Studies of the Association of the Eighth and Ninth Components of Human Complement within the Membrane-bound Cytolytic Complex*

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The association of the eighth (C8) and ninth (C9) components of human complement within membrane-bound C5b-9 was investigated using the photosensitive cross-linking reagent N-succinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate. Reaction of this reagent with either the purified α-γ or β subunit of C8 resulted in the incorporation of photosensitive 6-(4'-azido-2'-nitrophenylamino)hexanoate (ANH) as an intrinsic ligand on each protein. The resulting ANH-(α-γ) or ANH-(β) was capable of recombining with equimolar amounts of β or α-γ, respectively, to yield ANH-C8. Parallel modifications of purified C9 resulted in incorporation of 3-4 mol/mol of ANH-ligand. Both ANH-C8 and ANH-C9 retained their ability to incorporate into C5b-9. Two approaches were used to determine the proximity of C8 subunits to C9 within C5b-9. In one, the complex was assembled on erythrocytes by incubating EAC1-7 cells separately with each of ANH-C8 and subsequently saturating with 125I-C9. After lysis, membranes were irradiated, solubilized, and analyzed by gel electrophoresis. Cross-linking was assessed by a shift in electrophoretic mobility of 125I-C9 to a higher molecular weight. Results using either form of ANH-C8 in C5b-9 showed that, although at least 30% was involved in cross-linking, none was cross-linked to C9. Similar results were obtained using a second approach in which cross-linker and radiolabel were transposed between C8 and C9. Here, EAC1-7 cells were incubated first with 125I-C8 containing either 125I-(α-γ) or 125I-(β) and subsequently with ANH-C9. Although at least 48% of ANH-C9 in C5b-9 was involved in cross-linking in these experiments, no cross-linking to either subunit of C8 was detected. These results suggest that C8 is not in close physical association with C9 within membrane-bound C5b-9.

Human C8\(^1\) and C9 are serum glycoprotein components of C5b-9, the membrane-bound cytolytic complex formed from terminal complement proteins C5b, C6, C7, C8, and C9 (1). Formation of C5b-9 on cell membranes occurs as a consequence of complement activation and proceeds by a sequential mechanism involving several intermediate complexes as follows:

\[
\text{C5b} \rightarrow \text{C5b-6} \rightarrow \text{C5b-7} \rightarrow \text{C5b-8} \rightarrow C9 \rightarrow \text{C5b-9.}
\]

In this sequence, interaction of equimolar amounts of C5b and C6 leads to formation of C5b-6, a dimer which subsequently interacts with one C7 to yield the trimeric C5b-7 complex (2). This latter complex possesses a high affinity lipid-binding site which mediates interaction of the nascent complex with target membranes (3). Once tightly associated with the membrane bilayer, C5b-7 subsequently binds one C8 to produce C5b-8. This is followed by binding of several C9 molecules to yield C5b-9, the terminal complex which alters membrane permeability and produces cell lysis (4-10).

Assembly of C5b-9 on the membrane surface is an unusual biological process because association of the constitutive proteins appears to be noncovalent yet nearly irreversible under nondenaturing conditions. Moreover, interaction between these proteins is highly specific and their association occurs in an obligatory, sequential manner. These features of the assembly process suggest that each intermediate complex must contain a distinct recognition site for the protein to which it binds. In the case of membrane-bound C5b-7, we recently demonstrated the existence of a binding site which specifically recognizes the β subunit of human C8 (11, 12). Binding and incorporation of C8 into the nascent complex was found to be mediated strictly by this subunit. With regard to C5b-8 interaction with C9, evidence suggests that several C9 molecules must bind to form a lytically active C5b-9 complex. The number of C9 molecules bound per complex reported in the literature has varied between 3 (2, 6), 6 (9, 13), and 12 (14) when measured by a variety of methods. The significance of the multiplicity of C9 binding has only recently become apparent with the observation that aggregation or polymerization of C9 on the membrane may be essential to formation of functional C5b-9. Recent ultrastructural studies of C5b-9 on liposomes suggest that the major ringlike structure normally associated with C5b-9 may actually be a polymer of C9 (15). This structure resembles that formed by C9 alone as a consequence of heat-induced aggregation (16).

