

HUMAN C4-BINDING PROTEIN

II. Role in Proteolysis of C4b by C3b-Inactivator*

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The activated form of C4 (C4b)¹ is an essential component of two key enzymes of the classical pathway, C3 convertase (C4b2a) and C5 convertase (C4b2a3b). The function of these enzymes is limited by the decay of C2a, which is very labile at 37°C. There is also evidence that another serum enzyme, C3b-inactivator (C3bINA) can inhibit the activity of cell-bound C4b2a, probably by cleaving C4b (1). Some uncertainty exists regarding the activity of C3bINA on C4b. According to Shiraishi and Stroud (2) the activity of C3bINA on fluid phase C4b required a high molecular weight cofactor. Recent studies of Pangburn et al. (3) have shown that cleavage of C4b requires a different protein (β 1H) as an essential cofactor.

Mouse (4) and human serum (5) contain a high molecular weight protein with several combining sites for C4b. This protein, named C4-binding protein (C4-bp), differs from all known complement components and inactivators. Because in mouse and human serum most or all C4b generated by activation of the complement cascade is found in combination with C4-bp, we considered the possibility that C4-bp might be the cofactor which modulates the function and/or assembly of C3 and C5-convertase in the fluid phase.

Materials and Methods

Materials. *N,N'*-methylene-bis-acrylamide, acrylamide, *N,N,N',N'*-tetramethylene diamine (TEMED), ammonium persulfate, sodium dodecylsulfate (SDS), Bio-Rad Laboratories, Richmond, Calif; ovalbumin, β -galactosidase (*Escherichia coli*), catalase (beef liver), myoglobin (horse heart), alcohol dehydrogenase (yeast), phosphorylase A (rabbit muscle), 2-mercaptoethanol (2-ME), Sigma Chemical Co., St. Louis, Mo.; CNBr-activated Sepharose 4B, Pharmacia Fine Chemicals, Division of Pharmacia Inc., Piscataway, N. J.; X-omat R film XR-5, Eastman Kodak, Rochester, N. Y.; phosphate-buffered saline (PBS) (Dulbecco's PBS), Grand Island Biological Co., Grand Island, N. Y.

Antisera. Rabbit antiserum to C3bINA was purchased from Kent Laboratories, North Vancouver, British Columbia. This antiserum reacted against a single component from human serum by crossed immunoelectrophoresis and did not react with the serum of a patient genetically deficient in C3bINA. Anti- β 1H was a gift from Dr. D. Fearon, Harvard University School of Medicine, Boston, Mass. Anti-C4-bp was prepared as described previously (5).

Preparation of Immune Adsorbents. The IgG fractions of anti- β 1H and C4-bp, purified by

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¹ Abbreviations used in this paper: BSA, bovine serum albumin; C4b, activated form of C4; C4-bp, C4-binding protein; C3bINA, C3b inactivator; 2-ME, 2-mercaptoethanol; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecylsulfate polyacrylamide slab gel electrophoresis.

DEAE-cellulose chromatography, were coupled to CNBr-activated Sepharose 4B according to the method described in (6).

Purified Complement Components and Reagents. C1s and C4 were purified as described (7, 8). C4 was radiolabeled with ^{125}I by the method described in (4). Specific activity was $\cong 10^7$ cpm/ μg protein. C4b was prepared by adding 30 μg of C1s to 400 μg of C4, and incubating the mixture for 48 h at 0°C . Cleavage of the α -chain of C4 was ascertained by SDS-polyacrylamide slab gel electrophoresis (PAGE) under reducing conditions. β 1H and C3bINA were purified from human serum according to the method of Weiler et al. (9) and Fearon and Austen (10). To remove trace amounts of C4-binding protein, the preparation of β 1H was passed through an anti-C4-bp Sepharose column. The activity of β 1H was measured, as described in (9), by its inhibitory activity on the EAC4,3B intermediate. C4-bp was isolated as described previously (5). To remove trace contamination of β 1H, C4-bp was passed through an anti- β 1H Sepharose column.

The protein concentration of C4 was estimated by the method of Hartree (11), using bovine serum albumin (BSA) as standard. In the case of C3bINA, C4-bp, and β 1H, the protein concentration was calculated from their optical densities at 280 nm, assuming an absorption coefficient value of $E^{1\text{ cm}} = 1$.

