The amino-terminal module of the C4b-binding protein α-chain is crucial for C4b binding and factor I-cofactor function

Ylva HÄRDIG, Andreas HILLARP and Björn DAHLBÄCK*

Department of Clinical Chemistry, Lund University, University Hospital, Malmö, S-205 02 Malmö, Sweden

C4b-binding protein (C4BP) regulates the classical pathway C3-convertase of the complement system. Human C4BP is composed of seven identical subunits (α-chains) and one unique one (β-chain). Both types of chains contain homologous repeats called complement control proteins (CCPs); the α-chain contains eight CCPs and the β-chain three. Each α-chain contains a binding site for C4b although the detailed localization of this binding site is not known. We have used three different chimeric proteins, originally designed to localize the protein S-binding site on C4BP, to demonstrate the importance of the amino-terminal part of the α-chain for the complement-regulatory functions of C4BP. These recombinant proteins were composed of C4BP α-chains with one, two or three of the amino-terminal CCPs replaced by corresponding CCPs from the C4BP β-chain. Furthermore, seven different monoclonal antibodies were raised against C4BP and characterized using the recombinant chimeric proteins. Whereas all three recombinant chimeras bind protein S with the same affinity as plasma-purified C4BP, none of them bound to C4b. Three of the antibodies, which were found to bind to α-chain CCP 1 and CCP 2, completely inhibited the binding of plasma-purified C4BP to immobilized C4b. In addition, two of these antibodies totally blocked the factor I-cofactor activity of C4BP in a C4b-degradation assay. The binding site for one of the monoclonal antibodies was also studied using electron microscopy where it was confirmed that this antibody bound to the amino-terminal tip of the α-chain. These results show that the amino-terminal CCP of the C4BP α-chain (CCP 1) is crucial for the C4b binding and factor I-cofactor activity.

INTRODUCTION

Human C4b-binding protein (C4BP) is composed of two types of subunits, the α-chain (70 kDa) and the β-chain (45 kDa) [1–3]. Both types of chains contain internally homologous repeats called complement control proteins (CCPs), short consensus repeats or Sushi domains [4,5]. The α-chain contains eight CCPs and the β-chain three. The predominant form of C4BP in human plasma is composed of seven α-chains and one β-chain disulphide, linked to each other through cysteines in their non-repeat carboxy-terminal parts into a spider-like structure with extended tentacles (Figure 1) [6,7]. C4BP contains binding sites for the complement component C4b, the anti-coagulant protein S and the pentraxin serum amyloid P component (SAP) [1,8,9]. It has been shown that all four proteins, C4BP, C4b, protein S and SAP, can form a complex and associate with membranes, probably through the membrane-binding capacity of protein S [10]. More recently, however, SAP was demonstrated to interfere with the binding of C4BP to immobilized C4b (but not to fluid-phase C4b) and with the factor I-cofactor function of C4BP [11]. Each C4BP molecule contains six or seven C4b-binding sites, one on each α-chain, whereas the stoichiometry of its interaction with protein S and with SAP is 1:1 [2,12–14]. Protein S binds to the C4BP β-chain, whereas the binding site for SAP has been localized to the 160 kDa central core fragment of C4BP obtained after digestion by chymotrypsin. This fragment is composed of disulphide-linked parts of the α-chains, each one containing CCP 8 and the non-repeat carboxy-terminal region. C4BP regulates the complement system both by interfering with the formation and decay of the classical C3-convertase, C4b2α, and through its role as a cofactor for factor I in the proteolytic degradation of C4b [15,16]. Several studies concerning the localizations of regions important for the C4b binding and factor I-cofactor activity of human C4BP have been reported but the results are somewhat contradictory. Using electron microscopy, C4b was demonstrated to bind to the amino-terminal part of the α-chain, but a binding site involving CCP 6 and CCP 7 has also been suggested [7,17]. The factor I-cofactor activity site has similarly been assigned to residues 177–332 of the α-chain (involving CCP 3 to CCP 6) [17].

We have used three recombinant C4BP chimaeras, originally designed to localize the protein S-binding site on the C4BP β-chain, and seven different monoclonal antibodies (mAb) against C4BP, to study the interaction between C4BP and C4(H_2O) [C4(H_2O) was used instead of C4b in all binding assays performed]. The results obtained clearly demonstrate that the amino-terminal CCP of the C4BP α-chain is involved in the C4BP–C4b interaction.

MATERIALS AND METHODS

Proteins

C4BP [18], C4 [19] and factor I [20] were purified from human plasma as previously described. C4(H_2O), which was used instead of C4b in the ligand-binding studies and in the factor I-mediated degradation assays, was prepared by repeated freezing and thawing of purified C4 [21,22]. Concentrations of purified proteins were estimated by measuring absorbance at 280 nm. The following molar absorption coefficients (1%, 1 cm) were used: intact C4BP, 14.1 [23]; C4, 8.3 [24] and factor I, 14.3 [20].

Abbreviations used: C4BP, C4b-binding protein; CCP, complement control protein; SAP, serum amyloid P component; mAb, monoclonal antibody(ies).

* To whom correspondence should be addressed.

