THE ROLE OF C4-BINDING PROTEIN AND 
β1H IN PROTEOLYSIS OF C4b AND C3b*

BY TEIZO FUJITA AND VICTOR NUSSENZWEIG

From the Department of Pathology, New York University Medical Center, New York 10016

Clarification of the nature of the proteins involved in the cleavage of C3b and C4b is of great interest because these complement fragments are opsonins and, in addition, they modulate the activities of several key enzymes of the complement cascade. When these complement peptides are in solution, proteolysis by a serum enzyme, C3b-inactivator (C3bINA), requires cofactors: β1H in the case of C3b and C4-binding protein (C4-bp) or C3b-C4bINA cofactor in the case of C4b (1-4).

Some uncertainties remain regarding the activities of the cofactors. The observations of Whaley and Ruddy (5) strongly suggest that only β1H, among all serum proteins separated by molecular sieve chromatography, accelerates the inactivation of cell-bound C3b by C3bINA, whereas the results of Nagasawa and Stroud (4) indicate that, in addition to β1H, the C3b-C4bINA cofactor can also function in the cleavage of fluid phase C3b by C3bINA. Other sources of complication are that preparations of the cofactors are frequently cross-contaminated and that C4-bp contains two proteins that are antigenically identical but display slight differences in molecular weight and net charges (6).

In the present paper we report the isolation of both forms of C4-bp and compare their activities, as well as that of β1H, on cell-bound and fluid phase C3b and C4b. In addition, we studied the relationship between C4-bp and the C3b-C4bINA cofactor.

Materials and Methods


Buffers

DGVB: gelatin veronal buffer, pH 7.4 containing 5% dextrose, 0.1% gelatin, 0.15 mM CaCl2, and 0.5 mM MgCl2.

EDTA-DGVB: gelatin veronal buffer containing 0.01 M EDTA.

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Abbreviations used in this paper: A, antibody; C3bINA, C3b inactivator; C4-bp, C4-binding protein; C4-bp low, or high, C4-binding protein of low or high molecular weight; CIE, crossed immunoelectrophoresis; DFP, diisopropylfluorophosphate; DGVB, dextrose gelatin veronal buffer; E, sheep erythrocytes; RIE, rocket immunoelectrophoresis; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis.
Antisera. Rabbit antisera to human C3bINA and IgM were purchased from Kent Laboratories, North Vancouver, British Columbia, and Behring Diagnostics, American Hoechst Corp., Somerville, N. J., respectively. Anti-β1H and anti-C4-bp were prepared in our laboratory by injecting rabbits with the purified proteins incorporated in complete Freund's adjuvant. Anti-C3b-C4bINA cofactor was a gift from Dr. Robert J. Wyatt, Children's Hospital Research Foundation, Cincinnati, Ohio.

Preparation of Immunoadsorbents. Purified C4b and the IgG fractions of anti-β1H, C4-bp, and IgM, obtained by DEAE-cellulose chromatography were coupled to CNBr-activated Sepharose 4B according to the directions of the manufacturer.

Purified Complement Components. Guinea pig C1 and C2 (7), human C4 (8), C3 (9), P (10), B (11), D (12), β1H (13), and C3bINA (14) were purified by previously published techniques. Guinea pig serum diluted 1:50 in EDTA-GVB served as a source of C3-C9.

C4b and radiolabeled C4b were prepared as described previously (2). C3b was prepared from purified C3 as described by Bokisch et al. (15). C3b was radiolabeled with 125I by the method described in (16). Specific activity was 4 × 10^6 cpm/μg protein.

Two forms of C4-bp which differ slightly in molecular weight (C4-bp low or high) were separated by the method previously described (6) with minor modifications. The starting material, a euglobulin fraction of serum obtained by 1/5 dilution in water containing 2 mM EDTA, pH 7.4, was subjected to chromatography on DEAE Sephadex A-50. The fractions enriched in C4-bp low or high were further purified by passage on Bio-Rex 70 (Bio-Rad Laboratories, Richmond, Calif.) and molecular sieve chromatography on Sepharose 6B. Additional contaminants, such as β1H and IgM were removed by passage through Sepharose 4B conjugated with the IgG fraction of monospecific antisera.

