

INTERACTION OF C4-BINDING PROTEIN WITH CELL-BOUND C4b

A Quantitative Analysis of Binding and the Role of C4-binding Protein in Proteolysis of Cell-bound C4b

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The classical pathway C3 convertase (C4b2a) is responsible for the cleavage of C3 and the subsequent assembly of the membrane attack complex (C5-9). The function of the C4b2a enzyme is limited by the decay of C2a, which is very labile at physiological temperature (1). There is evidence that two control proteins, C4-binding protein (C4-bp)¹ and C3b/C4b inactivator (I), modulate the activity of cell-bound C4b2a (2-4); C4-bp accelerates the decay of C2a and I blocks the capacity of C4b to form the C3 convertase by cleaving peptide bonds in this molecule. In addition, the C4-bp serves as an essential cofactor for I in the cleavage of fluid-phase C4b (5, 6).

The mechanism by which C4-bp produces these effects is of great interest. The most likely explanation is that C4-bp displaces C2a from specific binding sites on C4b, and that the direct binding of C4-bp to C4b leads to allosteric changes of C4b. Although C4-bp displays a specific binding affinity for fluid-phase C4b (7), a direct demonstration of the binding of C4-bp to cell-bound C4b has not yet been achieved. In this paper we describe the specific binding of C4-bp to cell-bound C4b and its quantitative analysis, and examine the effect of fluid-phase C4b and C2 on this binding. In addition, we show that the efficient binding of C4-bp to cell-bound C4b enhances the proteolytic activity of I.

Materials and Methods

Buffers. Isotonic veronal-buffered saline containing 0.1% gelatin, 0.5 mM MgCl₂ and 0.15 mM CaCl₂ (GVB); a mixture of equal volumes of GVB and 5% dextrose in water containing 0.1% gelatin, 0.5 mM MgCl₂ and 0.15 mM CaCl₂ (DGVB); and gelatin veronal-buffered saline containing 10 mM EDTA (EDTA-GVB) were used.

Purified Complement Components. Guinea pig C1 (8), human C1s (9), C4 (10), C2 (11), and I (12) were purified as described. C4-bp was purified by the method described (7), with minor modifications (13,14). The supernatants from barium citrate adsorption of human plasma were used as starting materials, followed by precipitation with polyethylene glycol and chromatog-

¹ *Abbreviations used in this paper:* C4-bp, C4-binding protein; DGVB, veronal-buffered saline with 2.5% dextrose containing 0.1% gelatin, 0.5 mM MgCl₂ and 0.15 mM CaCl₂; EA, sheep erythrocytes sensitized with rabbit IgG antibody; EAC1, EAC4, EAC14, cellular intermediates prepared with EA and bearing either C1, C4, or both; EAC4C4-bp, cellular intermediates prepared by incubating EAC4 with CH-bp; EDTA-GVB, veronal buffer containing 0.1% gelatin and 10 mM EDTA; GVB, isotonic veronal-buffered saline containing 0.1% gelatin, 0.5 mM MgCl₂ and 0.15 mM CaCl₂; I, C3b/C4b inactivator; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

raphy on ion exchanges. Analysis of the purified preparation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) revealed two bands that were identified as C4-bp (14). C4b was prepared by incubation of 400 μg C4 with 15 μg C1s at 37°C for 2 h. The protein concentrations of purified C4, C4-bp, and C2 were estimated by the method of Lowry et al. (15) using bovine serum albumin (BSA) as a standard.

Radiolabeling. Highly purified C4-bp was labeled with ^{125}I or ^{131}I (Amersham International Limited, Amersham, United Kingdom) by means of Enzymobeads (Bio-Rad Laboratories, Richmond, CA). Free iodine was removed by dialysis. The specific activities were 2.6×10^6 cpm/ μg protein and 2.1×10^5 cpm/ μg protein for ^{125}I and ^{131}I , respectively. C4 was labeled by the same method used for C4-bp. The specific activities were 2.3×10^6 cpm/ μg protein and 2.1×10^5 cpm/ μg protein for ^{125}I and ^{131}I , respectively. The correspondence between ^{125}I and ^{131}I counts and numbers of molecules of C4 or C4-bp were calculated from the specific activities of the iodinated proteins, using Avogadro's number and molecular weights of 200,000 and 500,000 for C4 and C4-bp, respectively.

Cellular Intermediates. EAC1 were prepared from antibody (IgG)-sensitized sheep erythrocytes (EA) by reacting with excess guinea pig C1 (5,000 site-forming units/cell). EAC14 were prepared by incubating EAC1 with purified C4 (either labeled or unlabeled) at a concentration of 20 $\mu\text{g}/1 \times 10^8$ cells. In some experiments, EAC14 cells bearing varying numbers of C4b-molecules/cell were also prepared by incubating EAC1 with different amounts of C4. The amount of C4b deposited on the EAC14 was determined by an uptake of labeled C4. To prepare EAC4, the EAC14 were suspended in EDTA-GVB and incubated for 15 min at 37°C. After washing twice, the EAC4 were resuspended in the same buffer.