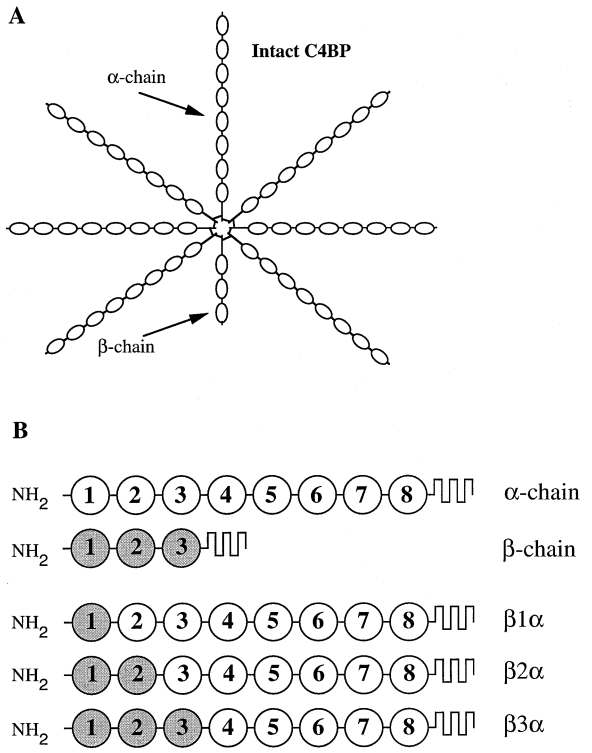

Figure 1 Schematic drawing of C4BP and the recombinant α/β-chains

(**A**) Intact C4BP containing seven α-chains and one β-chain. (**B**) Schematic representation of intact α-chain, intact β-chain and the three recombinant chains. Each circle represents a CCP module. White circles correspond to the α-chain and shaded circles to the β-chain.

C4BP and C4(H2O) were radiolabelled with ^{125}I (Amersham) using Iodo-beads™ (Pierce Chemical Co.) and purified as described previously [25].

Expression of recombinant C4BP α/β-chimaeras

The α/β-chimaeras were constructed, expressed and characterized as described [26]. They were composed of α-chains with one, two or three of the amino-terminal CCPs replaced by corresponding CCPs from the β-chain (Figure 1). The recombinant proteins, called $\beta1\alpha$, $\beta2\alpha$ and $\beta3\alpha$ respectively, were all retained within the cells. The cells were lysed with a single-detergent buffer containing 50 mM Tris/HCl (pH 8.0), 0.15 M NaCl, 1% (v/v) Nonidet P40, 0.02% (v/v) NaN_3, phenylmethylsulphonyl fluoride (100 μg/ml) and leupeptin (2 μg/ml), and the cell lysates were used in all binding assays performed.

Monoclonal antibodies

Balb/c mice were immunized with 10 μg of purified human C4BP in Freund's complete adjuvant and after 1 and 3 weeks the mice were boosted with the same amount of antigen. Spleen cells (10^8 cells) from the immunized mice were fused with myeloma cells (10^8 cells) from the cell-line Sp 2/0-Ag 14. Fusion and hybridoma selection were performed according to standard procedures. Six high-titre master clones were detected using an ELISA with C4BP-coated microtitre plates. The master clones were subcloned three times by limiting dilution (1.0, 0.5 and 0.05 cells/well) in the presence of mouse peritoneal macrophages. The positive clones were injected intraperitoneally into pristine-treated Balb/c mice. Ascites fluid was collected from 12–15 mice per master clone 9–11 days after injection and the antibodies precipitated with ammonium sulphate. After centrifugation, the precipitates were dissolved and dialysed against 20 mM Tris/HCl/20 mM NaCl, pH 7.8, and then chromatographed through a column (1.5 cm × 16 cm) packed with DEAE–Sephacel (Pharmacia, Uppsala, Sweden) equilibrated in the same buffer. After the column had been washed with equilibration buffer, the adsorbed proteins were eluted with a linear salt gradient (0.02–0.5 M NaCl). Fractions were monitored by agarose-gel electrophoresis and by ELISA. The six different mAb are referred to as mAb 67, 70, 92, 96, 102 and 104. In addition, a mAb against the C4BP β-chain was used. This mAb, called mAb 36, has previously been shown not to interfere with protein S binding to C4BP although its binding epitope has not been characterized in detail [27].

Characterization of the mAb

Determination of isotypes

The isotypes of the purified mAb were determined by double-antibody sandwich ELISA using isotype-specific rabbit anti-mouse antisera in conjunction with peroxidase-conjugated goat anti-rabbit IgG.

Competition between the mAb for binding to C4BP

Plasma-purified C4BP was coupled to immunobeads (Bio-Rad) at pH 7.8 as recommended by the manufacturer (35 mg of protein/g of beads). The immunobeads were kept at 4 °C in 0.1% NaN_3 until use. Before assay, the beads were preincubated for 30 min at room temperature with 1% (w/v) BSA in assay buffer (10 mM potassium phosphate/0.15 M NaCl, pH 7.2) to saturate non-specific binding sites. The beads were sedimented by centrifugation and resuspended in assay buffer to a final concentration of 1 mg/ml. For each sample, 100 μl of beads (0.1 mg) and approx. 2.5 nM radiolabelled mAb were used (final volume 200 μl). Increasing concentrations of unlabelled mAb were added and the samples were incubated for 90 min at room temperature. Before separation of bound and free tracer, the beads were diluted with assay buffer (final volume 1.2 ml). The beads were sedimented by centrifugation and the supernatant discarded. The amount of radioactivity associated with the pellet was measured in a γ-spectrometer (NE 1600, Nuclear Electronics).

Epitope mapping using the chimaeric proteins

The mAb were tested for binding to the recombinant proteins as described earlier [26]. Briefly, microtitre plates were coated with the various mAb, plasma-purified C4BP or the recombinant proteins (1 μg/ml) were added and the amount of bound protein was detected using biotinylated mAb 67, streptavidin-conjugated horseradish peroxidase and 1,2-phenylenediamine.

