Functional Domains of the α Subunit of the Eighth Component of Human Complement: Identification and Characterization of a Distinct Binding Site for the γ Chain†

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ABSTRACT: The purified γ subunit of the eighth component of human complement (C8) was used to characterize its site of interaction within C8 and to probe the ultrastructure of membrane-bound C5b-8 and C5b-9 complexes. Purification of γ was accomplished by separating the disulfide-linked α–γ subunit from the noncovalently associated β chain and subjecting the former to limited reduction, alkylation, and ion-exchange chromatography. Upon mixing, purified α and γ exhibited a high affinity for each other, as evidenced by their ability to form a noncovalent, equimolar complex at dilute concentrations and in the presence of excess serum albumin. Purified γ also exhibited an affinity for C8′, a previously described derivative that is functionally similar to C8 although it is composed of only α and β. These results indicate that α possesses a specific site for interaction with γ and that this site is preserved in the isolated subunit. Furthermore, this site remains accessible when α is associated with β. In related experiments, γ was found to specifically associate with membrane-bound C5b-8′ and C5b-(8′)9 complexes. These results indicate that the site for γ interaction remains accessible on α in C5b-8′ and is not shielded by C9 within C5b-(8′)9. It is concluded that the γ subunit of C8 is located on the surface of membrane-bound C5b-8 and C5b-9.

The cytolytic C5b-9 complex of complement is formed on target membranes by sequential association of C5b, C6, C7, C8, and C9 (Bhakdi & Tranum-Jensen, 1983; Podack & Tschopp, 1984). Among these proteins, C8 is the most structurally complex in that it is composed of three nonidentical subunits: α (M_r 64000), β (M_r 64000), and γ (M_r 22000) (Kolb & Müller-Eberhard, 1976; Steckel et al., 1980). These are arranged as a disulfide-linked α–γ dimer that is noncovalently associated with β.

Recent studies aimed at delineating structure–function relationships within C8 revealed the existence of distinct functional domains that facilitate subunit interactions with each other and with constituents of the cytolytic complex. The β subunit contains at least two such domains. One interacts specifically with the C8 binding site on C5b-7 and thereby mediates incorporation of C8 into this intermediate complex (Monahan & Sodetz, 1980). A second domain that is distinct from the first facilitates association of β with α–γ (Monahan & Sodetz, 1981). At least three functional domains have also been identified in α. One mediates the noncovalent interaction between α–γ and β, as evidenced by the fact that α alone can associate with an equimolar amount of β. This association yields C8′,[1] a derivative that is lacking γ yet is functionally similar to C8 (Brickner & Sodetz, 1984). A second domain of α has been shown to insert directly into the lipid bilayer during assembly of C5b-8 on target membranes (Steckel et al., 1983). The preceding paper in this issue describes a third domain on α that is capable of binding an equimolar amount of C9 (Stewart & Sodetz, 1985). This domain has a direct role in incorporating C9 into the precursive C5b-8 complex. In contrast to α and β, γ appears to have no direct role in cytolysis since C8′ was found to express normal C8 function.

In this study, we have identified a fourth domain on α that is distinct from those above. This domain is capable of binding γ noncovalently and with high affinity. We utilized this affinity to establish the accessibility of the binding site and therefore γ within C8 and within the ultrastructure of the membrane-bound cytolytic complex. Based on results from this study and the preceding paper in this issue (Stewart & Sodetz, 1985), a more detailed model can be proposed for the arrangement of C8 subunits within C5b-8.

Experimental Procedures

Materials. Human Cohn fraction III was kindly provided by Cutter Laboratories, Berkeley, CA. Bovine serum albumin, 4-vinylpyridine, DEAE-Sephadex, and dithiothreitol were purchased from Sigma. 4-Vinylpyridine was distilled prior to use.