By SDS-polyacrylamide gel electrophoresis (PAGE), the preparation of C4-bp high (0.3 mg/μl) showed a single band. However, C4-bp low was contaminated with C4-bp high. The amount of contamination was estimated to be 23% by counting the radioactivity in the stained bands of radiolabeled C4-bp low after SDS-PAGE. When SDS-PAGE was performed under reducing conditions, a single polypeptide chain, mol wt 75,000, was observed in the preparations of both C4-bp high and low (Fig. 1).

The protein concentrations of purified C4-bp and C4 were estimated by the method of Lowry using bovine serum albumin as the reference protein (17). In the case of C3bINA, β1H, B, D, and P, the protein concentrations were calculated from their optical densities at 280 nm, assuming that an absorption coefficient value of 1.0 equals 1 mg/ml.

Measurement of the Interaction between β1H with Cell-Bound C3b. The assay which we used was based on the ability of β1H to accelerate the inactivation of cell-bound C3b by C3bINA (5).

Two different types of C3b-bearing cells were used: (a) EAC4b3b cells with limited C3b sites, or (b) EC3b cells were prepared by mixing an equal volume of E(1 × 10⁹/ml) and purified C3 (400 μg/ml) with 1% trypsin (wt/wt of C3). After incubation at 37°C for 3 min, the reaction was stopped by adding 1% soybean trypsin inhibitor and the cells washed three times with DGVB.

Fractions containing β1H and C4-bp were assayed as follows: 100 μl of EAC4b3b or EC3b cells (1.5 × 10⁹/ml), 50 μl of C3bINA (2 μg/ml), and 50 μl of test samples were incubated in EDTA-GVB at 37°C for 30 min. The cells were washed twice with DGVB and resuspended in 0.3 ml of DGVB containing B (6 μg/ml) and D (0.2 μg/ml). After incubation at 37°C for 15 min, the resulting cells were further incubated at 37°C for 1 h with 1.2 ml of C3–C9. Controls consisted of EAC4b3b or EC3b incubated with C3bINA alone or with EDTA-GVB. The tubes were centrifuged, and the hemoglobin in the supernate was measured spectrophotometrically at 414 nm.

On the basis of the percent hemolysis observed, the average number of hemolytic sites per cell (Z) was calculated. Inhibition of hemolysis, which reflects inactivation of C3b, was expressed as percentage inhibition of hemolytic sites.

Analytical Procedures. Crossed (CIE) and rocket (RIE) immunoelectrophoresis and SDS-PAGE were performed as described previously (2, 16, 18).

Results

Presence of Two Distinct Cofactors for Cleavage of C3b by C3bINA in the Euglobulin Fraction of Human Serum. In view of the report that, in addition to β1H, a high molecular
weight protein could also function as a cofactor in the cleavage of C3b by C3bINA (4), we examined the possibility that these two activities could be separated by gel filtration. 50 ml of serum treated with DFP were diluted five times with water containing 2 mM EDTA, pH 7.4. After 60 min at 0°C, a euglobulin fraction was separated by centrifugation. Fig. 2 shows the results of assays performed in fractions obtained after the passage of this euglobulin fraction through a Sepharose CL-6B column.

Three protein peaks were obtained. C4-bp protein first appeared in the ascending portion of the second protein peak with an apparent mol wt of 1,200,000. β1H protein eluted between the second and third protein peaks with a mol wt of 300,000.

Fractions were first tested for their ability to enhance the activity of C3bINA on EAC4b3b cells (5). The peak of activity coincided precisely with the position of β1H. No activity was associated with C4-bp. To rule out the possibility that the lack of activity of C4-bp on cell-bound C3b was a result of the presence of C4b on the EAC4b3b cells we repeated the assay using EC3b. Again, β1H produced a strong dose-dependent inhibition of the hemolytic activity of EC3b, whereas C4-bp had no effect even at high concentrations (Fig. 3).

Next, the same fractions were incubated with radiolabeled C3b and C3bINA at 37°C for 1 h. After denaturation and reduction of the proteins with SDS, urea, and 2-mercaptoethanol, the mixtures were subjected to SDS-PAGE. Fig. 2, D shows the radioautography. It is clear that the α′-chain of C3b was cleaved when incubated with fractions containing β1H (76–80) or C4-bp (56–60).

