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Immunology

Membrane attack complex of complement: Distribution of subunits between the hydrocarbon phase of target membranes and water

(protein–phospholipid complexes/supramolecular organization/membrane damage/photolabel)

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ABSTRACT **Membrane destruction by complement is effected by the membrane attack complex (MAC) which is the dimer of a fusion product of the complement proteins C5b, C6, C7, C8, and C9. Phospholipid bilayer vesicles were used as target membranes for the MAC and its intermediate complexes. The subunits of these membrane-bound complexes were explored as to their relative exposure to the hydrocarbon phase of the lipid bilayer and to water surrounding the lipid vesicles. Protein exposed to the aqueous phase was labeled with ^{125}I; protein exposed to the hydrocarbon phase was labeled by using tritiated azido phospholipids and irradiation. Analysis of the membrane-bound MAC showed that subunits C5b, C8β, and C9 were exposed to the aqueous phase. The subunits C8α-γ and C9 were primarily in contact with the hydrocarbon phase. C6 and C7 were little exposed to either phase, suggesting that these proteins are inaccessible within the MAC. Analysis of the intermediate complexes showed that C5b was the subunit most exposed to water in membrane-bound C5b-7, and C5b and C8β were the water-exposed subunits in C5b-8. Subunit exposure to the hydrocarbon phase of the lipid bilayer changed during MAC assembly. Whereas all three subunits of C5b-7 carried the phospholipid photolabel; most of the label was bound to the C8 subunit in C5b-8 and to C9 in the MAC. It is proposed that contact with the hydrocarbon core of membranes is established by C5b-7 through each of its subunits, by C5b-8 through C8, and by the MAC through C8 and, particularly, C9.**

The membrane attack complex (MAC) of complement forms on target membranes upon complement activation. It is directly responsible for complement-dependent membrane damage and cytolysis. The manner in which the MAC exerts its cytolytic and membranolytic functions has been the object of many recent studies which included the determination of the apparent pore size of the MAC-induced membrane lesion (1–8), the effect of the MAC on lipid bilayer structure (9–12), and the structural analysis of the MAC itself (13–19). The aim of the present study was to identify those subunits of the MAC and its intermediate complexes that are in direct physical contact with the hydrocarbon core of the target membrane and thus are probably responsible for the expression of membranolytic activity.

The MAC is assembled from five precursor proteins, C5, C6, C7, C8, and C9 (20, 21). Assembly commences with the enzymatic production of fragment C5b, which forms with C6 the bimolecular complex C5b-6. Together with C7, C5b-6 forms the intermediate complex C5b-7 which exhibits a metastable membrane binding site. Addition of C8 leads to formation of C5b-8 and addition of C9 leads to formation of C5b-9 which, upon spontaneous dimerization, becomes the MAC (16–18). Previous studies have shown that the MAC and its membrane-bound intermediate complexes exhibit a marked phospholipid binding capacity (12) and that the interaction of the MAC with ordered lipid bilayers results in reorientation of the bilayer lipids (11). Ultrastructural analysis provided suggestive evidence for the concept that the MAC anchors itself in the target membrane through C8 and particularly through C9 (17).

Insertion of MAC subunits into the hydrocarbon phase of the lipid bilayer of phospholipid vesicles was assessed by the formation of covalent bonds between tritiated azido phospholipid and protein upon irradiation. Exposure of subunits to the aqueous phase was assessed by labeling MAC-dioleoyllecithin vesicle complexes with ^{125}I. The results suggest the occurrence of a dynamic reorganization of hydrophobic domains in the subunits during MAC assembly. They also indicate that C9 and C8α-γ provide the structures through which the MAC establishes intimate contact with the hydrophobic interior of target membranes. Some of these results have been published in abstracts (22, 23).

MATERIALS AND METHODS

Complement Proteins, Protein Complexes and the Photolabel. C5b-6 (24), C7 (25), C8 (26), C9 (27), MAC (16), and SC5b-9 (21) were purified as described. ^3H-Labeled L-α-1-(palmitoyl / stearoyl)-2- (18 -azidolinoleoyl) phosphatidylcholine (photolabel) was synthesized according to Stoffel *et al.* (28, 29).