Purification of Proteins. Human C8 (Steckel et al., 1980) and C9 (Biesecker & Müller-Eberhard, 1980) were isolated from Cohn fraction III by using previously published procedures. The α–γ and β subunits of C8 were purified by molecular sieve chromatography as described elsewhere (Rao & Sodetz, 1985). A trace amount of β remaining in the α–γ pool was removed by affinity chromatography using anti-β immunoglobulin G (IgG) resin. Molar concentrations of proteins were determined from published molecular weights and previously determined $\epsilon^{1\%}_{280}$ values (Monahan et al., 1983). Radioiodinations were performed with iodogen and routinely

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[1] Abbreviations: C8′, a derivative of C8 formed by combining α with β; EAC1-7, sheep erythrocytes carrying membrane-bound C5b-7; EAC1-8, EAC1-8′, EAC1-9, and EAC1-(8′)9, sheep erythrocytes carrying membrane-bound C5b-8, C5b-8′, C5b-9, and C5b-(8′)9, 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–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid.

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yielded specific radioactivities of (2–4) × 10^7 cpm/nmol of protein.

Purification of α and γ was accomplished by ion-exchange chromatography after limited reduction and modification of α–γ. For preparation of radiolabeled subunits, samples of ^{125}I-(α–γ) at 0.4–0.6 mg/mL in 0.1 M sodium phosphate, pH 7.0, were reduced and alkylated as previously described (Brickner & Sodetz, 1984). The solution was then adjusted to 0.5 mg/mL bovine serum albumin and applied to a DEAE-Sephadex column (0.7 × 5 cm) equilibrated in 0.1 M sodium phosphate, pH 7.0. The column was eluted at 25 °C with 4–6 volumes of 0.1 M sodium phosphate/0.01 M NaCl, pH 7.0. This was followed by 20–30 volumes of 0.1 M sodium phosphate/0.05 M NaCl, pH 7.0. Lastly, the column was eluted with 5–7 volumes of 0.1 M sodium phosphate/0.2 M NaCl, pH 7.0. Fractions containing ^{125}I-α or ^{125}I-γ were identified by the presence of radiolabel and pooled. Each pool was adjusted to 1 mg/mL bovine serum albumin and separately dialyzed vs. 0.1 M sodium phosphate, pH 7.0 at 4 °C, for 12 h. The molar specific radioactivities of ^{125}I-α and ^{125}I-γ were assumed to be 80% and 20% of the starting ^{125}I-(α–γ), respectively (Brickner & Sodetz, 1984). When unlabeled α and γ were desired, the same purification procedure was applied to α–γ that contained only trace amounts of ^{125}I-(α–γ).

Preparation of Highly Purified C8′ for Binding Assays. As reported previously, C8′ is prepared by combining purified α with β (Brickner & Sodetz, 1984). However, a small amount of residual α–γ in α also combines with β to yield a trace contaminant of C8. This contaminant interferes with interpretation of results and must be removed by adsorption to EAC1-7 as follows. Stock solutions of subunits were prepared which contained 8 μg/mL ^{125}I-α in 0.1 M sodium phosphate/1 mg/mL bovine serum albumin, pH 7.0, or 0.8 mg/mL β in 0.1 M sodium phosphate, pH 7.0. To prepare C8′, ^{125}I-α was mixed with an equimolar amount of β and incubated for 15 min at 25 °C. The resulting mixture was then incubated for 30 min at 37 °C with an amount of EAC1-7 cells such that β was 6-fold over the total number of C8 binding sites on the cells. Preparation of EAC1-7 and measurement of the number of binding sites were performed as described previously (Monahan & Sodetz, 1981). This adsorption step completely removes contaminating C8 with only a minimal loss of C8′ (~10%). This was verified as described previously by SDS–PAGE analysis of the supernatant after adsorption with EAC1-7 (Brickner & Sodetz, 1984). The supernatant containing C8′ was collected after centrifugation and used in binding studies.

Binding Assays. Direct binding of radiolabeled C8, C8′, and C8′ + γ to EAC1-7 was measured as described earlier for C8 (Monahan & Sodetz, 1981). The C8′ + γ used here was prepared by incubating the above purified C8′ with a 3-fold molar excess of unlabeled γ. In a typical binding assay, increasing amounts of ligand in 0.3 mL of 0.1 M sodium phosphate/1 mg/mL bovine serum albumin, pH 7.0, were incubated for 30 min at 37 °C with 0.1 mL of EAC1-7 prepared at 1 × 10^9 cells/mL GIGB. Washing and corrections for nonspecific binding were performed as described earlier. Moles of ^{125}I-C8 bound were determined from the molar specific radioactivity of C8. Moles of C8′ and C8′ + γ were calculated on the basis of the specific radioactivity of ^{125}I-α contained in each protein. To directly confirm the presence of γ on membranes, parallel experiments were performed in which C8′ was mixed with a 3-fold molar excess of ^{125}I-γ and incubated with EAC1-7 as above. Membranes were collected by centrifugation, washed with 5 mM EDTA, pH 8.0, and subjected to SDS–PAGE analysis as described below.

