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Topology of the membrane-bound form of complement protein C9 probed by glycosylation mapping, anti-peptide antibody binding, and disulfide modification

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Abstract

The two N-linked oligosaccharides in native human C9 were deleted by site-specific mutagenesis. This aglycosyl-C9 did not differ from its native form in hemolytic and bactericidal activity. A new N-glycosylation site (K311N/ E313T) was introduced into the turn of a helix-turn-helix [HTH] fold that had been postulated to form a transmembrane hairpin in membrane-bound C9. This glycosylated form of human C9 was as active as the native protein suggesting that the glycan chain remains on the external side of the membrane and that translocation of this hairpin is not required for membrane anchoring. Furthermore, flow cytometry provided evidence for the recognition of membrane-bound C9 on complement-lysed ghosts by an antibody specific for the HTH fold. A new N-glycosylation site (P26N) was also introduced close to the N-terminus of C9 to test whether this region was involved in C9 polymerization, which is thought to be required for cytolytic activity of C9. Again, this glycosylated C9 was as active as native C9 and could be induced to polymerize by heating or incubation with metal ions. The two C-terminal cystines within the MACPF domain could be eliminated partially or completely without affecting the hemolytic activity. Free sulfhydryl groups of unpaired cysteines in such C9 mutants are blocked since they could not be modified with SH-specific reagents. These results are discussed with respect to a recently proposed model that, on the basis of the MACPF structure in C8 α , envisions membrane insertion of C9 to resemble the mechanism by which cholesterol-dependent cytolysins enter a membrane.

Keywords

Complement; Complement 9; MACPF; (poly)C9; monotopic membrane protein; membrane protein anchoring

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1. Introduction

Human complement protein C9 is a hydrophilic serum glycoprotein responsible for the efficient expression of cytolytic and bacteriolytic functions of complement. It assembles on the surface of a target cell together with C5b, C6, C7, and C8 to form the C5b-9 or membrane attack complex (MAC1) and in the process refolds to become an integral membrane protein (Esser, 1994; Müller-Eberhard, 1986; Plumb and Sodetz, 1998). Based on amino acid sequence alignments it is evident that C6, C7, C8 α , C8 β and C9 share a homologous central region called the MACPF domain (Plumb and Sodetz, 1998) and a MACPF fragment of C8 α expressed in bacteria could substitute for the complete C8 α molecule in hemolytic and bacteriolytic assays (Slade et al., 2006). Although C5b-8 precursor complexes at high concentrations are weakly hemolytic, killing of nucleated cells and Gram-negative bacteria is dependent on C9. With respect to bacterial killing it has been useful conceptually to consider the C5b-8 complex as a receptor to which C9 binds and then translocates across the outer membrane to reach the periplasm (Dankert and Esser, 1987; Taylor, 1992). Bacterial cell death can be produced solely by C9 (Dankert and Esser, 1987). Strong evidence has been published showing that C5b-8 by itself is not a transmembrane structure but is situated in the membrane as a monotopic complex (McCloskey et al., 1989). Many investigators favor the view that polymerization of C9 leads to formation of transmembrane protein channels that are responsible for complement-mediated cytotoxicity (Podack and Tschopp, 1982; Tschopp et al., 1982; Tschopp et al., 1985). Early models designed to explain how C9 anchors the MAC in a membrane postulated that amphipathic α -helices generate transmembrane structures in membrane-bound C9 (Peitsch et al., 1990). The formation of disulfide bonds between adjacent C9 molecules in the assembled MAC has also received much attention (Hatanaka et al., 1994; Ware and Kolb, 1981; Yamamoto and Migita, 1983) and glycosylation of C9 was thought to be important for function (Kontermann and Rauterberg, 1989).

In order to test these models we decided to seek further experimental evidence for the topography of membrane-bound C9. After removing the two consensus N-glycosylation sites in C9 we used glycosylation mapping to probe the surface topology of C9. Thus, new N-linked glycosylation sites were created by site-directed mutagenesis and glycosylation of these new sites was verified by lectin blotting. We also generated several mutants with completely deleted disulfide bonds and tested their importance for hemolytic activity and those having an unpaired cysteine were tested for their potential use in cysteine-scanning experiments. Recently two groups reported the crystal structure of the C8 α MACPF domain and noticed that it shares a common folding pattern with cholesterol-dependent cytolysins (Hadders et al., 2007; Slade et al., 2008). On the basis of this common folding pattern it was proposed (Hadders et al., 2007; Rosado et al., 2008) that C9 polymerizes and enters a target membrane in a fashion similar to CDC proteins. According to this model the membrane-inserting regions are two β -strands that are connected by disulfide bonds. While our results are not in conflict with this newly proposed membrane insertion mechanism some details, however, are difficult to reconcile with earlier results from our laboratory and others.

