Structural Requirements for the Complement Regulatory Activities of C4BP*

Received for publication, March 19, 2001, and in revised form, May 17, 2001
Published, JBC Papers in Press, May 21, 2001, DOI 10.1074/jbc.M102445200

Anna M. Blom‡, Lena Kask, and Björn Dahlbäck
From the Lund University Wallenberg Laboratory, Department of Clinical Chemistry, University Hospital Malmö, S-205 02 Malmö, Sweden

C4b-binding protein (C4BP) is a regulator of the classical complement pathway C3 convertase (C4b2a complex). It is a disulfide-linked polymer of seven α-chains and a unique β-chain; the α- and β-chains are composed of eight and three complement control protein (CCP) domains, respectively. To elucidate the importance of the polymeric nature of C4BP and the structural requirements for the interaction between C4b and the α-chain, 19 recombinant C4BP variants were created. Six truncated monomeric variants, nine polymeric variants in which individual CCPs were deleted, and finally, four variants in which double alanine residues were introduced between CCPs were functionally characterized. The smallest truncated C4BP variant still active in degrading C4b on cell surfaces. All three N-terminal CCP domains contributed to the binding of C4b and were important for full functional activity; CCP2 and CCP3 were the most important. The spatial arrangements of the first CCPs were found to be important, as introduction of alanine residues between CCPs 1 and 2, CCPs 2 and 3, and CCPs 3 and 4 resulted in functional impairment. The results presented here elucidate the structural requirements of individual CCPs of C4BP, as well as their spatial arrangements within and between subunits for expression of full functional activity.

Human C4b-binding protein (C4BP) is a high molecular mass (570 kDa) plasma glycoprotein that efficiently inhibits the classical pathway of complement activation. Apart from preventing the assembly of the C3-convertase (C4b2a complex), it also accelerates the natural decay of the complex (1). In addition, C4BP binds C4b and serves as a cofactor to the plasma serine protease factor I in the cleavage of C4b both in fluid phase and when C4b is deposited on cell surfaces (2). C4BP belongs to a gene family of related proteins named the regulators of complement activation family binds C4b and/or C3b and is important for the inhibition of the classical and/or alternative pathways of complement activation. All these proteins contain variable numbers of tandemly arranged domains, which are denoted complement control protein (CCP) repeats. These domains are cysteine-rich and ~60 amino acid residues long, and each is composed of a hydrophobic core that is wrapped by β-strands (4). The major form of C4BP in plasma consists of seven identical α-chains (eight CCPs each) and one β-chain (three CCPs), and all of the chains are linked together by disulfide bridges (2, 5). Electron microscopic analysis of C4BP demonstrated an octopus-like conformation, with the seven α-chains forming extended tentacles (6).

We have recently localized the C4b binding site to the interface between CCP1 and CCP2 of the α-chain and identified a cluster of positively charged amino acids that are important for the binding (7, 8). This region of C4BP is also important for binding of C4BP to heparin (7), Bordetella pertussis (9), and M-proteins of Streptococcus pyogenes (10). The aim of the present study was to further characterize the structural requirements of C4BP for its binding to C4b and for its factor I cofactor activity. Recombinant C4BP lacking individual CCP domains and truncated monomeric C4BP variants were created and functionally characterized. The elongated conformation of α-chains and the fact that they are exclusively organized into repetitive, individually folding domains makes C4BP particularly suitable for this kind of studies. A total of 19 C4BP variants were studied, and based on their properties, we conclude that CCP2 and CCP3 are the most important domains, being crucial for binding of C4b and for the functional activity of C4BP. In addition, CCP1 was found to be important, as was the length of the linkers between the first four CCPs.

EXPERIMENTAL PROCEDURES

Proteins—Human plasma C4BP (11), C1 (12), C2 (13), C4 (14), and factor I (15) were purified as described in their respective references. C3 and C4b were purchased from Advanced Research Technologies. C1 and C2 were only functionally pure, i.e. they were devoid of other complement factors. C4BP, C4, C4b, C3, and factor I were at least 95% pure, as judged by Coomassie staining of proteins separated by polyacrylamide gel electrophoresis (PAGE) performed in the presence of SDS. All proteins were stored at ~80 °C. Protein concentrations were determined from absorbance at 280 nm or from amino acid analysis following 24 h hydrolysis in 6 M HCl. C4b and C4BP were labeled with 125I using the chloramine T method. The specific activity was 0.4–0.5 MBq/μg of protein.

cDNA Clones for Recombinant Proteins—Full-length cDNA encoding human C4BP α-chain was cloned to pcDNA3 (Invitrogen), a eucaryotic expression vector. This was used as template for the mutagenesis. To create monomeric, truncated C4BP variants, stop codons were introduced after Trp492 (CCP1–8), Lys433 (CCP1–7), Glu313 (CCP1–6), Glu248 (CCP1–5), and Gly187 (CCP1–4), using the QuikChange kit (Stratagene). The recombinant, overlapping extension po

* This study was supported by a Senior Investigators Grant from the Strategic Foundation, research grants from the University Hospital in Malmö, and grants from the Swedish Medical Research Council, the Swedish Natural Science Research Council, Tore Nilson’s Trust, the Crafoord Trust, and the Royal Physiographic Society in Lund. The costs of publication of this article were defrayed in part by the payment of page charges. This advertisement must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 46-40-33-82-33; Fax: 46-40-33-70-44; E-mail: Anna.Blom@klkemi.mas.lu.se.