Epitope mapping using electron microscopy

C4BP was incubated with mAb 104 (molar ratio 1:3) in 0.1 M NH_4OAc/0.05 M NH_4HCO_3, pH 7.4, at 37 °C for 1 h and the proteins were then diluted to approx. 10 μg/ml. Within 5 min of dilution the proteins were adsorbed to carbon film as described [28] and then were negatively stained with 1.5% (v/v) uranyl formate by the pleated-sheet technique [29,30]. To stabilize their structure, the proteins were also incubated with 0.25% (v/v) glutaraldehyde for 15 min just before adsorption to the carbon films, a procedure described by Valentine et al. [28]. The photographs were taken at a primary magnification of 59000 in

a JEOL 200 CX transmission electron microscope, operating at 80 kV with a 60-mm objective aperture.

C4(H_2O)-binding assays

Recombinant proteins

In the direct binding assay, microtitre wells were coated with C4(H_2O) (50 μl, 10 μg/ml) in 75 mM sodium carbonate buffer (pH 9.6), at 4 °C overnight. The next day, the wells were washed three times in 50 mM Tris/HCl (pH 7.5)/0.15 M NaCl (TBS) containing 0.1 % (v/v) Tween 20. The plates were then quenched in 1 % BSA for 30 min at room temperature. After washing, increasing concentrations (0.0001–7 nM) of plasma-purified C4BP (in the same lysis buffer as the recombinant proteins) or the recombinant proteins were added and the plates were incubated for 2 h at room temperature. After incubation, the plates were washed, incubated with biotinylated mAb 67, washed again, and then developed using streptavidin, biotinylated horseradish peroxidase, and 1,2-phenylenediamine (Dakopatts AB) according to the manufacturer's instructions. The competition assay was performed essentially as described [11]. Briefly, microtitre plates were coated with C4(H_2O) (50 μl, 10 μg/ml) whereafter plasma-purified C4BP (0.02–320 nM) or the recombinant α/β-chain chimaeras (0.0035–35 nM) were added together with a trace amount of ^{125}I-labelled C4BP (final volume, 50 μl). After incubation and washing, the amount of bound C4BP was measured in a γ-counter.

Monoclonal antibodies

Microtitre wells were coated with C4(H_2O) (50 μl, 0.5 mg/ml) in 50 mM Tris/HCl/5 mM EDTA/0.15 M NaCl, pH 7.5 (coating buffer) for 2 h at 37 °C. The wells were then washed in coating buffer containing 0.25 % BSA and 100 μl of the BSA solution was added to each well and incubated for 30 min at 37 °C. The wells were emptied and increasing concentrations of the various mAb (0.03–300 nM), containing a trace amount of ^{125}I-labelled C4BP (approx. 2.8 nM), were added and left for 2 h at 37 °C. The total volume was 35 μl/well. After a final washing procedure the wells were dried and the amount of bound C4BP was measured in a γ-counter.

C4(H_2O)-degradation assay

The degradation assay was performed essentially as described previously [31]. C4BP (250 nM) and mAb (1750 nM) were incubated in TBS, pH 7.5, for 1 h at room temperature. C4(H_2O) (250 nM), factor I (60 nM) and a trace amount of ^{125}I-labelled C4(H_2O) were added to a final reaction volume of 50 μl and each sample was further incubated for 1 h at 37 °C. The reactions were terminated by the addition of sample-preparation buffer containing SDS and 0.1 mM dithiothreitol. The samples were boiled for 2 min, alkylated with 1 mM iodoacetamide, and subjected to 10 % PAGE in the presence of SDS. The gels were dried and analysed using a PhosphorImager SI (Molecular Dynamics).

RESULTS

Binding of the recombinant proteins to C4(H_2O)

The construction and characterization of the recombinant C4BP α/β-chain chimaeras called $\beta1\alpha$, $\beta2\alpha$ and $\beta3\alpha$ have been described previously [26]. Since the recombinant proteins were retained within the cells, the cells were lysed and the cell lysates were used in all assays performed. The concentrations of recombinant proteins were determined using an ELISA. All three chimaeras were polymerized into high-molecular-mass proteins composed of seven and eight chains as judged from SDS/polyacrylamide gels [26]. To find out whether the replacement of CCP 1, CCP 1 + CCP 2 or CCP 1 + CCP 2 + CCP 3 of the C4BP α-chain with their β-chain counterparts had any effect on the C4(H_2O) binding, both a direct binding assay and a competition assay were performed (Figure 2). In the direct binding assay, plasma-purified C4BP in lysis buffer or cell lysates containing the various chimaeras were added to immobilized C4(H_2O) and the amount of bound protein was detected using biotinylated mAb 67. In contrast with plasma-purified C4BP, none of the recombinant proteins was able to bind to C4(H_2O). The same result was obtained when the ionic strength was decreased (25 mM Tris/HCl/75 mM NaCl) (results not shown). In the competition assay, plasma-purified C4BP or the α/β-chain recombinants were allowed to compete with a ^{125}I-labelled C4BP tracer for binding to immobilized C4(H_2O). While plasma-purified C4BP in fluid phase was able to displace all binding of ^{125}I-labelled C4BP tracer to the immobilized C4b, none of the recombinant proteins had any effect on the binding.

Characterization of the mAb

All mAb against C4BP were IgG antibodies; mAb 67, 96 and 102 were of IgG 1 isotype while mAb 70, 92 and 104 were of IgG 2α isotype. The isotype of mAb 36 was not determined.