Although binding and polymerization of C9 is mediated through interaction with C5b-8, it remains to be established whether C8 participates directly in this process. It is not known if C8 itself contains the C9 binding site on C5b-8 or if it comprises any portion of the site at all. Furthermore, it has not been determined whether C8 and C9 are physically associated by gel electrophoresis; GIBB, buffer containing 5 mM imidazole, 72.7 mM NaCl, 0.15 mM CaCl\(_2\), 0.5 mM MgCl\(_2\), 2.5% glucose, 0.06% gelatin, 5 mg/ml bovine serum albumin, pH 7.3 at 4°C.
mediated within C5b-9. Our laboratory is presently concerned with these questions and with delineating the topographical arrangement of protein components within the various intermediate complexes leading to formation of membrane-bound C5b-9. Our purpose is to identify those residing in close proximity to each other within each complex to the exclusion of other components. Several of the protein-specific binding sites for the constitutive proteins incorporated into nascent C5b-9. Such information is essential to understanding the molecular basis for the recognition of specific proteins by each complex. In this report, we describe results from our investigation into the spatial relationship between C8 and C9 in membrane-bound C5b-9. Our approach uses purified C8 and C9, one of which carries a radiolabel and the other a photosensitive cross-linker. Both approaches can be incorporated into membrane-bound C5b-9 and after irradiation, light-induced cross-linking between C8 and C9 can be measured. By transposing the intrinsic cross-linker and radiolabel between C8 and C9, we have established that most or all of the C9 within C5b-9 is not in close physical proximity to C8. A preliminary report of this work has appeared elsewhere (17).

EXPERIMENTAL PROCEDURES

Purification of Proteins—Human C8 (18) and C9' were isolated from Cohn Fraction III and evaluated for purity and hemolytic activity as described elsewhere. The hemolytic specific activity of purified C8 and C9 was found to be 3.6 ± 0.5 × 10^6 serum CH50 units/mg and 3.3 ± 0.5 × 10^6 serum CH50 units/mg, respectively. Normal serum used as a reference to assign these activities contained approximately 2.8 ± 0.4 × 10^6 CH50 units/mg and 1.5 ± 0.3 × 10^6 CH50 units/mg of C8 and C9 activity, respectively, when measured under our assay conditions (18). Isolation of the noncovalently associated α-γ and β subunits of C8 was performed in the presence of SDS as described elsewhere but with significant improvements incorporated into the purification procedure (18). Specifically, high pressure liquid chromatography using molecular sieve columns was substituted for conventional gel filtration. For a typical subunit preparation, C8 was prepared at 1 mg/ml in 0.1 M sodium phosphate, pH 7.0, and adjusted to 1 mm diisopropyl fluorophosphate. After incubation for 30 min at 25 °C, the sample was adjusted to 0.1% SDS. After further incubation for 45 min at 37 °C, the sample was concentrated by ultrafiltration to 5-7 mg/ml. Separation of α-γ and β was accomplished using a Varian 5000 high performance liquid chromatograph equipped with two TSK-G3000 SW columns (0.75×50 cm) in a tandem configuration. Sample volumes of 0.5 ml were applied to the columns which were equilibrated and developed in 0.1 M sodium phosphate, 0.1% SDS, 0.02% NaN3, pH 7.0, at 25 °C. Separated α-γ and β were pooled and the SDS was removed as previously described (18). Subunits purified by this procedure exhibited the same properties after renaturation as those isolated by our earlier method. Both were homogeneous as judged by SDS-PAGE under nonreducing conditions, and when mixed in a 1:1 mole ratio, they physically recombined to yield functional C8. As reported earlier, this recombined C8 possessed 80-90% of the hemolytic specific activity associated with an equivalent mole quantity of native C8.

Radioiodinations of C8, α-γ, β, and C9, were performed under mild conditions using agarose-bound lactoperoxidase (19). Specific radioactivities were expressed on a mol basis using experimentally determined values of ε280 and published molecular weights for each protein (18, 20). Measurement of ε280 was determined by quantitive amino acid analyses. Values found were 14.9, 11.8, 13.7, and 9.88 for C8, α-γ, β, and C9, respectively, in 10 mm sodium phosphate, 0.15 M NaCl, pH 7.0. Specific radioactivities for each protein were between 2 and 7 × 10^4 cpm/mg. To prepare 125I-C8 containing radiiodine in only one of its noncovalently linked subunits, 125I-(α-γ) or 125I-(β) was mixed with an equimolar amount of unmodified or, respectively, reconstituted:

Incorporation of ANH into C8 and C9—The photosensitive cross-linking reagent N-succinimidyl 6-(4′-azido-2′-nitrophenylamino)-hexanoate was purchased from Pierce Chemical Co. Incorporation of the photolabile 6-(4′-azido-2′-nitrophenylamino)hexanoate moiety into protein and all subsequent steps using ANH-modified proteins were performed under filtered light. To prepare ANH-C8 containing cross-linker in one subunit, purified α-γ or β was prepared at 0.3-0.4 mg/ml in 10 mm sodium phosphate, 0.15 M NaCl, pH 7.0, and incubated with 1% (v/v) of 0.05 M ANH prepared in dry dimethylformamide. This mixture contained an approximately 100-fold molar excess of reagent over protein. After 10 min at 25 °C, the reaction was quenched by adjusting to 0.02 mg/l. L-lysine, respectively. Because of its aromatic properties, we observed that ANH-L-lysine generated from reaction with excess SANH remained associated with the protein. Although noncovalently bound, this amount of reagent interfered with the ability of ANH-(α-γ) or ANH-(β) to fully recombine to form ANH-C8. It also precluded determination of the amount of ANH which were actually bound covalently. Consequently, reaction mixtures were gel filtered on a Bio-Gel P-10 column in 10 mm sodium phosphate, 0.15 M NaCl, 4 mM urea, pH 7.0, to remove free and noncovalently bound ANH-L-lysine. The recovered ANH-(α-γ) and ANH-(β) subunits were renatured by dialysis at 25 °C against 10 mm sodium phosphate, 0.15 M NaCl, pH 7.0, and stored at 0 °C. The molar amount of ANH covalently bound was determined from absorbance at 450 nm of each ANH-protein and the corresponding molar extinction coefficient of 5400 M⁻¹ cm⁻¹ for ANH. To prepare ANH-C8 by recombination, equimolar amounts of ANH-(α-γ) or ANH-(β) were incubated with the unmodified subunits, respectively.

Preparation of 125I-ANH-C8 containing both 125I label and ANH in the same subunit was performed similarly by substituting 125I-(α-γ) or 125I-(β) for the unlabeled subunits in the above reactions with SANH.

For the preparation of ANH-C9, purified C9 was adjusted to 1 mg/ml in 0.1 M sodium phosphate, pH 7.0, and incubated as above with 0.3 mg of 0.1 M SANH in dimethylformamide (18). The sample contained an approximately 6-fold molar excess of reagent over C9. The reaction was quenched by addition of L-lysine-HCI as described above. When the reaction mixture was filtered in ures to remove noncovalently bound reagent, the resulting ANH-C9 was found to be inactive. Control gel filtration experiments using unmodified C9 showed this was a result of irreversible denaturation by urea. Analysis of the reaction mixture by sucrose density gradients also showed that ANH modification induced a significant amount of C9 to form aggregates. These problems were circumvented by fractionating the reaction mixture by sucrose density gradient centrifugation. Linear 50% sucrose gradients were used for ANH-C8, and 50% sucrose gradients were used for ANH-C9. After quenching, samples of reaction mixture were applied to a TSK-G3000 SW column (0.75×50 cm) in 0.1 M sodium phosphate, 0.02% NaN3, pH 7.0, at 25 °C. This column completely separated ANH-C9 monomer from its aggregates and from all noncovalently bound reagent. Moles of ANH bound to C9 were quantitated as described above. To prepare 125I-ANH-C9, 125I-C9 was substituted for C9 in the above reaction with SANH.

Characterization of ANH-C8 and ANH C9—Hemolytic assays of ANH-C8 prepared by recombination of ANH-(α-γ) or ANH-(β) with their complementary unmodified subunit and assays of ANH-C9 were performed as described elsewhere (18). The hemolytic specific activity of each sample was determined and compared to equimolar amounts of unmodified control protein in the same assay. The recombining potential of ANH-(α-γ) and ANH-(β) was also examined by sucrose density gradient centrifugation. Linear 5-10% sucrose gradients were prepared in 4 ml of 25 mm imidazole, 0.15 M NaCl, 1 mg/ml bovine serum albumin, pH 7.2. All samples were diluted in the same buffer. Centrifugation was performed in a Sorvall TV-865 vertical rotor at 48,000 rpm for 2 h at 4 °C. In addition to hemolytic assays, ANH-C8 was tested for its ability to interact with the C5-binding site on EAC1-7 cells. This type of cellular intermediate was prepared as described elsewhere (12). Assays to measure binding of C8 to EAC1-7 were performed by incubating 0.3 ml of 10 cells/ml in GIBB buffer with increasing amounts of 125I-C8 or 125I-ANH-C8 contained in 0.1 ml of the same buffer. Incubations were for 30 min at 37 °C. The resulting EAC1-8 or EAC1-7(ANH-C8) were washed by repeated centrifugation and bound radiolabeled protein was quantitated as described previously (12). Nonspecific binding was measured by substituting EA for EAC1-7 and subtracted from the results as before. In all cases, moles of 125I-C8 or 125I-ANH-C8 bound were determined from the known specific radioactivity of each preparation.

The ability of ANH-C8 to support C9 incorporation into the cytolytic complex was also assessed in separate binding assays using 125I-C9 and EAC1-8 or EAC1-7(ANH-C8). These cellular intermediates were prepared as above by incubating EAC1-7 with saturating amounts of unmodified C8 or ANH-C8. To make the target cell suspension, each cell type was prepared at 1 × 10^6 cell/ml and mixed with increasing amounts of 125I-C9 in 0.1 ml of GIBB buffer. Incubations

were for 35 min at 37 °C. To correct for nonspecific binding, parallel samples were prepared which contained EAC1-7 cells. After incubation, cells and lysed membranes were washed by repeated centrifugation at 31,000 × g. Bound 125I-C9 was quantitated after correcting for nonspecific binding to EAC1-7 cells.