The abbreviations used are: C4BP, C4b-binding protein; CCP, complement control protein repeat; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

Downloaded from http://www.jbc.org/ on 2018-07-18
Complement Regulation by C4BP

Description of 19 C4BP mutants used in the present study

| Modification of amino acid sequence in the α-chain of C4BP | Primer sequence (sense) |
|----------------------------------------------------------|------------------------|
| CCP1-8: Expressed Asn¹–Trp² | 5'-CCC AAG TGT GAG TAG TGG TAG ACC CCC GAA GCC TGT |
| CCP1-7: Expressed Asn¹–Lys³³ | 5'-GCC CTC CAA TGA AAA TAG TCG TGC CCG AAA CCA |
| CCP1-6: Expressed Asn¹–Gly22 | 5'-AGA CCA TCA TGT GGA TAG ATT TGC AAT TCT CTC |
| CCP1-5: Expressed Asn¹–Ala29 | 5'-CAA CAA GGA TAA ATT ACC TGC CAG AAG |
| CCP1-4: Deleted Asn¹–Glu28 | 5'-CCT CTC CTT GGT CCC TTA TGT TGC CCT GAA |
| CCP1-3: Deleted Asn¹–Glu187 | 5'-TAC CCA GGA TGT GAG GGC GAC ATT TGC AAT CTT |
| ΔCCP1: Deleted Asn¹–Tyr62 | 5'-ACA CCA TCA GGT GAC CTG TCG TGC CGG AAA |
| ΔCCP2: Deleted Lys²³–Ile¹²⁴ | 5'-GCA TGT GAA ATT GCA GCA GTC AAG TGT AAT AAT |
| ΔCCP3: Deleted Val¹²⁵–Ile¹⁸⁶ | 5'-GCC CCA TGT GAA ATT GCA GCA GTC AAG TGT AAT AAT |
| ΔCCP4: Deleted Lys¹⁸⁶–Asn²⁴⁹ | 5'-GCA TGT GAG CCC TTA TGT TGC CCT GAA |
| ΔCCP5: Deleted Asn²⁴⁹–Ala³¹⁴ | 5'-AGC CCA TGT GAG GGC GAC ATT TGC AAT CTT |
| ΔCCP6: Deleted Leu³¹⁵–Gly³⁷⁵ | 5'-GCA TGT GAG GGC GAC ATT TGC AAT CTT |
| ΔCCP7: Deleted Asp³⁷⁶–Lys⁵³ | 5'-ACA CCA TCA GGT GAC CTG TCG TGC CGG AAA |
| ΔCCP8: Deleted Cys³⁵⁶–Trp⁶² | 5'-GCA TGT GAG GGC GAC ATT TGC AAT CTT |
| ΔCCP1–2: Deleted Asn¹–Ala³¹⁴ | 5'-GCA TGT GAG GGC GAC ATT TGC AAT CTT |
| AACC1–2: Two alanines inserted between Lys⁶⁵ and Arg⁹⁴ | 5'-GCA TGT GAG GGC GAC ATT TGC AAT CTT |
| AACC2–3: Two alanines inserted between Ile¹²⁴ and Val¹³² | 5'-GCA TGT GAG GGC GAC ATT TGC AAT CTT |
| AACC3–4: Two alanines inserted between Lys¹⁸⁶ and Ile¹⁹⁰ | 5'-GCA TGT GAG GGC GAC ATT TGC AAT CTT |
| AACC4–5: Two alanines inserted between Pro¹²⁴ and Asn²⁴⁹ | 5'-GCA TGT GAG GGC GAC ATT TGC AAT CTT |

Cloning of Recombinant Proteins—Human kidney 293 cells (ATCC number 1573-CRL) were transfected with the various C4BP constructs using Lipofectin, according to the manufacturer’s instructions (Life Technologies, Inc.). The neomycin analogue, G418, at a concentration of 100 μg/ml (Life Technologies), was added (20 kcpm/well), together with the various unlabeled proteins diluted in 50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, 0.1% bovine serum albumin, pH 7.5. The samples were incubated for 4 h at room temperature, the wells were washed five times, and the amount of radioactivity bound in each well measured in a gamma counter.

C4β Degradation Assay—C4BP (200 μm) was mixed with 250 μm C4b, 60 μm factor I, and trace amounts of I²-labeled C4b in 50 mM Tris-HCl, 150 mM NaCl, pH 7.4. The samples were incubated for 1.5 h at 37 °C, and the reaction was terminated by the addition of SDS-PAGE sample buffer with reducing agent (dithiothreitol). The samples were then incubated at 95 °C for 3 min and applied to a 10–15% gradient SDS-PAGE. The separated proteins were visualized and quantified using a PhosphorImager (Molecular Dynamics).

Circular Dichroism—In a Cary-Glass cuvette, the absorbance readings were recorded at 0.5 nm intervals from 260 to 210 nm. The spectra were calculated in terms of mean residue ellipticity per residue (° cm² dmol⁻¹) at 222 nm. The data were analyzed using the program of Fourier (Boehringer, diluted 1:3000 in DGVB +). The results were expressed as the mean of three independent experiments, each of which was repeated at least three times. The mean residue ellipticity was calculated from the absorbance readings at 222 nm.

Liverrase chain reaction technique was used to construct mutants lacking individual CCPs. The polymerase chain reaction products were cloned into HindII and NotI sites of pcDNA3. The amino acids deleted in each construct, and the sequences of the primers that were used for the polymerase chain reactions are given in Table I. All mutations were confirmed by automated DNA sequencing (PerkinElmer Life Sciences).

Purification of Recombinant Proteins—Human kidney 293 cells were centrifuged, and lysis of the erythrocytes was determined spectrophotometrically.