Binding Assays. EAC4, prepared with ^{131}I -C4, were incubated for 30 min at 37°C with various amounts of ^{125}I -C4-bp. The cells were then sedimented by centrifugation, and the supernatants were removed. After washing once with EDTA-GVB, the cells were counted for radioactivity, and the amount of ^{125}I -C4-bp bound was determined. The experimental data were then plotted according to the Scatchard method (16): $r/c = nK - rK$, where r represents the number of C4-bp molecules bound per ^{131}I -C4b molecule, K is the average association constant, n is the number of binding sites per C4b, and c is the concentration of free bindable ^{125}I -C4-bp. In the final Scatchard plots, the best straight line for the experimental points was computed by the method of least squares using an HP-85 computer (Hewlett Packard, Corvallis, OR). K was obtained from the slope of this line and was converted to the more familiar liters per molar unit by using Avogadro's number and a molecular weight of 500,000 for C4-bp. The maximum number of C4-bp binding sites (n) was obtained from the intercept of the line with the abscissa.

Structural Analysis of Cell-bound ^{125}I -C4b. SDS-PAGE was performed according to Laemmli (17), and two-dimensional slab gel electrophoresis according to Law and Levine (18). Hydroxylamine treatment of gel strips was done as previously described (19). In all cases, a 3% stacking gel and 5–15% gradient running gels were used. Cellular intermediates, carrying ^{125}I -labeled C4, were lysed in 50 mM Tris-HCl, pH 6.8, containing 2% Na₂SO₄, 6 M urea, 10% glycerol, and 10% 2-mercaptoethanol. The samples were heated for 3 min in boiling water and subjected to SDS-PAGE. After electrophoresis, the gels were stained and dried. Radioautography was carried out by exposing the dried gel to a Fuji X-ray film Rx (Fuji Photo Film Co., Ltd., Japan) at –70°C overnight. Supernatant fluids were analyzed in the same manner. For estimation of the molecular weight of the bands, the following standards were used: 94,000 phosphorylase B; 68,000 BSA; 43,000 ovalbumin; 30,000 carbonic anhydrase; 20,100 soybean trypsin inhibitor.

Results

Binding of C4-bp to Cell-bound C4b. ^{125}I -C4-bp was incubated with varying numbers of EA, EAC1, EAC14, or EAC4 cells for 30 min at 37°C. After washing once with GVB, the amount of ^{125}I -C4-bp bound to cells was determined. As seen in Fig. 1, C4-bp bound specifically to both EAC14 and EAC4 cells, and the binding curves were virtually identical. Since the amount of C4-bp bound nonspecifically to EA is smaller than that to EAC1, we used EAC4 in further studies.

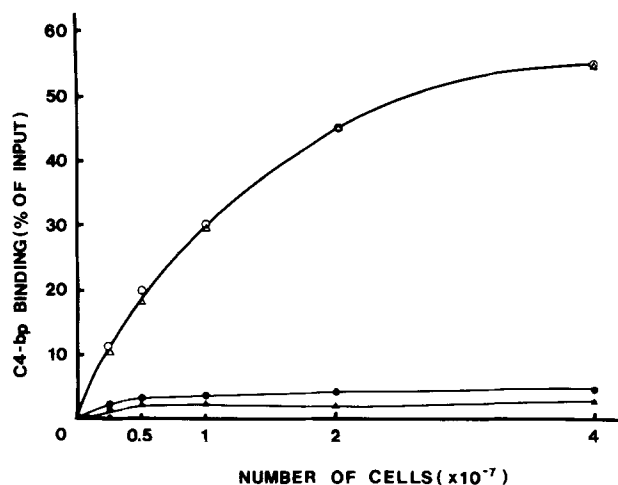


FIG. 1. Percentage of ^{125}I -C4-bp bound by varying numbers of EA (Δ), EAC1 (\bullet), EAC4 (Δ), or EAC14 (\circ). $10\ \mu\text{l}$ of ^{125}I -C4-bp ($1\ \mu\text{g}/\text{ml}$) was incubated for 30 min at 37°C with $400\ \mu\text{l}$ of varying numbers of EA, EAC1, EAC4, and EAC14. The tubes were centrifuged and the cell pellets were washed once with GVB. The percent of counts incorporated onto cells was determined by counting pellets and supernatant fluids.

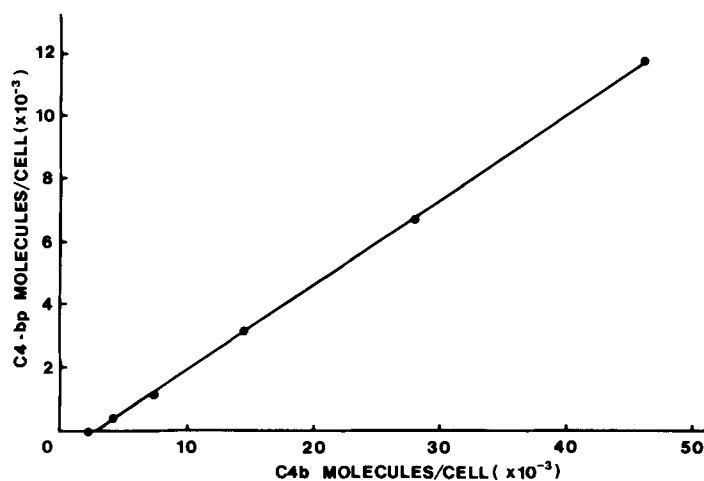


FIG. 2. Stoichiometry of interaction of C4-bp and cell-bound C4b. $10\ \mu\text{l}$ of ^{125}I -C4-bp ($20\ \mu\text{g}/\text{ml}$) was incubated with $50\ \mu\text{l}$ of EAC4 (1×10^8 cells/ml) bearing 2,160, 4,160, 7,600, 14,700, 27,800, or 45,900 C4b molecules/cell. After 30 min incubation at 37°C , the cells were separated by centrifugation and washed once with EDTA-GVB. The amount of C4-bp binding to cells was determined and the number of C4-bp molecules bound per cell was calculated.