Serum from patients with C4-deficiency and C3b-inactivator deficiency were gifts from Dr. H. Ochs, University of Washington School of Medicine, Seattle, Wash.; and Dr. C. Alper, Harvard University School of Medicine, Boston, Mass., respectively.

Analytical Procedures. SDS-PAGE was performed as described by Laemmli (12). The stacking and separating gel combinations used were either 3 and 7.5% or 3 and 10%. Gradient gels, from 5 to 18% acrylamide, were used in some experiments. After electrophoresis, the gels were stained with 0.04% Coomassie blue in 10% methanol and 7% acetic acid. Destaining was carried out with a 30% methanol, 7% acetic acid solution. Photographs of the stained gel were taken at this stage. Radioautography was carried out by exposing the dried gel to an X-omat R film X R-5 at -70°C , for 12–72 h.

To calibrate the acrylamide gels for molecular weight estimations, the following protein markers were used and included in every gel: 94,000 phosphorylase A; 130,000 β -galactosidase; 67,000 BSA; 60,000 catalase; 41,000 alcohol dehydrogenase.

Results

Native C4 is composed of three polypeptide chains, α , β , and γ . Treatment of C4 with C1s cleaves a fragment (C4a) from the α -chain. The remaining peptide, named C4b, consists of a fragment of the α -chain (α'), as well as the intact β - and γ -chains.

Treatment of 6 μg C4b with buffer, 0.6 μg C3bINA, 4 μg β 1H, 4 μg C4-bp, in a total volume of 40 μl , or by mixtures of β 1H and C4-bp, or C3bINA and β 1H at 37°C for 1 h had no effect on the structure of the C4b molecule (Fig. 1). However, incubation of C4b with a mixture of C3bINA and C4-bp resulted in the fragmentation of the α' chain of C4b into three peptides, with mol wt of 47,000 daltons (α 2), 25,000 daltons (α 3), and 17,000 daltons (α 4). The β - and γ -chains remained intact (Table I). This reaction was not affected by treatment of the reagents with diisopropylfluorophosphate (5×10^{-3} M). Treatment of native C4 with C3bINA and C4-bp was without effect.

In view of the report that β 1H may function as a cofactor in the cleavage of C4b at high β 1H/C4b ratios (3), an additional experiment was performed. 0.05 μg in 20 μl radiolabeled C4b was mixed with 0.6 μg in 5 μl C3bINA and 6 μg in 30 μl β 1H. The mixture was incubated at 37°C for 1 h and analyzed by SDS-PAGE under reducing conditions. We found no effect on the structure of C4b. However, β 1H, which had not been passed through an anti-C4-bp Sepharose column, cleaved C4b in the presence of C3bINA, indicating that trace amounts of C4-bp were present.

When labeled C4b, treated with C3bINA and C4-bp, was subjected to SDS-PAGE without the reducing agent 2-ME, two fragments, of mol wt 150,000 and 47,000, were

