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Immunology

Modulation of the classical pathway C3 convertase by plasma proteins C4 binding protein and C3b inactivator

(C4b cleavage/C2a decay)

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ABSTRACT **We recently described the isolation from human serum of a serum protein (C4 binding protein) that functions as an essential cofactor for C3b inactivator in the proteolysis of fluid-phase C4b and, to a much lesser extent, C3b. We show here the role of C4 binding protein in the formation and function of the classical pathway C3 convertase ($C\overline{42}$). C4 binding protein interferes with the assembly of the membrane-bound C3 convertase of the classical pathway and accelerates the decay of $C\overline{42}$ in a dose-dependent fashion. Its removal from serum by means of specific immune absorption promotes the vigorous consumption of C3 after addition of $C\overline{1}$; this effect is abolished by reconstitution with purified C4 binding protein.**

Although C4 binding protein inhibits the hemolytic function of cell-bound C4b, we did not detect any change in the structure of C4b even after prolonged incubations of $EAC\overline{1}4$ with C4 binding protein. For this reason, and on the basis of studies of the time required for maximal reactivity (T_{max}) of cellular intermediates generated in the presence of C4 binding protein and limited amounts of C2, we conclude that the effects of C4 binding protein are probably mediated by displacing C2a from specific binding sites on C4b. In addition, C4 binding protein enhances the cleavage by C3b inactivator of the α' chain of cell-bound C4b. When $EAC\overline{1}4$ cells were incubated with both control proteins, the T_{max} of the cells was prolonged and the lysis was markedly diminished. We conclude that C4 binding protein and C3b inactivator control the C3 convertase of the classical pathway in a fashion similar to that described for β1H and C3b inactivator in the alternative pathway.

The classical complement pathway is initiated by the interaction of antigen–antibody complexes with the first component of complement, C1 (1, 2). The bound C1 develops proteolytic activity ($C\overline{1}$) (3) that is responsible for the activation of C4 (4) and C2 (5) and the formation of an enzymatic active complex (C4b2a or $C\overline{42}$), the C3 convertase (6), which mediates the cleavage of C3 into C3a and C3b. The $C\overline{42}$ enzyme is labile due to the decay of $C\overline{2}$ (C2a) (7), but it can be regenerated on the residual $C\overline{4}$ (C4b) by the uptake of additional C2 and its cleavage by $C\overline{1}$ (7).

The orderly progression of the classical pathway is modulated, in part, by this instability of the $C\overline{42}$ enzyme (7). In this paper we describe a previously unrecognized control of the classical C3 convertase by a normal serum protein, C4 binding protein (C4-bp) (8). This protein serves as a cofactor for the enzyme C3b inactivator (C3bINA) in the further degradation of fluid-phase C4b (9, 10) and, to a lesser extent, C3b (11, 12). The experiments described here demonstrate that C4-bp acts as a modulator of the $C\overline{42}$ enzyme, accelerating the decay of C2a. In addition, they show that C4-bp participates, in conjunction with C3bINA, in the inactivation of the hemolytically active C4b bound to the erythrocyte membrane, thereby preventing the regeneration of the classical pathway convertase.

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MATERIALS AND METHODS

Buffers. Veronal-buffered saline (pH 7.5), 0.15 M, containing 0.1% gelatin (GVB); GVB containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (GVB^{2+}); 75 mM dextrose/veronal-buffered saline ($DGVB^{2+}$); and 40 mM EDTA buffer in GVB (EDTA GVB) were prepared as described (13).

Purified Complement Components. Guinea pig (13) and human $C\overline{1}$ and C1s (14), human C4 (15), C2 (16), oxidized C2 (17), C4-bp (8), β1H, and C3bINA (18, 19) were purified by published techniques. C4 was radiolabeled with ^{125}I as described (20); the specific activity was $\approx 3 \times 10^5$ cpm/μg of protein. These proteins were quantitated either by hemolytic techniques or by determination of their protein concentration by the method of Lowry *et al.* (21) or by their absorbance at 280 nm, assuming that an absorptivity 1.0 equals 1 mg of protein per ml.