In experiments where cross-linker was also used to compare binding of C9 and ANH-C9 to EAC1-8. The EAC1-8 cells used here contained saturating amounts of unmodified C8. Moles of 125I-C9 or 125I-ANH-C9 bound were quantitated from their known specific radioactivities.

Incorporation of ANH-Proteins into Membrane-bound C5b-9 and Photolysis—In experiments where cross-linker was to be located on C8, saturating amounts of ANH-C8 containing either ANH-(α-γ) or ANH-(β) were prepared in 2 ml of GIGB buffer and incubated for 30 min at 37 °C with 6 ml of EAC1-7 prepared at 1 × 10⁶ cells/ml. The resulting EAC1-7(ANH-C8) cells were washed by centrifugation, resuspended in the same volume of GIGB buffer, and incubated for 35 min at 37 °C with saturating amounts of 125I-C9. Membranes from the resulting lysed cells were washed with GIGB buffer by repeated centrifugation at 31,000 × g and suspended in 4 ml of 10 mM sodium phosphate, 0.15 M NaCl, pH 7.0. Each sample was then divided equally with one to be photoscavenged and the other to be used as a nonphotolysed control. Another pair of samples identical with this was also prepared by substituting C8 for ANH-C8 in the above procedure.

In experiments which estimated the extent of cross-linking between C8 subunits themselves, ANH-C8 contained ANH-(α-γ) and 125I-(β) or 125I-(α-γ) and ANH-(β). This form of ANH-C8 was used to prepare EAC1-7(ANH-C8) cells which were subsequently incubated with unlabeled C9 as above. Photoactivities of these and parallel control cells containing 125I-C8 instead of ANH-C8 were performed as described above.

In experiments where cross-linker was to be located on C9 and cross-linking to C8 measured, saturating amounts of ANH-C9 in 2 ml of GIGB buffer were incubated with 6 ml of EAC1-8 cells prepared at 1 × 10⁶ cells/ml. The C8 used to prepare these cells contained either 125I-(α-γ) or 125I-(β). Lysed membranes resulting from these incubations were prepared for photosynthesis as described above. Parallel control samples were prepared similarly in which unmodified C9 was substituted for ANH-C9.

Photolysis of membrane suspensions was performed while gently stirring for 2 h in a thermostatted bath at 25 °C. A 120-Watt flood lamp was utilized as the light source at a distance of 10 cm. Both photolyzed and nonphotolyzed samples were subsequently centrifuged at 31,000 × g to quantitatively recover membranes. The membrane pellet was solubilized by incubating for 12 h at 37 °C with an equal volume of 10 mM sodium phosphate, 10% SDS/40 mM dithiothreitol, pH 7.2. Samples were subsequently analyzed on 5% polyacrylamide gels in the presence of 0.1% sodium dodecyl sulfate by determining the percentage of recovered radiolabel migrating with the labeled protein added.

Parallel experiments were performed to measure total cross-linking of each of the above ANH-modified proteins in membrane-bound C5b-9. In these experiments, both the ANH-moiety and 125I-label were located on the same components. These components were incorporated into C5b-9 as described above and cross-linking was evaluated from the shift in electrophoretic mobility of the radiolabeled ANH-protein on SDS gels. As before, controls were prepared similarly using components which contained only 125I-label.

RESULTS

Properties of ANH-C8 and ANH-C9—Modification of purified α-γ or β with SANH as described above resulted in incorporation of 6–8 mol of photolabile ANH/mol of subunit. When incubated with equimolar amounts of complementary unmodified subunit, both ANH-(α-γ) and ANH-(β) recombined to yield functional C8. Physical evidence of this is presented in Fig. 1 where sucrose density gradient analyses show that complete recombination of 125I-ANH-(α-γ) or 125I-ANH-(β) occurs with β or α-γ, respectively. Hemolytic assays indicated that the specific activity of either form of ANH-C8 was equal to that of C8 formed by recombination of the unmodified subunits. In addition, ANH-C8 was tested for its ability to interact with the precursor C5b-7 complex. Assays using EAC1-7 cellular intermediates were performed to compare the binding of C8 and each form of ANH-C8 to membrane-bound C5b-7. Results from these experiments in Fig. 2, A and B, show that, regardless of which subunit is modified, ANH-C8 exhibits binding properties comparable to C8. Both modified and unmodified C8 have similar affinities for EAC1-7 and similar mole quantities of each are bound at saturation. Experiments in Fig. 2, C and D, also compare the abilities of ANH-C8 and C8 to promote C9 binding subsequent to their