Quantitative Analysis of C4-bp Binding. In the experiments shown in Fig. 2, we estimated the stoichiometry of interaction between C4-bp and cell-bound C4b. A constant amount of ^{125}I -C4-bp was incubated for 30 min at 37°C with EAC4 cells bearing increasing numbers of C4b molecules. The number of C4-bp molecules bound per cell was calculated. As illustrated, the number of C4-bp molecules bound per cell is proportional to the number of C4b molecules on the cell surface. However, only very small amounts of C4-bp bound to those EAC4 bearing $<3,000$ C4b-molecules/

cell on their surface, indicating that a relatively high C4b-density per cell is necessary for the binding of C4-bp.

Next, increasing amounts of ^{125}I -C4-bp were incubated with a constant number of EA or EAC4 bearing 46,000 C4b-molecules/cell. The specific binding of C4-bp to EAC4 is shown in Fig. 3A. The binding curve is convex to the abscissa indicating a saturable reaction. To estimate the binding constant of this reaction, the experimental data were subjected to a Scatchard analysis (16). As shown in Fig. 3B, Scatchard plots were linear. An associate constant (K) of $4.6 \times 10^8 \text{ L/M}$ was obtained from the slope of the line, and extrapolation to the abscissa indicates a maximum of 0.43 C4-bp molecules bound per C4b molecule, equivalent to an average of one molecule of C4-bp per two or three molecules of cell-bound C4b. From these findings, together with the previous results, it seems likely that one C4-bp molecule can not bind to single molecules of cell-bound C4b and requires two or more molecules of C4b in close proximity on the cell surface for binding.

Influence of Fluid-phase C4b on Binding of C4-bp to Cell-bound C4b. We investigated the specificity of interaction between C4-bp and cell-bound C4b. A constant number of EAC4 cells was mixed with increasing concentrations of either native C4 or C4b and incubated with ^{125}I -C4-bp for 30 min at 37°C . After washing, the binding of C4-bp to cells was determined. Fig. 4 shows that C4b prevented ^{125}I -C4-bp binding to the cells in a dose-dependent fashion, whereas we found that native C4 had little effect on

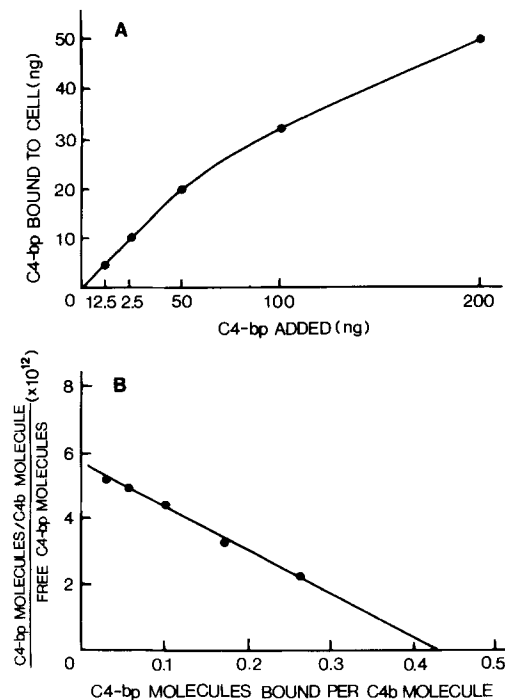


FIG. 3. Quantitative analysis of binding of ^{125}I -C4-bp to EAC4. Increasing amounts of ^{125}I -C4-bp (12.5–200 ng) were incubated for 30 min at 37°C with 5×10^6 cells of EA or EAC4 bearing 46,000 C4b molecules/cell in a total volume of 100 μl . After washing once with EDTA-GVB, the cells were counted for radioactivity. Specific binding of ^{125}I -C4-bp to EAC4 was calculated from the binding to EAC4 by subtracting the binding to EA. In the upper panel (A) the amount of ^{125}I -C4-bp is shown; the lower panel (B) is a Scatchard analysis of the same data.