Cellular Intermediates and Assays. EA and $EAC\overline{1}$ were prepared from sheep erythrocytes as described and converted to $EAC\overline{1}4$ by using highly purified C4 at a concentration of 6 μg/1 $\times 10^8$ cells (22). The specific activity of C4 was 1.1×10^{10} units/μg of protein. The C4 deposited on the cellular intermediate represented 10% of the C4 input, as assessed by uptake of ^{125}I-labeled C4. C4, C2, and C3 were measured by hemolytic titrations (23). Rat serum diluted 1:20 in EDTA GVB was used as a source of C3–C9. C5, C6, C7, C8, and C9 used in C3 titrations were purchased from Cordis Laboratories (Miami, FL).

Depletion of C4-bp from Normal Human Serum. Rabbit antiserum against C4-bp was prepared by injecting rabbits with purified C4-bp incorporated in complete Freund's adjuvant. By crossimmunoelectrophoresis the antiserum reacted against a single protein in human serum and against purified C4-bp. The IgG fraction of this antiserum, obtained by DEAE-cellulose chromatography, was coupled to CNBr-activated Sepharose 4B (Pharmacia), following directions from the manufacturer.

Serum depleted of C4-bp was obtained by passage of 2 ml of fresh normal human serum containing 20 mM EDTA through an anti-C4-bp immunoabsorbent column equilibrated with EDTA GVB. The effluent was concentrated to the original serum volume by ultrafiltration.

Structural Analysis of Fluid-Phase and Cell-Bound ^{125}I-Labeled C4. Slab-gel sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide electrophoresis was performed as described by Laemmli (24).

Cellular intermediates carrying ^{125}I-labeled C4 were lysed

Abbreviations: C4-bp, C4 binding protein; C3bINA, C3b inactivator; $C\overline{42}$, classical pathway C3 convertase; $DGVB^{2+}$, half-isotonic Veronal-buffered saline/0.1% gelatin/ 0.5 mM $MgCl_2$/0.15 mM $CaCl_2$/2.5% dextrose; DGVB-EDTA, half-isotonic Veronal-buffered saline/0.1% gelatin/10 mM EDTA/2.5% dextrose; EDTA GVB, Veronal-buffered saline/40 mM EDTA; $NaDodSO_4$, sodium dodecyl sulfate.

in 50 mM Tris-HCl, pH 6.8/2% $NaDodSO_4$/6 M urea/5% 2-mercaptoethanol. The samples were heated for 3 min in boiling water and applied to 7.5% $NaDodSO_4$/polyacrylamide gels. After electrophoresis, the gels were stained, dried, and sliced into 1-mm segments, and radioactivity was measured. Immunoprecipitation was performed as described (22).

RESULTS

Inhibition of Hemolytic Activity and Structural Modification of Cell-Bound C4b (EAC$\overline{14}$) by C4-bp and C3bINA. As shown previously, fluid-phase C4b is cleaved by the serum enzyme C3bINA only in the presence of C4-bp (10). To elucidate whether these proteins also participate in the inactivation of cell-bound C4b, we performed the following experiments. EAC$\overline{14}$ cells, prepared with ^{125}I-labeled C4, were divided into four 4-ml samples at a concentration of 1×10^8/ml and incubated with 1 ml of either $DGVB^{2+}$ alone or $DGVB^{2+}$ containing 20 μg of C4-bp, 3 μg of C3bINA, or C4-bp and C3bINA. After 2 hr at 37°C, the cells were centrifuged and the radioactivity of the supernatants was measured. The cells were washed three times with cold $DGVB^{2+}$ and resuspended in the same buffer at 1×10^8/ml. Aliquots of each cell sample were saved for structural analysis of the deposited ^{125}I-labeled C4b. The hemolytic activity of each sample of EAC$\overline{14}$ cells was measured by determining its ability to form C$\overline{42}$ in the presence of a limited C2 input as follows. The four samples of EAC$\overline{14}$ cells, warmed to 30°C, were mixed with an equal volume of C2, diluted to generate approximately two hemolytic sites, and incubated at 30°C. At zero time and intervals thereafter, 0.5-ml samples were removed from each mixture and added to 0.75 ml of rat complement diluted in EDTA GVB to measure C3 convertase activity. After 60 min of incubation at 37°C, lysis of the erythrocytes was determined.