**Fig. 1.** Sucrose density gradient analysis of ANH-C8. Samples of 125I-ANH-(α-γ) and 125I-ANH-(β) were analyzed on sucrose density gradients separately and after recombination with β or α-γ, respectively. Linear 5–10% gradients in 25 mM imidazole, 0.15 M NaCl, 1 mg/ml bovine serum albumin, pH 7.2, were prepared as described in the text. Top, sedimentation profile of 125I-ANH-(α-γ) alone (○) and after incubation for 15 min at 25 °C with an equimolar amount of β (○). The arrow shows the position of 125I-C8 marker. Bottom, sedimentation profile of 125I-ANH-(β) alone (○) and after incubation with an equimolar amount of α-γ (○). The arrow indicates the position of 125I-C8.

**Fig. 2.** Comparison of C8 and ANH-C8 binding properties. A, comparison of 125I-C8 (○) and 125I-ANH-C8 (●) binding to EAC1-7 is shown. The 125I-ANH-C8 used here contained equimolar amounts of 125I-ANH-(α-γ) and β. Increasing amounts of each radiolabeled form of C8 were incubated with EAC1-7 and specific binding was quantitated as described in the text. B, corresponding results obtained when experiments in A are performed using 125I-ANH-C8 which contains α-γ and 125I-ANH-(β). C, comparison of 125I-C8 binding to EAC1-8 (○) and EAC1-7(ANH-C8) (●). The cellular intermediates were prepared by incubating EAC1-7 with saturating amounts of C8 or ANH-C8 which contains equimolar amounts of ANH-(α-γ) and β. Incubations and quantitation of specific binding were performed as described in the text. D, corresponding results obtained when experiments in C are performed with ANH-C8 composed of α-γ and ANH-(β).
interaction with EAC1-7. Experiments here show the binding resulted in covalent incorporation of saturation that ANH-C8 and C8 have a similar ability to each form of EAC1-7(ANH-C8). It is evident from these curves and the amount of 125I-C9 bound in each case at saturation that ANH-C8 and C8 have a similar ability to mediate C9 binding.

Incubation of C9 with a 6-fold molar excess of SANH resulted in covalent incorporation of 3-4 mol of ANH/mol of C9. When examined on sucrose density gradients, 15-25% of the ANH-C9 formed high molecular weight aggregates as a result of modification. Such behavior was not unexpected considering the reported tendency of C9 to self-aggregate (15, 16). Functional characterization of ANH-C9 aggregates recovered from density gradients indicated that they had no C9 hemolytic activity nor did they bind EAC1-8 when tested in C9-binding assays. Nevertheless, the monomeric form of ANH-C9 was separated from the aggregates on a preparative scale by high performance liquid chromatography and used in cross-linking experiments. Results from density gradient experiments in Fig. 3 confirm that ANH-C9 isolated in this manner is indeed monomeric. When tested in hemolytic assays, monomeric ANH-C9 was found to possess 10 ± 5% of the specific activity associated with C9 prior to modification. Importantly, results from binding assays in Fig. 3 indicate that, despite this lower activity, ANH-C9 retains its ability to bind to the C9 binding site on EAC1-8. Saturation of this site occurs when approximately the same molar amount of either C9 or ANH-C9 is bound and, as expected, binding was observed to be nearly reversible in both cases.

Cross-linking of ANH-C8 to C9—To examine whether C8 and C9 are in close proximity within membrane-bound C5b-9, we utilized an experimental system in which the photosensitive cross-linker was located on C9 and radiiodine on C8. Both components were incorporated into C5b-9 on erythrocyte membranes. After photoactivation and SDS-PAGE analysis of these membranes, the appearance of high molecular weight forms of 125I-C9 was used as evidence of cross-linking between C8 and C9. By placing the cross-linker on either α-γ or β, it was also possible to determine which of these C9 subunits, if any, is in close proximity to C9. An essential requirement of this approach is the ability to demonstrate that ANH-C8 does indeed cross-link to something, i.e. membrane proteins and/or constituents of C5b-9. Measurement of such total cross-linking was accomplished by parallel experiments in which both ANH and radiiodine are located on the same component. Typical SDS-PAGE data from experiments using ANH-C8 containing ANH-(α-γ) are shown in Fig. 4 along with results from corresponding control experiments. Analyses such as these were used to quantitate the extent of ANH-(α-γ) cross-linking. Quantitative results from experiments which measured total cross-linking of ANH-(α-γ) are shown in the upper half of Table I. These results indicate that 28% of ANH-(α-γ) undergoes cross-linking while present in C5b-9. This net value was obtained by subtracting the apparent cross-linking observed in photoactivated controls containing 125I-(α-γ) from that observed in samples containing ANH-(α-γ). Quantitative results from corresponding experiments which specifically measured cross-linking of ANH-(α-γ) to C9 are shown in the lower portion of Table I. Here, no net cross-linking to 125I-C9 is observed after apparent cross-linking in control samples containing α-γ is subtracted from those containing ANH-(α-γ). It is noted that, in each case, controls containing no cross-linker were subtracted to arrive at a value for net cross-linking. This method of correction was adopted when it became apparent from results such as those in Table 1 that irradiation conditions alone can influence behavior of the radiolodinated component on SDS-PAGE, regardless of whether cross-linker is present.