To find out whether the various mAb were able to stimulate or inhibit each other's binding, a series of competitive immunoradiometric assays were performed (Table 1). mAb 67 did not influence the binding of any of the other mAb. Only one pair of mAb could displace each other: unlabelled mAb 70 was able to displace 70 % of the binding of C4BP to radiolabelled mAb 96, whereas mAb 96 only showed a weak displacement (30 %) of radiolabelled mAb 70. Three pairs of mAb, mAb 70–mAb 104, mAb 92–mAb 102 and mAb 70–mAb 92, showed a 'co-operative' action. The tracer binding could be increased by up to 60 % by adding a co-operative antibody. According to a Scatchard analysis (mAb 70 and mAb 104 studied), the number of binding sites for mAb 70 was found to remain constant, whereas the affinity of the mAb 70 tracer for C4BP increased from a K_A of 1×10^8 M to a value of 2.5×10^8 M (not shown).

The recombinant α/β-chain chimeras were used to determine the epitopes for the mAb in an ELISA system (Table 2). mAb 67 recognized all the constructs equally well, whereas mAb 96 was able to bind to one of the constructs, $\beta1\alpha$, and mAb 36 only bound to the recombinant protein containing β-chain CCP 3, $\beta3\alpha$. The other four antibodies did not recognize any of the recombinant proteins. We therefore concluded that mAb 36 bound to CCP 3 of the β-chain, mAb 96 to α-chain CCP 2 and the epitopes for mAb 70, 92, 102 and 104 included α-chain CCP 1. Since mAb 67 does not recognize the central core region of C4BP (A. Hillarp and B. Dahlbäck, unpublished work), its binding epitope must be located in the middle of the α-chain (CCP 4–CCP 7).

The binding of one of the mAb, mAb 104, to plasma-purified C4BP was also studied using electron microscopy (Figure 3). C4BP was visualized as a spider-like molecule with highly flexible, elongated tentacles, each tentacle representing an α-chain. The length of the α-chain was found to be approx. 300 Å, which is in agreement with earlier reported data [7]. mAb 104 can be seen as a V-shaped molecule bound to the amino-terminal tip of two or more of the α-chains on each C4BP molecule shown. The distance between the centre of the C4BP molecule and the beginning of the mAb suggests a binding site in CCP 1 of the α-chain.

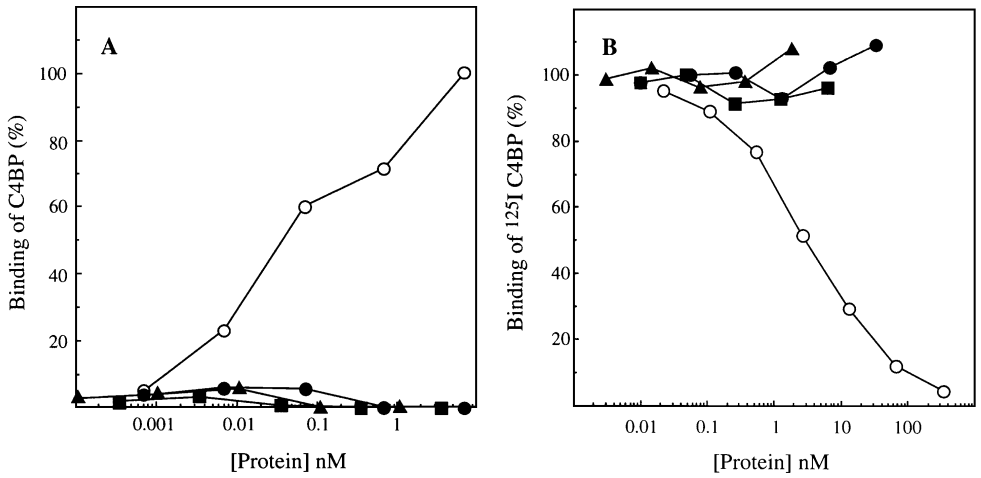

Figure 2 C4(H_2O) binding to $\beta1\alpha$, $\beta2\alpha$, and $\beta3\alpha$

(**A**) Results of the direct binding assay performed as described in the Materials and methods section. 100% corresponds to the maximum A_{490} value obtained. (**B**) Competition assay. Increasing concentrations of C4BP or recombinant proteins in fluid phase were allowed to compete with ^{125}I-labelled C4BP for binding to immobilized C4(H_2O). 100% binding was estimated in the absence of fluid-phase competitor. Results of three different binding experiments. (○) plasma-purified C4BP; (●) $\beta1\alpha$; (■) $\beta2\alpha$; (▲) $\beta3\alpha$.

Table 1 Competition between the various monoclonal antibodies for binding to C4BP

Unlabelled mAb were allowed to compete with ^{125}I-labelled mAb for binding to immobilized C4BP. The table shows the results from experiments using 2.5 nM radiolabelled mAb and 300 nM unlabelled mAb. The values are expressed as the percentage of tracer binding when no competitor was added.

	Unlabelled mAb					
^{125}I-labelled mAb	67	70	92	96	102	104
67	15	100	100	100	100	100
70	95	20	120	70	105	130
92	90	160	50	100	160	105
96	70	30	100	10	105	120
102	95	110	155	100	10	100
104	100	110	100	100	100	5

Table 2 Epitope mapping of the monoclonal antibodies using chimaeric proteins

Immobilized mAb were tested for their ability to bind to the various recombinant proteins. Values represent absorbance at 490 nm (mean from three different experiments).