As seen in Fig. 1, the number of SC$\overline{42}$ generated ($\overline{Z}$) was markedly reduced in the samples in which the EAC$\overline{14}$ cells were incubated with C4-bp and C3bINA. Moreover, the time of maximal reactivity (T_{max}) of the EAC$\overline{14}$ cells, which reflects the number of hemolytically active EAC14, was prolonged to more than 6 min as compared to the 3.5 min observed in the sample incubated with buffer. Cells treated with C4-bp alone, although their T_{max} did not change, consistently exhibited a lower lysis than controls. C3bINA slightly diminished the reactivity of the EAC$\overline{14}$ cells. This inhibition was concentration dependent. However, even at concentrations higher than present in normal serum, C3bINA inhibited only 30% of hemolysis of EAC$\overline{14}$ and the T_{max} of the cells remained the same as that of the control.

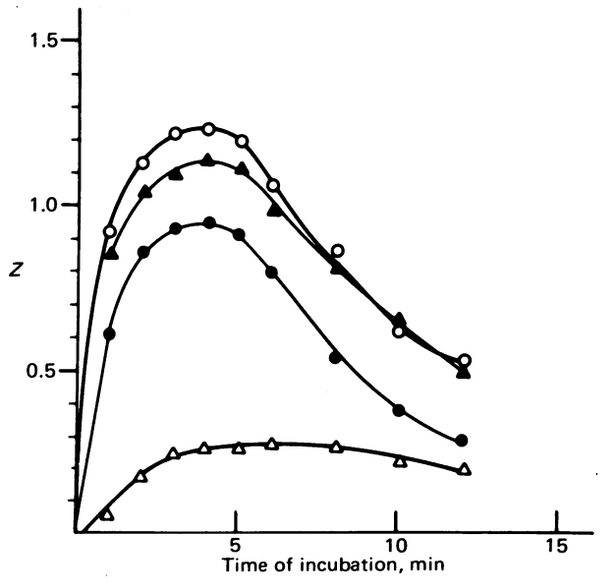

FIG. 1. Time of maximal reactivity (T_{max}) of EAC$\overline{14}$ (^{125}I-labeled C4) after treatment with $DGVB^{2+}$ alone (○) and $DGVB^{2+}$ plus C3bINA (▲), C4-bp (●), or C4-bp and C3bINA (△). Cells were washed and mixed at 0 time with limited C2. Final concentrations of C4-bp and C3bINA were 20 and 3 μg/ml, respectively. Z represents the number of C$\overline{142}$ formed and equals the natural negative log of (1 − % lysis).

The structural changes of cell-bound C4b mediated by C3bINA and C4-bp are shown in Fig. 2. Aliquots of the EAC$\overline{14}$ (^{125}I-labeled C4) cells used in the experiment above were treated as described in *Materials and Methods* and applied to $NaDodSO_4$/polyacrylamide gels. After electrophoresis, the gels were dried and cut into 1-mm slices, and radioactivity was measured in a gamma counter. The results showed the expected α', β, and γ chains of C4b in the samples incubated with buffer alone and with C4-bp, whereas the samples incubated with either C3bINA or with C3bINA and C4-bp revealed three additional bands of radioactivity (α2, α3, and α4). No high molecular weight material was observed on top of the gel. The supernatants corresponding to the latter contained C4c (α3, α4, β, and γ chains), as demonstrated by immunoprecipitation with antibody against C4, followed by $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography (not shown). The supernatant of cells incubated with buffer or C4-bp contained only C4b (α', β, and γ chains).

