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Antigenic crossreactivity of the α subunit of complement component C8 with the cysteine-rich domain shared by complement component C9 and low density lipoprotein receptor

(mosaic proteins/antipeptide antibody/protein family/conserved sequence)

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ABSTRACT **Complement component C9 contains two distinct cysteine-rich domains exhibiting high sequence resemblance to a domain present in the low density lipoprotein (LDL) receptor and epidermal growth factor precursor, respectively. Antibodies were raised against a peptide corresponding to the most conserved region of the LDL receptor/C9-homology segment. The antibodies were shown by immunoblotting to bind specifically to C9 but also to crossreact with C8α, the α subunit of complement component C8. Moreover, a monoclonal antibody to a neoantigen present in polymerized C9 bound to an epitope exposed on C8 within the C5b–8 complex but buried in monomeric C8, suggesting that C8 and C9 undergo similar conformational changes during membrane-attack-complex assembly. Isolated C8α–γ exhibited the propensity to polymerize in the presence of Zn^{2+} and urea, as already demonstrated for C9. These data indicate that C8α is closely related, both structurally and functionally, to C9.**

Amino acid sequence data have revealed that several plasma proteins are evolutionarily related to each other. For instance, serine proteases of the coagulation, fibrinolysis, and complement systems are similar to other serine proteases such as trypsin or chymotrypsin (1). The genes coding for most of these proteins are thought to have arisen from gene duplication, and their products exhibit similar functions. More recently it has become evident that whole exon units from different genes can be recombined to give rise to new, so-called mosaic proteins (1, 2). In such proteins, segments borrowed from other proteins are put together to form molecules with new functions. These modular segments are most likely to correspond to entire domains, each of which is encoded by an individual exon. It is becoming apparent that such exon-encoded domains fall into several groups (1, 3). Most of them are rich in disulfide bridges, which contribute to their high structural stability. They have a unit length of approximately 40–80 amino acids. The most common modular unit to date is a cysteine-rich domain, present in epidermal growth factor (EGF), low density lipoprotein (LDL) receptor, factors IX and X and protein C of the coagulation cascade, tissue plasminogen activator, urokinase, complement component C9, and several viral proteins (1). Another modular segment, the "C9-type" cysteine-rich domain, has been found so far, apart from C9, only in the LDL receptor, as seven repeated domains.

Although proteins having a common domain are related by amino acid sequence, they are not necessarily related in function. For instance, no functional resemblance is evident between LDL receptor and C9: LDL receptor is responsible for the binding and internalization of LDL (4), whereas C9 is the crucial protein for target-cell lysis by complement. Membrane damage occurs after binding of C9 to the target-cell-bound complex composed of C5b, C6, C7, and C8 (C5b–8) (5). Consequently, C9 rearranges, exposes its hydrophobic domain, and circularly polymerizes, generating the typical "complement lesions" (6, 7). These lesions contain, apart from C5b–8, C9 oligomers of different sizes which traverse the lipid bilayer by exposing hydrophobic segments on one side and hydrophilic domains on the other side (8, 9). This spatial arrangement creates transmembrane channels of 1–10 nm inner diameter, depending on the number of C9 polymers.

The exact mechanism by which C9 polymerizes and inserts into the lipid bilayer is not known. Since the C9-type cysteine-rich domain is directly involved in the interaction of LDL receptor with LDL, we were interested in a possible function of this domain within C9. Antibodies against a peptide derived from this segment were raised in an attempt to investigate this hypothesis.

MATERIALS AND METHODS

Proteins. C9 was purified (10) using polyethylene glycol precipitation and DEAE-Sephacel and hydroxylapatite chromatography as purification steps. C8 was isolated according to Kolb and Müller-Eberhard (11). Other complement proteins were isolated as described (12). C9 fragments were generated either by thrombin (13) or by trypsin (3). C8α–γ was isolated by chromatographing purified C8 on a Mono P column (Pharmacia, FPLC system) in a buffer containing 7 M urea, 1 mM EDTA, and 25 mM bis(2-hydroxyethyl)imino-[tris(hydroxymethyl)]methane (Bis-Tris) at pH 7. The subunits were eluted with a buffer containing 7 M urea, 1 mM EDTA, and Polybuffer (Pharmacia, pH 4.0). C8α–γ was eluted from the column at pH 4.8.