Experiments similar to the above were also performed using ANH-C8 containing ANH-(β). Results from SDS-PAGE analyses are shown in Fig. 5 while corresponding quantitative
measurements of total ANH-(β) cross-linking and cross-linking to C9 are presented in Table II. Here, 30% of total ANH-(β) in C5b-9 is cross-linked but no measurable cross-linking to C9 is observed.

Control experiments similar to those in Tables I and II were performed to assess the extent of α-γ to β cross-linking within C8. Here, ANH-C8 contained cross-linker on one subunit and radiolabel on the other. Regardless of which subunit contained ANH-ligand, the level of intersubunit cross-linking was found to be 3–5% while total cross-linking was routinely 2–8–30%. Thus, of the total observed cross-linking, only ~10–16% involves the other subunit. This indicates that only a small

<table>
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<th>Components bound to EAC1-7*</th>
<th>Phototacted</th>
<th>Recovery of label applied to gel</th>
<th>Label recovered above noncross-linked marker</th>
<th>Net cross-linking of labeled component</th>
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<tbody>
<tr>
<td>(α-γ)* + (β) + C9</td>
<td>–</td>
<td>91</td>
<td>4</td>
<td>%</td>
</tr>
<tr>
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<tr>
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<td>6</td>
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<td>ANH-(α-γ) + (β) + C9*</td>
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<td>92</td>
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<tr>
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<td>+</td>
<td>93</td>
<td>13</td>
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* *, radioiodinated components.

Table II
Quantitation of ANH-(α-γ) cross-linking within C5b-9
Results from SDS-PAGE analyses shown in Fig. 4 were used to quantify the extent of cross-linking of ANH-(α-γ) within C5b-9. The amount of radiolabel migrating above α or C9 on each gel was used as an indication of cross-linking and is expressed as a percentage of total label recovered from the gel. Net cross-linking was determined by subtracting background observed in corresponding samples which did not contain cross-linker. Results in the upper half of the table measure total cross-linking of ANH-(α-γ) and the bottom half measures cross-linking to C9.

<table>
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<tr>
<th>Components bound to EAC1-7*</th>
<th>Phototacted</th>
<th>Recovery of label applied to gel</th>
<th>Label recovered above noncross-linked marker</th>
<th>Net cross-linking of labeled component</th>
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<tr>
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<tr>
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<td>48</td>
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<td>6</td>
<td>0</td>
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</table>

* *, radioiodinated component.

Fig. 5. SDS-PAGE analysis of ANH-(β) cross-linking within C5b-9. Experiments similar to those shown in Fig. 4 were performed using C8 or ANH-C8 containing ANH-(β). Photolabeling and SDS-PAGE analyses were performed as described in the text. The top of each gel is indicated by an arrow. Solid bars show results obtained from experiments which measured total cross-linking of ANH-(β). The C5b-9 was formed with C8 containing either 125I-(α-γ) or 125I-(β) and unlabeled C9. The electrophoretic mobility of noncross-linked β is shown for reference. Hatched bars show results from corresponding experiments which measured cross-linking of ANH-(β) to C9. Here, C5b-9 was formed with either C8 (top) or C8 containing ANH-(β) (bottom) and 125I-C9. The mobility of noncross-linked C9 is shown for reference.

Table III
Quantitation of cross-linking between C8 and ANH-C9 within C5b-9
Radiolabeled C8 containing either 125I-(α-γ) or 125I-(β) was incorporated along with ANH-C9 into membrane-bound C5b-9 by incubation with EAC1-7. Parallel samples for measuring total cross-linking contained unlabeled C8 and 125I-ANH-C9. Photolabeling was performed as described in the text and membranes were subjected to SDS-PAGE analysis as in Fig. 4. Quantitation of cross-linking was performed as described in Table I by measuring the amount of label migrating above the noncross-linked radiolabeled component. The upper portion of the table shows results obtained for total ANH-C9 cross-linking. The bottom portion shows those obtained for cross-linking of ANH-C9 to α-γ or β.