Acceleration of Decay Rate of Classical Pathway C3 Convertase by C4-bp. The effect of C4-bp on the decay of EAC$\overline{142}$ was investigated kinetically. EAC$\overline{142}$ were generated by incubating EAC$\overline{14}$ cells at 1×10^8/ml with an equal volume of C2 diluted in $DGVB^{2+}$ (2.5 hemolytic units/ml). The cells were washed in ice-cold $DGVB^{2+}$, resuspended at 1×10^8 cells per ml, and divided into four equal samples. One aliquot received 6 ml of $DGVB^{2+}$, the other three received equal volumes of $DGVB^{2+}$ containing C4-bp at final concentrations of 3, 1.5, and 0.75 μg/1×10^8 cells. The mixtures were incubated at 30°C, and 1-ml samples were removed from each tube at time intervals to measure the residual C3 convertase activity. As shown in Fig. 3*A*, the half-life of the C$\overline{42}$ enzyme in buffer alone was 7.5 min, and it diminished with increasing concentrations of C4-bp. The loss of convertase activity followed first-order kinetics at each concentration of C4-bp.

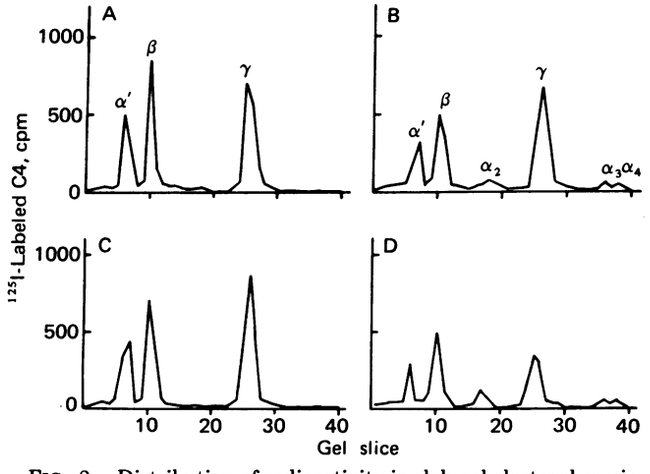

FIG. 2. Distribution of radioactivity in slab-gel electrophoresis of cellular intermediates carrying ^{125}I-labeled C4 (EAC$\overline{14}$) after treatment with $DGVB^{2+}$ alone (*A*) and $DGVB^{2+}$ plus C3bINA (*B*), C4-bp (*C*), and C4-bp and C3bINA (*D*). Concentrations of C4-bp and C3bINA are as for Fig. 1.