Preparation of Protein–Lipid Complexes. Dry dioleoyllecithin (DOL) (Applied Science Laboratories, State College, PA) was dissolved in an equal amount (wt/wt) of 1% sodium deoxycholate (Mallinckrodt) in 20 mM Tris acetate, pH 8.1/90 mM NaCl/2 mM EDTA/0.02% sodium azide. To the mixed micelles produced, equimolar amounts of C5b-6 and C7 were then added at a lipid/protein ratio of 5:1 (wt/wt). This mixture was passed over a 1.5 × 30 cm column of Sephadex G-50 (Pharmacia), equilibrated with the same buffer without deoxycholate, at a flow rate of 4 ml/hr at 4°C (30). The void volume fractions containing C5b-7–DOL vesicles were pooled. C5b-8–DOL vesicles were prepared by adding an equimolar amount of C8 (relative to C5b-7) to C5b-7–DOL vesicles. C5b-9–DOL vesicles were prepared by adding a 6-fold molar excess of C9 (relative to C8) to C5b-8–DOL vesicles. Free protein was separated from vesicle-bound protein by flotation of the vesicles through discontinuous sucrose density gradients consisting of 1 ml of 60% (wt/vol) sucrose, 1.5 ml of 40% sucrose containing the sample, 2 ml of 35% sucrose, and 0.5 ml of 10% sucrose. The sucrose was dissolved

Abbreviations: MAC, membrane attack complex of complent (C5b-9 dimer); SC5b-9, cytolytically inactive and monomeric C5b-9 complex containing S protein; C5b-6, C5b-7, and C5b-8, protein complexes of C5b with C6, with C6 and C7, or with C6, C7, and C8, respectively; C5b-7–DOL, C5b-8–DOL, C5b-9–DOL, and MAC–DOL, protein complexes in association with dioleoyllecithin single-bilayer vesicles; photolabel, ^3H-labeled L-α-1-(palmitoyl/stearoyl)-2-(18-azido linoleoyl)phosphatidylcholine.

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in 0.15 M saline buffered with 3.3 mM Veronal (pH 7.4) containing 1.5 mM $MgCl_2$ and 0.5 mM $CaCl_2$. Centrifugation was performed at 4°C for 16 hr in a Beckman SW 50.1 rotor at 35,000 rpm. The floating vesicles were recovered with a Pasteur pipette and characterized by electron microscopy (not shown) and $NaDodSO_4$/polyacrylamide gel electrophoresis.

Radiolabeling of Protein–Lipid Complexes with Iodine from the Aqueous Phase. Four procedures were used to radiolabel protein–lipid complexes: (*i*) iodination by the chloramine-T method (31); (*ii*) iodination by solid-state lactoperoxidase (32) (Enzymobeads, Bio-Rad); (*iii*) iodination with the iodogen procedure (Pierce) (33); (*iv*) modification with the Bolton–Hunter procedure (34). The last procedure was used for the SC5b-9 complex only. Methods i–iii which modify tyrosine residues and method *iv* which labels lysine residues gave similar results. In control experiments, the MAC and its intermediate complexes were first dissociated with 1% $NaDodSO_4$/4 M urea and then subjected to iodination. Diminished iodination of the subunits in the protein–lipid complexes compared to that of the dissociated complexes was interpreted to indicate protection of tyrosine or lysine residues by other subunits in the complex or by phospholipid.

Radiolabeling of Protein–Lipid Complexes with Phospholipid Photolabel from the Hydrocarbon Phase. Protein–lipid complexes were prepared in the dark with DOL and [^{3}H]azidolecithin at a 3:1 molar ratio. Covalent interaction of the photolabel with proteins was achieved by irradiation of the complexes at a wavelength >300 nm as described (28, 29). Samples were flushed with Ar for 2 hr in the dark and then irradiated under Ar at 4°C for 15 min in borosilicate glass tubes at 3 cm from a 125-W Hg lamp. In controls, DOL–[^{3}H]azidolecithin vesicles were irradiated together with C5b-6 or with free C7, C8, and C9; 2% (wt/vol) $NaDodSO_4$ and 4 M urea (final concentrations) were then added and the samples were boiled for 2 min.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Iodine-labeled or photolabeled complexes were analyzed according to Weber and Osborne (35) in 12-cm-long 7.5% polyacrylamide gels which were subsequently sliced into 1-mm segments with an automatic gel fractionator (Gilson) to determine the distribution of radioactivity. Total uptake of photolabel was estimated from the sum of covalently bound radiolabel, the protein content, and the specific radioactivity of the photolabel. Samples were also analyzed by $NaDodSO_4$/polyacrylamide slab gel electrophoresis and autoradiography.

