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The region Ser^{333}–Arg^{356} of the α-chain of human C4b-binding protein is involved in the binding of complement C4b

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Human C4b-binding protein (C4BP) functions as a cofactor to factor I in the degradation of C4b and accelerates the decay rate of the C4b2a complex. In this study we describe a monoclonal antibody directed against the α-chain of C4BP that inhibits the binding of C4b to C4BP. In order to identify the structural domain of the α-chain of C4BP that interacts with C4b, tryptic fragments of C4BP were generated. Amino acid sequence analysis of the fragments revealed that the residues Ser^{333}–Arg^{356} of the α-chain of C4BP contain the epitope of this antibody, and as a consequence, that this part of the α-chain of C4BP is likely to be involved in the interaction with C4b.

Complement regulation; C4b-binding protein; Complement C4

1. INTRODUCTION

C4b-binding protein (C4BP) primarily functions as a cofactor to factor I in the degradation of C4b, but in addition accelerates the decay rate of the C4b2a complex and interacts with the vitamin K-dependent protein S [1,2]. Recently, it has been reported that C4BP can bind to serum amyloid P, but the importance of this latter interaction remains to be determined [3]. The major form of C4BP is composed of 7 identical α-chains (70 kDa) and a single β-chain (45 kDa) that are linked by disulfides [4–6]. Each of the α-chains consists of 549 amino acids and forms a tentacle with the N-terminus located at the periphery of the tentacle, whereas the C-termini are located in a central core [5,7]. The binding sites for C4b were located on the peripheral half of each tentacle [8,9], whereas one single binding site for protein S was localized on the β-chain [10–12]. The β-chain consists of 235 amino acids and the 175 N-terminal residues can be divided into 3 short consensus repeats (SCRs) homologous to the 8 SCRs in the α-chain of C4BP [11,13–15].

Complement C4 (189 kDa) is a glycoprotein consisting of 3 disulfide-linked α (88 kDa), β (72 kDa) and γ (32 kDa) chains [16–19]. The proteolytic cleavage by C1s in the N-terminal part of the α-chain of C4 leads to the generation of the activation peptide C4a (8 kDa) and the activated C4b (181 kDa). The cleavage of C4b by factor I and C4BP in the α′-chain of C4b leads to the generation of the C4c and C4d fragments [20]. The N-terminal end of the α′-chain of C4b between Ala^{738} and Arg^{826} is involved in C4BP binding [21,22].

Limited proteolysis of C4BP by chymotrypsin results in cleavage of the α-chain, at Tyr^{395} and Trp^{425}, yielding a 48 kDa fragment, constituting the major part of the α-chain, and a 160 kDa fragment representing the central core of C4BP [5,23]. Proteolysis of C4BP with trypsin results in cleavage in the middle of each α-chain of C4BP thereby generating fragments of 36–48 kDa. The tryptic fragments remain disulphide-linked, in contrast to the chymotrypsin digestion [24]. The C4b-binding and factor I cofactor activity are located in the chymotryptic 48 kDa fragments [23] and the region for C4b binding was tentatively assigned to residues 332–395 of the α-chain of C4BP [25]. However, as no direct C4b-C4BP binding studies could be performed the residues actually important for C4b binding remained to be identified. The positioning of the C4b binding site to the C-terminal half of the α-chain seemed not to be compatible with the results obtained from electron microscopic imagery of C4BP–C4b complexes [5], although it is possible that the N-termini of the α-chains of C4BP fold inwards towards the core leaving the putative cofactor sites at the extremities of the molecule. Anti-C4BP-specific mAbs have been described that were found to react with the 48 kDa chymotryptic fragment of the α-chain of C4BP and abolished C4b binding [9,26]. However, the C4b-binding site on C4BP could not be identified by these mAb in detail, because these apparent discontinuous epitopes were not available on reduced C4BP. In the

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Abbreviations: C4BP, C4b-binding protein; mAb, monoclonal antibody; SCR, short consensus repeat.

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present study, we have obtained a mAb that reacted with the reduced α-chain of C4BP and inhibited the binding of C4BP to C4b that enabled us to identify a region of the α-chain of C4BP that is involved in the binding of C4b.

