Function of Subunits within the Eighth Component of Human Complement: Selective Removal of the γ Chain Reveals It Has No Direct Role in Cytolysis†

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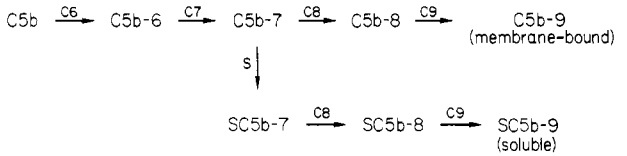
ABSTRACT: The eighth component of human complement (C8) consists of a disulfide-linked α–γ dimer that is noncovalently associated with β. Previous results from this laboratory established that two of these subunits have distinct roles in the cytolytic function of C8. Binding of C8 to the precursive C5b-7 complex is mediated strictly through β while interaction between C8 and the lipid bilayer of target membranes occurs primarily through α. In the present study, we examined the importance of the γ subunit in cytolysis by characterizing functional properties of C8′, a derivative of C8 that lacks γ. Preparation of this derivative was accomplished by limited cleavage of disulfide bonds in purified α–γ and separation of α from γ. When mixed, purified α and β combined to form C8′. When tested for functional similarity to normal C8, the following results were obtained. (1) Specific and saturable binding of C8′ to the C8 binding site on C5b-7 could be achieved. Significantly, the resulting C5b-8′ supported subsequent C9 binding and cell lysis, which was equivalent to that observed with C5b-8. (2) Complement activation of (C8 + C9)-depleted serum containing C8′ resulted in formation of SC5b-8′. Inclusion of C9 in the serum resulted in further conversion to SC5b-(8′)9. These observations indicate that C8′ is functionally similar to C8. Furthermore, they indicate that unlike α and β, the γ subunit has no direct role in facilitating C8 interaction with the nascent cytolytic complex or in mediating C9 binding and membrane lysis. On the basis of these and earlier results, a model is proposed that depicts the arrangement of C8 subunits within membrane-bound C5b-8.

Human C8[1] is a serum glycoprotein constituent of C5b-9, a macromolecular cytolytic complex of C5b, C6, C7, C8, and C9 (Müller-Eberhard, 1980; Esser, 1982). Assembly of this complex on target membranes begins with formation of C5b and proceeds in a sequential manner as follows:



Upon formation of C5b-7, a lipid binding site develops that facilitates association of the nascent complex with cell membranes. In subsequent steps, C8 and C9 bind sequentially to yield C5b-9, the fully assembled complex that produces membrane lysis. Activation of complement in the absence of membranes yields an alternative intermediate (SC5b-7) as a consequence of interaction between the S protein in serum and the lipid binding site on C5b-7. Although this interaction prevents binding of the nascent complex to membranes, sequential association of C8 and C9 still occurs to yield SC5b-9, the soluble, inactive counterpart of the cytolytic complex (Podack et al., 1977; Podack & Müller-Eberhard, 1980).

Our laboratory is investigating the chemical basis for the highly specific interactions between C5b-9 constituents themselves as well as the basis for their interaction with the membrane bilayer during cytolysis. Our studies have focused on human C8 and on defining structure–function relationships within this unusual protein. Human C8 contains three nonidentical subunits referred to as α (M_r 64 000), β (M_r 64 000), and γ (M_r 22 000) (Kolb & Müller-Eberhard, 1976; Steckel et al., 1980). These subunits occur as a disulfide-linked α–γ dimer that is noncovalently associated with β. Recently, we reported procedures for dissociating α–γ from β and purifying each in high yield (Steckel et al., 1980; Monahan et al., 1983). While neither subunit alone was functional, mixing equimolar amounts of each resulted in their physical recombination and complete restoration of C8 activity (Monahan & Sodetz, 1980). Our studies also showed that β alone has a high affinity for C5b-7, a finding which established that interaction of C8 with its binding site on C5b-7 is mediated strictly through β (Monahan & Sodetz, 1981). More recently, a distinct role for α has also been identified. A photosensitive, membrane-restricted probe was used to identify constituents of the cytolytic complex that insert into the lipid bilayer during lysis of natural membranes (Steckel et al., 1983). In both C5b-8 and C5b-9, α was found to be one of the predominant components inserted. Insertion of β was minimal and no evidence of γ insertion was detected. These results indicate that α has a major role in mediating C8 interaction with the membrane bilayer after incorporation into the cytolytic complex.

We have now evaluated the importance of γ in C8 function by preparing a derivative which lacks this subunit and characterizing its functional properties. Our results described in this report indicate that unlike α and β, γ is not essential for C8 function and thus has no direct role in the cytolytic process. A preliminary report of these results has appeared elsewhere (Brickner & Sodetz, 1983).

