Glutathione-catalyzed Disulfide-linking of C9 in the Membrane Attack Complex of Complement*

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The membrane attack complex of complement (the dimeric C5b-9 complex) is a multimolecular assemblage of five proteins (C5b, C6, C7, C8, and C9) which are held together by noncovalent forces. We found that C9 molecules in the complex can be covalently cross-linked (disulfide-linked) by glutathione. In this experiment, the tetramolecular C5b-8 complex bound to phospholipid vesicles was first prepared from purified C5b-6, C7, and C8. The vesicle-bound C5b-8 complex was then incubated (37 °C, 20 min) with an excess of 131I-C9 in the presence of 1 mM glutathione; an average of 5.3 molecules of C9 per C5b-8 were bound and the C5b-9 complex formed was predominantly a dimeric C5b-9 complex. About one-third of C9 in this C5b-9 complex was found to be in a disulfide-linked dimeric form. The C5b-9 complex, having only an average of 0.9 molecules of C9 per C5b-8, was also prepared in the presence of glutathione; this C5b-9 preparation contained both monomeric and dimeric C5b-9 complexes, and about one-fifth of the C9 subunits was in a cross-linked dimeric form. By contrast, C9 in the absence of the C5b-8 complex was not significantly cross-linked by glutathione. These results indicate that C9 has a unique property to associate with itself upon reaction with the C5b-9 complex.

Membrane attack by C1 is mediated by the five terminal C components (C5b, C6, C7, C8, and C9) that become assembled on a target cell membrane upon C activation (1, 2). Although all of these C components are hydrophobic serum proteins, the assemblage of these proteins (the C5b-9 complex) is an amphiphilic macromolecule and causes cell lysis by perturbing membrane organization without apparent degradation of covalent structures in the lipid bilayer (3-6).

Several recent studies have shown that the C5b-9 complex as assembled on target membranes has a dimeric C5b-9 structure consisting of equimolar amounts of C5b, C6, C7, and C8, and of multiple C9 per C5b-8 (7-9). C9 plays an essential role in the final assembly of this complex, since the dimerization of the C5b-9 complex occurs after the binding of C9 to the C5b-8 complex (8) and appears to involve C9-C9 interaction (10). Also it has been shown recently that C9 constitutes a major lipid-binding site of the C5b-9 complex (11, 12). Thus, these studies have indicated an important role for C9 in the structure and function of the C5b-9 complex, and are consistent with the previous observations that the C5b-8 complex in the absence of C9 is ineffective in inducing membrane damage (13) and that C9 plays a critical role in determining the cytolytic efficiency of the C5b-9 complex (14).

Another interesting and, potentially, important property of C9 has been reported recently by Ware and Kolb (15). They found that a fraction of C9 in the C5b-9 dimer isolated from C-lysed sheep erythrocyte membranes was in a disulfide-linked dimeric form and suggested that the quaternary structure of C5b-9 dimers is stabilized by the disulfide-linking of C9. However, it remains unclear how C9 is disulfide-linked and whether the disulfide-linking of C9 represents an intrinsic process in C5b-9 assembly rather than an artificial reaction.

We show here that C9 molecules in the C5b-9 dimer formed on phospholipid vesicles are not covalently linked but can be disulfide-linked by glutathione. The results of the present study, therefore, indicate that the disulfide-linking of neighboring C9 molecules in the C5b-9 dimer does not occur spontaneously and requires non-C substances such as glutathione. In addition, C9 was found to be disulfide-linked by glutathione even under conditions when only an average of less than one molecule of C9 per C5b-8 is bound, indicating that C9 binds in a cluster.

EXPERIMENTAL PROCEDURES

Purified Human C Proteins—C5b-6 (16, 17), C7 (18), C8 (19), and C9 (20) were purified by reported methods. C8 and C9 were radiolabeled with 125I or 131I using lactoperoxidase (22). The specific radioactivity of 125I-labeled C8 (125I-C8) and 131I-labeled C9 (131I-C9) were 7.5 × 10^5 cpm/pg and 1.9 × 10^5 cpm/pg, respectively, and both of these labeled proteins retained 95% of their initial hemolytic activity. In addition, more than 85% of these labeled proteins was incorporated into the SC5b-9 complex upon incubation with C5b-6 in the presence of other terminal C components (supplied as C8-depleted or C9-deficient human serum).

Preparation of the C5b-9 Complexes Bound to Phospholipid Vesicles—Unilamellar phospholipid vesicles were prepared from DMPC as described (17). DMPC vesicles were incubated at 37 °C for 10 min with C5b-6 (80 µg) and C7 (40 µg), and then with 125I-C8 (65 µg) for an additional 10 min at 37 °C (a final protein/lipid ratio of 1.29, w/w). The C5b-8 complex formed on DMPC vesicles was then separated from unassociated proteins by floatation in discontinuous sucrose density gradients as described (17).