RESULTS

Identification by Iodination of MAC Subunits Exposed to the Aqueous Phase. Fig. 1 shows the radioiodine uptake by the MAC–lipid complexes and by the same complexes after their dissociation into subunits. The solid-state iodogen and lactoperoxidase procedures gave identical results. Samples of C5b-7–DOL, C5b-8–DOL, and C5b-9–DOL either were labeled with ^{125}I or were first dissociated with 2% $NaDodSO_4$/4 M urea and then labeled with ^{131}I. Dissociation in $NaDodSO_4$/urea and subsequent labeling was done to ascertain that the lack of labeling of subunits in the native complex was caused by complex formation and not due to inherent properties of the isolated subunit. Corresponding samples of ^{125}I- or ^{131}I-labeled material were combined and the mixtures were subjected to $NaDodSO_4$/

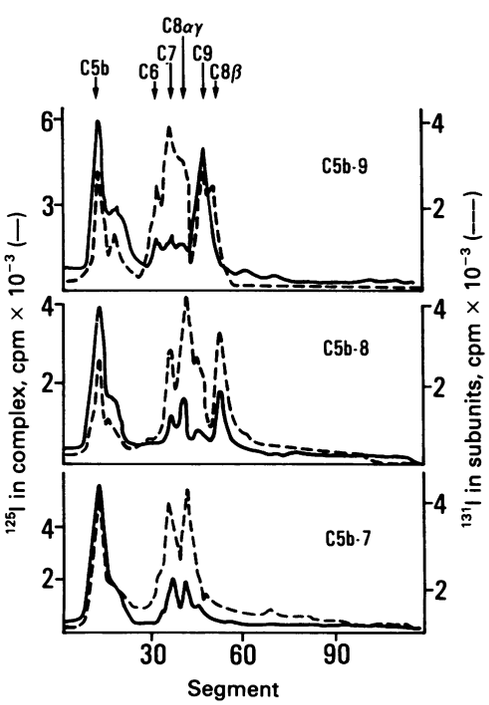

FIG. 1. Radioiodination of C5b-7, C5b-8, and MAC in association with phospholipid vesicles. Solid line, subunit distribution of radiolabel in C5b-7, C5b-8, and C5b-9 after labeling by the iodogen procedure; broken line, subunit distribution of radiolabel from $NaDodSO_4$-dissociated complexes labeled by the chloramine-T procedure. Samples were analyzed by coelectrophoresis on 7.5% $NaDodSO_4$/polyacrylamide gels under nonreducing conditions.