Peptide Synthesis. A peptide corresponding to residues 101–111 of the C9 sequence was synthesized. The exact amino acid sequence was Asp-Asn-Asp-Cys-Gly-Asp-Phe-Ser-Asp-Glu-Asp-Tyr. The carboxyl-terminal tyrosine was added for coupling the peptide to ovalbumin (see below). The peptide was synthesized according to a modified Merrifield method (14). 9-Fluorenylmethoxycarbonyl (Fmoc)-protected amino acids were purchased from Bachem (Bubendorf, Switzerland). A semi-automatic peptide synthesizer (Labortec, Bubendorf, Switzerland) was used for the synthesis. The peptide was separated from the resin with trifluoroacetic acid/CH_2Cl_2 (1:1) and was purified by size-exclusion chromatography (Sephadex G-25) and by DEAE-Sephacel (Pharmacia) chromatography. The composition of the peptide was checked by amino acid analysis. The peptide was coupled to ovalbumin (Sigma) via the tyrosine as follows.

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Abbreviations: LDL, low density lipoprotein; BSA, bovine serum albumin.

C9 (76–116)	A E D D C G N D – F Q C S T G R C I K M R L R C N G D N D C G D F S D E D D C E S E
LDL receptor (91–122)	P P K T C S Q D E F R C H D G K C I S R Q F V C D S D R D C L D G S D E A S C P V L
Peptide (101–111)	D N D C G D F S D E D

FIG. 1. Comparison of homologous sequences of C9 and LDL receptor. Standard one-letter amino acid abbreviations are used.

Ovalbumin (1 mg) dissolved in 100 μl of borate buffer (0.16 M borate/0.13 M NaCl, pH 9.0) was added to peptide (2 mg) dissolved in 400 μl of borate buffer at 0°C. Bis(diazo)benzidine solution (150 μl) was added and the pH was adjusted to 9.0 with 0.5 M NaOH. The mixture was stirred for 2 hr at 4°C and then dialyzed versus Tris-buffered saline (TBS: 0.15 M NaCl/10 mM Tris Cl, pH 7.4) to remove excess peptide. The solution containing the peptide-ovalbumin conjugate was stored at −20°C until use. Bis(diazo)benzidine was prepared by dissolving 0.23 g of benzidine·HCl (Fluka) in 45 ml of 0.2 M HCl and then adding 0.175 g of $NaNO_2$ in 5.0 ml of H_2O. The reaction mixture, which immediately became orange, was stirred intermittently at 4°C for 60 min. Aliquots (1 ml) were then quickly frozen and stored at −60°C.

Immunization. To obtain the antipeptide antibodies, two rabbits were injected subcutaneously on days 0, 20, and 34 with an emulsion of 250 μg of peptide-ovalbumin conjugate in 500 μl of TBS and 500 μl complete Freund's adjuvant (day 0) or incomplete adjuvant (days 20 and 34). Rabbits were bled on day 44.

Biochemical Techniques. $NaDodSO_4$/PAGE was carried out using an 8% or 10% polyacrylamide gel according to the procedure described by Laemmli (15). The protocol outlined by Towbin *et al.* (16) was followed for immunoblots, using horseradish peroxidase-coupled second antibody (Sigma) and H_2O_2 plus 4-chloro-1-naphthol as substrate. The hemolytic activity of C9, C8, and C8α–γ were determined exactly as described by Steckel *et al.* (8).

Electron Microscopy. The protein was adsorbed to carbon-coated grids (6). The grids were washed twice with a volatile buffer (0.15 M ammonium acetate, pH 7.4) and negatively stained with 1.5% uranyl formate (Eastman Kodak). The micrographs were taken at ×35,000 initial magnification.