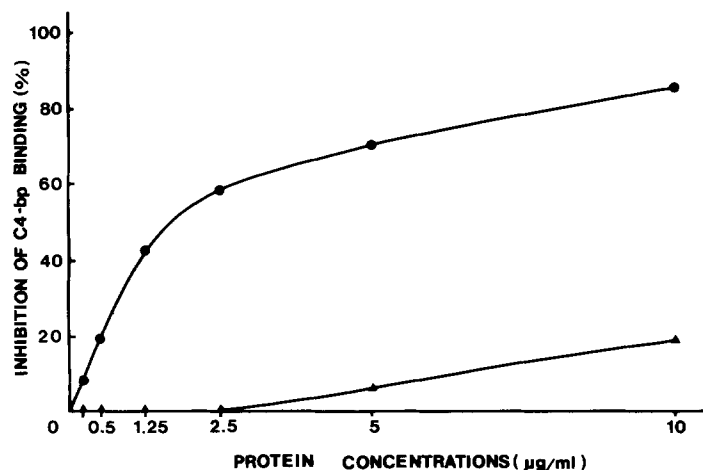


FIG. 4. Inhibition of ^{125}I -C4-bp binding to EAC4 by increasing amounts of C4 (▲) or C4b (●). Serially diluted C4 or C4b was added to 2×10^7 EAC4 bearing 39,600 C4b-molecules/cell. The mixtures were incubated for 30 min at 37°C with 24 ng of ^{125}I -C4-bp in a total volume of 200 μl . After washing once with EDTA-GVB, the cells were counted for radioactivity. Controls consisted of ^{125}I -C4-bp and EA or EAC4. Specific binding of ^{125}I -C4-bp to EAC4 was determined from the binding to EAC4 by subtracting the binding of EA and then the percentage of inhibition was calculated. The final concentrations of C4 or C4b (0.25–10 $\mu\text{g/ml}$) are shown in the abscissa, and the total amount of cell-bound C4b in the mixture was 1.32 $\mu\text{g/ml}$.

the incorporation of ^{125}I -C4-bp on EAC4 cells. In the presence of 1.7 $\mu\text{g/ml}$ of fluid-phase C4b, 50% of ^{125}I -C4-bp binding to cells was inhibited. This concentration is very close to the total amount of cell-bound C4b in the mixture, 1.32 $\mu\text{g/ml}$.

Influence of C2 on Binding of ^{125}I -C4-bp to Cell-bound C4b. In view of the reports (2, 3) that C4-bp accelerates the decay of C2 from EAC142 cells, we studied the effect of C2 on the binding of C4-bp to cell-bound C4b. ^{125}I -C4-bp was mixed with increasing concentrations of either C2 or, for comparison, unlabeled C4-bp. EAC14 cells were then added and after 10 min at 30°C , the amount of ^{125}I -C4-bp bound was determined. Fig. 5 shows that C2 inhibited the binding of ^{125}I -C4-bp to cells in a dose-related fashion; on a molar basis 27-fold more C2 than unlabeled C4-bp was required for 50% inhibition of ^{125}I -C4-bp binding, indicating that C4-bp binds to cell-bound C4b with higher affinity than C2.

Subsequent experiments shown in Fig. 6 demonstrated that C2 enhances the release of C4-bp from EAC4,C4-bp cells. EAC4,C4-bp cells were prepared by incubating EAC4 cells with ^{125}I -C4-bp for 30 min at 37°C . After washing, the EAC4,C4-bp cells were mixed with serially diluted C2, and then incubated for 30 min at 30°C . Supernatants and cells were separated by centrifugation and assessed for radioactivity. In the absence of C2, 8% of the bound C4-bp was released. The percentage of bound ^{125}I -C4-bp released was related to the concentration of C2 added. However, the maximum release was $\sim 50\%$, suggesting that a considerable amount of C4-bp remains bound to C4b on the cell surface even in the presence of high concentration of C2. On the basis of these results, it is clear that C4-bp and C2 compete for the same or similar binding sites on C4b.

Cofactor Activity of C4-bp for the Cleavage of Cell-bound C4b by I. In light of the previous experiments showing that binding of C4-bp to EAC4 cells required a relatively high

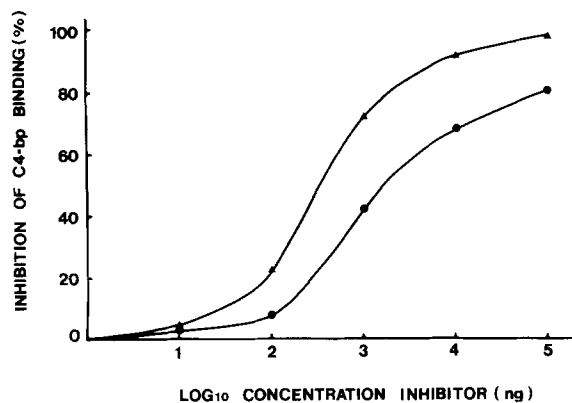


FIG. 5. Inhibition of ^{125}I -C4-bp binding to EAC14 by unlabeled C4-bp (▲) or C2 (●). 100 ng of ^{125}I -C4-bp was mixed with increasing concentrations of either C2 or unlabeled C4-bp. To these mixtures, 5×10^7 cells of EAC14 in DGVB were added in a total volume of 200 μl and incubated at 30°C . After 10 min, the cells were washed and counted for radioactivity. The percent inhibition of binding was calculated. The concentrations of unlabeled C4-bp and C2 required for 50% inhibition of ^{125}I -C4-bp binding to EAC14 were 300 and 1,600 ng, respectively.