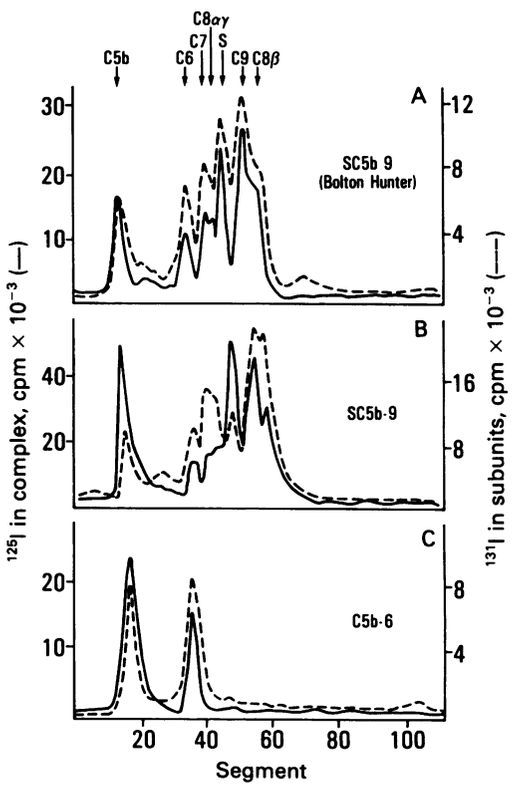

FIG. 2. Radioiodination of the hydrophilic complexes SC5b-9 and C5b-6. (*A*) Subunit distribution of radioactivity of Bolton-Hunter modified SC5b-9 (solid line) and of SC5b-9 modified after dissociation with SDS (broken line). (*B*) Subunit distribution of radioactivity in SC5b-9 labeled by the lactoperoxidase procedure (solid line) and in $NaDodSO_4$-dissociated SC5b-9 labeled by the chloramine-T procedure (broken line). (*C*) Subunit distribution of radioactivity of C5b-6, labeled by the iodogen procedure (solid line) and of C5b-6 labeled by the chloramine-T method after dissociation in $NaDodSO_4$ (broken line). All samples were analyzed by coelectrophoresis on 7.5% $NaDodSO_4$/polyacrylamide gels under nonreducing conditions.

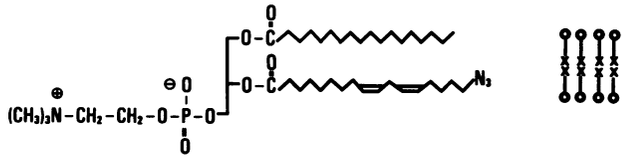

FIG. 3. Structure of the photoaffinity probe used to measure labeling of MAC complexes from the hydrocarbon phase of phospholipid vesicles.

polyacrylamide gel electrophoresis under non reducing conditions. Compared to the $NaDodSO_4$/denatured complexes, intact C5b-7, C5b-8, and C5b-9 in association with phospholipid vesicles showed a markedly different distribution of label among the subunits. Within the complexes, C6 and C7 took up much less radiolabel than when the complexes were dissociated. Similarly, uptake of radiolabel by complex-bound C8 was markedly diminished, the C8α-γ subunit being more affected than the C8β subunit. In the case of C5b and C9, however, the extents of iodine uptake were similar regardless of whether labeling occurred within the MAC or after dissociation of the complex. These results suggest that C5b, C8β, and C9 are more exposed to water than are other subunits of the vesicle bound MAC. No difference in the distribution of label was seen when phospholipid vesicle-bound complexes were compared with the corresponding DOC complexes (not shown).

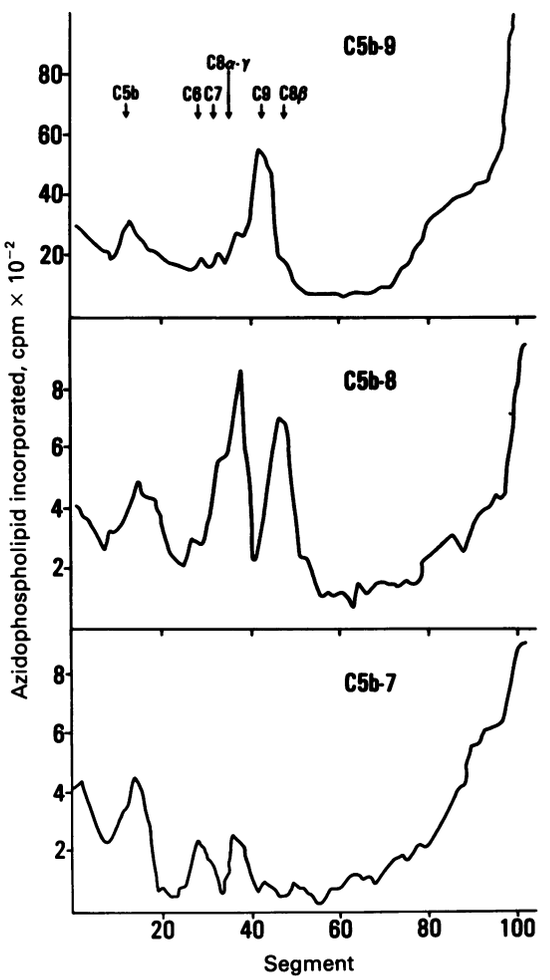

FIG. 4. Covalent labeling of subunits of C5b-7, C5b-8, and MAC with tritiated azidophospholipid. The complexes were assembled on DOL vesicles containing the photolabel. Covalent binding of the probe was achieved by irradiation at 4°C in an Ar atmosphere. After dissociation with $NaDodSO_4$/urea, samples were analyzed on 7.5% $NaDodSO_4$/polyacrylamide gels. Direction of migration was to the right; the radioactivity at the dye front (extreme right) represents free photolabel.