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[1] Abbreviations: complement proteins are named in accordance with recommendations in *Bull. W. H. O.* (1968); C8′, a derivative of C8 formed by combining ^{125}I-α with β; C8R, a derivative of C8 formed by combining ^{125}I-(α–γ) with β; EAC1-7, sheep erythrocytes carrying membrane-bound C5b-7; EAC1-8, EAC1-8′, EAC1-8R, and EAC1-7(β), sheep erythrocytes carrying membrane-bound C5b-8, C5b-8′, C5b-8R, and C5b-7(β), respectively; SC5b-8′ and SC5b-(8′)9, complexes of SC5b-7 and C8′ or C8′ and C9, respectively; GIGB, buffer containing 5 mM imidazole, 72.7 mM NaCl, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2.5% glucose, 0.05% gelatin, and 1 mg/mL bovine serum albumin, pH 7.3, at 25 °C; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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Experimental Procedures

Materials. Human Cohn fraction III was kindly provided by Cutter Laboratories, Berkeley, CA. Bovine serum albumin, zymosan, 4-vinylpyridine, and dithiothreitol were purchased from Sigma. 4-Vinylpyridine was distilled prior to use. Amino acid and *S*-(pyridylethyl)cysteine standards were purchased from Pierce.

Purification of Proteins. Human C8 (Steckel et al., 1980) and C9 (Biesecker & Müller-Eberhard, 1980) were isolated from Cohn fraction III by using procedures described elsewhere. The α–γ and β subunits of C8 were purified by molecular sieve chromatography in the presence of SDS as described previously (Steckel et al., 1980; Monahan et al., 1983). Molar concentrations were determined from published molecular weights and previously determined $\epsilon_{280}^{1\%}$ values of 14.9, 11.8, 13.7, and 9.88 for C8, α–γ, β, and C9, respectively.[2] These values were determined by quantitative amino acid analyses of samples in 10 mM sodium phosphate/0.15 M NaCl, pH 7.0. Radioiodinations were performed using immobilized lactoperoxidase and routinely yielded specific radioactivities of (2–4) × 10^7 cpm/nmol of protein.

Separation of α from γ was accomplished by molecular sieve chromatography after limited reduction and modification of α–γ. For a typical preparation, a sample of ^{125}I-(α–γ) at 0.2–0.3 mg/mL in 0.1 M sodium phosphate, pH 7.0, was incubated under N_2 with a 140-fold molar excess of dithiothreitol for 30 min at 25 °C. A 3-fold molar excess of 4-vinylpyridine relative to dithiothreitol was subsequently added and the solution was incubated under N_2 for 2 h at 25 °C. The solution was adjusted to 0.5 mg/mL bovine serum albumin and applied to a TSK-G3000SW HPLC column (0.7 × 50 cm) equilibrated in 0.1 M sodium phosphate, pH 7.0. Fractions containing ^{125}I-α or ^{125}I-γ were identified by the presence of radiolabel and were adjusted to 1 mg/mL bovine serum albumin prior to further use. The molar specific radioactivity of ^{125}I-α was assumed to be ~80% of the starting ^{125}I-(α–γ). This assumption was based on SDS–PAGE analysis of reduced ^{125}I-(α–γ), which indicated the distribution of radiolabel between α and γ is ~4:1.

To estimate the number of disulfides modified, parallel samples of unlabeled α–γ were treated similarly and then dialyzed against 1% acetic acid. These were lyophilized, hydrolyzed in 6 N HCl at 110 °C for 24 h, and subjected to amino acid analyses. The number of reduced and modified sulfhydryls was determined from the content of *S*-(pyridylethyl)cysteine.

Preparation of C8′ and C8R. Stock solutions of subunits were prepared that contained ^{125}I-α at 0.01 mg/mL or β at 0.12 mg/mL in 0.1 M sodium phosphate/1 mg/mL bovine serum albumin, pH 7.0. To prepare C8′, a fixed amount of ^{125}I-α was incubated with increasing amounts of β in a final volume of 250 μL. After 15 min at 25 °C, formation of C8′ was detected by centrifugation on 5–10% sucrose density gradients.

To obtain C8′ that was free of trace C8 contaminants, it was necessary to perform an adsorption step using EAC1-7. As noted later, this step takes advantage of the higher affinity of C8 for these cells relative to C8′ and permits complete removal of residual C8 with only minimal loss of C8′. In this procedure, ^{125}I-α was incubated as above with a 1.5-fold molar excess of β. This amount was required to ensure that ^{125}I-α association with β was complete. The resulting mixture of C8′ and excess β was then diluted with GIGB buffer such that 0.4 mL of mixture contained a total amount of β equal to twice the number of C8 binding sites on 1.2 × 10^9 EAC1-7 cells. Preparation of EAC1-7 and measurement of the number of C8 binding sites per cell were performed as described previously (Monahan & Sodetz, 1981). A volume of 0.4 mL of diluted mixture was then incubated at 37 °C for 30 min with 0.6 mL of EAC1-7 prepared at 2 × 10^9 cells/mL in GIGB. The supernatant containing C8′ was collected by centrifugation and used in further studies. Where analysis of membrane-bound radioactivity was desired, pelleted cells were resuspended, washed 3 times with GIGB, and then lysed with 5 mM EDTA, pH 8.0. Membranes were collected by centrifugation, washed with 5 mM EDTA, pH 8.0, and subjected to SDS–PAGE analysis as described below.

Control samples containing C8R were prepared by substituting ^{125}I-(α–γ) for ^{125}I-α in the above procedure. These were incubated with EAC1-7 under the same conditions as described for C8′.