<table>
<thead>
<tr>
<th>Components bound to EAC1-7*</th>
<th>Phototacted</th>
<th>Recovery of label applied to gel</th>
<th>Label recovered above noncross-linked marker</th>
<th>Net cross-linking of labeled component</th>
</tr>
</thead>
<tbody>
<tr>
<td>(α-γ) + (β) + C9</td>
<td>–</td>
<td>95</td>
<td>7</td>
<td>%</td>
</tr>
<tr>
<td>(α-γ) + (β) + ANH-C9</td>
<td>–</td>
<td>94</td>
<td>6</td>
<td>%</td>
</tr>
<tr>
<td>(α-γ) + (β) + ANH-C9</td>
<td>+</td>
<td>98</td>
<td>29</td>
<td>48</td>
</tr>
<tr>
<td>(α-γ) + (β) + ANH-C9*</td>
<td>+</td>
<td>93</td>
<td>73</td>
<td>48</td>
</tr>
<tr>
<td>(α-γ) + (β) + C9</td>
<td>–</td>
<td>92</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>(α-γ) + (β) + ANH-C9</td>
<td>–</td>
<td>85</td>
<td>6</td>
<td>%</td>
</tr>
<tr>
<td>(α-γ) + (β) + C9</td>
<td>+</td>
<td>84</td>
<td>24</td>
<td>2</td>
</tr>
<tr>
<td>(α-γ) + (β) + ANH-C9</td>
<td>+</td>
<td>90</td>
<td>26</td>
<td>&lt;0</td>
</tr>
<tr>
<td>(α-γ) + (β) + C9</td>
<td>–</td>
<td>91</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>(α-γ) + (β) + ANH-C9</td>
<td>+</td>
<td>95</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>(α-γ) + (β) + C9</td>
<td>+</td>
<td>91</td>
<td>22</td>
<td>&lt;0</td>
</tr>
</tbody>
</table>

* *, radioiodinated component.

portion of available cross-linker is consumed by intersubunit cross-linking.

We next performed experiments in which radiolabel was located on C8 and cross-linker on C9. These experiments were intended to complement those above and establish whether significantly different results would be obtained if the cross-linker and radiolabel were transposed. Here, EAC1-7 cells were prepared using C8 which contained either 125I-(α-γ) or 125I-(β). These were subsequently incubated with saturating amounts of ANH-C9, washed, and irradiated. Total cross-linking was measured in parallel samples containing unlabeled C8 and 125I-ANH-C9. Cross-linking analyses were performed by SDS-PAGE as described for Figs. 4 and 5. Quantitative
results from these analyses are presented in Table III. Results in the upper portion of Table III measure total cross-linking and indicate that approximately 48% of ANH-C9 undergoes covalent cross-linking to molecular species which alter C9 electrophoretic mobility. Significantly, results in the lower portion indicate that this cross-linking does not involve either the α-γ or β subunits of C8.

**DISCUSSION**

The cross-linker used in this study has several advantages over nonphotosensitive bifunctional cross-linking reagents which are frequently used to study protein-protein interactions. These advantages enabled us to approach the question of C8-C9 association in a direct manner. One notable feature of this reagent is that it can be introduced in its precursive form as an intrinsic ligand on the protein under study. Prior knowledge of which protein carries the ANH-ligand means that, if cross-linking of any radiolabeled component is observed as a result of irradiation, it must necessarily be cross-linked to that particular protein. Such a definitive interpretation of cross-linking results is usually not possible with bifunctional reagents which are added extrinsically. Addition of these reagents to such multicomponent complexes as C5b-9 would likely result in cross-linking or polymerization of the entire complex with no meaningful indication as to relative proximities of the constituent proteins. A related advantage is the fact that the ANH-ligand requires photoactivation. This is important because it enables the ANH-protein and other constitutive proteins to be incorporated into C5b-9 prior to initiating cross-linking. Consequently, cross-linking of proteins within C5b-9 can be accomplished in situ and, thus, a more meaningful indication of the spatial arrangement of components as they actually reside in the complex can be obtained.

Another advantage of the ANH-ligand is that it does not alter the ability of C8 and C9 to incorporate into C5b-9, a requirement which is essential to the validity of our approach. Regarding C8, our results show that ANH-(α-γ) and ANH-(β) can recombine with their complementary unmodified subunits to yield ANH-C8 which is functionally similar to C8. The ability to modify these subunits separately and retain recombining potential allowed us to examine whether one particular subunit of C8 is in close proximity to C9. Our results show that ANH-C8 formed by recombination interacts with C5b-7 on EAC1-7 and supports subsequent binding of C9. With regard to modification of C9, incorporation of ANH induces some aggregation. However, the monomeric form of ANH-C9 can be isolated and, although this preparation has a lower specific activity as a consequence of modification, it retains the ability to bind C5b-8. Importantly, results from binding experiments indicate that comparable molar amounts of ANH-C9 and C9 are bound to EAC1-8 at saturation. These results provide evidence that ANH-C9 binds specifically to the C8 binding site on C5b-8 and that comparable amounts become incorporated into C5b-9. These observations support the validity of using ANH-C9 to probe the spatial arrangement of the C9-binding site on C5b-8.