The C5b-9 complexes containing different numbers of C9 per C5b-8 were prepared as follows. The DMPC vesicle-bounded C5b-8 complex (11 µg) was incubated at 37 °C for 20 min with either an excess (23 µg) or a limiting amount (1.4 µg) of 131I-C9. When desired, a reduced form of GSH (Sigma) was included in the reaction mixture at concentrations as indicated. The C5b-9 complexes formed were then subjected to separation by floatation in discontinuous sucrose density gradients. The vesicle-bound C5b-9 complexes were pooled and freed of sucrose by dialysis against 20 mM Tris-acetate buffer (pH 8.1) containing 0.1 M NaCl, 0.1 mM EDTA, and 0.01% sodium azide (TBS).

Sucrose Density Gradient Ultracentrifugation—A 10-50% (w/w)
linear sucrose density gradient was prepared in TBS containing 1% sodium deoxycholate. Centrifugation was performed in a Beckman SW 50.1 rotor at 36,000 rpm for 16 h (∼4 °C).

**SDS-Polyacrylamide Gel Electrophoresis**—[^11C9]C9 in the C5b-9 complexes was analyzed on 5% gel according to the method of Weber and Osborn (22). The samples were treated with 2% SDS containing 20 mM dithiothreitol. After electrophoresis, the gels were stained with Coomassie blue, or were sliced into 2-mm segments for radioactivity measurement. The stained gels were scanned at 600 nm for estimation of the stain intensity of protein components.

### RESULTS AND DISCUSSION

A fraction of C9 in the C5b-9 dimer isolated from C-lysed sheep erythrocyte membranes was in the form of disulfide-linked C9 dimers (15). However, since C9 did not contain any reactive free sulfhydryl group as measured by incorporation of radiolabeled iodoacetoamide or N-ethylmaleimide into C9 in the presence or absence of 6 M guanidine hydrochloride (data not shown), spontaneous formation of disulfide-linked C9 dimers through oxidation of free sulfhydryl groups in C9 is unlikely to occur. In fact, as shown in Fig. 1A, C9 in the C5b-9 dimer formed on DMPC vesicles migrated predominantly as a C9 monomer ($M_r = 76,000$) on SDS-polyacrylamide gel electrophoresis under nonreducing conditions. Since erythrocytes contain a sizable amount of GSH (23), a possibility that GSH catalyzes the disulfide-linking of C9 in the C5b-9 complex through thiol-disulfide exchange reaction was then examined. The DMPC vesicle-bound C5b-8 complex (11 µg) was incubated at 37 °C for 20 min with an excess of [11C9]C9 (23 µg) in the presence of either 0.1 mM, 1 mM, or 10 mM of GSH. The C5b-9 dimers formed were then isolated through discontinuous sucrose density gradients and were analyzed on SDS-polyacrylamide gel electrophoresis under nonreducing conditions. As shown in Fig. 1, B–D, the C5b-9 dimers formed in the presence of GSH contained a significant amount of covalently linked C9 dimers ($M_r = 151,000$); 13% (Fig. 1B), 32% (Fig. 1C), and 29% (Fig. 1D) of the total [11C9]C9 were converted to C9 dimers by 0.1 mM, 1 mM, and 10 mM of GSH, respectively. In addition, some [11C9]C9 were found in the origin of the gel and in the $M_r = 200,000$ component (in Fig. 1D), for example, 10% and 4% of the total [11C9]C9 were present in the origin of the gel and in the $M_r = 200,000$ component, respectively suggesting the formation of C9 oligomers larger than a C9 dimer. Since these covalently linked C9 oligomers including C9 dimers were not seen when SDS-polyacrylamide gel electrophoretic analysis was done under reducing conditions (data not shown), the formation of C9 oligomers involved disulfide-linking.

Although as much as 43% of C9 bound to the C5b-8 complex was covalently cross-linked by GSH as shown above, C9 in the absence of the C5b-8 complex was not significantly cross-linked by GSH. In this experiment, isolated C9 was incubated at 37 °C for 20 min at a final concentration of 3.2 mg/ml in the absence or presence of 1 mM GSH. After dialysis against TBS at 4 °C overnight, the samples were analyzed on SDS-polyacrylamide gel electrophoresis under non-reducing conditions. Only 3.8% of C9 was found to be converted to covalently linked C9 dimers by GSH (data not shown). This is consistent with a recent report by Podack and Tschopp (24). They have shown that spontaneous polymerization of C9 is

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**Fig. 1. Glutathione-catalyzed covalent linking of C9 in the C5b-9 dimer.** The C5b-9 dimers were prepared by incubating (37 °C, 20 min) the phospholipid vesicle-bound C5b-8 complex (11 µg) with an excess of [11C9]C9 (23 µg) in either the absence (A) or presence of 0.1 mM (B), 1 mM (C), or 10 mM (D) of GSH. Analysis by SDS-polyacrylamide gel electrophoresis was done under non-reducing conditions. The percentage of each component is indicated above the respective peaks. Only 10% of the total [11C9]C9 in the C5b-9 dimer formed in the absence of GSH was covalently linked, while 21-43% of the total [11C9]C9 was covalently linked in the presence of 0.1-10 mM GSH.
markedly temperature- and time-dependent, and is negligible below 40 °C within 2 h.

