the mutants had no effect. Taken together, this suggests that the K126Q/K128Q and F144S/F149S mutants bind C3b and C4b but do not induce conformational changes required for binding/cleavage of FI. Additional support for this hypothesis comes from the experiment in which we have tested cofactor activity of C4BP in
degradation of C4b and C3b in the presence of increasing concentrations of FI. No cofactor activity was observed even at very high FI concentrations (results not shown), excluding the possibility that the mutants have impaired ability to bind FI, as effects of decreased affinity would be alleviated by increased concentration of FI.

Our mutagenesis data provide the first set of information discriminating the ligand binding sites and the cofactor sites of C4BP. However, full understanding of these reactions will require further structure-function studies. For instance, our future plans include determination of the structure of CCP1–4 of the C4BP α-chain by NMR spectroscopy and computer docking experiments, which will allow further interpretation of our mutagenesis results. Investigations of interaction between C4BP and its ligands should have important implications for understanding the mechanisms involved in the inhibition of the complement system and in microbial pathogenesis.

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