Binding Assays. Direct binding of C8, C8′, C8R, α, or β to EAC1-7 was measured as described earlier for C8 (Monahan & Sodetz, 1981). In a typical assay, increasing amounts of radioiodinated ligand in 0.33 mL of GIGB were incubated for 30 min at 37 °C with 0.07 mL of 1 × 10^9 EAC1-7 cells/mL in the same buffer. Controls for nonspecific binding contained hemolysin-treated sheep erythrocytes instead of EAC1-7. Washing and correction for nonspecific binding were performed as described earlier. Moles of ^{125}I-C8, ^{125}I-α, or ^{125}I-β bound to EAC1-7 were determined from their respective molar specific radioactivities. Moles of C8′ or C8R bound were calculated based on the specific radioactivity of ^{125}I-α or ^{125}I-(α–γ) contained in each protein.

Binding of ^{125}I-C9 to EAC1-8, EAC1-8′, and EAC1-8R was performed by using procedures similar to those described earlier (Monahan et al., 1983). In order to facilitate direct comparisons of C9 binding, each type of cellular intermediate was prepared such that it carried approximately the same mole quantity of C8, C8′, or C8R. The exact quantity of each ligand required to achieve the desired amount bound was determined from the binding assays above. In a typical assay, increasing amounts of ^{125}I-C9 in 0.1 mL of GIGB were incubated for 35 min at 37 °C with 0.3 mL of each cell suspension prepared at 1 × 10^9 cells/mL. Cells and lysed membranes were collected and washed with GIGB by repeated centrifugation at 31000*g*. The mole quantity of ^{125}I-C9 bound was calculated from its known specific radioactivity and corrected by subtracting nonspecific binding to EAC1-7. The absorbance at 541 nm of supernatants from each incubation mixture was measured and used to correlate hemolysis with C9 binding.

Incorporation of C8′ into Soluble Complexes. Human serum depleted of both C8 and C9 was prepared by sequential passage of normal serum through affinity resins containing purified C8 antibodies or C9 antibodies (Monahan & Sodetz, 1980; Steckel et al., 1983). The ability of C8′ to interact with SC5b-7 was tested by mixing 150 μL of C8′ at 0.33 μg/mL in GIGB with 500 μL of (C8 + C9)-depleted serum. Controls were prepared by substituting ^{125}I-C8 or ^{125}I-α for C8′. Complement activation was initiated by addition of 50 μL of a 100 mg/mL suspension of zymosan in 0.01 M sodium phosphate–0.15 M NaCl, pH 7.2. Nonactivated controls received 50 μL of 0.14 M EDTA in the same buffer. After 1 h at 37 °C, 0.3 mL of mixture was analyzed by centrifugation on 15–40% sucrose density gradients.

To test for the ability of SC5b-8′ to bind C9, C8′ was mixed with (C8 + C9)-depleted serum as above but purified C9 was

[2] J. B. Monahan and J. M. Sodetz, unpublished results.

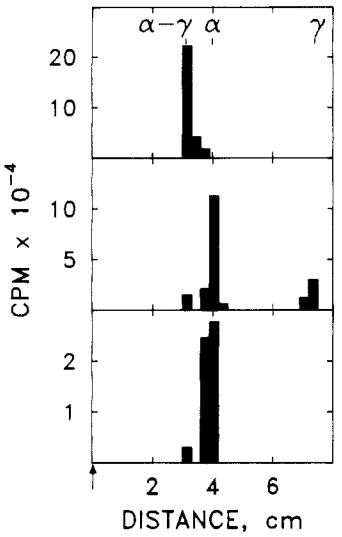

FIGURE 1: SDS–PAGE analysis of purified α. A sample of ^{125}I-(α–γ) was reduced, modified with 4-vinylpyridine, and then chromatographed on a molecular sieve column to separate α from γ. Results show radioactivity profiles from SDS–PAGE analysis of nonreduced samples obtained at different stages of the isolation procedure. Gel tops are indicated by an arrow while the inset shows the mobility of α–γ, α, and γ markers. Top: ^{125}I-(α–γ) prior to modification. Middle: ^{125}I-(α–γ) after reduction and modification. Bottom: Pool of ^{125}I-α after separation from ^{125}I-γ by HPLC.