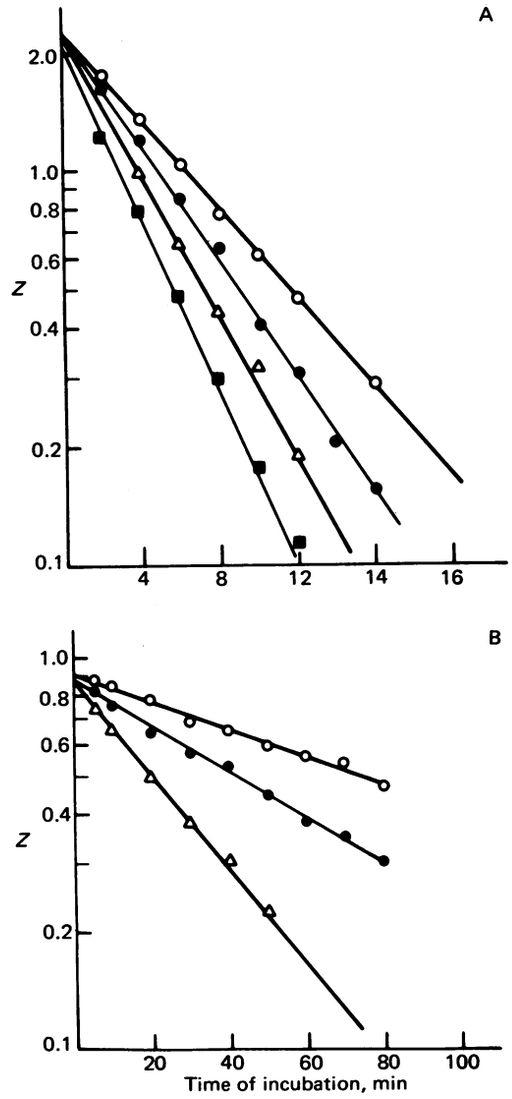

FIG. 3. (*A*) Decay rate at 30°C of EAC$\overline{142}$ incubated with DGVB^{2+} alone (○) or with DGVB^{2+} plus C4-bp at concentrations of 3.0 (■), 1.5 (△), and 0.75 (●) μg/1 × 10^8 cells. (*B*) Decay rate at 30°C of EAC$\overline{142}$ prepared with oxidized C2. Cells were incubated with DGVB^{2+} alone (○) or with DGVB^{2+} plus C4-bp at concentrations of 10 (●) and 25 (△) μg/ml. In *A* and *B*, the ordinate depicts the hemolytic activity of the EAC$\overline{142}$ during the decay time, which is represented by the abcissa.

In a similar experiment, we demonstrated that C4-bp accelerated the decay of the more stable C3 convertase prepared with oxidized C2 (Fig. 3*B*). The half-life of the stabilized convertase in buffer alone (80 min) was shortened to 20 min in the presence of C4-bp (25 μg/ml). At the same concentration, β1H, which is the accelerating factor for decay of the alternative pathway C3 convertase, failed to affect the decay rate of both EAC$\overline{142}$ and EAC$\overline{14}^{oxy}2$.

Effect of C4-bp on Formation and Regeneration of Classical Pathway C3 Convertase. Because C4-bp accelerated the functional decay of stabilized and unstabilized C3 convertase, its capacity to prevent the formation and regeneration of the C$\overline{42}$ convertase was examined. EAC$\overline{142}$ cells (1 × 10^8/ml), prepared with a large excess of C2, were incubated for 2 hr in DGVB^{2+} alone or in DGVB^{2+} containing C4-bp (10 μg/1 × 10^8 cells). Samples of EAC$\overline{14}$ cells were similarly treated. The four reaction mixtures were washed twice in cold DGVB^{2+} and resuspended in the same buffer, warmed to 30°C, which contained C2 at a concentration of approximately 1 hemolytic unit per cell. Incubation of the mixtures at 30°C was initiated immediately upon mixing and the formation of C3 convertase was measured at time intervals.

As shown in Fig. 4, both the EAC$\overline{142}$ that had been incubated in buffer and the original EAC$\overline{14}$ cells were equally lysed and had a T_{max} of 2.5 min. On the other hand, much less lysis was observed in the tubes containing EAC$\overline{14}$ or EAC$\overline{142}$ cells decayed in the presence of C4-bp. The T_{max} of these cells was, however, unaltered. These data suggest that C4-bp remains bound to the cells carrying C4b even after several washings and inhibits the expression of the C3 convertase probably by accelerating the decay of the enzyme or preventing the binding of C2 (or both). That this may be the case is shown by the following experiment. EAC$\overline{14}$ cells were incubated for 1 hr at 30°C with either buffer alone or buffer and C4-bp at a concentration of 10 μg/1 × 10^8 cells, washed several times, and converted to EAC$\overline{142}$ with a relative excess of C2. After washing, the half-life at 30°C of the convertase formed on the control cells was 9 min, whereas that formed on the cells that had been incubated with C4-bp and washed prior to the addition of C2 was 5 min.

The efficiency of C4-bp in preventing the formation of the C3 convertase was concentration dependent. Duplicate samples of a total of 5 × 10^8 EAC$\overline{14}$ cells in volumes of 0.5 or 5 ml were incubated at 37°C for 2 hr with 1 ml of C4-bp (10 μg) or of buffer. The cells were washed, and each sample was resuspended to a concentration of 1 × 10^8/ml and used to measure T_{max}. Marked inhibition of C$\overline{42}$ formation occurred only in those samples prepared with higher concentrations of cells and C4-bp per unit volume. C4-bp was effective at concentrations of less than 10 μg/ml, which represents about 4% of the normal serum levels (±250 μg/ml).