2. EXPERIMENTAL

2.1. *Proteins*

Human C4BP and C4b were purified as described [12,27]. Protein concentrations were determined by measuring the absorbance at 280 nm using an extinction coefficient (1%, 1 cm) of 14.3, 14.1 and 8.7 for IgG, C4BP and C4, respectively [28]. C4BP was radiolabeled with $Na^{125}I$ (Amersham, Buckinghamshire, UK) using Iodobeads (Pierce Chemical Co.) [26].

2.2. *mAbs*

MAbs specific for C4BP were obtained as described [26] using either C4BP or its central core as the antigen. Hybridoma supernatants were screened for the presence of mAbs by an ELISA in which reduced C4BP was used as the antigen. Positive clones were subcloned by limiting dilution, expanded, and antibody-rich ascites fluid was obtained [26]. Murine IgGs were isolated by protein G–Sepharose 4FF (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden).

2.3. *Electrophoretic and immunochemical techniques*

SDS-PAGE was performed according to Laemmli [29], and the gels were either stained with Coomassie brilliant blue R-250 or immunoblotted [30]. The Immobilon-P membranes (Millipore, Molsheim, France) were either blocked with 5% (w/v) non-fat dry milk in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, incubated with anti-C4BP mAb and visualized with peroxidase-conjugated rabbit anti-mouse IgG (Dako, Glostrup, Denmark), or stained with Coomassie brilliant blue. Prestained molecular weight markers were from Bio-Rad Labs. (Richmond, CA, USA).

2.4. *C4b–C4BP binding assay*

Direct binding of C4BP to C4b was measured as described [26]. Briefly, microtiter plates (Dynatech remova strips, Plockingen, FRG) were coated with C4b, radiolabeled C4BP was applied and the plates were incubated overnight at 4°C. The wells were washed and the amount of C4BP bound to C4b was measured. The various anti-C4BP mAbs were added together with the radiolabeled C4BP to examine their effect on the binding of C4BP to C4b.

2.5. *Competition between different mAbs for C4BP*

Microtiter plates were coated with each mAb, washed and blocked as described [26]. The various mAbs (35 μg/ml final concentration) were added to parallel mixtures of non-labeled (1 μg/ml final concentration) and radiolabeled C4BP, transferred to the wells and incubated overnight at 4°C. The wells were washed and the amount of bound C4BP was measured.

2.6. *Peptide mapping by SDS-PAGE and immunoblotting*

C4BP (1 mg/ml) was incubated with 3% (w/w) TPCK-trypsin (Worthington Biochemical Corporation, NJ) at 37°C for 2 h. The digestion was terminated by incubating the samples for 5 min at 90°C with 5% (v/v) 2-mercaptoethanol, 10 mM Tris-HCl, 2% SDS, 0.01% (w/v) Bromophenol blue, 10% (v/v) glycerol, pH 6.8. The digest was separated by SDS-PAGE under reduced conditions and subsequently transferred to an Immobilon membrane. The membranes were incubated with the anti-C4BP mAbs and parallel stained with Coomassie brilliant blue. The fragments detected by Coomassie brilliant blue staining were excised and their N-terminal amino acid sequencees were determined with an Applied Biosystems model 477A gas-phase sequencer, connected to an on-line 120A PTH Analyzer [31].

3. RESULTS

3.1. *Production of mAbs against C4BP*

Previously, we reported on the preparation and characterization of mAbs against C4BP that were prepared by immunizing mice with either C4BP or the 160 kDa chymotryptic central core [26]. In the present study, we rescreened the remaining anti-C4BP positive clones for reactivity with reduced C4BP. Using this strategy 7 new clones which reacted with reduced C4BP were obtained and characterized for their effect on the binding of C4BP to C4b. One mAb, designated C4BP-7F2, was selected and characterized in detail.

To determine whether the mAbs were directed against similar or different epitopes, competition experiments were performed. For this, the various mAbs were mixed with [^{125}I]C4BP and added to microtiter wells containing the immobilized mAbs C4BP-3B9 and 7B4, that previously were found to inhibit C4b binding [26]. From the data obtained (not shown) the anti-C4BP mAbs C4BP-3B9 and 7B4, resulted in strong inhibition of [^{125}I]C4BP binding to the mAb C4BP-7F2, suggesting that the epitopes of the mAbs C4BP-3B9, 7B4 and 7F2 are in proximity.