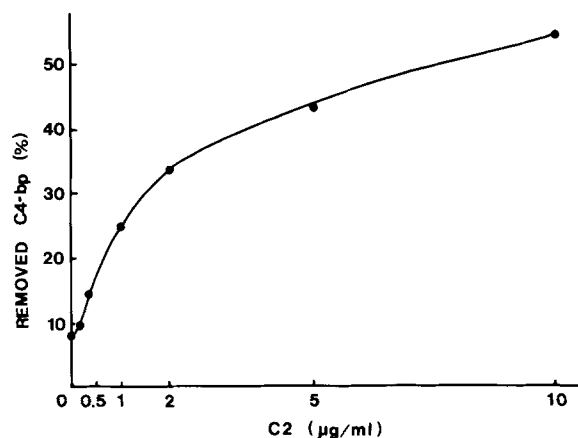


FIG. 6. Removal of ^{125}I -C4-bp from EAC4,C4-bp by C2. 1 ml of EAC4 ($1 \times 10^9/\text{ml}$) was incubated with 100 μl of ^{125}I -C4-bp (80 $\mu\text{g/ml}$) at 37°C . After 30 min, the cells were washed twice with EDTA-GVB, once with DGVB, and then resuspended in DGVB. 10.5% of the ^{125}I -C4-bp was incorporated into the cells. 100 μl of the EAC4,C4-bp cells was incubated with 100 μl of serially diluted C2 at 30°C for 30 min. After incubation, 2 ml of DGVB was added to each sample and the cells and supernatant fluids were separated by centrifugation. The percentage of counts removed from EAC4,C4-bp cells was determined by counting the cells and supernatant fluids.

C4b-density per cell, we prepared EAC4 cells carrying small or large amounts of C4b and evaluated cofactor activity of C4-bp in the cleavage of cell-bound C4b by I. Two sets of EAC4 bearing small or large amounts of C4b, prepared with ^{125}I -labeled C4, were incubated with ^{131}I -labeled C4-bp or buffer. After 30 min of incubation at 37°C , the cells were centrifuged, washed twice with ice-cold EDTA-GVB, and counted for radioactivity. The number of C4-bp molecules bound per cell was determined from the radioactivity. Each EAC4 was divided into three aliquots and incubated with buffer, I alone, or I plus C4-bp. EAC4,C4-bp was divided into two aliquots and incubated with either buffer or I. After 1 h at 37°C , the supernatant fluids were

removed, and the cells were washed twice with EDTA-GVB. Subsequently the radioactivity of the cells and supernatants was measured. The cells then lysed, the membranes were solubilized in SDS-urea, and the membranes and supernatants were subjected to SDS-PAGE followed by radioautography. As shown in Table I, only a very small amount of C4-bp bound to EAC4 bearing 3,000 C4b-molecules/cell. Almost the same amounts of radioactivity were released from EAC4 and EAC4,C4-bp by I even in the presence of C4-bp. In contrast, a considerable amount of C4-bp bound to EAC4 bearing 19,000 C4b-molecules/cell, and as a result of the binding C4-bp encouraged the release of C4c ($\alpha 3$, $\alpha 4$, β , and γ) by I, whereas the release of C4c from EAC4 by I alone was incomplete (Table I and Fig. 7).

SDS-PAGE analysis of the membranes revealed high molecular weight bands, presumably caused by an ester linkage of the α' chain of C4 with the membrane (19). To clarify the extent of cleavage of the α' chain, the gel strips were treated with hydroxylamine after electrophoresis of the identical samples, and a second electrophoresis perpendicular to the first dimension was performed. The results are shown in Fig. 8. Analysis of EAC4 revealed that the high molecular weight bands in the first dimension were completely displaced to the location of the α' chain of C4b, indicating that the α' chain had been bound to the erythrocyte membrane through an ester bond (Fig. 8A). These off-diagonal bands of the α' chain were completely cleaved by I in the presence of C4-bp. Consequently, the $\alpha 2$ fragment (C4d) remained associated with the membrane (Fig. 8C) and the $\alpha 3$, $\alpha 4$, β , and γ peptides (C4c) were found in the supernatant (Fig. 7). On the other hand, treatment of EAC4 with I alone resulted in incomplete proteolysis of the α' chain and an intermediate cleavage product, α -75 fragment, was observed in the same position of the β -chain (Fig. 8B). Therefore, we conclude that the direct binding of C4-bp to C4b on the cell surface enhances the enzymatic activity of I on cell-bound C4b.

TABLE I
Release of Radioactivity from EAC4 and EAC4 C4-bp by I*

Reaction mixture	C4b molecules/cell	Released counts of ^{125}I -C4
		%
EAC4 + buffer	3,000	7.1
EAC4 + I	3,000	41.4
EAC4 + I + C4-bp	3,000	47.3
EAC4,C4-bp‡ + buffer	3,000	4.1
EAC4,C4-bp + I	3,000	41.9
EAC4 + buffer	19,000	9.0
EAC4 + I	19,000	36.7
EAC4 + I + C4-bp	19,000	66.9
EAC4,C4-bp§ + buffer	19,000	8.1
EAC4,C4-bp + I	19,000	68.3

* Intermediate cells were prepared with ^{125}I -C4 and ^{131}I -C4-bp. 100 μl of various cells ($1 \times 10^9/\text{ml}$) were incubated at 37°C for 1 h with 30 μl of buffer containing either I (120 $\mu\text{g}/\text{ml}$), I + C4-bp (533 $\mu\text{g}/\text{ml}$), or alone.

‡ 120 molecules of C4-bp bound per cell.

§ 4,200 molecules of C4-bp bound per cell.