	Protein chimeras			Control
	$\beta1\alpha$	$\beta2\alpha$	$\beta3\alpha$	Plasma C4BP
mAb 36	0.02	0.02	**3.08**	1.16
mAb 67	**2.87**	**2.95**	**3.01**	2.96
mAb 70	0.02	0.01	0.02	3.49
mAb 92	0.04	0.01	0.01	2.29
mAb 96	**3.57**	0.01	0.03	3.82
mAb 102	0.02	0.01	0.02	3.92
mAb 104	0.01	0.00	0.01	3.37

Inhibition of C4BP-C4(H_2O) binding by the mAb

The mAb were tested for inhibitory capacity in a C4BP–C4(H_2O)-binding assay (Figure 4). The final concentration of radiolabelled C4BP was approx. 2.8 nM. Since each C4BP molecule contains six to seven C4b-binding sites, the concentration of mAb must be 6–7 times higher to saturate the binding sites. Addition of 20 nM mAb 96, mAb 102 and mAb 104 resulted in over 80% inhibition of the binding of C4BP to the immobilized C4(H_2O), whereas the other three mAb had a weaker effect. Furthermore, mAb 96, 102 and 104 were all able to almost completely inhibit C4(H_2O) binding. mAb 36, which was directed against the C4BP β-chain, did not interfere with the C4BP–C4(H_2O) interaction.

C4(H_2O)-degradation assay

The α-chain of C4(H_2O) is cleaved by factor I, together with C4BP, resulting in three fragments: $\alpha2$ (C4d) (45 kDa), $\alpha3$ (25 kDa) and $\alpha4$ (13 kDa) [21,32]. To find out whether any of the mAb were able to inhibit the factor I-cofactor function of C4BP, they were preincubated with C4BP before the addition of C4(H_2O) [containing a trace amount of ^{125}I-labelled C4(H_2O)] and factor I as described in the Materials and methods section. To saturate the binding sites on the C4BP molecule, we used an antibody/C4BP ratio of 7:1. After 1 h, the reaction was terminated, the samples run on SDS/PAGE and analysed using a phosphorimager (Figure 5). The degradation pattern showed that mAb 96 and mAb 104 totally blocked the degradation of C4(H_2O), whereas mAb 102 interfered with the formation of the C4d fragment as shown by the appearance of two bands instead of one. None of the other antibodies seemed to have any effect on the degradation.

DISCUSSION

Each α-chain contains a binding site for C4b. Under physiological conditions, however, only four of these binding sites can be simultaneously occupied, whereas binding of additional C4b

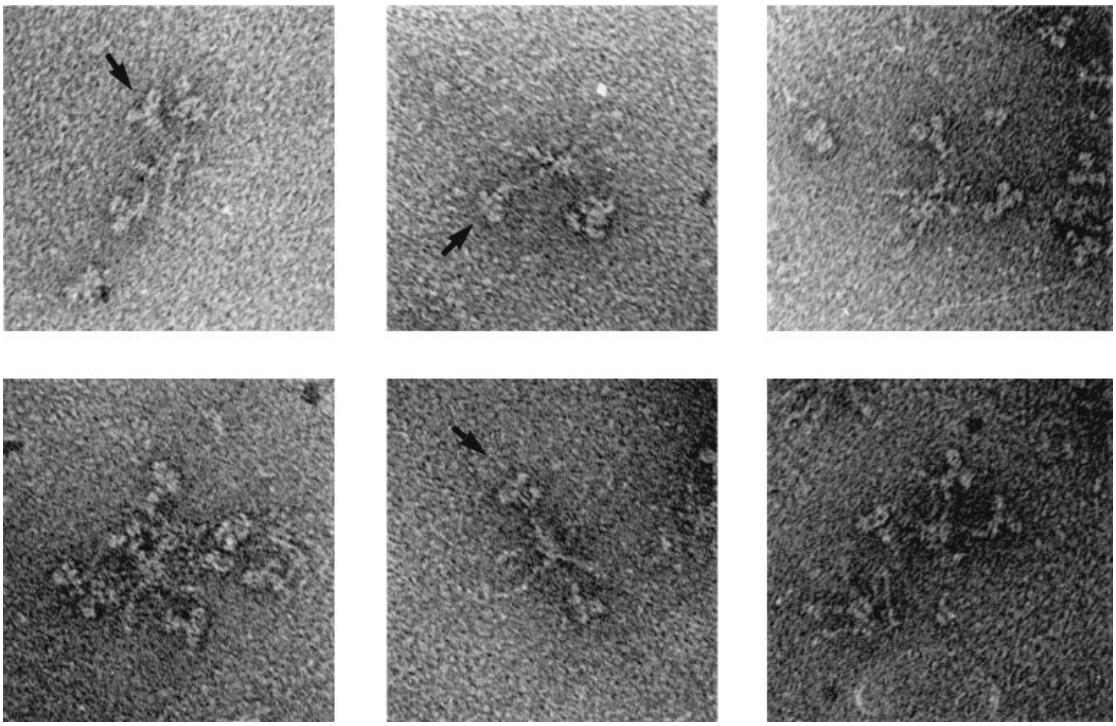

Figure 3 Electron microscopy images of C4BP and mAb 104

Selected images demonstrating the C4BP–mAb 104 complexes. The mAb can be seen as a V-shaped molecule at the peripheral tip of each C4BP α-chain. The arrows indicate some of the antibodies.