RESULTS

Antibodies Against a Synthetic Peptide Corresponding to C9-Type Cysteine-Rich Domain Recognize C9. C9 contains a cysteine-rich domain near the amino terminus (residues 76–116) (3, 13). A synthetic peptide corresponding to the most conserved region of this C9-type unit (residues 101–111) was synthesized. Six out of eleven amino acids of this segment are identical within C9 and the LDL receptor, the only other protein now known to contain the C9-type domain (Fig. 1). Antiserum was raised against this peptide and checked by immunoblots. Purified C9 was subjected to $NaDodSO_4$/PAGE and transferred to nitrocellulose. The antiserum reacted with C9 (Fig. 2, lanes 1), whereas preimmune serum failed to recognize C9 (data not shown). When C9 was cleaved by thrombin at position 220, yielding two fragments of apparent molecular mass 34 kDa and 37 kDa (17), only the 34-kDa fragment corresponding to residues 1–220 was detected by the antiserum to the peptide (Fig. 2, lanes 2). Similarly, when C9 was cleaved by trypsin, which cleaves at position 423, only the 53-kDa fragment containing the C9-type unit was immunostained (lanes 3). To examine further the specificity of this antiserum, we incubated blots of C9, ovalbumin, and bovine serum albumin (BSA) with the antiserum in the presence or absence of peptide at 0.1 mg/ml (≈80 μM). The immunostaining of unreduced C9 was completely inhibited by addition of the peptide (Fig. 3). The antiserum also reacted with ovalbumin, since the peptide was coupled to ovalbumin for the immunization. This interaction, however, was not blocked by the peptide. BSA was not immunostained at all. Similar results were obtained when reduced samples were analyzed. In this case, the background staining was higher and two faint bands were occasionally seen in all three proteins tested. However, staining of these bands could not be inhibited by addition of the peptide, indicating their nonspecificity (Fig. 3, *Right*).

The C9-Type Cysteine-Rich Domain Occurs Also in C8α. When the antiserum to the C9 peptide was tested against other complement proteins, C8 also reacted with the antiserum (Fig. 4). C8 is known to bind to membrane-bound C5b–7, thereby exposing the binding site for C9. C8 contains three polypeptide chains, C8α (68 kDa), C8β (66 kDa), and C8γ (20

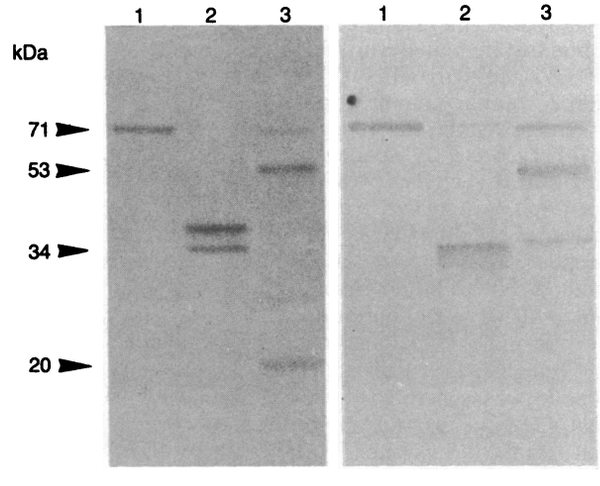

FIG. 2. Characterization of anti-C9-peptide antiserum. One microgram of C9 (lanes 1), of C9 split by thrombin (lanes 2), and of C9 split by trypsin (lanes 3) were analyzed by $NaDodSO_4$/PAGE, electrophoretically transferred to nitrocellulose, and stained with Ponceau S (*Left*) or immunostained using the anti-C9-peptide antiserum (*Right*).