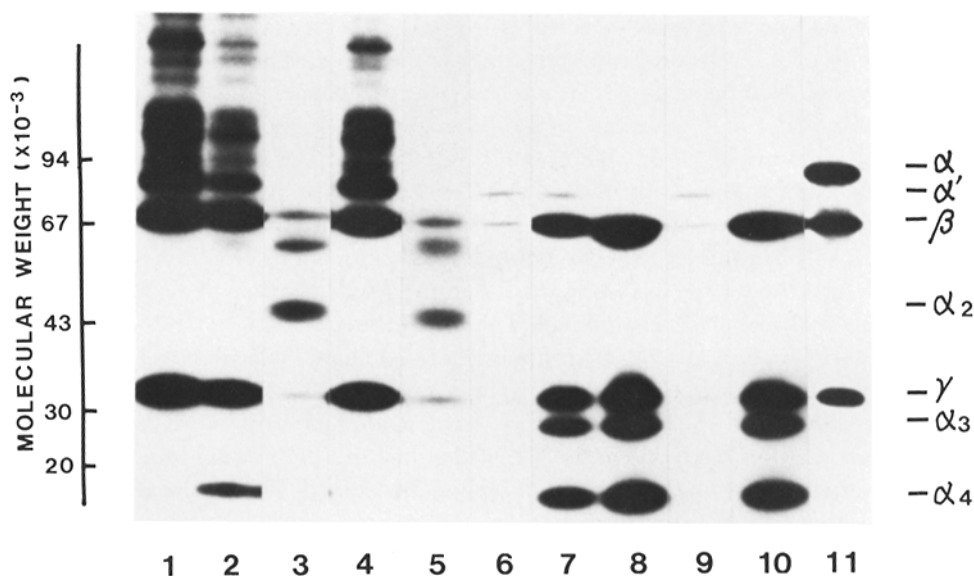
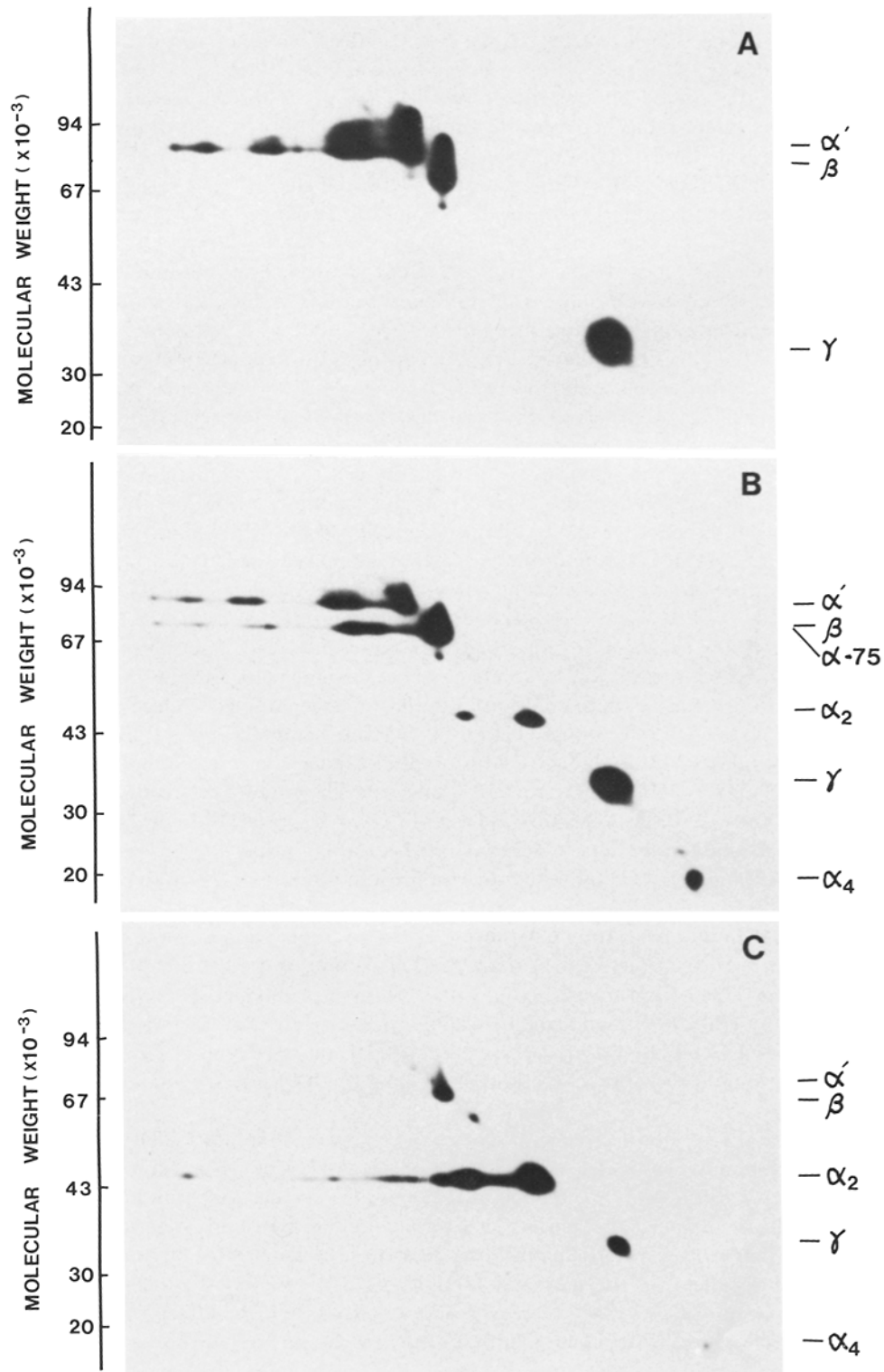