Binding of ^{125}I-γ to EAC1-8 and EAC1-8′ was measured by procedures similar to those above. Cellular intermediates carrying saturating amounts of ^{125}I-C8 or C8′ containing ^{125}I-α were prepared at 1 × 10^9 cells/mL GIGB. In a typical assay, increasing amounts of ^{125}I-γ in 0.6 mL of GIGB were incubated for 30 min at 37 °C with 0.2 mL of each cell suspension. After washing, cells were collected by centrifugation, and membranes were lysed with 5 mM EDTA, pH 8.0, and subjected to SDS–PAGE analysis as described below. The mole ratio of ^{125}I-γ to either ^{125}I-C8 or ^{125}I-α within C8′ was determined by quantitating the amount of each radiolabeled component on the gel.

Binding of ^{125}I-γ to EAC1-9 and EAC1-(8′)9 was measured by using similar procedures. EAC1-8 and EAC1-8′ intermediates were prepared as described above but were further incubated with saturating amounts of C9 for 35 min at 37 °C. The resulting EAC1-9 and EAC1-(8′)9 cells and membranes were washed with GIGB at 4 °C by repeated centrifugation at 31000*g*. Remaining intact cells were lysed with 5 mM EDTA, pH 8.0. The resulting EAC1-9 and EAC1-(8′)9 membranes were washed and resuspended in GIGB to a volume equivalent to one-third of the starting volume of cells. In a typical assay, increasing amounts of ^{125}I-γ in 0.2 mL of GIGB were incubated for 30 min at 37 °C with 0.2 mL of each membrane suspension. Washing was done as described above for preparing EAC1-9 and EAC1-(8′)9. Membranes were collected by centrifugation, washed with 5 mM EDTA, pH 8.0, and subjected to SDS–PAGE analysis as described below. The mole ratio of ^{125}I-γ to either ^{125}I-C8 or ^{125}I-α within C8′ was determined as above.

Polyacrylamide Gels. Membrane-bound proteins were analyzed by solubilizing membranes with two volumes of 20 mM sodium phosphate/5% SDS, pH 7.2. Electrophoresis was performed on 5% SDS–PAGE under nonreducing conditions (Weber & Osborn, 1969). Gels were sliced into 3-mm sections to determine the location and quantity of radioactivity.

RESULTS

Purification of ^{125}I-γ and Interaction with α and C8′. In the procedure for purifying ^{125}I-γ, this subunit elutes from DEAE-Sephadex with 0.1 M sodium phosphate/0.01 M NaCl while the wash with 0.1 M sodium phoshate/0.05 M NaCl contains a mixture of ^{125}I-(α–γ), ^{125}I-α, and ^{125}I-γ (data not shown). Eluent from the 0.1 M sodium phosphate/0.2 M NaCl wash contains ^{125}I-α. Figure 1 shows results from SDS–PAGE analysis of α and γ purified in this manner. It is apparent they can be completely separated from each other as evidenced by an absence of ^{125}I-γ in the α pool or ^{125}I-α in the γ pool. It is noted, however, that both contain a small amount of residual ^{125}I-(α–γ). This is only a concern when preparing C8′ from ^{125}I-α since it can associate with β to yield a C8 contaminant. Importantly, this contaminant can be removed by adsorption to EAC1-7 as described above.

Figure 2 shows results obtained when purified ^{125}I-γ is incubated with increasing amounts of α, α–γ, or β and analyzed for formation of stable complexes by sucrose density gradient centrifugation. It is noted that, with increasing amounts of α, the sedimentation position of ^{125}I-γ shifts to a position corresponding to α–γ. Association of ^{125}I-γ is complete when α is present at a 3-fold molar excess. These results indicate α is capable of binding a stoichiometric amount of γ to yield a stable, dimeric complex. Binding appears to be unique to α since control experiments in Figure 2 show no interaction between γ and as much as a 30-fold excess of α–γ or β.