2. Materials and Methods

2.1 Complement Proteins and Assays

Hemolytic activity of complement proteins was assayed according to standard procedures and bactericidal activity using the *Escherichia coli* strain C600 was assayed as described (Tomlinson et al., 1993). Cell survival is presented as the ratio of CFU/mL incubated with serum (sample) to CFU/mL of cells incubated in buffer (control).

2.2 Construction of prokaryotic C9 expression plasmids

All DNA manipulations were carried out using standard techniques (Sambrook et al., 1989). The previously characterized C9 vectors pSL301HuC9 (Tomlinson et al., 1993) and pYW29 (Wang et al., 2000), which contains two mutations (L532S/K538R) and a His6 C-terminal tag, were used as the starting plasmids for the expression vectors listed in Figure 1. A KpnI – SacI fragment from pSL301HuC9 was cloned into pSelect-1 to eliminate the two existing N-glycosylation sites individually using the “Altered Sites Mutagenesis System” (Promega, Madison, WI). The mutated genes were then transferred into pSVL (Pharmacia) as XbaI – SacI fragments to generate pYW30 and pYW31, respectively. Plasmid pYW32 was prepared by exchanging the XbaI – NruI fragment in pYW31 with the corresponding one from pYW30. The hexahistidine tag was added by exchanging the XhoI – Tth111I fragment in pYW29 with the respective XhoI – Tth111I fragment of pYW32 to generate pYW33.

In order to introduce new glycosylation sites into aglycosyl-C9 a KpnI – SacI fragment of pYW33 was transferred into pSelect-1 and mutated at the desired positions. The mutated genes were transferred back into pYW33 by exchanging the respective XbaI – SacI fragments to yield pYW34, pYW35, and pYW36. To generate the P26N mutation an XbaI – SacI fragment was moved from pYW33 to pUC19 and mutagenesis was performed with the “Transformer Site-directed Mutagenesis System” from Clontech (Madison, WI) according to the manufacturers protocol. The mutated gene was then transferred back into pSVL as an XbaI – SacI fragment to create pVR11. All C9 mutants are listed in Figure 1 together with a sketch showing the location of the various glycosylation sites within the C9 domain structure.

2.3 Construction of Recombinant Baculovirus

A baculovirus shuttle vector, or bacmid system (Luckow et al., 1993) kindly provided by Dr. Verne Luckow (Monsanto Corporation, Chesterfield, MO) was used for the generation of recombinant viruses. In brief, XbaI – SacI fragments were excised from pYW33, pYW34, and pVR11 and inserted individually between the NheI and SacI sites of the donor plasmid pMON27045. Transposition of the various C9 genes was carried out by transforming the recombinant donor plasmids into *E. coli* DH10B harboring the transposition helper plasmid pMon7124 and the bacmid bMON14272. Selection of the correct composite bacmids was performed as described (Tomlinson et al., 1993) and purified bacmid DNA was used for the transfection of insect cell lines.

2.4 Expression of C9 mutants in COS-7 and Insect cells

COS-7 cells were transfected with the pSVL-derived expressions vectors by electroporation and the different proteins were isolated as described previously (Tomlinson et al., 1993). C9 mutants were expressed at 27 °C by baculovirus-infected Sf9 cells growing in suspension in serum-free medium contained in 250 mL shaker flasks rotating at 135 rpm as described (Rossi et al., 1998). Protein purifications were performed as published (Tomlinson et al., 1993) except those that carried a C-terminal hexahistidine tag were purified by metal-affinity chromatography using the manufacturer's (Qiagen, Valencia, CA) protocol.