| Description of 19 C4BP mutants used in the present study | Primer sequence (sense) |
|----------------------------------------------------------|------------------------|
| CCP1–8: Expressed Asn¹–Trp² | 5'-CCC AAG TGT GAG TAG TGG TAG ACC CCC GAA GCC TGT |
| CCP1–7: Expressed Asn¹–Lys³³ | 5'-GCC CTC CAA TGA AAA TAG TCG TGC CCG AAA CCA |
| CCP1–6: Expressed Asn¹–Gly22 | 5'-AGA CCA TCA TGT GGA TAG ATT TGC AAT TCT CTC |
| CCP1–5: Expressed Asn¹–Ala29 | 5'-CAA CAA GGA TAA ATT ACC TGC CAG AAG |
| CCP1–4: Deleted Asn¹–Glu28 | 5'-CCT CTC CTT GGT CCC TTA TGT TGC CCT GAA |
| CCP1–3: Deleted Asn¹–Glu187 | 5'-TAC CCA GGA TGT GAG GGC GAC ATT TGC AAT CTT |
| ΔCCP1: Deleted Asn¹–Tyr62 | 5'-ACA CCA TCA TGT GGA GCT TCG TGC CGG AAA |
| ΔCCP2: Deleted Lys²³–Ile¹²⁴ | 5'-GCA TGT GAG GGC GAC ATT TGC AAT CTT |
| ΔCCP3: Deleted Val¹²⁵–Ile¹⁸⁶ | 5'-GCC CCA TGT GAA ATT GCA GCA GTC AAG TGT AAT AAT |
| ΔCCP4: Deleted Lys¹⁸⁶–Asn²⁴⁹ | 5'-GCA TGT GAG GGC TTA TGT TGC CCT GAA |
| ΔCCP5: Deleted Asn²⁴⁹–Ala³¹⁴ | 5'-GCA TGT GAG GGC GAC ATT TGC AAT CTT |
| ΔCCP6: Deleted Leu³¹⁵–Gly³⁷⁵ | 5'-GCA TGT GAG GGC GAC ATT TGC AAT CTT |
| ΔCCP7: Deleted Asp³⁷⁶–Lys⁵³ | 5'-ACA CCA TCA TGT GGA GCT TCG TGC CGG AAA |
| ΔCCP8: Deleted Cys³⁵⁶–Trp⁶² | 5'-GCA TGT GAG GGC GAC ATT TGC AAT CTT |
| ΔCCP1–2: Deleted Asn¹–Ala³¹⁴ | 5'-GCA TGT GAG GGC GAC ATT TGC AAT CTT |
| AACC1–2: Two alanines inserted between Lys⁶⁵ and Arg⁹⁴ | 5'-GCA TGT GAG GGC GAC ATT TGC AAT CTT |
| AACC2–3: Two alanines inserted between Ile¹²⁴ and Val¹³² | 5'-GCA TGT GAG GGC GAC ATT TGC AAT CTT |
| AACC3–4: Two alanines inserted between Lys¹⁸⁶ and Ile¹⁹⁰ | 5'-GCA TGT GAG GGC GAC ATT TGC AAT CTT |
| AACC4–5: Two alanines inserted between Pro¹²⁴ and Asn²⁴⁹ | 5'-GCA TGT GAG GGC GAC ATT TGC AAT CTT |
antibody and then washed twice in DGVB\(^+\). A fluorescein isothiocyanate-conjugated rabbit anti-mouse antibody (Dakopatts) was used during the second incubation, and the cell-associated fluorescence was then detected in a flow cytometer (Becton Dickinson). Based on the results of the C4 titration, two batches of C3-convertases were prepared with 1 or 32 \(\mu\)g/ml of C4. EAC142 cells were generated by incubating EAC14 cells at 2 \(\times\) 10\(^5\) cells/ml with an equal volume of C2 diluted in DGVB\(^+\) (final C2 concentration, approximately 20 \(\mu\)g/ml). The cells were incubated for 5 min, centrifuged at 4 °C, and resuspended in 11 \(m\)l aliquots were removed after 5 min and either added to 100 \(m\)l of cold guinea pig serum diluted 1:30 in GVB-EDTA or placed on ice. The aliquots that were mixed with the guinea pig serum were incubated at 37 °C for 1 h, and the amount of erythrocyte lysis was determined spectrophotometrically after centrifugation. To the aliquots that were placed on ice, C3 (40 \(\mu\)g/ml) was added, and the samples were then incubated for 30 min at 30 °C. After washing, the cells were incubated with fluorescein isothiocyanate-conjugated rabbit anti-mouse antibodies and deposited C3 was detected in a flow cytometer.

**RESULTS**

**Expression and Characterization of Recombinant C4BP Variants**—To define which of the eight CCP domains in the \(\alpha\)-chain of C4BP are functionally involved in the regulation of C3-convertase activity, 19 recombinant C4BP variants were created. In series A of C4BP variants (Fig. 1), stop codons were introduced after CCP1–3. B, polymeric mutants of C4BP lacking individual CCP domains, the last one missing both CCP1 and CCP2. C, polymeric mutants of C4BP with two alanine residues introduced between CCP domains.

The purified recombinant, polymeric forms of C4BP lacking single CCP domains (series B) and mutants having Ala residues introduced between the CCP domains (series C) were used in this study. A, monomeric forms of \(\alpha\)-chain were truncated at the C terminus by introduction of stop codons. Six variants were constructed and expressed, the shortest encompassing CCP1–3. B, polymeric mutants of C4BP lacking individual CCP domains, the last one missing both CCP1 and CCP2. C, polymeric mutants of C4BP with two alanine residues introduced between CCP domains. The purified recombinant, polymeric forms of C4BP lacking single CCP domains (series B) and mutants having Ala residues introduced between the CCP domains (series C) were subjected to SDS-PAGE. Recombinant C4BP and the mutants (~2 \(\mu\)g/well for Coomassie staining and 0.04 \(\mu\)g/well for immunoblotting) were separated by SDS-PAGE on 10–20% gel (Tricine buffer). Proteins were then subjected to Coomassie staining (A and B) or transferred to a membrane (C) and allowed to react with mAb 104 directed against CCP1.
analyzed by SDS-PAGE under both reducing and nonreducing conditions (Fig. 3). All of the variants of both series B and C formed polymeric C4BP similarly to wild type C4BP, and the molecular weight differences between the C4BP variants on nonreduced gels were those that could be expected from the introduced mutations. This suggests that the polymerization process was unaffected by the introduced mutations. The expression levels of all of the recombinant C4BP variants were similar to that of wild type C4BP, which is consistent with proper folding during synthesis. The mutants were also probed with a panel of monoclonal antibodies directed against a chain of C4BP (mAbs 67, 70, 92, 96, 102, and 104). The mutants were immobilized in wells of microtiter plate and incubated with increasing concentrations of antibodies. Bound antibodies were detected with a rabbit anti-mouse antibody conjugated with horseradish peroxidase. Approximately 50% binding to immobilized wild type C4BP was observed at 0.4–1 nM concentration of each antibody (not shown). Similar results were obtained for all variants of C4BP except when the deleted CCP domain contained antibody epitope. We mapped antibody epitopes to CCP1 (mAbs 70, 92, 96, 102, and 104) and CCP4 (mAb 67). In general, the mutations did not affect the recognition of the C4BP by the monoclonal antibodies, suggesting that the overall conformation of the C4BP variants was correct. Circular dichroism analysis of C4BP variants yielded very similar spectra, once again confirming that mutagenesis did not cause folding changes. Results are presented as strength of the signal relative to the lowest point (millidegrees/s) measured (Fig. 4). The results are expressed as the percentage of the lowest point seen in each spectrum. A and B, polymeric mutants lacking single CCP domains; C, polymeric mutants with two alanine residues introduced between CCP domains.