3.2. *Immunoblotting analysis of C4BP*

To examine the reactivity of the anti-C4BP mAbs with the disulfide-linked α- and β-chains, C4BP was reduced and analyzed by SDS-PAGE and immunoblotting. Table I shows the immunoblotting characteristics of the anti-C4BP mAbs and together with the competition experiments, the mAbs can roughly be subdivided into 3 groups: group A, C4BP-2E9 and 7F2; group B, C4BP-5C11 and 6B1; and group C, C4BP-3F9, 6B11 and 9F6. All mAbs from groups A–C were shown to react with the reduced α-chain, but remarkably, the mAbs from group B in addition seemed to react with the β-chain. This may suggest that the mAbs from group B recognize a common epitope that could be present on

Table I

Immuno-blotting analysis of C4BP

Group	mAb	Reduced chains	
		α	β
A	2E9	++	–
	7F2	++	–
B	5C11	+	+
	6B1	+	+
C	3F9	+	–
	6B11	+	–
	9F6	+	–

The different mAbs were immobilized in microtiter wells and the binding of [^{125}I]C4BP was measured. The influence of the different mAbs on C4BP binding to each solid-phase mAb was examined by adding the various mAbs to the [^{125}I]C4BP. – indicates no, + indicates weak and ++ indicates strong staining with the mAb.

the SCRs of both the α- and β-chains as the sequence homology between the 3 SCRs of the β-chain and the 8 SCRs of the α-chain has been found to be between 17 and 35% [11].

3.3. *The effect of mAbs on the C4BP binding to C4b*

The same mAbs were examined for their effect on the binding of C4BP to C4b. For this purpose C4b was coated on microtiter plates and incubated with [^{125}I]C4BP in the presence of an excess of anti-C4BP mAb. Table II demonstrates that the mAb C4BP-7F2 (group A) inhibits the binding of [^{125}I]C4BP to C4b almost completely, whereas the other mAbs showed no or minor effect. Some significant inhibition was also observed by the presence of mAb C4BP-9F6.

Table II

Effect of the mAbs on the complex formation of C4BP with C4b

Group	mAb	Inhibition of binding to C4BP for C4b*
A	2E9	20
	7F2	87
B	5C11	0
	6B1	0
C	3F9	30
	6B11	0
	9F6	54

*The percentage of inhibition of C4BP binding to solid-phase C4b was determined with radiolabeled C4BP in the presence of an excess of the different anti-C4BP mAbs, as described in section 2. The percentage of inhibition is expressed as % of the binding of C4BP alone. The data represent the average of 3 determinations.

3.4. *C4b-binding site on C4BP*

Our strategy for determining the structural domain(s) of C4BP that bind C4b involved a tryptic fragmentation of the molecule. SDS-PAGE of the reduced tryptic fragments of C4BP showed the liberation of 5 fragments derived from the α-chain of C4BP with apparent M_r's of about 48 kDa (T1), 45 kDa (T2), 36 kDa (T3), 32 kDa (T4) and 30 kDa (T5) (Fig. 1A). As the β-chain hardly stains with Coomassie brilliant blue [6], its presence was not detected. Immunoblotting analysis of the tryptic cleavage products of C4BP with the mAb C4BP-7F2 (Fig. 1B) revealed that this mAb reacts strongly with T3, and in addition recognized T4 and T5. To locate the tryptic fragments containing a possible C4b binding fragment within the structure of the C4BP molecule, the N-terminal sequences of the excised bands were determined. The N-terminal sequence obtained for T3 matched the sequence derived from the cDNA studies [13,14,32] and indicated that T3 started at residue Ser^{333}. The observation that the generation of the tryptic fragment T3 is due to cleavage by trypsin at Lys^{332} is in agreement with an apparent similar tryptic T3 fragment obtained by limited proteolysis studies of C4BP by Chung and Reid [25]. The N-terminal sequences obtained for the tryptic fragments, T4 and T5, both started at position His^{138}. On the basis that there are only 2 Arg residues present (positions 356 and 370) in the fragment between Ser^{333} and Tyr^{395} (the latter representing the cleavage site for chymotrypsin for the generation of the 48 kDa tentacles of C4BP), where trypsin has been