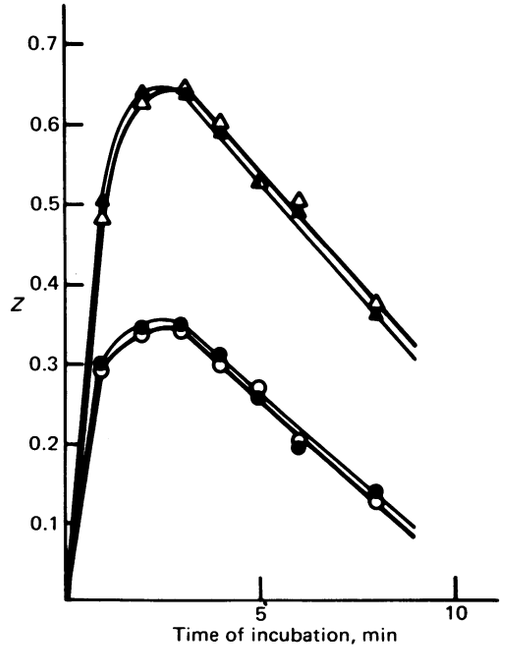

FIG. 4. T_{max} of EAC$\overline{14}$ (△,○) and EAC$\overline{142}$ (▲,●) incubated either in DGVB^{2+} alone (△,▲) or in DGVB^{2+} plus C4-bp (10 μg/1 × 10^8 cells) (○,●). After the cells were washed, they were mixed with a limited concentration of C2. Samples were removed at time intervals and lysed with rat serum diluted 1:20 in EDTA GVB. The ordinate represents the number of hemolytically active EAC$\overline{142}$ generated at each time of sampling.

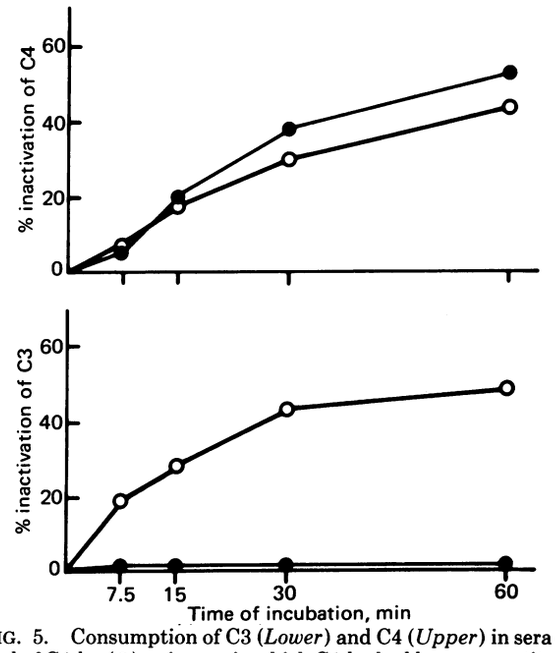

FIG. 5. Consumption of C3 (*Lower*) and C4 (*Upper*) in sera depleted of C4-bp (○) or in sera in which C4-bp had been reconstituted (●). Complement activation was initiated by addition of 5 μg of $\overline{C1s}$.

Formation of C3 Convertase in Normal Human Serum Is Controlled by C4-bp. The previous experiments demonstrate that C4-bp controls the assembly and function of the classical pathway C3 convertase generated with purified reagents, but did not address themselves to the function of C4-bp in normal human serum. This question was studied in the following experiment. Duplicate samples of 40 μl of serum depleted of C4-bp by passage through a specific immunoabsorbent column were incubated at 37°C. One sample was reconstituted with 40 μl of GVB^{2+} containing purified C4-bp (15 μg) and the other with buffer alone. Five micrograms of $\overline{C1s}$ was added to both samples in a volume of 10 μl. At zero time and at time intervals thereafter, 10-μl samples were removed and titrated for residual C4 and C3 activity. As seen in Fig. 5, C4 was inactivated by $\overline{C1s}$ equally in the presence or absence of C4-bp. In contrast, C3 was consumed only in serum samples from which C4-bp had been removed. Addition of C4-bp to the depleted serum prevented cleavage of C3. Taken together, these data demonstrate that both with purified reagents and in normal human serum C4-bp controls the expression of the classical pathway C3 convertase.