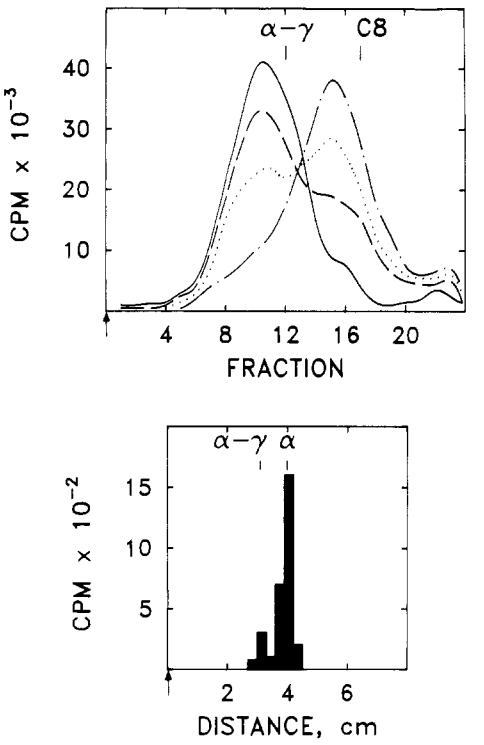

FIGURE 2: Analysis of the interaction of α with β to form C8′. Top: A 2-μg sample of ^{125}I-α was incubated as described in the text with increasing amounts of β in a final volume of 250 μL. Aliquots of 200 μL were analyzed on linear 5–10% (w/w) sucrose gradients prepared in 4.0 mL of 0.1 M sodium phosphate/1 mg/mL bovine serum albumin, pH 7.0. Centrifugation was performed at 4 °C in a Sorvall TV-865 vertical rotor at 202000*g* for 2 h. The top of each gradient is designated by an arrow. The inset shows the sedimentation position of α–γ and C8 markers. (—) ^{125}I-α; (---) a 1:0.5 molar ratio of ^{125}I-α to β; (···) a 1:1 molar ratio of ^{125}I-α to β; (-·-) a 1:1.5 molar ratio of ^{125}I-α to β. Bottom: SDS–PAGE analysis of C8′ formed by the association of ^{125}I-α with β. Fractions 15 and 16 from the above gradient analysis of a 1:1.5 mixture of ^{125}I-α and β were pooled and analyzed by SDS–PAGE under nonreducing conditions. The mobilities of α–γ and α markers are shown for reference.

also added to a final concentration of 0.50 μg/mL prior to activation. A radiolabeled SC5b-9 marker was prepared by substituting normal, unlabeled C8 for C8′ and ^{125}I-C9 for C9 in the above incubation mixtures. Activated and nonactivated samples were analyzed on sucrose density gradients as above.

Polyacrylamide Gels. SDS–PAGE was performed under nonreducing conditions on 5% polyacrylamide gels (Weber & Osborn, 1969). For analysis of C8′ in those supernatants remaining after adsorption with EAC1-7, samples were first adjusted to 5 mM EDTA and then 10 mM sodium phosphate/1% SDS, pH 7.2, prior to electrophoresis. For analysis of membrane-bound ligands, pelleted membranes were solubilized with 2 volumes of 20 mM sodium phosphate/5% SDS, pH 7.2. In all cases, radiolabeled components were located after slicing gels into 3-mm sections.

Results

Preparation of C8′. The C8′ used in these studies was prepared by combining ^{125}I-α with unlabeled β. In the initial step of this procedure, purified ^{125}I-(α–γ) was subjected to limited reduction to cleave interchain disulfide bond(s) that was (were) subsequently modified with 4-vinylpyridine. Quantitation of *S*-(pyridylethyl)cysteine after hydrolysis and amino acid analysis of modified α–γ indicated that one to three disulfide bonds were cleaved by the procedure. This mixture was then subjected to molecular sieve chromatography to separate α from γ. Figure 1 summarizes results from SDS–PAGE analysis of samples at various stages of the procedure. It is apparent that nearly complete conversion of α–γ to α and γ can be achieved under our modification conditions. Furthermore, α can be completely separated from γ by HPLC as evidenced by the absence of any ^{125}I-γ in the final pool. It is noted, however, that a trace amount of ^{125}I-(α–γ) remaining after reduction and modification is not separated by this step.

Having prepared α that was free of γ, we next tested its ability to interact with β. Such a possibility seemed reasonable since we previously observed that intact α–γ readily recombines with β at equimolar ratios to yield functional C8. The top panel of Figure 2 shows results obtained when samples of ^{125}I-α were incubated with increasing amounts of β and analyzed for formation of stable complexes by sucrose density gradient centrifugation. It is noted that with increasing amounts of β, the sedimentation position of ^{125}I-α shifts to a higher molecular weight. Complete assocation of ^{125}I-α occurs when β is present at a 1.5-fold molar excess.

Because α–γ and β readily associate to form C8, we were concerned that the presence of residual ^{125}I-(α–γ) in our ^{125}I-α preparation might affect results in Figure 2. Specifically, we were concerned that complexes detected on gradients may result from preferential association of β with residual ^{125}I-(α–γ) rather than ^{125}I-α. To examine this possibility, samples of complexes formed on gradients were subjected to SDS–PAGE analysis to confirm the presence of ^{125}I-α. Results from these experiments are shown in the bottom of Figure 2. As expected, some ^{125}I-(α–γ) is present as a consequence of association with β; however, the major component observed is ^{125}I-α. These results show conclusively that C8′ does form as a result of the stable association of α with β.