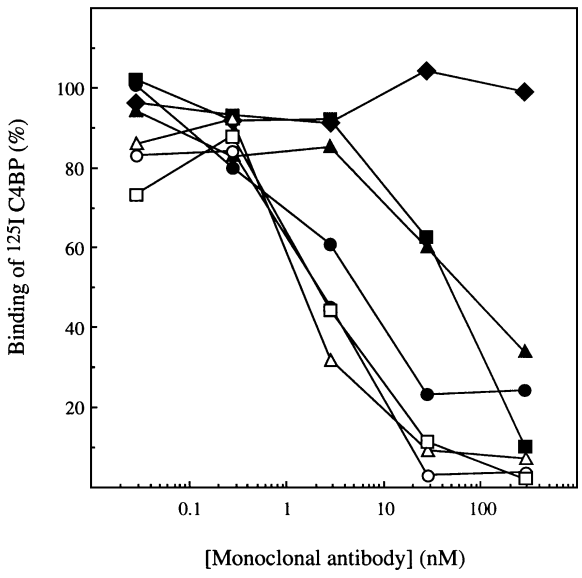

Figure 4 Competition between the mAb and plasma-purified C4BP for C4(H_2O) binding

Increasing concentrations of the various mAb were allowed to compete with ^{125}I-labelled C4BP for binding to immobilized C4(H_2O). 100% binding was estimated in the absence of fluid-phase competitor. Results of three different experiments using mAb 36 (◆), mAb 67 (▲), mAb 70 (●), mAb 92 (■), mAb 96 (□), mAb 102 (△) and mAb 104 (○).

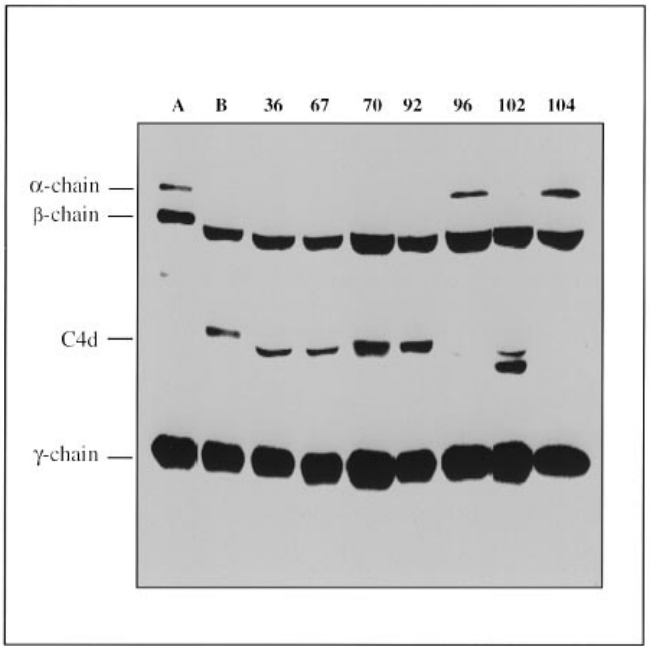

Figure 5 C4(H_2O)-degradation assay

C4BP was preincubated with the various mAb. Thereafter factor I, C4(H_2O) and a trace amount of ^{125}I-labelled C4(H_2O) were added. Factor I-mediated cleavage of C4(H_2O) in the presence of C4BP results in fragmentation of the α-chain into three new fragments. Only the largest fragment, C4d, can be seen on this autoradiography. In the controls, either C4BP, factor I and mAb (lane A), or only mAb (lane B), were omitted.

molecules is probably sterically hindered [33]. We have shown earlier that neither the β-chain nor the N-linked carbohydrates are involved in the C4b binding and factor I-cofactor function of C4BP [34]. Several regions of C4BP have been suggested to be important for its complement-regulatory functions. Limited proteolysis of C4BP by chymotrypsin results in cleavage of the α-chain at Tyr 395 and Trp 425, giving two major fragments, one 48 kDa fragment constituting the major part of the α-chain, and one 160 kDa fragment composed of multiple disulphide-linked 25 kDa peptides, each representing the carboxy-terminal part of the α-chains [12]. The amino-terminal 48 kDa fragment retained the factor I-cofactor activity, whereas the C4b-binding capacity seemed to be lost [12,35]. In a competition assay, however, the 48 kDa fragment obtained after digestion with chymotrypsin has been shown to be able to completely inhibit binding of C4BP to immobilized C4b [36]. Chung and Reid [17] have suggested amino acids 332–395 (involving parts of CCPs 6 and 7) to be important in C4b binding and residues 177–322 (CCPs 4 and 5 plus parts of CCPs 3 and 6) to be involved in factor I-cofactor activity. Furthermore, an mAb that inhibited the binding of C4b to C4BP and whose epitope was localized to residues 333–356 has been reported [37]. Contrary to all this, visualization of C4BP together with C4b by electron microscopy suggested that C4b binds close to the amino-terminal part of the α-chain [7]. Murine C4BP α-chain contains only six CCPs and, on the basis of sequence similarities, it is the region corresponding to CCPs 5 and 6 that is missing [38]. Since C4BP, from both mouse and human origin bind to human C4b, a binding site in this region can be questioned [39–41]. Moreover, a chimaeric construct containing the three amino-terminal CCPs of murine C4BP α-chain, has been demonstrated to bind to human C4b in a specific and high-affinity way [41]. So far, the sequences of five different mammalian C4BP α-chains have been reported: rat [42], human [4], mouse [38], rabbit [43] and bovine [44]. Whereas sequence comparisons showed that most of the amino acid identities were scattered throughout the sequences, a few regions manifesting more than 70% identity were identified: residues 27–37 in CCP 1, 84–101 in CCP 2, 434–444 at the junction between CCPs 7 and 8 and finally residues 521–533 in the carboxy-terminal non-repeat region [42]. In pairwise comparisons, CCP 2, CCP 3 and CCP 8 (CCP 6 in mouse) manifested the greatest identity. These inter-species similarities suggest that the most amino-terminal and the most carboxy-terminal parts of the α-chain are physiologically important. While it seems plausible that the amino-terminal CCPs are involved in the C4b interaction, CCP 8 and the carboxy-terminal region are likely to play a role in binding to SAP and in the polymerization of the chains. SAP has been shown to bind to the central core region of C4BP [13]. An explanation for the finding that SAP inhibits binding of C4BP to immobilized C4b can be that binding of SAP leads to a change in the overall molecular structure of the C4BP molecule.