FIG. 7. Cleavage of C4b in EAC4 and EAC4,C4-bp by I. EAC4 bearing 19,000 C4b-molecules/cell, prepared with ^{125}I -C4, were incubated with buffer or ^{131}I -C4-bp ($8 \mu\text{g}/10^9$ cells) for 30 min at 37°C . The cells were washed twice with EDTA-GVB and resuspended in the same buffer. $200 \mu\text{l}$ of EAC4 (1×10^9) and EAC4,C4-bp (1×10^9) were each divided into several equal samples; one sample of each received $30 \mu\text{l}$ of either EDTA-GVB or I ($120 \mu\text{g}/\text{ml}$) and in addition, EAC4 received $30 \mu\text{l}$ of buffer containing both I ($120 \mu\text{g}/\text{ml}$) and C4-bp ($533 \mu\text{g}/\text{ml}$). After 1 h at 37°C , the samples were centrifuged and the supernatant fluids were carefully removed from the cells. After washing twice with EDTA-GVB, the cells were lysed and the membranes were solubilized in SDS-urea. These samples were analyzed by a 5–15% gradient SDS-PAGE under reducing conditions, followed by radioautography. EAC4 were treated with buffer (track 1), I (track 2), and I + C4-bp (track 3). EAC4,C4-bp were treated with buffer (track 4) and I (track 5). The supernatant of EAC4 treated with buffer (track 6), I (track 7), and I + C4-bp (track 8), and those of EAC4,C4-bp treated with buffer (track 9) and I (track 10) are also shown. Track 11 depicts ^{125}I -C4 used to prepare the cellular intermediates. C4b is composed of three polypeptide chains, α' , β , and γ . In addition to these chains, high molecular weight bands were observed in the lysates of EAC4 and EAC4,C4-bp. The counts of ^{131}I -C4-bp were exhausted and resulted in no band.

Discussion

It has been previously reported that a normal serum protein C4-bp, which has several binding sites for fluid-phase C4b (7), functions as an essential cofactor for I in the proteolysis of fluid-phase C4b (5). It has been also shown that C4-bp accelerates the decay rate of C4b2a (2, 3) by dissociating C2a from cell-bound C4b. Therefore,

FIG. 8. Two-dimensional electrophoresis of EAC4 and EAC4,C4-bp treated with I. Aliquots of the lysates of EAC4 and EAC4,C4-bp cells used in the previous experiments were subjected to a 15–15% SDS-PAGE under reducing conditions. Gel strips were cut out, treated with 1 M hydroxylamine, and dialyzed against 0.8 M Tris pH 8.6 containing 2% SDS, 6 M urea, and 10% 2-mercaptoethanol. These strips were then placed on top of a 5–15% gradient polyacrylamide gel and electrophoresis was performed. The radioautograph of EAC4 treated with EDTA-GVB (A) or I (B) and that of EAC4,C4-bp treated with I (C) are shown. Analysis of EAC4 revealed that the high molecular weight bands in the first dimension were completely displaced to the location of the α' chain of C4b, 90,000 mol wt. Identical results were obtained in EAC4,C4-bp treated with buffer (not shown). Treatment of EAC4 with I resulted in incomplete cleavage of the α' chain of C4 and an intermediate cleavage product (α -75) was observed in the same position of the β chain. In EAC4,C4-bp treated with I, spots of the α' chain disappeared and horizontal spots corresponding to the position of the α 2 chain were observed, which shows that the α' chain was completely cleaved by I in the presence of C4-bp, and that the α 2 fragment remained associated with the membrane.



an interaction between C4-bp and cell-bound C4b should occur, but the direct binding of C4-bp to cell-bound C4b has not actually been demonstrated. The main finding of this report is that C4-bp does bind to cell-bound C4b.

Evidence that the C4-bp is binding to the C4b and not to the cell membrane is seen in the direct relationship between the binding of ^{125}I -C4-bp and the amount of cell-bound C4b (Fig. 2). In addition, a very small amount of ^{125}I -C4-bp binds to the cell intermediate EA or EAC1 (Fig. 4), and the specificity of this reaction is also demonstrated by the inhibition of the C4-bp binding activity by fluid-phase C4b (Fig. 4).

The Scatchard method was chosen to analyze the C4-bp binding data. This approach has been used in many protein-ligand binding studies and, more recently, in cell receptor-protein binding situations (20, 21), and in a cell-bound C3b- β 1H binding study (22). Our quantitative analysis of C4-bp binding reveals that the maximum number of C4-bp bound per C4b molecule is 0.43, close to an average of one molecule of C4-bp per two C4b molecules. Since C4-bp has several binding sites (7), this finding could be explained by assuming that two binding sites on C4-bp bind to two C4b-molecules in close proximity on the cell surface. This may mean that single molecules of cell-bound C4b can not form stable complexes with C4-bp. In other words, interaction of one binding site on the C4-bp with a single molecule of C4b on the cell surface may be very weak, so that C4-bp is dissociated during washing. This assumption would also explain our observation that only a small amount of C4-bp binds to EAC4 bearing <3,000 C4-molecules/cell and that C4-bp binds stoichiometrically to EAC4 bearing >3,000 C4b-molecules/cell. Another possibility is that one binding site on C4-bp binds to two C4-molecules on the cell surface. However, this would be viable only if fluid-phase C4b contained a large amount of dimer of C4b, because fluid-phase C4b formed stable complexes with C4-bp stoichiometrically (7), and inhibited the C4-bp binding capacity in a dose-related manner (Fig. 4). Whatever the mechanism involved, it seems likely that a relatively high C4b-density on the cell surface is required to establish two or more C4b-molecules in close proximity to enable the stable binding of one C4-bp molecule.