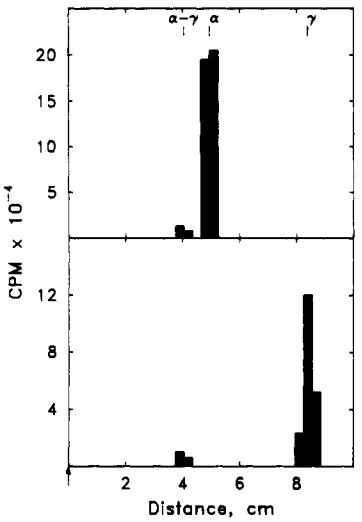

FIGURE 1: SDS–PAGE of purified α and γ. A sample of ^{125}I-(α–γ) was reduced, alkylated, and chromatographed on a DEAE-Sephadex column as described in the text. Results show radioactivity profiles from SDS–PAGE analysis of nonreduced samples of purified α and γ. Gel tops are indicated by an arrow while the inset shows the mobilities of α–γ, α, and γ markers. (Top) Pool of purified ^{125}I-α. (Bottom) Pool of purified ^{125}I-γ.

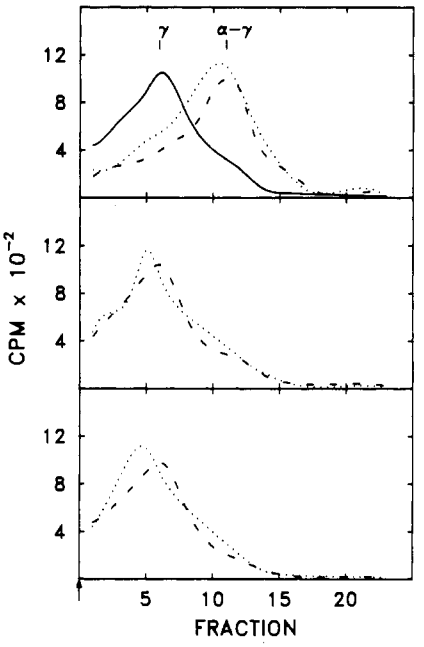

FIGURE 2: Analysis of the interaction of γ with C8 subunits. A 45-ng sample of ^{125}I-γ was incubated for 15 min at 25 °C with increasing amounts of α, α–γ, or β in a final volume of 250 μL of 0.1 M sodium phosphate/1 mg/mL bovine serum albumin, pH 7.0. Aliquots of 200 μL were analyzed on linear 5–10% (w/w) sucrose gradients prepared in 4.0 mL of the same buffer. 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 positions of α–γ and γ markers. (Top) Results show the sedimentation of ^{125}I-γ (—) and mixtures containing a 1:3 (···) and a 1:5 (- - -) molar ratio of ^{125}I-γ to α. (Middle) Control experiment using a 1:10 (···) and a 1:30 (- - -) molar ratio of ^{125}I-γ to α–γ. (Bottom) Control experiment using a 1:10 (···) and a 1:30 (- - -) molar ratio of ^{125}I-γ to β.

We next examined whether ^{125}I-γ could also associate with C8′. Figure 3 shows results obtained when samples of ^{125}I-γ were incubated with increasing amounts of C8′ or C8 and analyzed for formation of stable complexes by sucrose gradients. It is noted that, with increasing amounts of C8′, ^{125}I-γ shifts to a position corresponding approximately to the C8 marker. Complete association of ^{125}I-γ occurs when C8′ is