We next attempted to characterize the functional properties of C8′. Initial experiments were complicated by the fact that while forming C8′, residual ^{125}I-(α–γ) in our ^{125}I-α prepartion also combined with β to yield a small but significant amount of C8. The presence of even a trace of C8 was sufficient to interfere with an accurate evaluation of C8′ function. To circumvent this problem, an adsorption step using EAC1-7 was introduced. The presence of membrane-bound C5b-7 on these cells facilitated adsorption of residual C8 from the C8′

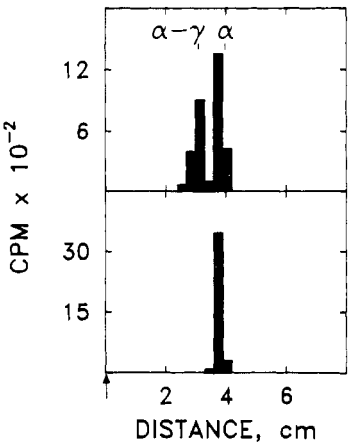

FIGURE 3: SDS–PAGE analysis of C8′ purified by adsorption with EAC1-7. Isolated ^{125}I-α, which still contained residual ^{125}I-(α–γ), was incubated with a 1.5-fold molar excess of β. Trace C8 formed in this mixture was separated from C8′ by incubating with a limited number of EAC1-7 as described in the text. Top: SDS–PAGE profile of a nonreduced sample of membranes recovered after incubating EAC1-7 with the above mixture. Bottom: SDS–PAGE profile of a nonreduced sample of the supernatant from this incubation.

preparation. The amount of EAC1-7 was adjusted such that all the C8 could be removed from solution with only minimal adsorption of C8′. This was possible because the affinity of C8 for EAC1-7 appeared to be substantially higher than that of C8′. Results that demonstrate the effectiveness of this adsorption are presented in Figure 3. The top of this figure shows SDS–PAGE profiles of solubilized membranes recovered after exposure of EAC1-7 to our C8′ preparation. Both the contaminating C8 and some C8′ become adsorbed to EAC1-7 as evidenced by the presence of ^{125}I-(α–γ) and ^{125}I-α on the membranes. Significantly, results in the bottom of Figure 3 show that only C8′ remains in the supernatant as indicated by the presence of ^{125}I-α and absence of ^{125}I-(α–γ). This procedure was used routinely to produce highly purified C8′ for use in all functional studies. Additionally, C8R was prepared as a control for this procedure by subjecting a mixture of ^{125}I-(α–γ) and excess β to the same adsorption conditions. Only C8R remaining in the supernatant was used in further studies.

Binding of C8′ to Membrane-Bound C5b-7. To test the ability of purified C8′ to interact with the C8 binding site on the nascent complex, binding assays were performed using increasing amounts of C8′ and a fixed amount of EAC1-7 as the source of membrane-bound C5b-7. Binding of C8′ was compared to that observed for normal C8 and C8R controls. Results shown in Figure 4 indicate that C8′ is indeed capable of specific binding to EAC1-7 although the amount bound at saturation is less than that observed for either C8 or C8R controls. This decreased binding is attributed to competition between C8′ and β. The source of this β is the excess used in preparing C8′ by combination with ^{125}I-α. A significant amount of it is retained along with C8′ in the supernatant after adsorption with EAC1-7. As noted earlier and as shown in a control experiment in Figure 4, β does bind specifically to the C8 site on EAC1-7 and thus could block the binding of C8′. Importantly, results in Figure 4 show that ^{125}I-α alone has no affinity for EAC1-7.

To confirm the identity of membrane-bound ligands in the above assay, samples of EAC1-8R and EAC1-8′ that were saturated with C8R and C8′, respectively, were lysed, solubilized, and analyzed by SDS–PAGE. The gel radioactivity profiles in the bottom of Figure 4 show that for EAC1-8R, only ^{125}I-(α–γ) and thus C8R is bound to the membranes. A

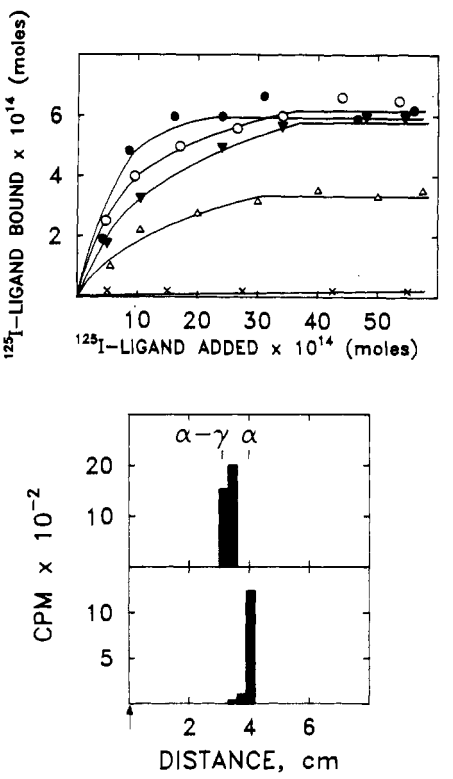

FIGURE 4: Binding of C8′ to EAC1-7. Top: Samples of C8′ were prepared by adsorption with EAC1-7 as described in Figure 3. Samples of C8R were prepared as controls by substituting ^{125}I-(α–γ) for ^{125}I-α in that procedure. Binding assays were performed as described in the text. Results show binding curves obtained for (●) ^{125}I-C8, (△) C8′, (○) C8R, (▼) ^{125}I-β, and (×) ^{125}I-α. Bottom: Samples of saturated EAC1-8′ or EAC1-8R were lysed, solubilized, and analyzed by SDS–PAGE to confirm the identity of membrane-bound ligand. Results in the upper panel show the gel profile obtained for EAC1-8R while the bottom shows corresponding results for EAC1-8′. The mobilities of α–γ and α markers are shown on the inset.

similar analysis of EAC1-8′ membranes indicates that only ^{125}I-α and thus C8′ is bound. These results provide further direct evidence that C8′ binds to the precursive C5b-7 complex on membranes.