2.5 Quantification of Secreted Proteins

Proteins were separated by 7.5% acrylamide SDS-PAGE and transferred to nitrocellulose using standard techniques. Transferred C9 was immunostained by means of mAb216 and anti-mouse IgG-alkaline phosphatase conjugate according to the protocol supplied by BioRad. Concentrations of recombinant wild type and mutant forms of C9 were determined by a dot blot procedure using the “ELIFA™” apparatus (Pierce, Rockford, IL). Samples were adsorbed to nitrocellulose, blocked with 5% “Blotto” solution and incubated overnight with mAb216 anti-C9 (20 µg/ml), washed with 0.3% Tween-20 TBS buffer, and then incubated for two hours

with goat anti-mouse 125I-IgG (10 µg/ml) as detection antibody. The amount of radioiodinated IgG bound to serial dilutions of respective test samples was then quantified in a PhosphorImager™ (Molecular Dynamics, Sunnyvale, CA). Purified human C9 was used to generate a standard curve from which the amounts of secreted C9 were determined. The hemolytic activity of the different recombinant C9 mutants was determined as published before (Tomlinson et al., 1993).

2.6 Analysis of Glycans on Secreted C9 Proteins

The presence of carbohydrates on native and secreted C9 mutants was detected by specific binding of lectins using the DIG Glycan Differentiation Kit purchased from Boehringer Mannheim (Indianapolis, IN) following the procedures supplied with the kit. Among the digoxigenin-labeled lectins only the *Sambucus nigra* agglutinin (SNA) recognized C9 purified from human serum demonstrating that it contains complex sialylated N-glycan chains.

2.7 Epitope Mapping by Sequence-specific Anti-peptide Antibodies

Antisera specific for two overlapping peptides, C-L₃₀₅PTTYEKGEYFAFLE₃₁₉ and C-K₃₁₁GEYFAFLETYGT₃₂₄, coupled via their N-terminal cysteines to keyhole limpet hemocyanin (KLH) were raised in rabbits as described (Laine and Esser, 1989a). Monospecific IgG was isolated individually from each antiserum by immunoaffinity chromatography using as an antigen the peptide C-TTYEKGEYFA coupled to ReactiGel (Pierce). Recognition of C9 on the surface of complement-lysed erythrocyte ghosts by these two antibodies was assayed by flow cytometry as published (Laine and Esser, 1989a) using a Becton-Dickson FacScan analyzer.

2.8 Modification of Disulfides in C9

Human C9 contains 12 disulfide bonds, three in each one of the four recognized domains (www.UniProt.org), that is, the TSP type-1 and the LDL-receptor class A at the N-terminus, the MACPF in the center, and the EGF-like domain at the C-terminus. The C9 gene constructs were first cloned into the PUC19 plasmid and the desired cystine changes within the MACPF domain were accomplished using the Clontech (Palo Alto, CA) Transformer Site-directed mutagenesis kit. The new constructs were then transferred either into a pSVL vector for expression in COS-7 cells or into pMON27045 for expression in insect cells.

2.9 Labeling of Free Sulfhydryl Groups

Fluorescence labeling of free sulfhydryls in C9 and mutants was attempted using reagents with either a methanthiosulfonate (MTS) reactive group, such as MTS- 4 fluoresceine, and MTS-pyrene (Toronto Research Chemicals, Inc.), or iodoacetamide [BODIPY-IA] and maleimide leaving groups (7-diethylamino-3-(4'-maleimidyl-phenyl)-4-methylcoumarin [CPM]), and also with monobromobimane (all purchased from Molecular Probes, Eugene, OR). Labeling reactions were done in 100 mM Hepes buffer at pH 7 – 7.5 and in the absence of any denaturing reagents.

3. Results

3.1 Cystine Modification in C9

Recently two groups reported the crystal structure of the C8α MACPF domain (residues 103 – 462) and noticed that it shares a common folding pattern with CDC proteins (Hadders et al., 2007; Slade et al., 2008). On the basis of this common folding pattern it was proposed (Hadders et al., 2007; Rosado et al., 2008) that C9 polymerizes and enters a target membrane in a fashion similar to CDC proteins, that is, residues 197-270 (TMH1) and 343-400 (TMH2) in C9 refold into amphipathic transmembrane β-sheets that provide both the anchoring and the walls of the

central aqueous pore in the poly(C9) ring structure. The MACPF domain in C9 contains three disulfide bonds and two of them are located within these putative transmembrane hairpin structures (Figure 2). Thus it was of interest to test whether any one of these cysteines is required for the functional activity of C9. Using site-specific mutagenesis the six cysteines were changed individually or together to serines. C9 mutants in which the disulfide bond between C121 and C160 was altered or deleted were not secreted from COS-7 cells or SF9 cells while alteration of the other two cysteines (at position 233/234 and 359/384 in the TMH1 and 2, respectively) was not detrimental and all secreted mutants retained hemolytic activity (Figure 3).