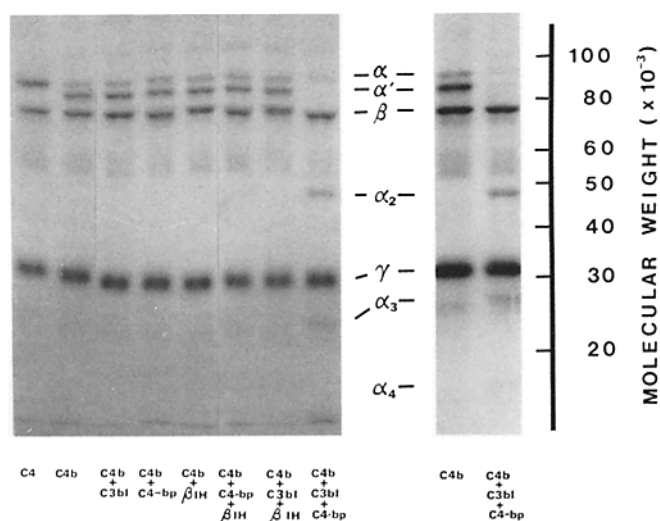


FIG. 1. Cleavage of C4b by C3bINA and C4-binding protein. ^{125}I -C4b (20 μl , containing 6 μg protein) were incubated with 5 μl , 0.6 μg C3bINA, 10 μl , 4 μg C4-bp, and 10 μl , 4 μg β 1H or with mixtures of C4-bp and β 1H, C3bINA and β 1H, or C3bINA and C4-bp for 60 min at 37°C in a total volume of 40 μl . Then 40 μl of a solution containing 2% SDS, 6 M urea, and 10% 2-ME, was added, and the mixture applied to a 10% SDS-PAGE. The slab gel was stained, dried, and subjected to radioautography. The radioautography shown in the panel on the left demonstrates that the α' -chain of C4 was cleaved only in the presence of C3bINA and C4-bp. The α_2 and α_3 fragments of the α' -chain are shown, but the smaller α_4 fragment migrates with the dye. To determine the molecular weight of the 4 fragment, ^{125}I -C4b was treated with buffer or with C3bINA and C4-bp under the same conditions and subjected to SDS-PAGE in a 5–20% gradient gel. The radioautogram in the panel on the right shows the α_2 , α_3 and α_4 fragments.

TABLE I
Fragments and Chains of C4b after Interaction with C3b Inactivator and C4-Binding Protein

Chain and fragment	mol wt (mean \pm 1 SD)
α_2	47,000 \pm 1,400
α_3	25,000 \pm 1,000
α_4	17,000 \pm 1,400
β -chain	75,000 \pm 1,200
γ -chain	31,000 \pm 1,300

detected (Fig. 2). Therefore, the α_2 fragment (probably C4d) is not covalently linked to the β - or γ -chains of C4.

In short, C3bINA and C4-bp cleave the α' -chain at two sites, producing three fragments, as shown schematically in Fig. 3.

In the experiment shown in Fig. 4, decreasing amounts of C4-bp were added to a constant amount of C4b and C3bINA. The reaction mixtures were incubated at 37°C for 60 min and subjected to SDS-PAGE. A stained gel is shown. The extent of cleavage of the C4b was dependent on the concentration of C4-bp. When cleavage was incomplete, the reaction did not proceed further by prolonging the time of incubation or increasing the concentration of C3bINA. Therefore, it appears that an optimal stoichiometric relationship between C4b and C4-bp is required for the activity of C3bINA.

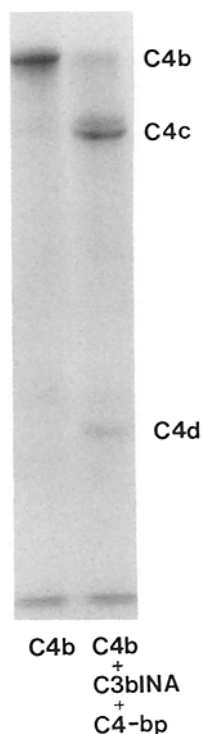


FIG. 2. 7.5% slab SDS-PAGE of unreduced samples of ^{125}I -C4b after treatment with buffer or with C3bINA and C4-bp. The samples were prepared as described in Fig. 1 except that 2-ME was not used. The mol wt of C4d is 47,000 daltons.

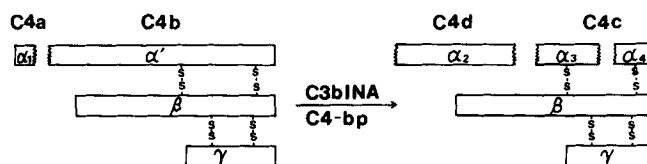


FIG. 3. Schematic representation of the cleavage of C4b by C3bINA and C4-bp.

We also investigated the specificity of interaction between C4b, C3bINA, and C4-bp. Radiolabeled C4b (containing $0.05 \mu\text{g}$ protein and 5×10^5 cpm) was incubated with the serum of a patient with genetic deficiency of C3bINA, or with a mixture of C3bINA-deficient serum and purified C3bINA. Also, radiolabeled C4b and C3bINA were incubated with C4-bp which had been passed through an anti-C4-bp Sepharose column. After incubation at 37°C for 1 h, the reaction mixtures were subjected to SDS-PAGE under reducing conditions followed by radioautography. The α' -chain of C4b was not cleaved when C4-bp was specifically removed, nor after incubation with C3bINA-deficient serum. However, after addition of purified C3bINA to the C3bINA-deficient serum, the α' -chain was cleaved and the α_2 , α_3 , and α_4 fragments were generated. Identical results were obtained when C4b was incubated in C4-deficient serum (Fig. 5).