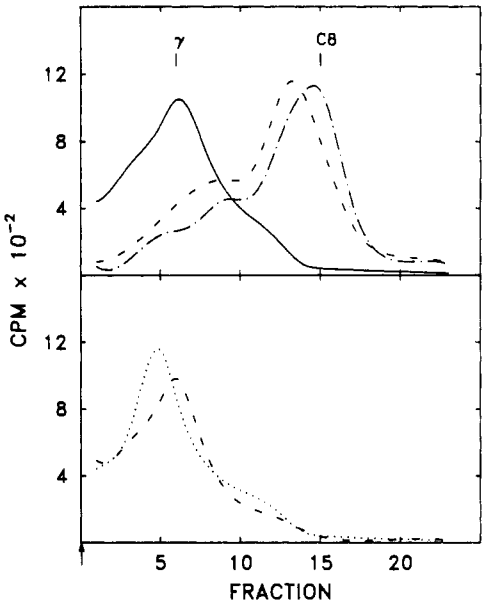

FIGURE 3: Analysis of the interaction of γ with C8′. C8′ was prepared by incubating 4.5 μg of α with a 3-fold molar excess of β for 15 min at 25 °C. A 45-ng sample of ^{125}I-γ was then incubated with increasing amounts of C8′ or C8 in a final volume of 250 μL. Aliquots of 200 μL were analyzed by sucrose density gradients as described in Figure 2. The top of each gradient is designated by an arrow while the inset shows sedimentation positions of γ and C8 markers. (Top) Sedimentation patterns of ^{125}I-γ (—) and mixtures containing a 1:5 (- - -) and a 1:10 (···) molar ratio of ^{125}I-γ to C8′. (Bottom) Control experiment using a 1:14 (···) and a 1:28 (- - -) molar ratio of ^{125}I-γ to C8.

present in a 5-fold molar excess. These results indicate that the γ binding site on α remains accessible within C8′. Importantly, no interaction of ^{125}I-γ with C8 was detected, a finding consistent with the inability of α–γ to bind additional γ.

Influence of γ on the Interaction of C8′ with Membrane-Bound C5b-7. To establish whether association of γ with C8′ alters its affinity for C5b-7, binding of C8′ + γ, C8′, and C8 to EAC1-7 was compared. Results shown in the top panel of Figure 4 indicate that C8′ + γ has a substantially higher affinity for the C8 binding site than does C8′ alone and this affinity approaches that of normal C8. Results shown in the bottom panel of Figure 4 confirm the identity of membrane-bound ligands in these experiments. Here, C8′ was mixed with ^{125}I-γ instead of unlabeled γ and added to EAC1-7 in saturating amounts. The gel profile of solubilized membranes shows that ^{125}I-α contributed from C8′ and the added ^{125}I-γ are both bound to the cells. Importantly, the mole ratio of α:γ is approximately 1:1.7, indicating that the ratio of C8′:γ on the membrane is at least 1:1. The slight excess of γ bound is attributed to nonspecific binding of ^{125}I-γ to the cell membrane itself (data not shown).

Interaction of γ with Membrane-Bound C5b-8′ and C5b-(8′)9. Experiments were performed next to determine if γ could associate with C8′ located within membrane-bound C5b-8′. Binding of γ to EAC1-8′ was measured and compared to EAC1-8 controls. Results shown in the top panel of Figure 5 indicate that γ does bind to EAC1-8′ while binding to EAC1-8 is negligible. When corrected for nonspecific binding, the ratio of bound γ to C8′ is 1:1, the value expected if γ is associating directly with α. Experiments shown in the bottom panel of Figure 5 examined the ability of γ to associate with C8′ within membrane-bound EAC1-(8′)9. Binding of γ to EAC1-(8′)9 was measured and compared to EAC1-9 controls. Results indicate that γ does bind to EAC1-(8′)9, and thus the