Since it was possible to create functional C9 mutant proteins with unpaired cysteines we attempted to label such cysteines with fluorescent reporter groups. After performing several control experiments it became evident, however, that even native C9 despite having only disulfide-paired cysteines could be labeled slightly with SH-specific labels in the native state but not under denaturing conditions, e.g., in the presence of 6M GuHCl, in agreement with observations published earlier (Yamamoto and Migita, 1983). The authors attributed the low level labeling to disulfide exchange within the native protein. The labile disulfide bond responsible for the exchange in the native state was shown to be the bond between C359 and C384 by Hatanaka et al. (Hatanaka et al., 1994). Thus, we created a C9 molecule having a single cysteine at position 234 and a complete deletion of the C359 to C384 disulfide bond. Surprisingly this mutant protein could not be labeled with a variety of SH-specific reagents even under denaturing conditions nor was it possible to label C359 when it was the single cysteine in C9-C384S. This is in contrast to bovine serum albumin, which contains a free cysteine and could be labeled successfully under the same conditions (data not shown). We analyzed the secreted proteins by SDS-PAGE under non-reducing conditions and also by gel chromatography and could not find any evidence for a disulfide-linked dimer or multimers (data not shown). We conclude that the sulfhydryl groups in these secreted mutant proteins were perhaps chemically blocked by glutathione or cysteine present in culture media or provided by the cells during secretion. Others encountered a similar situation with an engineered thrombin mutant in which an engineered free sulfhydryl group was blocked (Chen et al., 1996). Attempts to remove the blocking substance by limited reduction under mild conditions were not successful. Conditions that would remove it also inactivated the proteins.

3.2 Hemolytic and Bacteriocidal Activity of aglycosyl-C9

The two N-glycosylation sites in human C9 at N256 and N394 were mutated (T258M and T396M) individually and together to provide a non-glycosylated protein. This protein was expressed in COS-7 cells and secreted into the culture medium. The absence of the carbohydrate moiety had little effect on the hemolytic (Figure 4A) and the bacteriocidal activity of aglycosyl-C9 (Figure 4B). It is known that glycosylation can have an effect on the stability of some proteins and protect them against degradation. Aglycosyl-C9 was as stable as native C9 when incubated in buffer for several days at room temperature (data not shown).

3.3 Expression and activity of C9 with different glycosylation sites

The fact that aglycosyl-C9 retained its hemolytic and bacteriocidal activity made it possible to introduce new consensus glycosylation sites into human C9 and test for their effect on folding and function of the protein. Therefore, four new consensus sites, P26N, K311N/E313T, Y321N and E319N/Y321S, were introduced into human aglycosyl-C9. COS-7 cells did not secrete the last two mutants but the other two were and at rates similar to the wild type recombinant protein. To verify that the consensus N-glycosylation acceptor sites were indeed recognized by the oligosaccharyl transferase we used lectin blotting to demonstrate the presence of carbohydrate moieties on the C9 constructs. Using the DIG glycan differentiation kit we could confirm that serum C9 contains complex, sialylated carbohydrates (DiScipio et al., 1984) since it was recognized by *Sambucus nigra* agglutinin (SNA) (Figure 5, top panel, lane 1) as was rC9

secreted from COS-7 cells (data not shown). Proteins secreted from SF9 cells were not recognized by SNA (data not shown) consistent with many observations that indicate that insect cells produce glycoproteins without terminal sialic acid and with mostly terminal oligo-mannose structures. Indeed, a lectin specific for such structures, *Galanthus nivalis* agglutinin (GNA), recognizes C9 secreted from SF9 cells but not serum C9 (Figure 5, bottom panel, lanes 1 and 2) or rC9 when expressed in COS-7 cells (Figure 5, bottom panel, lane 5). However, both lectins detect the C9 mutants with newly created N-glycosylation sites demonstrating that these mutant proteins are indeed glycosylated. SNA binds to C9-258M/396M- K311N/ E313T when expressed in COS-7 (Figure 5, top panel, lane 6) and GNA binds to C9-258M/396M-P26N when produced in SF9 cells (Figure 5, bottom panel, lane 3) and the reverse is correct also, that is, the K311N/ E313T mutant is recognized by GNA when expressed in Sf9 cells (data not shown). Importantly, hemolytic assays indicated that the aglycosyl-C9 mutants and those with new N-glycosylation sites are as active as the recombinant wild-type C9 (Figure 4).