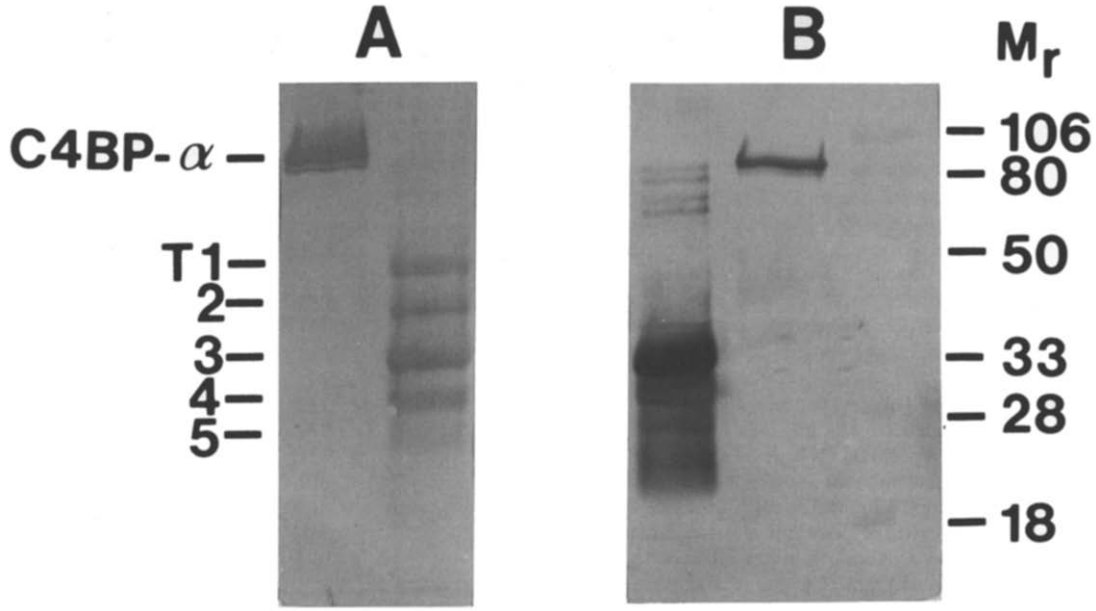

Fig. 1. SDS-PAGE and immunoblotting analysis of proteolytic fragments of C4BP. C4BP was digested with trypsin as described in section 2 and subsequently applied on 12.5% SDS-PAGE under reduced conditions. The gels with intact C4BP (α-chain) and the tryptic digest stained with Coomassie brilliant blue are shown to the left (A), and to the right (B) are the same polypeptides transferred to Immobilon membranes, which were analyzed with the mAb C4BP-7F2 (5 μg/ml).

found to perform enzymatic cleavage of the molecule [13], and since the apparent M_r of the T4 and T5 fragments are about 32 and 30 kDa respectively, it is reasonable to conclude that the domain of the α-chain of C4BP recognized by the inhibitory mAb C4BP-7F2 is identical to the domain of SCR 6 of the α-chain of C4BP, Ser^{333} through Arg^{356}. Additional support for this comes from a previous study [13] where a large population of succinylated, tryptic peptides of the α-chain of C4BP, that had been obtained after extensive trypsin cleavage, had been analyzed for their N-terminal sequences. They identified the fragments ST3-3 and ST9-9 starting at Thr^{371} and Phe^{357} respectively, thus suggesting that the T4 and T5 fragments are apparently generated by the same cleavages. The localization of the tryptic fragments and their overlapping region corresponding with the epitope of mAb C4BP-7F2 is demonstrated in Fig. 2. As a consequence of the above observations that the mAb C4BP-7F2 has been found to inhibit the interaction of C4b with C4BP, the residues Ser^{333}–Arg^{356} of the α-chain of C4BP are likely to be part of the putative C4b-binding site.