In the present work, we have used three recombinant α/β-chain chimaeras both as potential inhibitors in a C4BP–C4(H_2O) binding assay and as tools in the localization of the binding epitopes for seven different mAb against C4BP. All three recombinant proteins have earlier been shown to bind protein S with equally high affinity to that of plasma-purified C4BP, indicating that the recombinant proteins were correctly folded and functionally active. None of the recombinant chimeras were able to bind to immobilized C4(H_2O). Even though the low concentrations of recombinant proteins obtained from the cell lysates did not allow the addition of optimal amounts of chimaeras in the competition assay, the results were quite clear. The addition of 30 nM plasma-purified C4BP led to over 80% inhibition of binding of the tracer to immobilized C4b, whereas the same concentration of the $\beta 1\alpha$ construct did not have any effect on the binding. Five of the mAb described recognized the amino-terminal part of the C4BP α-chain, whereas mAb 67 probably binds to the middle part of the α-chain. mAb 96, 102 and 104 were effective inhibitors of C4BP binding to immobilized C4(H_2O). mAb 102 and mAb 104 did not recognize any of the recombinant proteins, whereas mAb 96 only recognized $\beta 1\alpha$. This suggests that mAb 96 binds CCP 2, whereas the binding sites for the others involve CCP 1. In a factor I-cofactor assay, mAb 96 and 104 completely inhibited the degradation of C4(H_2O), whereas mAb 102 interfered with the formation of the C4d fragment. Even though, as described previously, mAb 102 completely inhibited the binding of C4BP to immobilized C4(H_2O), it did not abolish binding and cleavage of C4(H_2O) in the fluid phase. The appearance of an additional, smaller sized, C4d fragment when mAb 102 was bound to C4BP is interesting though difficult to understand. It is possible that when this antibody is bound to C4BP, C4(H_2O) is presented to factor I in a different way with other parts exposed, leading to a new cleavage pattern. mAb 67, which binds to α-chain CCP 4 –CCP 7, was also able to partially inhibit the binding of C4BP to immobilized C4(H_2O), although not as efficiently as mAb 96, 102 and 104. C4BP is, however, a structurally complex molecule with six or seven extended α-chains and it is possible that saturation of the molecule by mAb binding to the middle part of the α-chain interferes with the binding of C4(H_2O) even though the antibodies do not directly compete for the same binding site as C4(H_2O). This may also be an explanation for the inhibitory capacity of the mAb (epitope in CCP 6) described by Hessing and co-workers [37]. We have focused our interest on the strong inhibitory effects of mAb 96, 102 and 104 and the results presented here demonstrate the importance of α-chain CCP 1 for the C4b binding and factor I-cofactor function of C4BP. The role of CCP 2 is more uncertain. mAb 96, which was found to bind to CCP 2, completely inhibited both the binding of C4(H_2O) and the factor I-mediated C4(H_2O) degradation, but it cannot be ruled out that this was a steric effect rather than a direct competition for the same binding site. In several other complement regulatory proteins (complement receptor 1, membrane cofactor protein and decay accelerating factor), however, two contiguous CCP modules have been shown to be involved in the binding to C4b, favouring the hypothesis that both CCP 1 and CCP 2 of the C4BP α-chain are involved in C4b binding [45–47]. In addition to the C4b-binding site, it is possible that there are regions important for the factor I-cofactor activity. Further studies are under way that will clarify whether the C4b-binding site is fully contained in CCP 1 or if other parts of the α-chain are involved in the C4b binding and factor I-cofactor function.

This work was supported by the Swedish Medical Council (grants 07143 and 11195) and by grants from the Alfred Österlund Trust, the Albert Påhlsson Trust, the Johan and Greta Kock Trust, the King Gustav V and Queen Victoria Trust, the King Gustav V 80th Birthday Trust, the Göran Gustafsson Trust, the Ax:son Johnson's Trust, the Magnus Bergvall Trust, the Crafoord Trust, research funds from the University Hospital, Malmö, and the Louis Jeantet Fondation. We thank Astra Andersson, Bergisa Hildebrand and Ulla Persson for expert technical assistance.