Earlier reports (Laine and Esser, 1989a; Scibek et al., 2002; Taylor et al., 1994) suggested that the N-terminal portion of C9 and specifically the few amino acids preceding the TSP-1 domain are important for the polymerization properties of C9. Our results with the P26N mutant indicate that addition of a glycan chain at position 26 does not affect the function of the protein (Figure 4) and the ability of the protein to aggregate when incubated with Zn^{2+} (Amiguet et al., 1985) to promote polyC9 formation (data not shown). This result is somewhat surprising since an antibody specific for amino acids 19-28 inhibited polymerization very strongly (Laine and Esser, 1989a).

3.4 Surface location of the EKGE peptide in membrane-bound C9

The observation that a carbohydrate structure at residue 311 in C9 did not alter its hemolytic activity suggested that the region around this carbohydrate attachment point remained on the surface of a target membrane during hemolysis. To test this possibility further we used binding of anti-peptide antibodies coupled with flow cytometry. We had used this methodology previously to locate C9 peptide regions on the surface of complement-lysed erythrocyte ghosts (Laine and Esser, 1989a). Two overlapping peptides with C-terminal cysteines covering the amino acid sequence between L305 and H324 were synthesized, coupled to KLH, and used as immunogens to raise antisera in two rabbits. Peptide-specific antibodies from both sera were purified separately on an immobilized antigen containing the overlapping region. As shown in Figure 6, both antibody preparations bound to complement-lysed ghosts indicating that this C9 region is located on the membrane surface.

4. Discussion

Although several different models, inspired by electron microscopy data, primary sequence analyses, or biochemical studies, have been proposed to explain the anchoring of complement protein C9 in a target membrane the details remain elusive. The current favorite is a model (Podack, 1984) in which C9, after binding to a C5b-8 complex, changes conformation and polymerizes into a ring structure called poly(C9) that not only anchors the MAC to a membrane but also forms the wall of a transmembrane pore structure that is responsible for its function. The latest entry is a model based on X-ray crystallographic studies on the core portion of the C8 α chain that support a role for β -sheets in the membrane anchoring of C9 within the C5b-9 complex.

The participation of TMH1 – also called the C9 hinge region in earlier publications - in these processes is indeed very attractive since it could provide answers to some intriguing unsolved puzzles. For example, when the primary structure of horse C9 was established it became immediately apparent that it was very similar to the human structure (Figure 7) although horse C9 lacked the ability to lyse erythrocytes (Esser et al., 1996). The only significant differences

noted were the two TMH sequences and indeed replacement of the hinge region in horse C9 with the human hinge rendered horse C9 hemolytic (Tomlinson et al., 1995). Furthermore, a peptide comprising residues 247-261 of human C9 was shown to be weakly hemolytic (Chang et al., 1994) indicating that TMH1 does have an affinity for membranes. The absence of hemolytic activity in horse C9 could be the result of an additional oligosaccharide structure at position N256 that prevents membrane insertion of TMH1 and formation of a lytic pore. However, in order for this explanation to hold true it would be necessary to verify that horse C9 is indeed glycosylated at this site and that other C9 hemolytic molecules from species such as rabbit, mouse, rat and trout that have similar potential glycosylation sites in this loop region, are secreted without such oligosaccharides (Figure 7). The alternating hydrophilic-hydrophobic pattern of the C-terminal part of horse TMH1 is also less pronounced compared to other C9 molecules but whether this could explain the lack of hemolytic power is not clear.

While it is extremely likely that the homologous MACPF core region in C9 is folded like the one in C8 α it does not necessarily follow that C9 uses the same structural elements to enter a membrane as similarly folded CDC proteins. First, cutting the peptide chain between H244 and G245 in TMH1 of human C9 has no effect on its lytic activity (Dankert and Esser, 1985). It is difficult to imagine how such a severed β -strand could insert and find its partner to form a functional transmembrane β -sheet. Second, previous photolabeling data by Ishida et al. (Ishida et al., 1982) and by Tschopp and collaborators (Amiguet et al., 1985; Peitsch et al., 1990) are in severe conflict with the proposed membrane association of TMH1 and TMH2. In these studies no evidence was obtained for any labeling of TMH2 and the N-terminal β -strand in TMH1 by membrane-restricted radioactive photolabels. The radioactivity was associated only with the stretch from G245 to S345 leaving out most of TMH1 and all of TMH2. Perhaps these two regions are more important for the C5b-8/C9 oligomerization process than the actual membrane insertion event, a possibility that is reinforced by the fact that cutting the protein in half at H244 prevents the formation of circular poly(C9) (Dankert and Esser, 1985). Since there is no direct experimental evidence that TMH1 and TMH2 are indeed located in a lysed target membrane it is therefore important to consider again the other modes of membrane attachment that were postulated earlier.