**Fig. 3. SDS-PAGE analysis of polymeric C4BP mutants lacking CCP domains.** Recombinant wild type C4BP and mutants were separated by SDS-PAGE. The polyacrylamide gel concentrations were 5 and 10% for unreduced (A) and reduced (B) samples, respectively. Proteins were then subjected to Coomassie staining.

**Fig. 4. Circular dichroism.** The circular dichroism spectra obtained for recombinant wild type C4BP and mutants are shown. The results are expressed as the percentage of the lowest point seen in each spectrum. A and B, polymeric mutants lacking single CCP domains; C, polymeric mutants with two alanine residues introduced between CCP domains.

Resistance of polymeric C4BP to unfolding (not shown). All polymeric C4BP variants shared this temperature resistance. The monomeric CCP1–8 demonstrated changes in ellipticity at 230 nm at ~70 °C. These changes were reversible as the normal spectra returned after overnight incubation of the protein at 4 °C.

**Monomeric C4BP a-Chains Equally Active as Polymeric C4BP in C4b Degradation of Fluid Phase—**It has been shown that each C4BP can bind four C4b molecules strongly and two additional C4b more weakly (16). To investigate whether isolated, monomeric a-chains were fully active in regulation of fluid phase C3 convertase, we compared the CCP1–8 variant with wild type C4BP. The abilities of the two proteins to inhibit the formation of C3 convertase, to accelerate its decay, and to act as cofactors to factor I were investigated. In a fluid phase C4b degradation assay, increasing concentrations of C4BP or CCP1–8 were incubated with C4b, factor I, and trace amounts of 125I-labeled C4b and then subjected to SDS-PAGE and autoradiography. To estimate the cofactor activity, the C4d bands were quantified by densitometry (Molecular Dynamics), and the C4d formation was plotted against the concentration of added protein (Fig. 5A). Both C4BP and CCP1–8 were active as factor I cofactors; ~100 nM C4BP was needed to reach 50% of C4d release, whereas 350 nM CCP1–8 was required to obtain a similar effect. The 3–4 times higher CCP1–8 concentration needed agrees well with the presence of four strong C4b binding sites in polymeric C4BP, whereas CCP1–8 only binds one C4b. The results suggest that CCP1–8 is equally efficient as intact C4BP in the degradation of fluid phase C4b, implying that there is no functional cooperativity between the a-chains of polymeric C4BP during C4b degradation in fluid phase.
Polymeric C4BP Is More Efficient Than CCP1–8 in Inhibition of Cell-bound C3-convertase—The efficiency of monomeric CCP1–8 to inhibit surface bound C3-convertases was compared with that of polymeric C4BP, thus testing the hypothesis that the polymeric nature of C4BP is important for regulation of surface-bound C3-convertases, in particular at high density of C3-convertases. In initial experiments, several batches of EAC14 cells were prepared using C4 concentrations ranging from 0.5 to 32 µg/ml. Deposited C4b was detected with fluorescein isothiocyanate-labeled monoclonal antibody against C4b using flow cytometry. At the highest concentration of C4 (32 µg/ml), the density of deposited C4b was at least 8-fold higher than that obtained using 1 µg/ml of C4 (Fig. 5B). EAC14 cells prepared at 1 µg/ml C4 and at 32 µg/ml C4 were used in the functional evaluation of C4BP and CCP1–8. The decay of C3-convertases was measured by two means, i.e., by the classical hemolytic assay (Fig. 5C) and by measuring C3b deposition on the cell surface using flow cytometry and fluorescein isothiocyanate-labeled C3-antibody (Fig. 5D). In both assays, polymeric C4BP was much more effective than CCP1–8 independently of the convertase density. Moreover, it was observed that lower concentrations of the C4BP were needed to reach similar relative inhibition at high density of C3-convertases as compared with low density, suggesting that the inhibition efficiency of polymeric C4BP increases with increased density of C3-convertases.

Binding Site for C4b on C4BP Involving CCP1–3—To evaluate the ability of all C4BP variants to bind C4b, a competition assay was used in which the various C4BP variants were allowed to compete with 125I-labeled wild type C4BP for binding to immobilized C4b (Fig. 6). In the absence of competitor, 25–35% of the added 125I-labeled C4b bound to the immobilized C4b, binding that was competed out by unlabeled C4BP (half-maximal inhibition observed at 5 nM C4BP). The ability of C4BP variants lacking CCP1, CCP2, CCP3, or CCP1–2 to compete was severely impaired, indicating that CCP1–3 is crucial for C4b binding. In particular, CCP2 was found to be important because deletion of this CCP completely abolished the C4b binding activity. In addition, the spacing between the CCPs was found to be important for the integrity of the C4b binding site, as illustrated by the disruptive consequence of alanine insertions between CCPs 1 and 2, CCPs 2 and 3, and CCPs 3 and 4. Deletion of CCP7 also resulted in 10-fold decreased ability to compete with C4BP binding to C4b.