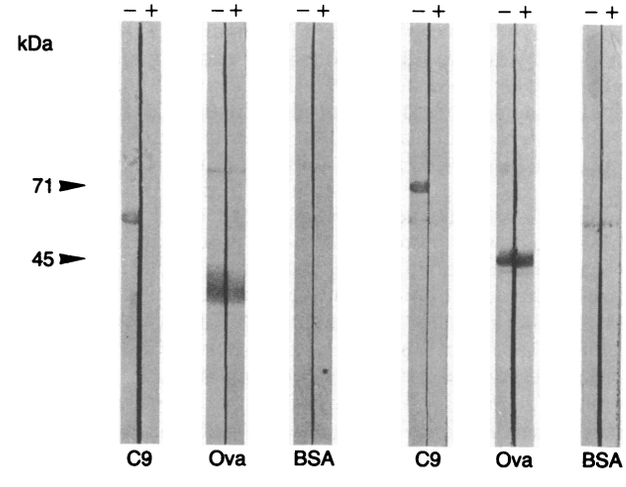

FIG. 3. Inhibition, by the C9 peptide, of the reaction of anti-C9-peptide antiserum with C9. C9, ovalbumin, and BSA (1 μg per lane) were analyzed by $NaDodSO_4$/PAGE under nonreducing (*Left*) or reducing (*Right*) conditions. Immunoblot analysis was carried out with (+) or without (−) C9-derived peptide present during incubation with antiserum.

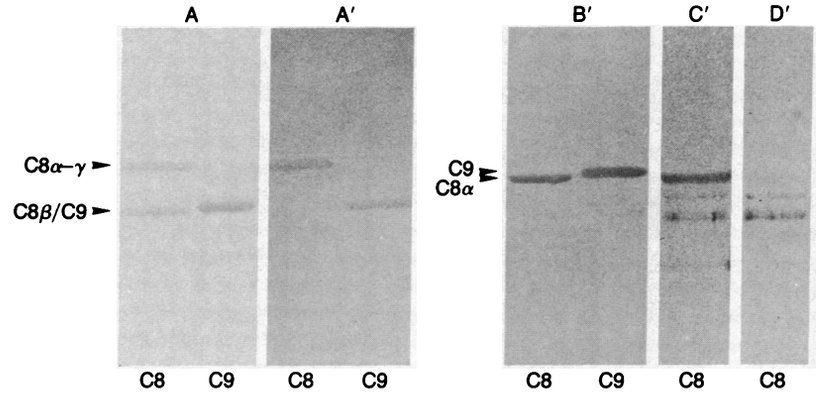

FIG. 4. Crossreactivity of anti-C9-peptide antibodies with C8α. C8 or C9 (1 μg per lane) was electrophoresed and stained with Ponceau S (*A*) or transferred to nitrocellulose and immunostained, with the antiserum specific for the C9 peptide, in the absence (*A'–C'*) or presence (*D'*) of the C9 peptide. The proteins were either not reduced (*A* and *A'*) or reduced (*B'–D'*) prior to electrophoresis. The immunoreaction is inhibited by the C9-derived peptide (*D'*).

kDa) (11). C8α and C8γ are disulfide-linked, whereas C8β is bound noncovalently. The antiserum reacted with C8α–γ (Fig. 4 *A* and *A'*) and, as seen when C8 was electrophoresed under reducing conditions, only C8α was immunostained (Fig. 4*B'*). This reaction was prevented by the C9 peptide, demonstrating its specificity (compare Fig. 4 *C'* and *D'*).

Exposure of an Antigenically Crossreactive Neoantigenic Site on C8 and C9. More evidence for crossreactive epitopes on C8 and C9 came from a monoclonal antibody raised against the fluid-phase terminal complements complex SC5b–9 (comprising C5b, C6, C7, C8, C9, and S-protein). This antibody (Ae 11) recognizes a neoantigen exposed on the assembled complex but fails to react with the individual precursor components (18). As shown by dot-blot analysis, this antibody immunostained polymerized C9, suggesting that the epitope detected by the antibody was part of C9 (18). Poly(C9) reacted with this antibody at a concentration of 2 ng per dot, whereas a 100 times higher concentration of monomeric C9 was required (Fig. 5 *Right*). BSA and C8 did not react with Ae 11. However, upon binding of C8 to C5b–7, forming the C5b–8 complex, the complexed C8 reacted with Ae 11, indicating the exposure of a neoantigenic site on C8 that crossreacts with the site exposed on C9. Other proteins tested were C5, C6, C7, and the complexes C5b–6 and C5b–7 (data not shown). All of them were negative even at high antigen concentrations, except C5b–7, which was detectable at 200 ng per dot, comparable to the detection limit for monomeric C9 but considerably higher than that for C5b–8.