In the topological model for membrane-bound C9 of Stanley and coworkers (Stanley et al., 1986) two amphipathic α -helices and three β -sheets transverse the membrane and two short and one longer loop of about 25-30 amino acids in the middle of the protein (between the major chymotrypsin and the single thrombin cleavage sites) protrude on the cytoplasmic side of the membrane. Peitsch combined secondary structure prediction, helix packing constraints, and energy minimization to construct a model for the membrane-spanning domain in C9 (Peitsch et al., 1990). In this model, residues 292-308 and 313-334 form an amphipathic α -helical hairpin, or HTH motif, that spans the membrane with the connecting short turn (E-K-G-E) protruding on the trans (cytoplasmic) side of the membrane (Figure 8A). Removing the two recognition sites N-X-T for N-glycosylation in human C9 by site-specific mutation without affecting the specific hemolytic and bactericidal activity of the secreted proteins allowed us to use glycosylation mapping to probe structural aspects of C9. This technique of placing novel carbohydrate structures has already shown its usefulness in defining the folding patterns of polytopic membrane proteins. It is based on the observation that N-linked carbohydrates are always located on the exoplasmic site of a membrane in animal cells. It has not been used to study the topography of soluble, membrane-inserting proteins but since it is energetically unfavorable to move a large hydrophilic mass across a lipid bilayer the same technique should also allow the identification of surface exposed peptide loops of the membrane-bound form of such proteins. Because the hemolytic activity of aglycosyl-C9 was unaffected by the addition of a new carbohydrate moiety at position 311 we assume that it is unlikely that an HTH structure as envisioned by Peitsch and coworkers spans the membrane in a membrane-bound C5b-9 complex. Additional support for a surface location of the K-E-G-E stretch was provided by the

recognition of the sequence K311-A316 by two different sequence-specific antipeptide antibodies. Since it is unlikely that the immobilized, short peptide that was used to purify the antibodies had much secondary structure we assume that these antibodies recognize non-helical segments and most likely a turn or random structures. Such a turn was indicated by the modeling experiments of Peitsch and indeed the homologous region in C8 α MACPF was shown recently to fold as such a helix-turn-helix motif (denoted as helix D and helix E in the topographical map of Slade et al.). Finally our earlier results (Laine and Esser, 1989b; Laine et al., 1988) identified residues 292-296 to be part of a discontinuous epitope that continues passed the EGF domain into the C-terminal end of C9 suggesting that the C-terminal end folds up against this putative hairpin.

However, we wish to emphasize that our results do not preclude the possibility that this pair of helices could lie more or less parallel to the membrane surface in the interfacial region analogous to the anchoring of monotopic membrane proteins (Picot and Garavito, 1994). Such topography is certainly correct for the membrane anchoring of the C5b-8 complex (McCloskey et al., 1989) and may be also correct for the initial C9 binding mode. The importance of this hairpin structure for C9 folding and function is evident from the mutagenesis studies of Dupuis et al. (Dupuis et al., 1993) who attempted to change its amphipathic character. All non-conservative amino acid replacements introduced on either side of the α helices – specifically residues 305, 318, 319 and 325 – resulted in non-secreted C9. Thus, the lack of secretion of the mutants we constructed with a consensus glycosylation site at position 319 (pYW35 and pYW36 in Figure 1) could be the result of replacing a tyrosine at position 321 with either a serine or an asparagine and not necessarily from an interfering glycan. Changing E319 to N319 does not affect secretion and has no influence on hemolytic activity (Dupuis et al., 1993).