REFERENCES

1 Scharfstein, J., Ferreira, A., Gigli, I. and Nussenzweig, V. (1978) J. Exp. Med. **148**, 207–222
2 Hillarp, A. and Dahlbäck, B. (1988) J. Biol. Chem. **263**, 12759–12764
3 Dahlbäck, B. (1991) Thromb. Haemostasis **1**, 49–61
4 Chung, L. P., Bentley, D. R. and Reid, K. B. M. (1985) Biochem. J. **230**, 133–141
5 Hillarp, A. and Dahlbäck, B. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 1183–1187
6 Hillarp, A., Hessing, M. and Dahlbäck, B. (1989) FEBS Lett. **259**, 53–56

7 Dahlbäck, B., Smith, C. A. and Müller-Eberhard, H. J. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 3461–3465
8 Dahlbäck, B. and Stenflo, J. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 2512–2516
9 de Beer, F. C., Baltz, M. L., Holford, S., Feinstein, A. and Pepys, M. B. (1981) J. Exp. Med. **154**, 1134–1149
10 Schwalbe, R. A., Dahlbäck, B. and Nelsesuen, G. L. (1990) J. Biol. Chem. **265**, 21749–21757
11 García de Frutos, P. and Dahlbäck, B. (1994) J. Immunol. **152**, 2430–2437
12 Nagasawa, S., Mizuguchi, K., Ichichara, C. and Koyama, J. (1982) J. Biochem. **92**, 1329–1332
13 García de Frutos, P., Härdig, Y. and Dahlbäck, B. (1995) J. Biol. Chem. **270**, 26950–26955
14 Härdig, Y., Rezaie, A. and Dahlbäck, B. (1993) J. Biol. Chem. **268**, 3033–3036
15 Gigli, I., Fujita, T. and Nussenzweig, V. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 6596–6600
16 Fujita, T., Gigli, I. and Nussenzweig, V. (1978) J. Exp. Med. **148**, 1044–1051
17 Chung, L. P. and Reid, K. B. M. (1985) Biosci. Rep. **5**, 855–865
18 Dahlbäck, B. and Müller-Eberhard, H. J. (1984) J. Biol. Chem. **259**, 11631–11634
19 Andersson, M., Hansson, A., Englund, G. and Dahlbäck, B. (1991) Eur. J. Clin. Pharmacol. **40**, 261–265
20 Crossley, L. G. and Porter, R. R. (1980) Biochem. J. **191**, 173–182
21 von Zabern, I., Bloom, E. L., Chu, V. and Gigli, I. (1982) J. Immunol. **128**, 1433–1438
22 von Zabern, I. and Gigli, I. (1982) J. Immunol. **128**, 1439–1442
23 Perkins, S. J., Chung, L. P. and Reid, K. B.M (1986) Biochem. J. **233**, 799–807
24 Nagasawa, S. and Stroud, R. M. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 2998–3001
25 Hillarp, A. and Dahlbäck, B. (1987) J. Biol. Chem. **262**, 11300–11307
26 Härdig, Y. and Dahlbäck, B. (1996) J. Biol. Chem. **271**, 20861–20867
27 García de Frutos, P., Mohammed Alim, R., Härdig, Y., Zöller, B. and Dahlbäck, B. (1994) Blood **84**, 815–822
28 Valentine, R., Shapiro, B. and Stadtman, F. (1968) Biochemistry **7**, 2143–2152
29 Seegan, G. W., Smith, C. A. and Schumaker, V. N. (1978) Proc. Natl. Acad. Sci. U.S.A. **76**, 907–911
30 Smith, C. A. (1979) Dissertation, University of California, Los Angeles, CA, U.S.A.
31 Dahlbäck, B. and Hildebrand, B. (1983) Biochem. J. **209**, 857–863
32 Nagasawa, S., Ichihara, C. and Stroud, R. M. (1980) J. Immunol. **125**, 578–582
33 Ziccardi, R. J., Dahlbäck, B. and Müller-Eberhardt, H. J. (1984) J. Biol. Chem. **259**, 13674–13679
34 Härdig, Y., García de Frutos, P. and Dahlbäck, B. (1995) Biochem. J. **308**, 795–800
35 Fujita, T., Kamato, T. and Tamura, N. (1985) J. Immunol. **134**, 3320–3324
36 Hessing, M., Vlooswijk, R. A. A., Hackeng, T. M., Kanters, D. and Bouma, B. N. (1990) J. Immunol. **144**, 204–208
37 Hessing, M., Kanters, D., Takaeya, H., van't Veer, C., Hackeng, T. M., Iwanaga, S. and Bouma, B. N. (1993) FEBS Lett. **317**, 228–232
38 Kristensen, T., Ogata, R. T., Chung, L. P., Reid, K. B. M. and Tack, B. F. (1987) Biochemistry **26**, 4668–4674
39 Kai, S., Fujita, T., Gigli, I. and Nussenzweig, V. (1980) J. Immunol. **125**, 2409–2415
40 Kaidoh, T., Natsuume-Sakai, S. and Takahashi, M. (1981) J. Immunol. **126**, 463–467
41 Ogata, R. T., Mathias, P., Bradt, B. M. and Cooper, N. R. (1993) J. Immunol. **150**, 2273–2280
42 Hillarp, A., Wiklund, H., Thern, A. and Dahlbäck, B. (1997) J. Immunol. **158**, 1315–1323
43 García de Frutos, P. and Dahlbäck, B. (1995) Biochim. Biophys. Acta **1261**, 285–289
44 Hillarp, A., Thern, A. and Dahlbäck, B. (1994) J. Immunol. **153**, 4190–4199
45 Krych, M., Clemenza, L., Howdeshell, D., Hauhart, R., Hourcade, D. and Atkinson, J. P. (1994) J. Biol. Chem. **269**, 13273–13278
46 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
47 Liszewski, K. and Atkinson, J. P. (1996) J. Immunol. **156**, 4415–4421

Received 18 October 1996/2 December 1996; accepted 4 December 1996