Factor I Cofactor Activity of C4BP Depending on CCP1–3—To elucidate whether the impaired C4b binding was matched by a decrease in factor I cofactor activity, the C4BP variants were tested in the C4b degradation assay (Fig. 7). During degradation of C4b, factor I cleaves two peptide bonds in C4b. Cleavage of Arg1217–Asn1318 gives rise to a small, 13-kDa peptide named α4. The second cleavage at Arg937–Asn938 results in release of the 45-kDa C4d fragment, which was quantified in the assay. The first three CCPs were found to be important for expression of factor I cofactor activity, and even the shortest monomeric form of C4BP (CCP1–3) was active. When ΔCCP1 was analyzed, the amount of generated C4d was decreased by 60% as compared with the wild type C4BP. However, it was noteworthy that most of the α-chain of C4b was gone after the incubation, suggesting that ΔCCP1 retains its ability to serve as cofactor for the cleavage of Arg1217–Asn1318 bond. Cleavage of this site releases the small α4 fragment, and the remainder of the α-chain co-migrates...
with the β-chain. The small α4 fragment could be quantified after overexposure of the gels. Almost 80–90% of α4 fragment, as compared with the amount formed using the wild type C4BP, was generated. Thus, deletion of CCP1 results in the specific loss of C4b-cleavage at Arg937-Asn938. Similar results were obtained using the C4BP variants in which two alanines had been inserted between CCP1 and CCP2 or between CCP3 and CCP4. In contrast, C4BP variants lacking both CCP1 and CCP2 were completely inactive (Table II). Similarly, C4BP variants lacking CCP2 and CCP3 were inactive as cofactors to factor I. Complete loss of function was also observed in variants having two alanine residues inserted between CCP2 and CCP3, whereas insertions of the alanines between CCP3 and CCP4 yielded an intermediate effect (60% inhibition). The AACCP4/CCP5 mutant was found to be equally active as the wild type C4BP. The factor I cofactor activity was completely inhibited by mAb 104, a monoclonal antibody that was directed against CCP1.

CCP1–3 Is Sufficient for Prevention of C3-convertase Assembly—C4BP is able to prevent the assembly of the classical C3-convertase. A hemolytic assay was used to elucidate whether disruption of the C4b binding site in C4BP correlated with impaired ability to prevent convertase assembly. EAC14 cells were mixed with C2 alone or together with C4BP and incubated at 30 °C. Aliquots were drawn at intervals and mixed with guinea pig serum that was a source of C3 and the terminal complement components. The samples were incubated at 37 °C for 1 h, and the degree of erythrocyte lysis was determined. The results were expressed as Z, which represents the number of C142 sites formed and equals the negative natural logarithm of (1 – % lysis). In the absence of C4BP, the convertase was efficiently assembled with maximal activity (Tmax) observed after 5 min (this Z value was considered as 100%). Recombinant wild type C4BP (44 nM) efficiently prevented the convertase assembly, decreasing the Z value to 42% (Table III). The monomeric forms of C4BP were tested at 6-fold higher molar concentrations than C4BP yielding similar molar levels of C4b binding sites. All monomeric C4BP variants prevented assembly of the C3-convertase, and CCP1–4 and CCP1–5 were even more active than C4BP (Table III). In contrast, deletion mutants ΔCCP1, ΔCCP2, ΔCCP3, and ΔCCP1–2, as well as alanine insertion variants AACCP1/2 and AACCP2/3, demonstrated decreased activity, suggesting that CCP1–3 contains the site responsible for inhibition of C3-convertase assembly.

**FIG. 6.** Interaction of C4BP with immobilized C4b. Competition assay: increasing concentrations of fluid phase recombinant C4BP or various mutants competed with trace amounts of 125I-labeled C4BP for binding of immobilized C4b. The 100% binding was estimated in the absence of fluid phase competitor. Results of two different experiments performed in doublets are shown.

**FIG. 7.** C4b degradation assay. A, truncated mutants C4BP (200 nM) were incubated with 250 nM C4b, 60 nM factor I, and trace amounts of 125I-labeled C4b for 1.5 h at 37 °C. Immediately afterward, a sample buffer with reducing agent was added, samples were heated at 95 °C, and the proteins were separated by SDS-PAGE (10–15% gradient gel). The gel was dried and subjected to autoradiography. In one sample, the CCP1–8 mutant was preincubated with an excess of mAb 104. As a control, factor I was omitted in the incubation mixture. B, mutants lacking individual CCP domains.

**TABLE II**

| Variant | C4d Intensity (mean ± S.D.) |
|---------|-----------------------------|
| Recombinant wild type | 100 ± 0 |
| ΔCCP1 | 40 ± 10 |
| ΔCCP2 | 6 ± 6 |
| ΔCCP3 | 4 ± 5 |
| ΔCCP4 | 111 ± 8 |
| ΔCCP5 | 107 ± 11 |
| ΔCCP6 | 86 ± 9 |
| ΔCCP7 | 104 ± 9 |
| ΔCCP8 | 105 ± 9 |
| ΔCCP1–2 | 2 ± 1 |
| AACCP1/2 | 22 ± 7 |
| AACCP2/3 | 5 ± 4 |
| AACCP3/4 | 40 ± 10 |
| AACCP4/5 | 84 ± 12 |
| Without factor I | 1 ± 1 |

Intensities of bands corresponding to the C4d fragment, released from C4b after cleavage by factor I in the presence of C4BP (Fig. 7), were determined by densitometry and are represented as mean values of three determinations ± S.D.
The cells were then centrifuged and resuspended in DGVB

C3-convertase decayed to 50% of its initial activity after

activity displayed by

classical pathway C3-convertase (Table III). The hypothesis

C4BP, including the CCP1–3 variant, accelerated decay of the

control were estimated. We found that all monomeric forms of

concentrations needed to accelerate 5 min decay down to 60% of

wild type C4BP enhanced the rate of decay. Dose-response

min in the presence of buffer alone (not shown). Recombinant

nM protein. Data represent mean ± S.D. of three experiments.