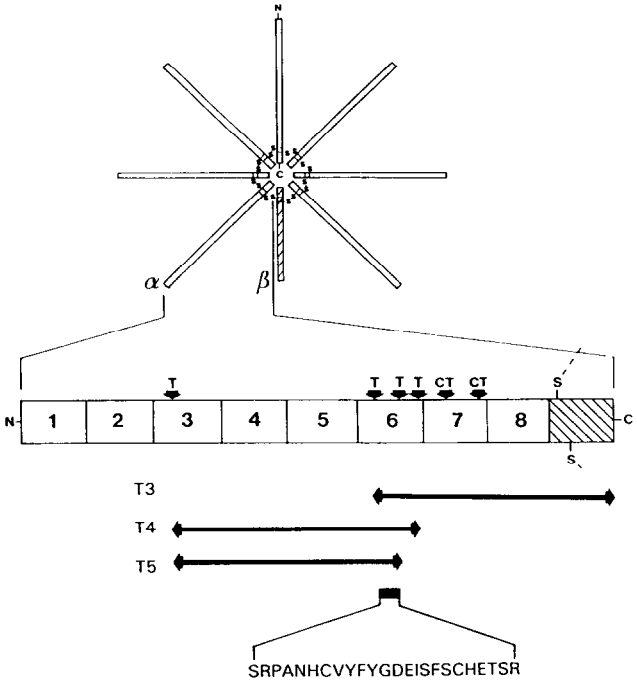

Fig. 2. Localization of the tryptic fragments containing the epitope of mAb C4BP-7F2 in a model of C4BP. (Upper part) The 7 α-chains and the distinct β-chain of C4BP (hatched bar) are disulfide linked to each other near their C-termini. The interchain disulfide bridges are tentatively placed between the non-homologous cysteins. (Lower part) One of the α-chains showing the 8 SCRs (denoted 1–8). The tryptic (T) and chymotryptic cleavage sites (CT) are indicated by the arrows. The proposed position for the epitope of the mAb C4BP-7F2 (Ser^{333}–Arg^{356}), and as a consequence the putative C4b-binding site, is indicated.

4. DISCUSSION

Human C4BP contains multiple binding sites for C4b that are localized on the chymotryptic 48 kDa fragments of the α-chain [23]. Fujita et al. [9] reported for the first time the preparation of anti-C4BP specific mAbs. One of these mAbs (TK 3) reacted with the 48 kDa chymotryptic fragments of the α-chain of C4BP and inhibited the binding of C4b. More recently, we have described anti-C4BP specific mAbs (C4BP-3B9, 7B4, 8C11, 9H3, 9H10) with apparent similar characteristics [26] that also abolished C4b binding to C4BP. However, the C4b-binding site on C4BP could not be identified by these mAbs in more detail, because their discontinuous epitopes were not available on the reduced molecule.

In this study an anti-C4BP-specific mAb, designated C4BP-7F2, directed against the reduced α-chain of C4BP, was obtained that inhibited the binding of C4b to C4BP. Tryptic proteolysis of C4BP and amino acid sequence analysis revealed that the residues Ser^{333}–Arg^{356} of the α-chain of C4BP contain the epitope of this mAb. As a consequence this part of the α-chain of C4BP is likely to be involved in the interaction with C4b. This result supports previous work [25] with the tentative placement of a putative C4b-binding site to residues 332–395 of the 6th SCR of the α-chain of C4BP. However, the C4b–C4BP inhibition experiments of this study and others [9,26] with 2 populations of mAbs with continuous and discontinuous epitopes, suggest that both the tertiairy structure of the α-chain of C4BP and at least the linear sequence Ser^{333}–Arg^{356} are important for a proper interaction of C4BP with C4b. Our experiments do not rule out the possibility that other sites (conformational or structural) in the α-chain of C4BP are also important for its interaction with C4b. One or more epitopes towards the N-terminus of the α-chain of C4BP might be required to support or facilitate the non-covalent binding with C4b. This latter may be in agreement with the electron microscopic studies [5] or with a previous suggestion that a region with an identical amino acid sequence (VTYSCDP) found in both the third SCR of C4BP and the membrane cofactor protein may be involved in C4b binding [26,33].

In conclusion, the present observations allow a tentative assignment of the C4b-binding site to the residues Ser^{333}–Arg^{356} of the 549 amino acid long α-chain of C4BP (Fig. 2), but a possible involvement of other parts of the molecule can not be excluded.

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