SC5b-9 is a hydrophilic complex of C5b-9 monomer and three molecules of S protein. The latter functions as the serum inhibitor of the forming MAC and prevents the attachment of C5b-9 to membranes as well as its dimerization. As judged by radioiodine labeling, the exposure of the subunits in SC5b-9 to water (Fig. 2) was similar to that of the subunits of the vesicle-bound MAC (Fig. 1). Subunits C6, C7, and C8α-γ are relatively concealed, whereas C5b, C9, and C8β are exposed. The strong labeling of the S protein suggests a relatively external disposition of this protein within SC5b-9. The labeling pattern of the SC5b-9 subunits was independent of the method of labeling used. The hydrophilic C5b-6 complex exhibited an unimpeded radiolabeling capacity of its subunits, indicating that major portions of both C5b and C6 are exposed to water.

Identification of MAC Subunits Exposed to the Hydrocarbon Phase by Photolabeling. Fig. 3 shows the structure of the photolabel used for these studies. The MAC and its intermediate complexes were assembled in the presence of the tritiated azidophospholipids and sodium deoxycholate. The detergent was subsequently removed by gel filtration on Sephadex G-50. To remove unbound protein, the protein–lipid complexes were subjected to flotation by discontinuous sucrose density gradient ultracentrifugation. The suspension of protein–lipid complexes was flushed with Ar, irradiated with UV light and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis under nonreducing conditions or by $NaDodSO_4$/polyacrylamide slab gel electrophoresis and autoradiography. Sixty-six percent of the radiolabel was found to be linked to C9, 15% to C8, and 19% to C5b, C6, and C7 (Fig. 4). C8α-γ carried more label than did C8β. In the C5b-8 complex, C8 carried the major portion of the radiolabel (45%), with C8α-γ carrying more than C8β. In the C5b-7 complex, approximately half of the radiolabeled azidophospholipid was linked to C5b and the other half was linked to C6 and C7. The number of covalently bound phospholipid molecules per complex was estimated from the total protein-bound radioactivity and the amount of protein added to the reaction mixture. C5b-7 and C5b-8 had 6–10 phospholipid molecules covalently attached and C5b-9 had 20–50. These data suggest that C9 causes an approximately 5-fold increase of covalent binding of the probe. Similar results were obtained in four replicate experiments.

The following control experiments were performed. Azidophospholipid vesicles were incubated with C5b-6 alone or with C7, C8, and C9 alone. Under these conditions, no firm association was observed between the proteins and the phospholipid when sucrose density gradient ultracentrifugation was used to separate the free proteins from the phospholipid vesicles. However, when the latter step was omitted, C9 became labeled by the azidophospholipid, indicating a reversible association of C9 with phospholipid bilayers sufficient to allow covalent labeling. None of the other proteins was labeled. Fig. 5 summarizes the observed labeling of the subunits of the MAC and its intermediate complexes from the hydrocarbon phase of phospholipid membranes and from the aqueous phase.

DISCUSSION

The MAC is regarded as an icosamolecular complex of five proteins with the probable subunit formula (C5b, C6, C7, C8, $C9_6)_2$ (17, 18, 36). The 20 noncovalently linked subunits contain a total of 26 polypeptide chains, of which 4 are disulfide linked (namely, C5α' to C5β and C8α to C8γ). The results of this study suggest that the portion of the MAC that is preferentially ex-

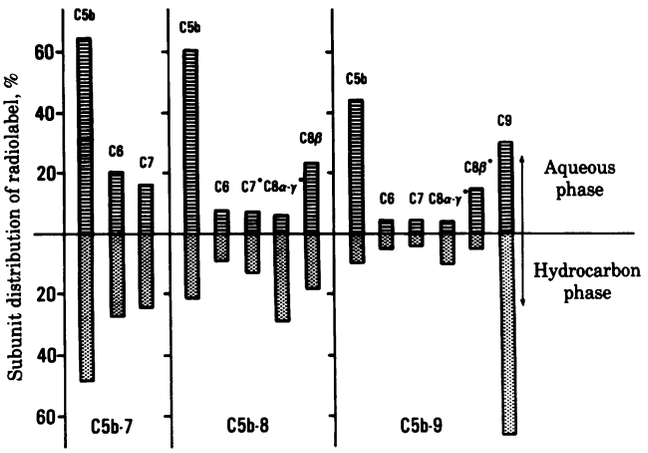

FIG. 5. Relative exposure of the subunits of DOL vesicle-bound C5b-7, C5b-8, and C5b-9 to the aqueous and the hydrocarbon phase. Radioactivity of subunits marked * was estimated by assuming a Gaussian distribution of radioactivity in the incompletely resolved protein bands after $NaDodSO_4$/polyacrylamide gel electrophoresis.