Next, we asked whether C4-bp was affected during the interaction of C4b/C4-bp complexes with C3bINA. A mixture containing $6 \mu\text{g}$ of C4b in $20 \mu\text{l}$, $0.5 \mu\text{g}$ of

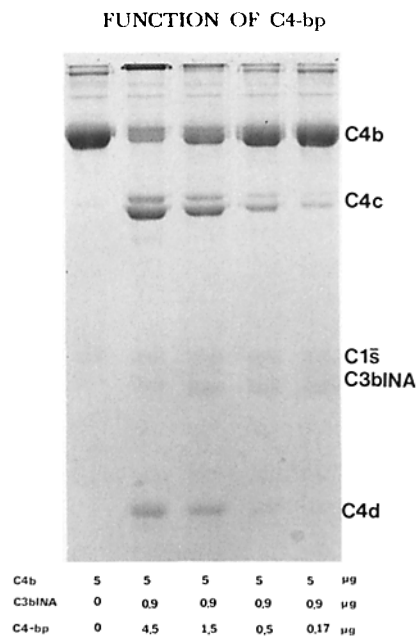


FIG. 4. Dose-response relationship between the concentration of C4-bp and the extent of cleavage of C4b. 5 μg of C4b in 10 μl and 0.9 μg of C3bINA in 8 μl were incubated with decreasing amounts of C4-bp for 1 h at 37°C in a total volume of 40 μl. The samples were mixed with equal volumes of 2% SDS and 6 M urea, and the mixtures were subjected to 7.5% SDS-PAGE under nonreducing conditions. A stained gel is shown. Proteolysis of C4b was directly proportional to the concentration of C4-bp in the mixture. When C4-bp was limiting, the extent of cleavage of C4b was not changed by increasing the time of incubation or the concentration of C3bINA. Three high molecular weight contaminants are seen in the preparation of C4b (C4 + C1s). One of them is an aggregate of C4b, which is also cleaved by C4-bp and C3bINA. C4-bp did not penetrate the gel.

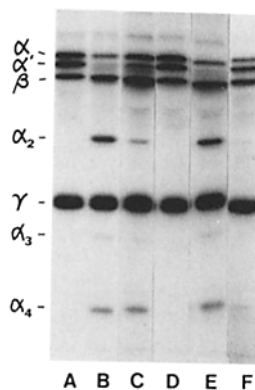


FIG. 5. Specificity of C4b, C4-bp, and C3bINA interaction. ^{125}I -C4b (20 μl, containing 0.05 μg protein) was incubated at 37°C for 1 h with 20 μl of 1/50 dilution of C3bINA-deficient serum. No cleavage of C4b was observed after SDS-PAGE and radioautography (D). However, when 0.2 μg of purified C3bINA was added, the α'-chain of C4b was cleaved into three fragments (E). When ^{125}I -C4b was incubated with C3bINA and a preparation of C4-bp which had been treated with anti-C4-bp Sepharose, no cleavage of C4b was observed (F). Controls consisted of ^{125}I -C4b alone (A), and a mixture of ^{125}I -C4b, purified C3bINA (0.2 μg) and C4-bp (0.6 μg) (B). Also shown is the cleavage of ^{125}I -C4b after incubation in serum from a patient with a genetically determined deficiency of C4 (C). Note that in all cases the cleavage of the α'-chain resulted in fragments of the same molecular weights.

C3bINA in 10 μ l, and 5 μ g of ^{125}I -C4-bp in 15 μ l was incubated at 37°C for 1 h. After denaturation and reduction with SDS, urea, and 2-ME, the mixtures were subjected to SDS-PAGE. The slab gel was stained, dried, and subjected to radioautography. The stained gel showed the characteristic pattern of degradation of the α' -chain of C4b. In contrast, the radioautography showed that C4-bp had not been cleaved. A single band, of mol wt \cong 75,000, was detected in the mixture containing ^{125}I -C4-bp, C3bINA and C4b, and in the control containing ^{125}I -C4-bp alone (not shown).