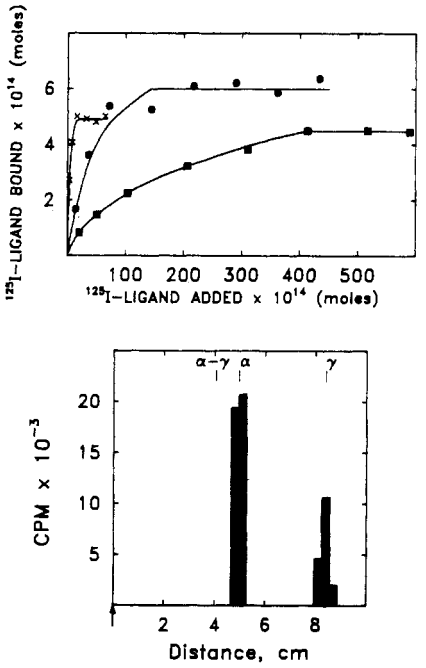

FIGURE 4: Binding of C8′ + γ to EAC1-7. (Top) Purified C8′ containing ^{125}I-α was incubated with a 3-fold molar excess of unlabeled γ for 15 min at 25 °C. Increasing amounts of this mixture were then incubated with a fixed amount of EAC1-7 as described in the text. Results show binding curves obtained for C8′ + γ (●), C8′ (■), and C8 (×). (Bottom) The above C8′ was incubated with a 3-fold molar excess of ^{125}I-γ and added to EAC1-7. Cells were then lysed, solubilized, and analyzed by SDS–PAGE under nonreducing conditions to confirm the identity of membrane-bound ligand. The mobilities of α–γ, α, and γ markers are shown in the inset.

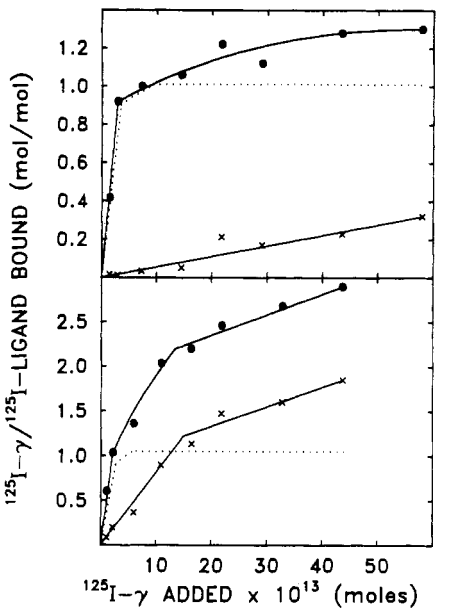

FIGURE 5: Accessibility of the γ binding site on membrane-bound C8′. (Top) Binding of ^{125}I-γ to EAC1-8 and EAC1-8′. Each cell type was prepared such that it carried the same mole quantity of ^{125}I-C8 or C8′ that contained ^{125}I-α. Binding of ^{125}I-γ was measured as described in the text. Results show the mole ratios of ^{125}I-γ to ^{125}I-C8 on EAC1-8 (×) and ^{125}I-γ to ^{125}I-α on EAC1-8′ (●). The dotted line shows the net mole ratio after the former curve is subtracted from the latter. (Bottom) Binding of ^{125}I-γ to EAC1-9 and EAC1-(8′)9. Each of the above cell types was further incubated with a saturating amount of unlabeled C9, to generate EAC1-(8′)9 and EAC1-9. Binding of ^{125}I-γ was measured as described in the text. Results show the mole ratios of ^{125}I-γ to ^{125}I-C8 on EAC1-9 (×) and ^{125}I-γ to ^{125}I-α on EAC1-(8′)9 (●). The dotted line shows the net mole ratio after the former is subtracted from the latter.

binding site on α remains accessible in this complex. It is noted, however, that γ binding to EAC1-9 is also significant. This level of nonspecific binding is attributed to the presence of lysed membranes. Importantly, when binding to EAC1-9 is subtracted, the ratio of γ to C8′ on EAC1-(8′)9 is approximately 1:1.

Discussion

Results in this study indicate that, in addition to the disulfide linkage that normally joins them, α and γ are capable of noncovalently associating through a single binding site on α. Their affinity must be substantial since association occurs in dilute solutions, is essentially complete at approximately equimolar concentrations of each, and persists during centrifugation through a density gradient. Binding of γ to α is specific since it occurs in the presence of a relatively high concentration of serum albumin. Furthermore, γ does not associate with α–γ or β at high molar excesses of each. Importantly, the ability of γ to associate with C8′ indicates that neither accessibility of the binding site on α nor the affinity for γ is significantly affected by the presence of β. This observation along with the known fact that α–γ can recombine with β suggests that α possesses separate sites for interaction with β and γ.