In view of these results, we proceeded to isolate the two varieties of C4-bp to determine whether both would function as cofactors in the cleavage of fluid phase C3b as well as C4b.
Cofactor activity for C3b-inactivator in fractions obtained after passage of serum euglobulin through a Sepharose CL-6B column. The euglobulin fraction was applied to a Sepharose CL-6B column (2.6 × 90) equilibrated with phosphate-buffered saline containing 2 mM EDTA. The OD of the fractions are shown in panel A. The arrows point to the positions of the peaks of IgM and IgG when chromatographed through the same column. C4-bp and β1H antigen were measured by rocket immunoelectrophoresis (panel B). The ability of fractions containing β1H to enhance the inhibitory activity of C3bINA on cell-bound C3b is shown in panel C. Panel D shows the cleavage of fluid phase C3b obtained by mixing purified C3bINA with the fractions. 125I-C3b (15 µl containing 2 µg of protein and 6 × 10⁴ cpm) were incubated with C3bINA (5 µl, 0.4 µg) and 30 µl of each individual fraction for 1 h at 37°C. Negative controls consisted of 125I-C3b alone (first column from left to right). Positive control (second column from left to right) was a mixture of 125I-C3b, C3bINA, and β1H (10 µl, 2 µg protein). After incubation, 30 µl of a solution containing 2% SDS, 6 M urea, and 10% 2-mercaptoethanol was added, and the mixtures were applied to 3–7.5% SDS-PAGE. The slab gel was stained, dried, and subjected to radioautography. Two peaks of activity, coinciding with the positions of β1H and C4-bp antigens, were detected (76–80 and 56–60).

Cofactor Activity of the Two Forms of C4-bp in the Cleavage of C4b and C3b. In the experiments shown in Fig. 4, decreasing amounts of the two varieties of C4-bp (C4-bp low, C4-bp high) were added to a constant amount of C4b and C3bINA. The reaction mixtures were incubated at 37°C for 1 h and subjected to SDS-PAGE. The α'-chain of C4b was cleaved into fragments with mol wt of 47,000 (α2), 25,000 (α3), and 17,000.
Fig. 3. Effect of C4-bp and β1H on the hemolytic activity of cell-bound C3b. Dilutions of purified C4-bp and β1H were incubated with EC3b bearing 1.02 C3b sites per cell in the presence of C3bINA. The residual average number of hemolytic C3b sites was determined as described in Methods. β1H, but not C4-bp, markedly inhibited the hemolytic activity of EC3b in the presence of C3bINA. C3bINA alone reduced the total number of hemolytic sites from 1.02 to 0.41. β1H or C4-bp alone had no effect on EC3b.

Fig. 4. Cleavage of C4b by C3bINA and C4-bp low (L) or C4-bp high (H). $^{125}$I-C4b (30 μl and 8 × 10⁶ cpm) and 5 μl of C3bINA were incubated with decreasing amounts of C4-bp low and C4-bp high for 1 h at 37°C in a 45-μl total volume. The reaction mixtures were applied to a 10% SDS-PAGE under reducing conditions, followed by radioautography. With either form of C4-bp, the α'-chain of C4b was cleaved to the same extent.
FIG. 5. Cleavage of C3b by C3bINA in the presence of β1H, C4-bp low (L), or C4-bp high (H).

125I-C3b (15 µl and 6 × 10⁴ cpm) and 5 µl C3bINA were incubated with decreasing amounts of β1H, C4-bp high, or C4-bp low for 1 h at 37°C in a 60-µl total volume. The mixtures were analyzed by 7.5% SDS-PAGE under reducing conditions. The radiography demonstrates that C3bINA and β1H cleaved the α′-chain of C3b into two peptides with mol wt of 65,000 (α′-65) and 40,000 (α′-40). Incubation with C4-bp high or C4-bp low resulted in the fragmentation of the α′-chain into three peptides; that is, α′-65, α′-40, and a minor band with an apparent mol wt of 45,000. This minor band disappeared when the amounts of C3bINA and C4-bp were increased in relation to 125I-C3b. This is shown in the last track on the right side of the figure.

The specific cofactor activities of C4-bp high or low are quite similar, as evaluated by the extent of cleavage of the α′-chain of C3b.