| Prevention of C3-convertase assembly | Decay acceleration of C3-convertase |
|-------------------------------------|-------------------------------------|
| % of Z for buffer at T_{max} | nM |
| Wild type | 42.6 ± 9 | 71 ± 5 |
| CCP1–8 | 29 ± 7 | 78 ± 18 |
| CCP1–7 | 31.3 ± 7 | 84 ± 22 |
| CCP1–6 | 23.4 ± 5 | 62 ± 14 |
| CCP1–5 | 10.6 ± 5 | 17.2 ± 4 |
| CCP1–4 | 5 ± 5 | 14.5 ± 6 |
| CCP1–3 | 20 ± 7 | 79 ± 44 |
| ΔCCP1 | 96 ± 6 | NA |
| ΔCCP2 | 89 ± 6 | NA |
| ΔCCP3 | 90 ± 9 | NA |
| ΔCCP4 | 28 ± 2 | 4.6 ± 4 |
| ΔCCP5 | 56 ± 5 | 17 ± 5 |
| ΔCCP6 | 59 ± 6 | 35 ± 8 |
| ΔCCP7 | 84 ± 10 | NA |
| ΔCCP8 | 58 ± 9 | 32 ± 7 |
| ΔCCP1–2 | 89 ± 7 | NA |
| AACCP1/2 | 87 ± 3 | NA |
| AACCP2/3 | 84 ± 5 | NA |
| AACCP3/4 | 37 ± 5 | 48 ± 19 |
| AACCP4/5 | 54 ± 10 | 57 ± 29 |

CCP1–3 and Decay Acceleration—The hemolytic assay was also used to elucidate whether the structural requirements for the decay accelerating property of C4BP were the same as those for C4b binding. For this purpose, EAC142 cells were generated by incubating EAC14 cells with C2 for 5 min (T_{max}). The cells were then centrifuged and resuspended in DGVB+ (control) or DGVB− containing C4BP or C4BP variants. The C3-convertase decayed to 50% of its initial activity after −15 min in the presence of buffer alone (not shown). Recombinant wild type C4BP enhanced the rate of decay. Dose-response curves were constructed for wild type C4BP and all of the C4BP variants. To compare the efficacy of the various proteins, the concentrations needed to accelerate 5 min decay down to 60% of control were estimated. We found that all monomeric forms of C4BP, including the CCP1–3 variant, accelerated decay of the classical pathway C3-convertase (Table III). The hypothesis that CCP1–3 is responsible for acceleration of C3-convertase decay was further supported by the observed low or absent activity displayed by ΔCCP1, ΔCCP2, ΔCCP3, ΔCCP1–2, as well as AACCP1/2 and AACCP2/3.

Heparin Binding Site Confined to CCP1–3—The interaction between C4BP and C4b can be inhibited by heparin, suggesting that the C4b and heparin binding sites overlap (17). To assess the capacity of the C4BP variants reported here to bind to heparin, heparin affinity chromatography was used. Recombinant wild type C4BP eluted from the column as a single peak (93.6%) at 27 mS/cm (Fig. 8). The heparin binding ability of C4BP was compromised by the removal of CCP2 and by insertion of two alanines between CCP1 and CCP2. In contrast to the dramatic effects on C4b binding, deletion of CCP3 and CCP1 had only minor effects on heparin binding, suggesting that CCP2 is the most important for the interaction.

Prevention of C3-convertase assembly

Decay acceleration of C3-convertase

| Prevention of C3-convertase assembly | Decay acceleration of C3-convertase |
|-------------------------------------|-------------------------------------|
| % of Z for buffer at T_{max} | nM |
| Wild type | 42.6 ± 9 | 71 ± 5 |
| CCP1–8 | 29 ± 7 | 78 ± 18 |
| CCP1–7 | 31.3 ± 7 | 84 ± 22 |
| CCP1–6 | 23.4 ± 5 | 62 ± 14 |
| CCP1–5 | 10.6 ± 5 | 17.2 ± 4 |
| CCP1–4 | 5 ± 5 | 14.5 ± 6 |
| CCP1–3 | 20 ± 7 | 79 ± 44 |
| ΔCCP1 | 96 ± 6 | NA |
| ΔCCP2 | 89 ± 6 | NA |
| ΔCCP3 | 90 ± 9 | NA |
| ΔCCP4 | 28 ± 2 | 4.6 ± 4 |
| ΔCCP5 | 56 ± 5 | 17 ± 5 |
| ΔCCP6 | 59 ± 6 | 35 ± 8 |
| ΔCCP7 | 84 ± 10 | NA |
| ΔCCP8 | 58 ± 9 | 32 ± 7 |
| ΔCCP1–2 | 89 ± 7 | NA |
| AACCP1/2 | 87 ± 3 | NA |
| AACCP2/3 | 84 ± 5 | NA |
| AACCP3/4 | 37 ± 5 | 48 ± 19 |
| AACCP4/5 | 54 ± 10 | 57 ± 29 |

DISCUSSION

The purpose of this study was to delineate complement regulatory sites within the eight CCP modules of the C4BP α-chain. Several different regions of the α-chains have been suggested to be important for binding of C4b. In the first report concerning this subject, an N-terminal 48-kDa α-chain fragment, formed by chymotrypsin digestion, was found to bind C4b and to express factor I cofactor activity (18, 19). This agreed well with electron microscopy images of C4BP-C4b complexes, which demonstrated binding of C4b at the peripheral end of each C4b tentacle (6, 20). Some later reports strengthened the concept that the three most N-terminal CCPs are necessary for binding of C4b (21–23). We recently demonstrated a cluster of positively charged amino acids at the interface between CCP1 and CCP2 to be crucial for binding of C4b and for the ability of C4BP to regulate the C3 convertase (7, 8, 10). We have now obtained additional data related to the structure-function relationships of C4BP based on studies of a panel of recombinant C4BP variants. Systematic deletions of CCPs from the C-terminal end of the α-chain in combination with several functional assays showed that all recombinant proteins that included CCP1–3 had functional activity. Furthermore, specific deletions of CCP2 and CCP3 entirely abolished the ability of C4BP to bind C4b and to inhibit the C3-convertase. Deletion of CCP1 had a significant effect; however, it did not entirely destroy functional activity of the α-chain. We therefore concluded that CCP2 and CCP3 are the most crucial domains for the functional activity of C4BP and that CCP1 also plays an important role. The pertinence of the native configuration of the three N-terminal CCPs was shown by mutants in which the relevant four CCPs were maintained but where the distance between them was increased by the introduction of two additional alanines. Variants having alanines inserted between CCP2 and CCP3 completely lacked biological activity, whereas the activities of the AACCP1/2 and AACCP3/4 variants were significantly impaired. Similar results were obtained in the C4b degradation assay, in which the ability of mutant proteins to act as factor I cofactors was assessed.