Ability of C8′ To Support Formation of a Functional Cytolytic Complex. Experiments were performed next to determine if C8′ within membrane-bound C5b-8′ could mediate C9 binding and yield a C5b-(8′)9 complex. Binding of C9 to EAC1-8′ was measured and compared to that of EAC1-8 and EAC1-8R controls. Results from these experiments are shown in the top panel of Figure 5. It is important to note that each cell type used in these experiments contained approximately the same mole quantity of bound C8, C8′, or C8R. Consequently, the results provide a direct indication of the relative amount of C9 that can bind to C5b-8, C5b-8′, or C5b-8R. The binding curves in Figure 5 are similar and therefore indicate that C8′ within the nascent complex can support a level of C9 binding that is comparable to that observed with normal C8. Importantly, control experiments using EAC1-7(β) show only negligible C9 binding even though these cells are saturated with β. This confirms that C9 binding cannot be mediated through the small amount of β that is present along with C8′ on EAC1-8′.

To establish if binding of C9 to EAC1-8′ leads to formation of a functional cytolytic complex, the extent of hemolysis was quantitated at each point in the above binding assays. Results in the bottom panel of Figure 5 show that binding of C9 to EAC1-8′ does produce hemolysis and thus confirms that a functional C5b-(8′)9 complex forms on these cells. Further-

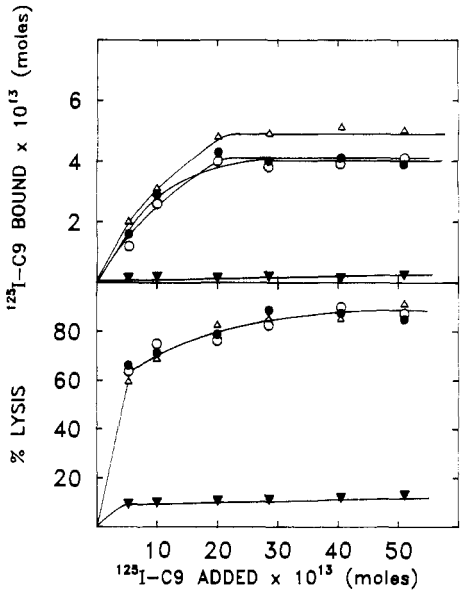

FIGURE 5: Binding of C9 to EAC1-8, EAC1-8′, and EAC1-8R and correlation with hemolysis. Data in Figure 4 were used as a guide for preparing each cell type such that it carried the same mole quantity of C8, C8′, or C8R. Typically, the amount of C8 or C8R on EAC1-8 or EAC1-8R was adjusted to be equivalent to the amount of C8′ bound to EAC1-8′ at saturation. As a control, EAC1-7(β), which carried saturating amounts of β, was also prepared. Binding assays were performed as described in the text. Hemolysis was quantitated from the absorbance of supernatants from each incubation mixture and expressed relative to the maximum expected for the number of cells used in the assay. Top: Results show binding of ^{125}I-C9 to (●) EAC1-8, (△) EAC1-8′, (○) EAC1-8R, and (▼) EAC1-7(β). Bottom: Hemolysis measured at each point in the above binding curves.

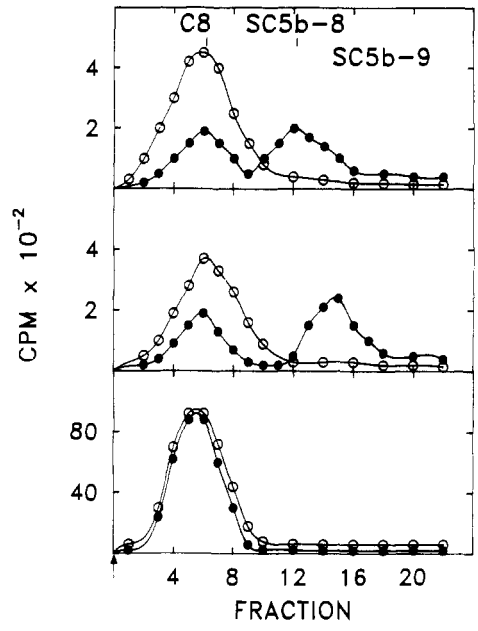

FIGURE 6: Sucrose density gradient analysis of the incorporation of C8′ into soluble terminal complexes in serum. Samples of purified C8′ or C8′ and C9 were incubated with (C8 + C9)-depleted serum and activated as described in the text. Analyses were performed on linear 15–40% (w/w) sucrose gradients prepared in 4.0 mL of 0.1 M sodium phosphate, pH 7.0. Centrifugation was performed at 4 °C on a Sorvall TV-865 vertical rotor for 2.5 h at 140000g. The top of each gradient is indicated by an arrow. The inset shows the sedimentation position of C8, SC5b-8, and SC5b-9 markers. Top: Results show sedimentation patterns of unactivated (○) and activated (●) (C8 + C9)-depleted serum containing C8′. Middle: Sedimentation patterns of unactivated (○) and activated (●) (C8 + C9)-depleted serum containing C8′ and supplemented with unlabeled C9. Bottom: Results from a control experiment showing the sedimentation patterns of unactivated (○) and activated (●) (C8 + C9)-depleted serum containing ^{125}I-α.