Further analysis showed that Ae 11 bound to purified C8α–γ on both immunoblots (Fig. 5) or dot blots (Fig. 6 *Left*).

Functional Resemblance of C8α–γ and C9. Purified, monomeric C9 can be induced to form amphiphilic, circular polymers [poly(C9)] in the presence of Zn^{2+} (20). Upon polymerization, C9 loses it capacity to bind to C5b–8, and the hemolytic activity of C9 is therefore abrogated. We observed the same effect when Zn^{2+} was added to purified C8α–γ. Incubation of C8α–γ (100 μg/ml) with 0.1 mM Zn^{2+} for 3 hr reduced its lytic activity by 70% (Table 1). Other agents known to polymerize C9, such as urea (21), also led to a loss of the hemolytic activity of C8α–γ. As in the case of C9, the diminution of C8α–γ activity was due to aggregation of the protein. Electron microscopic examination of purified C8α–γ exposed to 1 M urea at 37°C for 3 hr revealed nonspecific aggregates (Fig. 6 *Right*). Careful examination of the aggregates indicated the presence of elongated structures with an approximate length of 14–18 nm exhibiting several globular domains that resemble the domains found on C9 (19).

DISCUSSION

The C9-type cysteine-rich domain comprises amino acids 76–116 of C9 and is highly negatively charged. This cluster of negative charges is particularly strong in the sequence Asp-Cys-Gly-Asp-Phe-Ser-Asp-Glu-Asp-Glu-Glu-Cys (residues 103–114) and is also the sequence exhibiting the highest homology with the LDL receptor (Fig. 1). We therefore synthesized a peptide containing part of this particular amino acid sequence. Antibodies raised against this peptide reacted

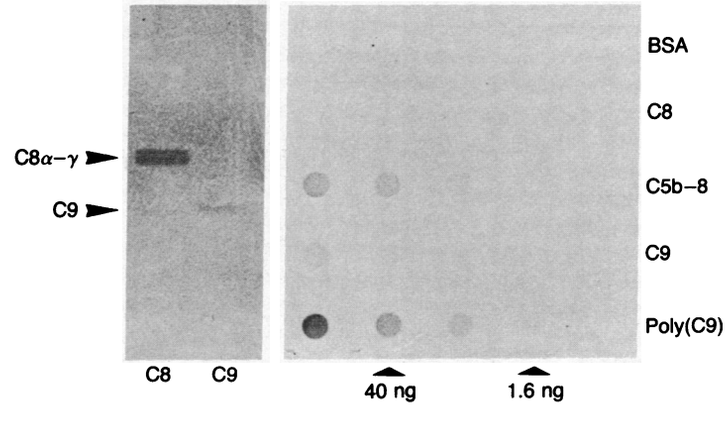

FIG. 5. Reactivity of monoclonal antibody Ae 11. (*Left*) NaDodSO$_4$/PAGE and immunoblot analysis of C8 and C9 (2 μg per lane), using Ae 11 as primary antibody. (*Right*) Dot immunoblot analysis of 200, 40, 8, 1.6, and 0.32 ng of BSA, C8, C5b–8 (protein quantity based on C8), C9, and poly(C9) (polymerized in the presence of 0.1 mM Zn^{2+}) adsorbed on nitrocellulose and immunostained with Ae 11.