Our results in Tables I and II indicate that, regardless of which C8 subunit carries the ANH-ligand, no cross-linking between ANH-C8 and C9 occurs. Approximately 30% of the total ANH-C8 in C5b-9 was found to be cross-linked to molecules of substantial molecular weight in these experiments. This percentage is considered reasonable since some cross-linking to membrane lipid is likely to occur as well as possible intrachain cross-linking of the ANH-subunit. Importantly, the small amount of intersubunit cross-linking observed within ANH-C8 indicates that utilization of cross-linker by this particular reaction was not substantial. In addition to these possibilities, some portion of the nitrene intermediate formed upon photoactivation of the ANH-ligand is likely to decay prior to reaction with a suitable nucleophilic donor on either protein or lipid. Alternative reactions such as these would not necessarily be detected by our SDS-PAGE method for measuring total cross-linking.

It is noted that the apparent low level of total ANH-C8 cross-linking to macromolecular species might be considered insufficient, if, as an extreme, they were assumed that only one C9 is in close proximity to C8 in C5b-9. If the C8:C9 ratio within C5b-9 was 1:3 as has been reported (2, 6), the maximum amount of 125I-C9 which could be cross-linked in this case would only be ~33%. Moreover, if only ~30% of the total ANH-C8 undergoes cross-linking, this value is reduced to ~11% of the total 125I-C9 in C5b-9. While this amount is small, we feel our experiments in Tables I and II were sufficiently sensitive and reproducible so as to detect even this low a percentage of 125I-C9 cross-linking to ANH-(α-γ) or ANH-(β). If the C8:C9 ratio were 1:6-12 as has also been suggested (9, 13, 14), then the maximum amount of 125I-C9 cross-linking which could occur would be reduced further and our ability to detect such cross-linking would be questionable. Significantly, the C8:C9 ratio on membranes used in this study was determined to be 1:3-4. This ratio was measured under saturating conditions of C8 and C9 added either sequentially or simultaneously to EAC1-7 and was found to be constant from 40-310 sites/cell.

Because of the possibility of relatively low percentages of 125I-C9 cross-linking when using ANH-C8, we substantiated our findings by an alternative and more sensitive approach using radiolabeled C8 and ANH-C9. Results in Table III show that 48% of ANH-C9 is involved in cross-linking to high molecular weight substances. Although total cross-linking is again <100% for reasons mentioned above, this experimental design is intrinsically more sensitive than using ANH-C8 to probe C8 and C9 interaction because the multiplicity of ANH-C9 binding. Only one of several ANH-C9 molecules in C5b-9 need be near C9 in order to potentially cross-link all the 125I-(α-γ) or 125I-(β) in the complex. Significantly, results in Table III show that no cross-linking between ANH-C9 and either C8 subunit occurs when this experimental approach is used.

Results in this study enable us to conclude that little or no C9 in membrane-bound C5b-9 is in close physical association with C8. While our experiments cannot completely exclude the possibility that a small percentage of C9 in the complex may be in contact with C8, such a possibility seems unlikely. For this to be true, it would require that all the cross-linker on both ANH-C8 and ANH-C9 be located at a site some distance from the point of physical contact between them, and consequently, no cross-linking could occur. We cannot completely eliminate this possibility at the present time because lack of structural and conformational data on C8 and C9 precludes identifying the exact location of the ANH-ligand in each protein. However, we did position the ligand on each of the two proteins whose cross-linking was under study in our system and in each case a substantial mole quantity of ANH-ligand was present and available for cross-linking. Furthermore, we demonstrated directly that each ANH-protein was in fact capable of cross-linking while in C5b-9. Considering this, it seems reasonable that, if C8 was physically associated with C9, some cross-linking between them should be detected when using at least one of the ANH-proteins.

Our results are significant because they provide new insight into the role of C8 in mediating C9 incorporation into C5b-9. There are several possible mechanisms by which C8 can perform this function once it is contained in the preclusive
C5b-8 complex. One possible mechanism would involve direct binding of one or more C9 molecules to C8 and formation of a closely associated and stable complex between these two proteins within C5b-9. Our results clearly do not support this particular mechanism because formation of a stable complex between C8 and C9 in the terminal complex should produce detectable cross-linking in our experiments. A second possible mechanism is that C8 within C5b-8 interacts directly with C9 but this interaction is transient as C9 binds and then is shunted into the membrane bilayer. Our results are compatible with this mechanism because they indicate that C8 is not located at the putative C9-binding site within the final C5b-9 complex. The absence of C8 at this site is also compatible with a third mechanism in which C8 may participate indirectly by simply inducing a conformational change in the nascent complex. This change may generate a site far removed from C8 and C9 and indicate that these components are not closely associated within the membrane-bound cytolytic complex.

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