more, the equivalent amount of hemolysis observed for EAC1-8′ and EAC1-8 in the presence of C9 is consistent with the binding data and provides direct evidence of the functional similarity between C8′ and C8.

Incorporation of C8′ into Soluble Terminal Complexes in Serum. To support our observations in the membrane-bound system, we next examined the functional capabilities of C8′ in the soluble system of complement. Here, C8′ was added to human serum that had been depleted of all endogenous C8 and C9. Following serum activation, the association of C8′ with SC5b-7 was monitored by sucrose density gradients. Results in the upper panel of Figure 6 show that C8′ incorporates into a high molecular weight complex that sediments in a position similar to that of SC5b-8. These results indicate formation of SC5b-8′ and are consistent with our observation that C8′ interacts strongly with the C8 binding site on membrane-bound C5b-7. The middle panel of Figure 6 shows results from a similar experiment except that both C8′ and C9 were added to the serum prior to activation. In the presence of C9, the complex containing C8′ sediments at a position similar to that of SC5b-9. This indicates SC5b-(8′)9 formation and, as with the membrane-bound system, establishes that C8′ is indeed capable of supporting C9 incorporation into the terminal complex. Importantly, the control experiment in the lower panel of Figure 6 shows that ^{125}I-α alone does not participate in these reactions.

Discussion

Results in this study indicate the α subunit of C8 is capable of interacting with β to yield C8′, a derivative lacking the γ chain. This interaction appears to be specific since it occurs in the presence of high concentrations of bovine serum albumin. Furthermore, the affinity between these subunits must be substantial since only a 1.5-fold molar excess of β is required for complete association and formation of a complex that is stable to density gradient centrifugation. A similar high affinity was reported previously for α–γ and β (Steckel et al., 1980; Monahan & Sodetz, 1980). These subunits were shown to readily recombine at equimolar ratios to yield a molecule that is functionally and physically indistinguishable from native C8. Importantly, the present results indicate that γ is not essential for α–γ and β interaction within C8 and that such interaction must involve primarily α and β.

Regarding the functional properties of C8′, our results show that it binds readily to the C8 site on either membrane-bound C5b-7 or soluble SC5b-7 complexes. This finding indicates that γ is not required for binding and suggests that it has no direct role in C8 interaction with the nascent complex. This observation is consistent with our previous conclusion that it is β that is recognized by the binding site and therefore mediates C8 incorporation into the cytolytic complex (Monahan & Sodetz, 1981). In view of this functional role for β, it is not surprising that C8′, which also contains β, retains the ability to bind C5b-7.

Although not required for binding, our results indicate that γ does influence the affinity of C8 for C5b-7. Qualitative evidence of this is provided by the adsorption experiments using EAC1-7 to separate trace amounts of C8 from C8′. Results from analyses of membrane-bound and supernatant material in Figure 3 show that virtually all residual C8 in the C8′ preparation binds to EAC1-7 and thus effectively competes for the limited number of binding sites. Such competition is particularly significant considering the initial ratio of C8′ to C8 in this preparation is ~20:1 yet all the C8 is removed.

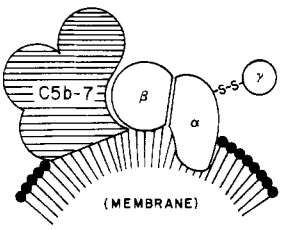

FIGURE 7: Schematic of C8 subunit arrangement within membrane-bound C5b-8.

Based on this, it seems reasonable to conclude that C8 has the higher affinity for C5b-7.

Regarding C8′ affinity for C5b-7, data in Figure 4 suggest it may be more similar to β than C8. Previous studies demonstrated that β is capable of binding to the C8 site on membrane-bound C5b-7. Therefore, depending on the amounts present and their relative affinities, β could inhibit C8′ binding in experiments such as those in Figure 4. Indeed, the lower saturation level observed for C8′ relative to that of normal C8 is attributed to competition with the substantial amount of residual β in the preparation. This is supported by experiments not shown in which C8′ was prepared by the addition of excess α to a limiting amount of β and then tested in a binding assay. Under these conditions, where no residual β was present, C8′ binding was equivalent to normal C8. It is of further interest to note in Figure 4 that although residual β is also present in the C8R preparation, it does not inhibit binding of this particular ligand at the levels used in these experiments. We attribute this to the fact that β has a significantly lower affinity for C5b-7 than either C8 or C8R.[3]