The C5b-9 complexes used in the above experiment contained an average of about 5 molecules of C9 per C5b-8 and were predominantly a dimeric C5b-9 complex. It is possible, therefore, that the GSH-catalyzed cross-linking of C9 in the C5b-9 complex depends on the binding of multiple C9 per C5b-8 and/or on C5b-9 dimer formation. In order to examine this possibility, the C5b-9 complexes containing different numbers of C9 per C5b-8 were prepared as follows. The DMPC vesicle-bound C5b-8 complex (11 µg) was incubated at 37 °C for 20 min with either an excess (23 µg), or a limiting amount (1.4 µg), of [35S]-C9 in the presence of 1 mM GSH. Under the conditions that an excess of C9 was offered, 28% of [35S]-C9 (6.4 µg) was bound to the C5b-8 complex, and the C5b-9 complex formed was predominantly a dimeric C5b-9 complex (Fig. 2A). At a limiting C9 input, 76% of [35S]-C9 (1.1 µg) was bound to the C5b-8 complex, and the C5b-9 complex formed was not homogeneous in size and contained both monomeric and dimeric C5b-9 complexes (Fig. 2B). These C5b-9 preparations were then analyzed by SDS-polyacrylamide gel electrophoresis under non-reducing conditions in order to determine the degree of formation of disulfide-linked C9 dimers. Fig. 3 shows the results of spectrophotometric analysis of the gels. The C5b-9 complex prepared with an excess of [35S]-C9 contained 0.84 molecules of C9 dimer and 3.6 molecules of C9 monomer per C5b-8 (Fig. 3A and Table I, column A). Therefore, this C5b-9 preparation incorporated an average of 3.3 molecules of C9 per C5b-8, 22% of these C9 molecules being in a dimer form. On the other hand, an average of 0.9 molecules of C9 per C5b-8 were bound to the C5b-8 complex at a limiting C9 input, 20% of these C9 being in a dimeric form (Fig. 3B and Table I, column B). The results were also consistent with the patterns of [35S]-C9 radioactivity distribution shown in Fig. 4. The above results, thus, indicate that C9 can be cross-linked by GSH even under the conditions that only a limiting amount of C9 is bound to the C5b-8 complex, though it is not clear whether C9 dimerization is a prerequisite to the cross-linking of C9. In preliminary experiments, however, about 20% of C9 in the monomeric C5b-9 complex formed on rabbit erythrocyte membranes (10) was found to be in a disulfide-linked dimeric form. C9 dimers were also formed in the monomeric C5b-9 complex containing S-protein (the SC5b-9 complex) which was generated in fluid phase by incubating C5b-6 with human serum in the presence of 1 mM GSH, a

<table>
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<tr>
<th>Subunit component</th>
<th>Observed molecular weight</th>
<th>Molar ratio a</th>
<th>b</th>
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<tr>
<td>C5b</td>
<td>173,000</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>C9</td>
<td>151,000</td>
<td>0.84</td>
<td>0.09</td>
</tr>
<tr>
<td>C6</td>
<td>114,000</td>
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<td>1</td>
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<tr>
<td>C7</td>
<td>102,000</td>
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<td>0.92</td>
</tr>
<tr>
<td>C8α-γ</td>
<td>96,000</td>
<td>0.6</td>
<td>0.54</td>
</tr>
<tr>
<td>C9</td>
<td>76,000</td>
<td>3.6</td>
<td>0.72</td>
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<tr>
<td>C8β</td>
<td>68,000</td>
<td>1.0</td>
<td>0.92</td>
</tr>
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</table>

a Molar ratios were estimated from densitometric scans of Coomassie blue stained gels and were expressed relative to C6.

b The C5b-9 preparations A and B were prepared by incubating (37 °C, 20 min) the DMPC vesicle-bound C5b-8 complex (11 µg) with an excess (23 µg) and a limiting amount (1.4 µg) of [35S]-C9, respectively, in the presence of 1 mM GSH.

K. Yamamoto, unpublished data.
although the SC5b-9 complex formed in the absence of GSH contained few C9 dimers (25).

In conclusion, we have shown that the disulfide-linking of C9 in the C5b-9 complexes requires non-C substances such as GSH. This suggests that the formation of the disulfide-linked C9 dimers as observed in the C5b-9 complex formed on erythrocyte membranes (15) is mediated by erythrocyte-derived GSH and does not represent an intrinsic process in C5b-9 assembly. On the other hand, the cross-linking of C9 in the C5b-9 complex as described is interpreted to indicate that C9 binds in a cluster. The present study thus revealed a molecule of C9 per C5b-8 on an average is bound, indicating that C9 binds in a cluster. The present study thus revealed a unique property of C9 which may be of importance in the structure and function of the C5b-9 complex.

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REFERENCES