Heparin interacts with several complement proteins, but the...
affinity of the binding of heparin to C4BP is higher than the affinities of heparin for other complement proteins (24). We have shown previously that heparin is able to block the interaction between C4b and C4BP and that Arg539, Arg64, and Arg66 play key roles in heparin binding (7). The same electropositive cluster of amino acids was also shown to be involved in binding of C4b. Analysis of the homology-based three-dimensional model of the whole α-chain of C4BP implied that the electropositive cluster between CCP1 and CCP2 is the only area of the α-chain that presents characteristics of a heparin binding site (7). In the present study, we found that the ability of C4BP to bind heparin, assessed by affinity chromatography, is strongly compromised by the removal of CCP2 and by insertion of two alamines between CCP1 and CCP2. Furthermore, the deletion of CCP1 and CCP3 had minor effects on the affinity for heparin. These results suggest that the binding site for heparin is located to CCP1–3, and CCP2 is the most crucial for the interaction. It appears that interaction sites for C4b and for heparin overlap to a large extent. In addition, both interactions are very sensitive to ionic strength and governed by charge-charge interactions.

Most published reports agree with the C4b binding site being located within CCPs 1–3, but other regions of the α-chain have been implicated to be involved in C4b binding as well. Thus, proteolytic fragments of C4BP were used to map the C4b binding and factor I cofactor activities to distinct regions spanning CCPs 6–7 and 3–6, respectively (25). Furthermore, a monoclonal antibody directed against CCP6 was found to block binding of C4b to C4BP (26). These results were difficult to reconcile with the observation that mouse C4BP lacking CCP6–5 binds human C4b (27). Recently, it was shown that a cryptic binding site for C3b is located near the C terminus of the α-chain (28). This site was only exposed in recombinant, monomeric, and cell-bound C4BP, which makes its physiological significance unclear. However, such a cryptic C3b binding site may also have the ability to interact with C4b, which could explain some of the disagreement in published reports. Interestingly, we found that C4BP lacking CCP7 displayed 10-fold lower apparent affinity for immobilized C4b in a competition assay. The ΔCCP7 mutant was also a bad inhibitor of convertase assembly, and it did not accelerate decay of C3-convertase. However, the mutation did not affect the ability of C4BP to serve as a cofactor to factor I.

For the other complement regulators, complement receptor 1, membrane cofactor protein, decay accelerating factor, and factor H, the involvement of individual CCP domains in complement regulatory function and binding of C4b/C3b have been investigated. In the case of 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 (29). In membrane cofactor protein (four CCPs), sites for C4b/C3b interaction have been mapped primarily to CCP2, CCP3, and CCP4 (30, 31). In factor H (20 CCPs), there are three C3b binding sites localized to CCP1–4 (32–35), CCP12–14, and CCP19–20 (36). Complement receptor 1 (28 CCPs) is organized into four repeats, each consisting of seven CCP units. Full ligand binding (C4b and C3b) and functional activity require the first four CCPs in each repeat (37–40). Taken together, our present analysis of C4BP and reports on other complement regulators suggest that a basic C3b/C4b binding unit consists of three or four CCP domains.

C4BP is the only complement regulator that is composed of multiple, identical subunits, and we aimed at elucidating the functional importance of the multimeric structure of C4BP. It has been suggested that the interaction between one binding site on C4BP and a single molecule of C4b on the cell surface may be very weak and that the multiple interactions are needed for efficient inhibition of cell-bound C3-convertases by C4BP (41). Indeed, we found that the polymeric wild type C4BP was much more efficient as inhibitor of C3-convertase than the monomeric α-chain. Furthermore, it appears that inhibition efficiency of wild type C4BP increases with the density of C3-convertase. This is most probably due to a fact that densely deposited C4b molecules will allow binding of several α-chains of C4BP simultaneously and will result in high affinity for the cell surface.

Our future plans include elucidation of the three-dimensional structure of CCP1–3 of the α-chain by NMR spectroscopy. Also, the mutants used in this study can be employed for determination of binding sites for other ligands of C4BP, such as streptococcal M-proteins and B. pertussis. We have recently reported that the binding site for porins of N. gonorrhoeae is localized to CCP1 of C4BP (42). This interaction is very relevant physiologically because it confers serum resistance to N. gonorrhoeae. The structural requirements for the functional activities of C4BP are of interest because C4BP is the major regulator of the classical pathway of complement, and it may have therapeutic potential. It has been shown recently that inhibition of the complement system in animal models of rheumatoid arthritis and reperfusion injury has considerably improved survival of the test animals (43, 44).

Acknowledgments—We thank Astra Andersson for excellent technical support. We are also grateful to Dr. Sara Linse, Lund Institute of Technology, for assistance with circular dichroism analysis.