Once C8′ is incorporated into membrane-bound C5b-8′, our results in Figure 5 show that C9 can bind to form a stable terminal complex. Significantly, the amount of C9 bound and the accompanying level of membrane lysis are comparable to those observed for C5b-8 and C5b-8R. It is important to note that this lytic activity of C8′ is not an artifact caused by contaminating C8. Direct evidence of this is provided in Figure 3. Here, SDS–PAGE analysis of purified C8′ indicates the complete absence of any α–γ and, therefore, C8. Further evidence of C8′ purity is provided by SDS–PAGE analysis of EAC1-8′ membranes in Figure 4. These were prepared by interaction of purified C8′ with EAC1-7, and we see only labeled α and thus C8′ bound to the membranes. If a significant level of contaminating C8 were present, labeled α–γ should also be detected on these membranes. Importantly, these same EAC1-8′ cells were used to test for C9 binding and hemolytic function in Figure 5. Since no C8 is detectable on these cells, the observed hemolysis must be due to C8′.

On the basis of the functional similarity between C8′ and C8, we conclude that γ is not essential for cell lysis and thus has no direct role in the cytolytic mechanism. This is consistent with results from photolabeling experiments in which we identified subunits of C8 that insert into the membrane bilayer during cytolysis (Steckel et al., 1983). These studies revealed that α has a key role in the lytic function of C8. Since α is present in C8′, it is not surprising that this derivative also exhibits lytic activity. Importantly, the fact that insertion of γ into membranes was not detected in photolabeling experiments further substantiates our conclusion here that it is not required for C8 function.

Using results from this and previous studies, we have developed a model depicting the arrangement of C8 subunits within membrane-bound C5b-8. Our model shown in Figure 7 is based on the existence of distinct functional domains in C8. The β subunit is shown to be residing in the C8 binding site because of its demonstrated ability to bind independently to the nascent complex and our conclusion that it mediates binding of the intact C8 molecule itself. Additionally, the extent of direct interaction between β and the membrane is depicted as minor. This is because photolabeling results indicated insertion of β in the bilayer is minimal in both C5b-8 and C5b-9. In contrast to β, α–γ does not bind independently to C5b-7 and therefore is not shown near the C8 binding site. Within α–γ, the α subunit is placed in direct contact with β because results in the present study revealed such an association occurs in the absence of γ and therefore is likely to exist within C8. Unlike β, the α subunit is depicted as having a sizable domain inserted in the membrane. This orientation of α is based on results from the previously mentioned photolabeling studies. Finally, the present study has established that γ is not required for C8 binding and therefore it is placed away from the binding site. The fact that it is not required for interaction of α–γ with β also favors placing it away from β. It is noted that γ is not positioned near the membrane surface because photolabeling experiments revealed it does not insert in the bilayer of target membranes.

Two variations of this model that are consistent with available data must also be considered. One is that α or γ or both may be directly associated with C5b-7. If so, neither α nor γ can reside in the C8 binding site and α must still be inserted in the membrane. The second variation is that γ could actually be physically associated with β in intact C8. If it is, such association is not required for C8 binding or the interaction between intact α–γ and β. While these and any further refinements in the model must await further data on the structure and function of C8, the evidence is conclusive that the subunits of C8 contain distinct functional domains. The ability to separate these domains should facilitate future studies of their role in the cytolytic process.

Registry No. Complement C8, 80295-58-5.

[3] A. Brickner and J. M. Sodetz, unpublished results.

References

Biesecker, G., & Müller-Eberhard, H. J. (1980) *J. Immunol. 124*, 1291–1296.

Brickner, A., & Sodetz, J. M. (1983) *Immunobiology 164*, 300.

Bull. W. H. O. (1968) *39*, 935.

Esser, A. F. (1982) in *Biological Membranes* (Chapman, D., Ed.) Vol.. IV, pp 277–326, Academic Press, London.

Kolb, W. P., & Müller-Eberhard, H. J. (1976) *J. Exp. Med. 143*, 1131–1139.

Monahan, J. B., & Sodetz, J. M. (1980) *J. Biol. Chem. 255*, 10579–10582.

Monahan, J. B., & Sodetz, J. M. (1981) *J. Biol. Chem. 256*, 3258–3262.

Monahan, J. B., Stewart, J. L., & Sodetz, J. M. (1983) *J. Biol. Chem. 258*, 5056–5062.

Müller-Eberhard, H. J. (1980) in *Progress in Immunology* (Fougereau, M., & Dausset, J., Eds.) Vol. IV, pp 1001–1024, Academic Press, New York.

Podack, E. R., & Müller-Eberhard, H. J. (1980) *J. Immunol. 124*, 1779–1783.

Podack, E. R., Kolb, W. P., & Müller-Eberhard, H. J. (1977) *J. Immunol. 119*, 2024–2029.

Steckel, E. W., York, R. G., Monahan, J. B., & Sodetz, J. M. (1980) *J. Biol. Chem. 255*, 11997–12005.

Steckel, E. W., Welbaum, B. E., & Sodetz, J. M. (1983) *J. Biol. Chem. 258*, 4318–4324.

Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.