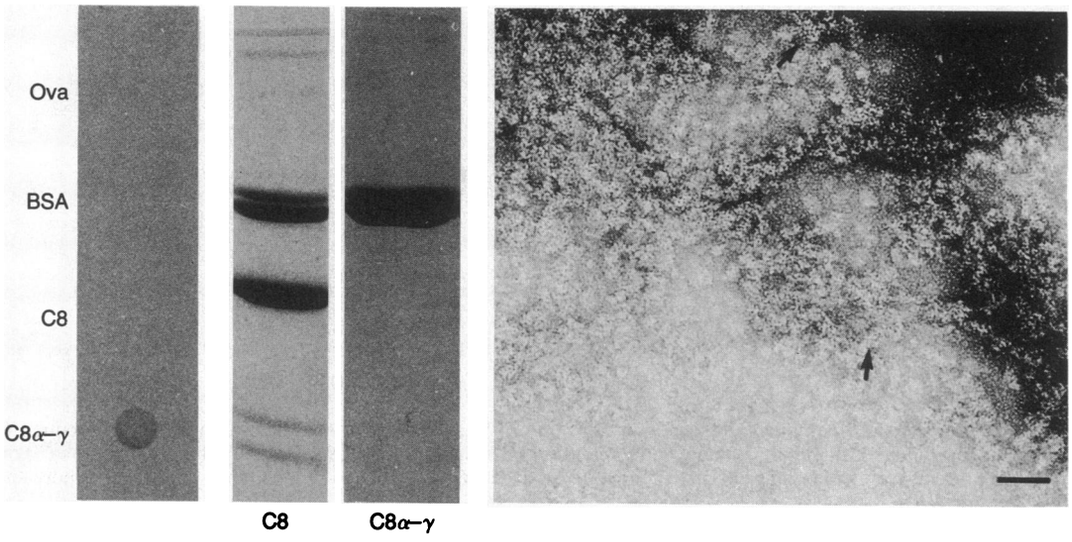

FIG. 6. Structural characteristics of purified C8α–γ. (*Left*) Purified C8α–γ, C8, BSA, and ovalbumin (200 ng) were adsorbed on nitrocellulose and immunostained with monoclonal antibody Ae 11. $NaDodSO_4$/PAGE analysis of isolated C8 and C8α–γ (stained with Coomassie blue) is also shown. (*Right*) Electron micrograph of isolated C8α–γ incubated for 3 hr at 37°C in the presence of 1 M urea. Arrows point to sphere-like domains with diameters of 3–4 nm, which are also observed when C9 is examined (19). (Bar = 50 nm.)

with C9 and C8α, indicating that an identical or similar sequence is also present in C8α. Since the amino acid sequence is part of a highly conserved cysteine-rich domain, it is very likely that the whole C9-type domain is contained within C8α. This would identify C8α as a third member of the superfamily including C9 and LDL receptor (Fig. 7 *Upper*). Other results support the structural resemblance between C8α and C9, suggesting that the homology may go beyond the conserved cysteine-rich domain. (*i*) The monoclonal antibody Ae 11, which binds to an epitope exposed on polymerized C9, crossreacts with C8α. Since this antibody binds to blotted C9 only weakly, a precise mapping of the recognized epitope within C9 is not possible. We cannot, therefore, exclude that Ae 11 binds to the C9-type cysteine-rich domain. (*ii*) C9 and C8α have virtually the same molecular weight and the same isoelectric point (pI 4.8) (22). (*iii*) C9 polymerizes at 37°C in the presence of 0.1 mM Zn^{2+} to form tubular poly(C9) and consequently becomes lytically inactive. C8α–γ loses its lytic activity under the same conditions, forming undefined aggregates. (*iv*) C9 exhibits a binding site for C9 (involved in C9 polymerization) and a binding site for C8α–γ (binding of C9 to C8). Since specific inhibitors of C9–C9 interaction also abrogate C9 binding to C8 (unpublished results) the corresponding binding sites for C8α–γ and C9, respectively, are likely to be similar. All these data indicate a high functional and structural resemblance of C8 and C9, implying the following concept of the assembly and function of the membrane attack complex. We propose that after the C8β-mediated binding of C8 (23) to C5b–7 (Fig. 7 *Lower*), C8α–γ rearranges, exposes its hydrophobic domain, and concomitantly inserts into the lipid bilayer, similar to the model originally proposed for C9 (6). Small transmembrane lesions