The ability of γ to bind C8′ prompted us to examine the effect of this association on the functional properties of C8′. Previous studies indicated C8′ was functionally similar to C8 in that it bound to C5b-7 and supported subsequent C9 incorporation and cell lysis (Brickner & Sodetz, 1984). However, its affinity for C5b-7 was markedly lower than that of C8. Results in Figure 4 show that addition of γ to C8′ increases its affinity for this complex to a level approaching that of C8. There are at least two possible explanations for this. One is that γ interaction with α induces a favorable conformational change that enhances β binding to C5b-7 and therefore increases the affinity of C8′. Alternatively, γ may interact directly with a constituent of membrane-bound C5b-7. This may stabilize C8 within its binding site, resulting in a higher affinity. In either case, γ is not essential for function, but its presence does have a favorable influence on C8 binding.

Interaction of γ with membrane-bound C5b-8′ and C5b-(8′)9 is significant with regard to establishing its location within the ultrastructure of these complexes. We found that 1 molar equiv of γ can associate with C8′ in each complex, indicating that the site of interaction on α is accessible to a substantially large ligand. This suggests that in either C5b-8 or C5b-9, the γ subunit is not shielded by the membrane, C5b-7, α, β, or C9. This, along with our previous observation that γ does not insert into the membrane, indicates it must be located on the surface of both complexes.

Although the γ binding site on α is functionally distinct from the three sites described previously, existing evidence suggests it is also physically distinct. For reasons described above, this site must be different from that which associates with β. The γ site also appears distinct from that portion of α which inserts in the bilayer of target membranes. Evidence for this is the ability of γ to bind to the exposed portion of α on C5b-8′ and C5b-(8′)9. Also supporting this is the fact that, within C5b-8 and C5b-9, γ does not interact with the membrane bilayer. As described in the preceding paper in this issue (Stewart & Sodetz, 1985), α also can bind 1 equiv of C9. This site must be distinct from that which binds γ since α–γ is also capable of binding one C9. Further evidence of this includes the fact that C5b-8′ can bind C9, which we now know involves α directly, and the resulting C5b-(8′)9 still binds γ. Both these observations indicate that γ and C9 can associate with α

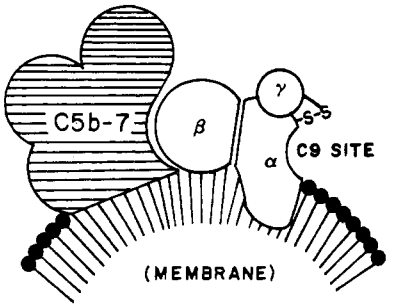

FIGURE 6: Arrangement of C8 subunits within membrane-bound C5b-8.

simultaneously and therefore at different sites.

The fact that distinct functional sites are retained on α after limited reduction, alkylation, and purification suggests they are quite stable and consequently must be structurally well-defined. In Figure 6, we present a model for membrane-bound C5b-8 that depicts the arrangement of C8 subunits and accommodates the existence of these sites on α. This is an expansion of an earlier model in which only two sites were recognized, one that associated with β and one that inserted in the membrane bilayer (Brickner & Sodetz, 1984). In that version, γ was placed on the surface of α simply because it was found to be unnecessary for C8 function. In the present study, we established that γ is indeed located on the surface. Additionally, we show the single C9 binding site on α that was identified in the preceding paper in this issue. Importantly, all sites of interaction on α are depicted as being distinct.

The accessibility of the γ site within C5b-(8′)9 is relevant to understanding the spatial relationship between C8 and C9 in C5b-9. Previous studies determined that few if any C9 molecules are in close proximity to C8 in the final complex (Monahan et al., 1983). Results in the present study and the preceding paper in this issue are consistent with these findings. According to our model for C5b-8, formation of C5b-9 would require simultaneous interaction of α with the membrane bilayer and β, γ, and C9, three substantially large proteins. On the basis of steric considerations, it seems unlikely that α could be associated with more than one C9 in the final C5b-9 complex. Indeed, the known ability of C9 to self-polymerize would not require more than one C9 associated with C8 at any time during C5b-9 formation (Podack & Tschopp, 1982). While at the present time ultrastructural information is inadequate to propose a detailed model of the molecular arrangement within C5b-9, the evidence is conclusive that γ must be located on the surface and that any direct interaction between C8 and C9 occurring within the fully assembled complex must be minimal.

Registry No. Complement C8, 80295-58-5; complement C5b-8, 82903-91-1; complement C5b-9, 82986-89-8; complement C9, 80295-59-6.

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