Discussion

We show here that C4b is cleaved in the presence of C4-bp and C3bINA. Because the activity of C3-convertase (C4b2a) and C5-convertase (C4b2a3b) is C4b-dependent, it is probable that C4-bp and C3bINA inhibit the function of these enzymes. This question is currently under investigation in our laboratory.

Three fragments of the α' -chain of C4b are generated after interaction with C4-bp and C3bINA: one of them, (α_2), has a mol wt of 47,000 and is noncovalently bound. The other two fragments (α_3 and α_4) remain associated through disulfide bonds to the rest of the molecule. It is very likely that the α_2 fragment is the previously described C4d fragment, whereas the disulfide bonded γ -, β -, α_3 , and α_2 peptides corresponded to the C4c fragment of C4.

The precise role of C4-bp and C3bINA in the cleavage of the α' -chain of C4b is unclear. The previous demonstration that C4b forms stable complexes with C4-bp and the results of Fig. 4 suggest a stoichiometric relationship between C4b and C4-bp. It is possible, therefore, that the protease is C3bINA, and that its substrate is the α' -chain, modified allosterically by the formation of C4/C4-bp complexes. Because three peptides are generated, C3bINA would have to act on two separate peptide bonds. Alternatively, C4-bp might act enzymatically after complex formation with C4b and C3bINA. Similar interaction have been described between other subcomponents of the enzymes of the complement system. For example, some investigators report that Clr is activated rapidly when complexed with AgAbClq and Cls, but not in the absence of either protein (13).

There is a striking homology between the requirements for cleavage of C3b and C4b in solution by C3bINA. In both cases an additional protein, with a specific affinity for the substrate, is necessary; β 1H in the case of C3b (3), and C4-bp in the case of C4b. Furthermore, β 1H and C4-bp share some physico-chemical properties, and purified preparations of either protein are frequently cross-contaminated. This may explain the results of Pangburn et al. (3) who showed that β 1H can function as a cofactor in the cleavage of both C3b and C4b. Although we confirmed that mixtures of β 1H (free of C4-bp) and C3bINA cleave C3b, the same preparations of β 1H were inactive in relation to C4b.

Our findings support the observations of Shiraishi and Stroud (2), who showed that a high molecular weight cofactor and C3bINA were necessary for the cleavage of fluid phase C4b into C4c and C4d. On the basis of its activity and some of its physico-chemical properties, the high molecular weight cofactor resembles C4-bp. In a subsequent publication, Nagasawa and Stroud (14) reported that their preparation of cofactor could also function, in conjunction with C3bINA, in the cleavage of the α' -chain of C3b. This cleavage resulted in the complete abrogation of the C3b-mediated activation of factor B of the alternative pathway. Their results suggest that

similarly to β 1H, the high molecular weight cofactor modulates the formation in the fluid phase of the alternative pathway C3-convertase (C3bB). Because the high molecular weight cofactor and C4-bp may be the same protein, we repeated Nagasawa and Stroud's experiments and found that our preparations of C4-bp could indeed serve as a cofactor for cleavage of C3b.² However, the interpretation of our findings is complicated by the presence in the preparations of C4-bp of two proteins with slight differences in molecular weights and net charges. As discussed elsewhere, both proteins react with the monospecific antiserum to C4-bp and bind to C4b. Whether they have similar or different functions in cleavage of C4b and C3b is currently under investigation.

Summary

We recently described the isolation from human serum of a high molecular weight protein with specific binding affinity for fluid-phase activated C4. We show here that the C4-binding protein (C4-Bp) functions as an essential cofactor in the proteolysis of C4b in the presence of C3b-inactivator (C3bINA). C4-bp, together with C3bINA, cleave the α' -chain of C4b into three fragments called α 2, α 3, and α 4, with mol wt of 47,000, 25,000, and 17,000 daltons, respectively. The α 2 fragment was dissociated from C4b without reduction, whereas the α 3 and α 4 fragments were disulfide bonded to the other chains of C4b.

The reaction did not occur when either C4-bp or C3bINA were omitted, nor in the presence of either protein in combination with β 1H. Native C4 was not affected by C3bINA and C4-bp.

C4b was not cleaved when incubated in serum of a patient with genetic deficiency of C3bINA. However, when purified C3bINA was added, the α' -chain of C4b was cleaved and fragments with the same molecular weight as α 2, α 3, and α 4 were generated.

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