Table 1. Loss of hemolytic activity of C9 and C8α–γ in the presence of Zn^{2+} or urea

		Remaining hemolytic activity, %		
	Treatment	0 hr	1 hr	3 hr
C8α–γ	Zn^{2+}	100	80	30
C9	Zn^{2+}	100	20	0
C8α–γ	Urea	100	80	30
C9	Urea	100	30	0

C8α–γ or C9 (0.1 mg/ml) were preincubated at 37°C for 0, 1, or 3 hr in 0.15 M NaCl/10 mM Tris Cl (pH 7.4) containing 0.1 mM Zn^{2+} or 1 M urea. Tests (described in ref. 8) were carried out at a final dilution of 1:100,000 for C8α–γ and 1:300,000 for C9.

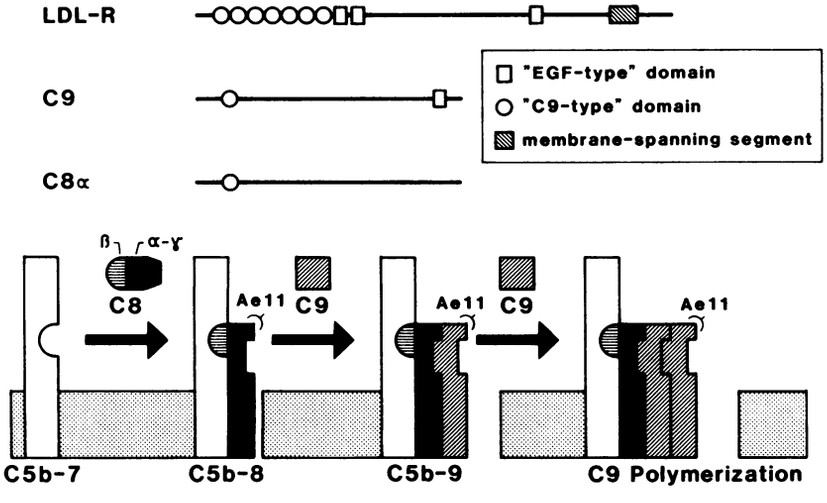

FIG. 7. (*Upper*) Domains of LDL receptor, C9, and C8α. All contain the C9-type module. The position of this domain indicated in C8α is hypothetical. EGF, epidermal growth factor. (*Lower*) Model of the assembly of the membrane-attack complex and its insertion into the lipid bilayer. Epitope recognized by monoclonal antibody Ae 11 is indicated.

could be created by C8α–γ; indeed, it is known that C5b–8 induces a slow lysis of erythrocytes due to the lipid-insertion of C8 (8, 24). It was postulated that this lysis is due to a slow aggregation of C5b–8 (25). The propensity of C8α–γ to aggregate may account for this complex formation. Binding of C9 to C8α–γ makes lysis more efficient. C9 then undergoes similar conformational changes as C8α–γ and consequently enlarges the transmembrane channel. Further addition of C9 then potentiates this effect. This model assumes a similar molecular rearrangement of C8α–γ and C9 during lipid insertion. That this is really the case is evidenced by the binding properties of monoclonal antibody Ae 11: it fails to bind native C9 or C8 but exhibits a high affinity for the amphiphilic form of these proteins. Removal of the β chain also exposes this neoantigen on C8α–γ, either by unmasking this site or by inducing a conformational change with C8α–γ similar to that induced by C5b–7 on the whole molecule.

We have to await the exact sequence of C8α–γ before drawing more conclusions concerning the extent of the resemblance between C8α–γ and C9. The sequence analysis may reveal not only that these two proteins share a highly conserved cysteine-rich domain but also that they may have arisen by duplication of the whole gene.

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