DISCUSSION

Control of the classical pathway C3 convertase is critical to the regulation of the activation of the complement system and may well determine whether the initial activation of the classical pathway leads to the sequential utilization of the late-acting components as well as the recruitment of the alternative pathway through the amplification loop. Because the proteolytic site for C3 cleavage in the classical pathway convertase resides on the C2a fragment (25) and is expressed only during the binding of this fragment to C4b, release of C2a in the form of C2i results in the loss of convertase function and serves as the inherent control of the enzyme (26).

We show here that the decay of the classical pathway C3 convertase is considerably augmented by the high molecular weight, normal serum protein C4-bp. The effect of C4-bp is dose related (Fig. 3*A*), and the accelerated decay of $\overline{C42}$ continues to follow first-order kinetics. This suggests an immediate and progressive action of C4-bp on the convertase, possibly by means of an increased disassociation of C2a from the C4b site. The capacity of C4-bp to accelerate loss of convertase function is also apparent on the stabilized enzyme prepared with oxidized C2 (Fig. 3*B*). The amount of C4-bp required is severalfold greater, though still within its physiological concentration. It seems likely that there is a relationship between C4-bp and two proteins described in guinea pig serum, a decay accelerating factor (27) and a serum α-globulin (28).

C4-bp also interferes with the formation and regeneration of the classical pathway C3 convertase (Fig. 4). Cell-bound $\overline{C42}$ that had been decayed in the presence of C4-bp could still express convertase function if incubated in the presence of fresh C2. However, the enzyme formed on the decayed and C4-bp-treated erythrocyte intermediate had a shorter half-life than expected. This suggests that C4-bp remained associated with the cells and inhibited subsequent expression of the convertase activity without altering the number of the available $\overline{C14}$ sites.

In contrast, irreversible loss of function of $EA\overline{C14}$ is brought about by the concerted action of C3bINA and C4-bp. The observed increase in the T_{max} (Fig. 1) indicates that the number of hemolytically active sites has been reduced. C3bINA and C4-bp also inhibited the ability of $EA\overline{C14}$ and $EA\overline{C4}$ cells to lyse upon subsequent exposure to C4-deficient sera, whereas these proteins failed to alter the hemolytic activity of $EA\overline{C1}$ or EA (not shown).

It is likely that the effects of C3bINA and C4-bp on $EA\overline{C14}$ are the consequence of the cleavage of the α′ chain of C4b and release in the fluid phase of the C4c fragments (Fig. 2). The activity of C3bINA alone on $EA\overline{C14}$ was much less pronounced. Only at relatively high concentration did it alter the total hemolytic activity of the cells without affecting its T_{max}. Also, although it cleaved the α′ of C4b, much less C4c was released in the supernatant. These findings confirm and extend the observations of Cooper (29) and Whaley and Ruddy (30) and are in sharp contrast with the observed lack of effect of C3bINA on the structure of fluid-phase C4b (10, 11). It is possible that the site on the α′ chain of C4b that is susceptible to cleavage by C3bINA is available only after binding to C4-bp or the cell membrane.

In view of the high concentrations of C4c found in the serum of patients with hereditary angioedema (31) in the presence of normal C3 levels, it was of interest to investigate the role that C4-bp may have in controlling the function of the classical pathway C3 convertase in whole serum. The results of those experiments are shown in Fig. 5. Depletion of C4-bp from whole sera prior to addition of $\overline{C1s}$ allows the cleavage of C3, whereas reconstitution of the sera with purified C4-bp inhibited the inactivation of C3; yet both samples yielded the same utilization of C4.

In short, the activity of C4-bp on C4b,2a appears to be very similar to that of β1H on the function of C3bBb, the alternative pathway C3 convertase (32, 33). The control proteins appear to inhibit enzymatic function by competitively displacing either C2a or Bb and by serving as cofactors for cleavage of C4b or C3b or both. These findings further support the idea that the proteins that form the C3 convertase of the classical and alternative pathways and those that control their functions are homologous and may have originated by gene duplication. If this is correct, C4-bp and β1H may have a common origin.

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