REFERENCES

1. Gigli, I., Fujita, T., and Nussenzevug, W. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 6598–6600
2. Scharfstein, J., Ferreira, A., Gigli, I., and Nussenzevug, W. (1978) J. Exp. Med. 148, 207–222
3. Hourcade, D., Lieszewski, M. K., Krych-Goldberg, M., and Atkinson, J. P. (2000) Immunopharmacology 49, 103–116
4. Norman, D. G., Barlow, P. N., Baron, M., Day, A. J., Sim, R. B., and Campbell, I. D. (1991) J. Mol. Biol. 219, 717–725
5. Hillarp, A., and Dahlback, B. (1988) J. Biol. Chem. 263, 12759–12764
6. Dahlback, B., Smith, C. A., and Muller Eberhard, H. J. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 3461–3465
7. Blom, A. M., Webb, J., Villoutreix, B. O., and Dahlback, B. (1999) J. Biol. Chem. 274, 19257–19265
8. Blom, A. M., Folyun-Zadura, A., Villoutreix, B. O., and Dahlback, B. (2000) Mol. Immunol. 37, 445–453
9. Berggard, K., Johnson, E., Mosi, F. R., and Lindahl, G. (1997) Infect. Immun. 65, 3638–3643
10. Blom, A. M., Berggard, K., Webb, J. H., Lindahl, G., Villoutreix, B. O., and Dahlback, B. (2000) J. Immunol. 164, 5328–5336
11. Dahlback, B. (1981) Biochem. J. 195, 279–286
12. Gigli, I., Porter, R. R., and Sim, R. B. (1976) Biochem. J. 157, 541–548
13. Dahlback, B., and Hultbrand, B. (1983) Biochem. J. 209, 857–863
14. Andersson, M., Hansson, A., Englund, G., and Dahlback, B. (1991) Eur. J. Clin. Pharmacol. 40, 261–265
15. Crossley, L., and Porter, R. (1986) Biochem. J. 193, 175–182
16. Ziccardi, R. J., Dahlback, B., and Muller Eberhard, H. J. (1984) J. Biol. Chem. 259, 13674–13679
17. Villoutreix, B. O., Haidar, Y., Wallqvist, A., Covell, D. G., Frutos, P. G. d., and Dahlback, B. (1998) Proteins Struct. Funct. Genet. 31, 391–405
18. Nagasawa, S., Mizuguchi, K., Ichihara, C., and Koyama, J. (1992) J. Biol. Chem. 92, 1329–1332
19. Fujita, T., Kamato, T., and Tamura, N. (1985) J. Immunol. 134, 3320–3324
20. Dahlback, B., and Muller Eberhard, H. J. (1984) J. Biol. Chem. 259, 11631–11634
21. Haidar, Y., Hillarp, A., and Dahlback, B. (1997) Biochem. J. 323, 469–475
22. Accardo, P., Sanchez Corral, P., Criado, O., Garcia, E., and Rodriguez de Cordoba, S. (1996) J. Immunol. 157, 4935–4940
23. Ogata, R. T., Mathias, P., Bradt, B. M., and Cooper, N. R. (1993) J. Immunol. 150, 2273–2280
24. Sahlu, A., and Pound, F. M. (1993) Mol. Immunol. 30, 679–684
25. Chung, L. P., and Reid, K. B. (1985) Nature 317, 855–860
26. Hessing, M., Kanters, D., Takeya, H., van’t Veer, C., Hackeng, T. M., Iwanaga, S., and Bouma, B. N. (1995) FEBS Lett. 377, 229–232
27. Kristen, T., Ogata, R. T., Chung, L. P., Reid, K. B., and Tack, B. F. (1987) Biochemistry 26, 4668–4674
28. Mikata, S., Miyagawa, S., Fukui, A., Murakami, Y., Shirakura, R., Iwamata, H., Hatanaka, M., Matsumoto, M., Saya, T., Suzuki, K., and Nagasawa, S. (1999) Mol. Immunol. 35, 537–544
29. Broddeick, G. W., Liu, D., Sperry, J., Mold, C., and Medof, M. E. (1996) J. Immunol. 156, 2528–2533
Complement Regulation by C4BP

30. Adams, E. M., Brown, M. C., Nunge, M., Krych, M., and Atkinson, J. P. (1991) J. Immunol. 147, 3005–3011
31. Iwata, K., Seya, T., Yanagi, Y., Pesando, J. m., Johnson, P. M., Okabe, M., Ueda, S., Ariga, H., and Nagasawa, S. (1995) J. Biol. Chem. 270, 15148–15152
32. Alsenz, J., Lambris, J. D., Schulz, T. F., and Dierich, M. P. (1984) Biochem. J. 224, 389–398
33. Gordon, D. L., Kaufman, R. M., Blackmore, T. K., Kwong, J., and Lublin, D. M. (1995) J. Immunol. 155, 348–356
34. Kuhn, S., Skerka, C., and Zipfel, P. F. (1995) J. Immunol. 155, 5663–5670
35. Kuhn, S., and Zipfel, P. F. (1996) Eur. J. Immunol. 26, 2383–2387
36. Jokiranta, T. S., Hellwage, J., Koistinen, V., Zipfel, P. F., and Meri, S. (2000) J. Biol. Chem. 275, 27657–27662
37. Klickstein, L. B., Bartow, T. J., Mileti, V., Rabson, L. D., Smith, J. A., and Fearon, D. T. (1988) J. Exp. Med. 168, 1699–1717
38. Krych, M., Hourcade, D., and Atkinson, J. P. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 4353–4357
39. Krych, M., Clemenza, L., Howdeshell, D., Hauhart, R., Hourcade, D., and Atkinson, J. P. (1994) J. Biol. Chem. 269, 3237–32378
40. Krych, M., Hauhart, R., and Atkinson, J. P. (1998) J. Biol. Chem. 273, 8623–8629
41. Fujita, T., and Tamura, N. (1983) J. Exp. Med. 157, 1239–1251
42. Ram, S., Cullinan, M., Blom, A. M., Gulati, S., McQuillen, D. P., Monks, B. G., O’Connell, C., Boden, R., Elkins, C., Pangburn, M. K., Dahlback, B., and Rice, P. A. (2001) J. Exp. Med. 193, 281–296
43. Linton, S. M., and Morgan, B. P. (1999) Mol. Immunol. 36, 905–914
44. Goodfellow, R. M., Williams, A. S., Levin, J. L., Williams, B. D., and Morgan, B. P. (2000) Clin. Exp. Immunol. 119, 210–216