(a4). On the basis of the extent of cleavage shown in the radioautography, and taking into account that C4-bp low is slightly contaminated with C4-bp high (Methods), we estimate that both forms of C4-bp have similar specific activities.

Next, we evaluated the effects of β1H, C4-bp low, and C4-bp high on the structure of C3b. When labeled C3b was treated with a constant amount of C3bINA and decreasing amounts of β1H, the extent of cleavage of C3b was dependent on the concentration of β1H (Fig. 5). The α′-chain of C3b was cleaved into two fragments with apparent mol wt of 65,000 and 40,000, and the β-chain remained intact.

Treatment of C3b with C3bINA and C4-bp low or C4-bp high resulted in the fragmentation of the α′-chain of C3b into three peptides (Fig. 5). Two of them (α′-65 and α′-40) were in identical positions to those resulting from the activity of β1H. An additional minor band was seen in a position corresponding to a mol wt of 45,000 (α′-45). When C3bINA and C4-bp were added to smaller amounts of C3b, this extra band disappeared (Fig. 5, additional track).

To rule out the possibility that other serine proteases contaminated our reagents, the same experiments were carried out in the presence of DFP (5 × 10⁻³ M). Results were identical.

In summary, we found no qualitative differences between β1H and the two forms of C4-bp in their role as cofactors in the cleavage of fluid phase C3b. Other investigators found that C3bINA and β1H cleave the α′-chain of C3b into two
fragments (1) which probably correspond to α'-65 and α'-40. The origin of the α'-45 fragment is not clear. One possibility is that it is an intermediate product which precedes the appearance of α'-40 as reported by Harrison and Lachmann (19).

On the basis of many titrations such as those shown in Fig. 5, we estimate that the efficiency of β1H to serve as cofactor for cleavage of C3b is ≈20-fold greater than C4-bp low or high on the basis of protein concentration.

**The Specificity of the Effects of C4-bp and C3b.** The possibility that the effects of C4-bp on C3b were a result of contamination with β1H was studied in additional experiments. C4-bp was further purified by affinity chromatography through Sepharose 4B-C4b or by passage through an anti-β1H Sepharose column. Its activity was not altered. When purified C4-bp was passed through an anti-C4-bp Sepharose column, the cofactor activities for both C4b and C3b were removed.

In addition, C4b inhibited the effect of C4-bp on the cleavage of C3b by C3bINA. In the experiment shown in Fig. 6, decreasing amounts of C4b were incubated with a constant amount of C4-bp at 37°C for 30 min. Then, radiolabeled C3b and C3bINA were added to the mixture. After an additional incubation at 37°C for 1 h, the samples were analyzed by SDS-PAGE and radioautography. The stained gel showed the characteristic pattern of degradation of the α'-chain of C4b (not shown). The radioautography demonstrates a dose-dependent inhibitory effect of C4b on the cleavage of C3b.

We conclude that C4-bp, as well as β1H, can serve as cofactors for the cleavage of fluid phase C3b, and that C4b and C3b compete for C4-bp binding sites.

**Identity between C4-bp and C3b-C4bINA Cofactor.** The results above show a striking functional similarity between C4-bp and the cofactor described by Stroud and his
associates (3, 4). C4-bp and C3b-C4b co-factor are high molecular weight β-globulins with sedimentation coefficients 10-11S, and consisting of subunits of mol wt between 70 and 80,000.

We recently compared the specificities of antisera to C4-bp and to the C3b-C4bINA cofactor. Both were monospecific, as shown by crossed immunoelectrophoresis of whole human serum or plasma. In double diffusion in agarose against purified C4-bp a reaction of identity between both antisera was obtained.

Discussion

As previously described, most or all normal human sera contain different proportions of a two forms of C4-bp (C4-bp low and C4-bp high), which differ slightly in molecular weight and net charge. Although the nature of this structural heterogeneity is unknown, both of them bind specifically to C4b and can be immunoprecipitated with antisera to C4-bp (6). We show here that C4-bp low and high cannot be distinguished functionally when assayed as cofactors for the cleavage of the α'-chain of C4b by C3bINA; the specific activities are quite similar and the fragments of C4b which are generated have identical molecular weights.