posed to the hydrocarbon phase of target membranes is composed of the 12 C9 subunits. In addition, the C9 subunits are also in contact with the aqueous phase, thus behaving like integral membrane proteins. C5b within the MAC is primarily exposed to the surrounding water whereas C6, C7, and C8α-γ are little accessible from the aqueous phase.

The analysis of the microenvironment of the subunits of the membrane-bound MAC must be considered tentative because of the inherent methodological limitations. Surface labeling of the MAC with radioiodine is dependent on the accessibility of tyrosine or lysine residues and therefore may not strictly correlate with exposure of the subunits to water. Unpublished observations in this laboratory do indicate, however, that, within the MAC, C6, C7, and C8 are less assessible to homologous antibody than are C5b and C9. The photolabeling procedure likewise has inherent limitations. Aromatic amino acid residues are photolabeled preferentially compared to aliphatic residues. The effeciency of labeling is low because of the exceedingly short life-time, 10 psec, of the aliphatic nitrene group (28).

The low degree of covalent labeling observed may be explained in part by the aforementioned technical limitations. Covalent binding accounted for only 2–5% of the previously determined noncovalent phospholipid binding capacity of the MAC and its precursor complexes. Because the critical azido group was located at the ω position of the linoleic acid used, only those MAC subunits that penetrated to the center of the hydrocarbon core were expected to be labeled. On the other hand, due to the flexibility of the hydrocarbon chain of linoleic acid, the azido group may not be entirely confined to the center of the hydrocarbon phase of the membrane. Proteins less deeply inserted may therefore also acquire some photolabel.

Covalent labeling with azidophospholipid increased approximately 5-fold with progression of assembly from the C5b-8 to the MAC stage. Of the total MAC label, 66% was found in the C9 subunits, and 81% in the C8 plus C9 subunits. The increase in labeling upon C9 binding may reflect both insertion of C9 into the bilayer core and the multiplicity of C9 molecules per MAC. Ultrastructural analysis of MAC assembly (17) and EPR studies (11) also support the view that the C9 subunits of the MAC are more intimately in contact with the interior of the bilayer membrane than are the other MAC subunits. Upon relating the amount of label to each individual subunit, no great differences are found: C5b, C6, and C7 carried approximately

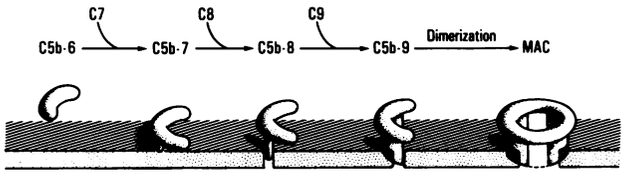

FIG. 6. Schematic representation of the MAC and its intermediate complexes in association with the lipid bilayer. This scheme combined information from the present study and electron microscopic examination of the complexes (17).

3% each, C8 carried 7.5%, and C9 carried 5.5%. However, due to the large multiplicity of C9 in the MAC (36), C9 bound two-thirds of the total label.

Electron microscopic visualization of the MAC and its precursor complexes has led to a formulation of the likely manner in which the MAC is assembled (17). Relevant to the present study is that the C5b-7 complex appears as a half-ring which is directly bound to the surface of the target membrane. Upon addition of C8, this half-ring is raised above the surface of the membrane, suggesting that C8 inserts itself between C5b-7 and the target membrane. C9 completes this process by increasing the mass of those structures of the complex that are in direct contact with the membrane and by causing dimerization (Fig. 6). The photolabeling data presented here constitute further evidence for the idea that all three subunits of C5b-7 are in contact with membrane lipids, that it is primarily C8 in C5b-8 that links the complex to the membrane, and that it is primarily C9 that constitutes the hydrophobic structures of the MAC that are in contact with the target membrane and thus directly involved in membranolysis.

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