The observation that C2 inhibited the equilibrium binding of C4-bp to C4b-coated cells (Fig. 5) is compatible with previous reports (2, 3) that C4-bp accelerates the decay of C4b2a by dissociating C2a from C4b. In addition, we now show that C4-bp has a much higher affinity to C4b than C2 (Fig. 5) and that a considerable amount of C4-bp remains bound to cell-bound C4b even in the presence of a large excess of C2 (Fig. 6). On the basis of these findings, it is likely that the mechanism of modulation of C4b2a by C4-bp is due to competitive displacement of C2a from C4b and to blocking of the uptake of additional C2 by the C4-bp that remains bound to C4b.

In addition, our findings clarify another mechanism of inhibition of C4b2a by C4-bp. Cell-bound C4b is cleaved by I alone in the absence of C4-bp (2), whereas the cofactor activity of C4-bp on cell-bound C4b remains unclear. According to Gigli et al. (2), C4-bp enhanced the activity of I on cell-bound C4b both functionally and structurally. However, a subsequent report (23) has shown that in the presence of I C4-bp has no effect on the hemolytic activity of EAC14 prepared with a limited concentration of C4. As shown in Figs. 7 and 8 and Table I, the efficient binding of C4-bp to cell-bound C4b encouraged the cleavage of C4b by I. In some situations,

therefore, C4-bp, in conjunction with I, limits the capacity of C4b to generate the C4b2a by cleaving C4b into C4c and C4d. As mentioned above, whether C4-bp binds to cell-bound C4b depends on the density of C4b on the cell surface. Thus, the effect of C4-bp, as well as β 1H (24, 25), is greatly influenced by the microenvironment in which the reaction takes place.

Another important finding is that the cleavage of cell-bound C4b consists of a two-step reaction. As shown in Figs. 7 and 8, the α' chain of C4b, which binds to the cell surface via a hydroxylamine-sensitive bond (19), is cleaved by I in the presence of C4-bp into three fragments, α 2, α 3, and α 4. The α 3, α 4, β , and γ peptides (C4c) were released from the erythrocytes, while the α 2 fragment (C4d) remained linked covalently to the cell membrane. On the other hand, cleavage of the α' chain by I alone was incomplete and an intermediate product (α -75 fragment) and an α 4 fragment were observed. On the basis of these findings, it appears that the first cleavage of the α' chain produces two fragments, α -75 and α 4, and subsequently a second cleavage occurs in the α -75, generating the cleavage products, α 2 and α 3. Similar findings have been previously reported using fluid-phase C4b (6).

The mechanisms by which the enzyme, I, alone is capable of cleaving cell-bound C4b and by which C4-bp enhances its reaction are unclear. Since C4b is the substrate of I, the C4b on the cell surface may be allosterically modified by the binding of the α' chain to the membrane via an ester bond, resulting in the exposure of the site for I cleavage. Subsequently, the first cleavage of the α' chain may induce additional allosterical changes and a concomitant exposure of the second cleavage site. Since there is no qualitative difference in cleavage of the α' chain by I between the presence and absence of C4-bp in spite of the great quantitative differences (Figs. 7 and 8), the binding of C4-bp to cell-bound C4b may enhance such allosterical changes.

Summary

Purified C4-binding protein (C4-bp) was shown to bind to cell-bound C4b by radioactive tracer techniques. With EAC4 bearing >3,000 C4b-molecules/cell, the number of C4-bp molecules bound was directly proportional to the number of C4b molecule on the cell surface; EAC4 bearing <3,000 C4b-molecules/cell bound a very small amount of C4-bp. Scatchard analysis of binding of C4-bp indicated an equilibrium constant of 4.6×10^8 L/M and a maximum of 0.43 C4-bp molecules bound per C4b molecule, equivalent to an average of one molecule of C4-bp per two or three molecules of C4b.

Fluid-phase C4b inhibited the binding of C4-bp to cell-bound C4b in a dose-dependent manner, whereas native C4 had little effect. C2 inhibited this binding and also released C4-bp from EAC4, C4-bp. However, C2 was 27 times less effective than unlabeled C4-bp on a molar basis and a considerable amount of C4-bp remained bound to C4b on the cell surface even in the presence of a large excess of C2.

We also examined the cofactor activity of C4-bp in the cleavage of cell-bound C4b by C3b/C4b inactivator (I). Cleavage of the α' chain of C4b on the cell surface by I alone was incomplete and an intermediate cleavage product, α -75, was observed. When C4-bp bound to C4b on the cell surface, the α' chain of the C4b cleaved into three fragments, α 2, α 3, and α 4. The α 3, α 4, β , and γ peptides (C4c) were released into the fluid phase, and the α 2 fragment (C4d) remained linked covalently to the

cell membrane via an ester bond. In some situations, therefore, C4-bp enhances the proteolytic activity of I on cell-bound C4b.

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