In addition, we found that both proteins can serve as cofactors for the cleavage of C3b in solution, and in this respect C4-bp resembles β1H (1). Because β1H and C4-bp share some physico-chemical properties, and purified preparations of either protein are frequently cross-contaminated, we considered the possibility that this activity of C4-bp was a result of contaminating β1H. However, we could not detect β1H in our preparations of C4-bp (300 µg/ml) by RIE, CIE, or by SDS-PAGE. The activity on C3b was fully preserved when C4-bp was passed through a Sepharose 4B-anti-β1H column, or after further purification of C4-bp by affinity chromatography on Sepharose 4B-C4b. Furthermore, highly purified C4b inhibited the cofactor activity of C4-bp on C3b, but it had no effect on the cofactor activity of β1H.

Although C4-bp and β1H, together with C3bINA, had similar qualitative effects on fluid phase C3b, several important quantitative differences in their activities were observed. For example, β1H diminished the hemolytic activity of EC3b, in the presence of C3bINA, at concentrations of 0.05 µg/ml, whereas C4-bp was without effect even at a 16 µg/ml concentration. Therefore, C4-bp is at least 320 times less effective than β1H when assayed for effects on cell-bound C3b. In relation to fluid phase C3b, C4-bp appeared to be about 20 times less effective than β1H on a weight basis. Because the concentration of β1H in normal serum (500 µg/ml) (13) is also higher than that of C4-bp (250 µg/ml, T. Fujita and V. Nussenzweig, unpublished observations) our results indicate that under physiological conditions the functions of C3b are mainly controlled by β1H.

The present findings show a striking homology between the requirements for cleavage for C3b and C4b in solution by C3bINA. In both instances an additional binding protein is necessary: β1H (or secondarily C4-bp) in the case of C3b, and C4-bp in the case of C4b. The mechanisms by which these regulatory proteins carry out their function are not entirely clear. Their effects may be mediated by the formation of complexes with C4b (6) or C3b (20, 21). The substrate of C3bINA may be the α'-chain of C3b or C4b modified allosterically by the interaction with β1H and C4-bp. If this concept is correct, our results could indicate that fluid phase C3b has a higher binding affinity for β1H than for C4-bp, and that cell-associated C3b exclusively...
binds $\beta_1H$. Whatever the reasons may be for the remarkable differences in susceptibility of cell-bound versus free C3b to C4-bp and C3bINA, our observations support the concept that the effects of the regulatory proteins are greatly influenced by the microenvironment in which the reaction takes place (21, 22).

We also examined the relationship between C4-bp and the C3b-C4bINA cofactor described by Stroud and his collaborators (3, 4). On the basis of its activity, the Stroud cofactor resembles C4-bp, as shown in this report and previous papers (2). In addition, we found that antiserum monospecific to the cofactor reacts with purified C4-bp and gives a reaction of identity with an antiserum to C4-bp. Therefore, by functional, physico-chemical and immunological criteria, C4-bp and the C3b-C4bINA cofactor are the same protein.

Summary

Two forms of C4-binding protein (C4-bp) (C4-bp low, C4-bp high), which differ slightly in net charge and apparent molecular weight, as determined by SDS-PAGE, were separated by ion-exchange chromatography and contaminants removed with specific antisera. Both forms of C4-bp served as cofactors for the cleavage of C4b in solution by C3b inactivator, and the resulting fragments of the $\alpha'$-chain of C4b had identical molecular weights. In addition, similarly to $\beta_1H$, C4-bp low or high served as cofactors for the cleavage of fluid phase C3b by C3bINA. However, important quantitative differences between the activities of C4-bp and $\beta_1H$ were observed. With regard to C3b in solution, the cofactor activity of $\beta_1H$ was $\approx$20 times greater than that of C4-bp on a weight basis. In relation to cell-bound C3b, the differences in activity were even more marked. Whereas $\beta_1H$ enhanced the effects of C3bINA on the erythrocyte intermediate EC3b, inhibiting the assembly of EC3bBb, C4-bp was without effect even at concentrations $\approx$300 times greater than $\beta_1H$. Therefore, under physiological conditions, it is likely that $\beta_1H$ is the key protein which controls the function of C3b, and that C4-bp activity is directed mainly toward the cleavage of C4b.

We also examined the relation between C4-bp and the C3b-C4bINA cofactor described by Stroud and collaborators (3, 4). By functional, physico-chemical and immunological criteria, they are the same protein.

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