Considering the combined evidence currently available it is clear that in membrane-bound C9 hydrophobic or amphipathic transmembrane α -helical hairpins are not possible. Thus, it is necessary to invoke either a monotopic arrangement, as shown in Figure 8B, or amphipathic transmembrane β -structures like those in the first Stanley model or those proposed for the membrane-inserted CDC proteins. However, it is also important to consider that in CDC proteins the transmembrane β -hairpins are α -helical or unordered in the native proteins and assume their new conformation already during pre-pore formation and before membrane insertion. Thus, it is tempting to speculate that in a C5b-9 complex containing only a few (less than four) C9 monomers these conserved α -helices D and E in C6, C7, C8, and in C9 anchor the complex in a monotopic fashion as shown in Figure 8 whereas in a fully formed poly(C9) structure other structural elements may refold and become the staves of a transmembrane barrel. Since we used normal human serum, or C9-deficient serum reconstituted with mutant C9 proteins, in which a C9:C8 ratio of 2:1 was never exceeded the experiments reported here apply to C5b-9 complexes containing very few if any poly(C9) structures. Therefore, our results may not be applicable when the majority of MACs contain poly(C9) rings.

Much of the success in understanding the membrane insertion of the CDC proteins is based on the ingenious use of cysteine-scanning mutagenesis combined with fluorescence labeling of these newly created sulfhydryls in CDC proteins by Tweten and Johnson and their collaborators (Heuck et al., 2001). Unfortunately, as our attempts to create such mutant proteins indicate, such a strategy may not be possible with C9 unless one can find culture conditions and/or cell lines that will not block free sulfhydryl groups during secretion. Alternatively, glycosylation scanning on a larger scale than presented here should allow identification of those amino acids that must enter a target membrane to achieve hemolysis and a more detailed analysis of C9 molecules with consensus glycosylation sites within the TMH1 and TMH2 regions should provide much useful information.

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Abbreviations

HTH	helix-turn-helix
MAC	membrane attack complex
PAGE	polyacrylamide gel electrophoresis
TBS	Tris buffered saline
SNA	<i>Sambucus nigra</i> agglutinin
GNA	<i>Galanthus nivalis</i> agglutinin
KLH	keyhole limpet hemocyanin
TMH	transmembrane β -hairpin
CDC	cholesterol-dependent cytolysin
wtC9	wild type C9
rC9	recombinant wtC9.

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Plasmid Name	Proteins	Tag	Secretion	Human C9 Domain Structure and N-Glycosylation Sites
pYW30	C9-T258M	None	Yes
pYW31	C9-T396M	None	Yes
pYW32	C9-T258M/T396M	None	Yes
pYW33	C9-T258M/T396M	His ₆	Yes
pYW34	C9-T258M/T396M-K311N/E313T	His ₆	Yes
pYW35	C9-T258M/T396M-K319N/Y321S	His ₆	No
pYW36	C9-T258M/T396M-Y321N	His ₆	No
pVR11	C9-T258M/T396M-P26N	His ₆	Yes
Serum C9	—	—	—

Figure 1.

C9 glycan mutants and location of the respective glycosylation sites.

Tag refers to the addition of a C-terminal hexahistidine sequence that was not removed from the individual proteins. Secretion indicates whether the proteins were secreted by COS-7 and also by Sf9 cells.

TMH1
 C8 α (201–256) DNANDLLSKVKDKSDSFGVTIGIGPAGSPLL-----VGVGVSQSQTSLNELNKYNEKK
 C9 (197–270) EEQIEAFKSIIEKTSNFNAISLKFTPETNKAEQCCETASSISLHGKGSFRFSYSKNETYQLFLSYSSKKE
 ::

TMH2
 C8 α (329–385) KAKMESLGITSRDITTFGGSLGIQYE-DKINVGGGLSGDHCCKKFGGG-KT-ERARKAMA
 C9 (343–400) KASMKRKGVELKDIKRLGYHLDVSLAFSEISVGAEFNKDDCVKRGEGR-RA-VNITSEN
 :.....:

Figure 2.

Putative TMH sequences in human C8 and C9 (Hadders et al., 2007).
 Dotted lines indicate disulfide bonds.

Figure 3.

Hemolytic activity and sulfhydryl labeling of C9-cysteine mutants.

C9 mutant proteins with modified cystine residues were expressed in COS-7 and Sf9 cells and tested for secretion into the culture fluid, hemolytic activity, and labeling by SH-specific fluorescent probes. All secreted proteins have a C-terminal His6 tag allowing isolation of the secreted proteins by affinity chromatography. Protein concentrations were determined by immunoblotting and equivalent amounts of proteins were used to determine hemolytic activities which were not significantly different from recombinant wild type protein. SH-specific labeling of C9 proteins was attempted using a variety of different reagents (listed in the Experimental Procedures section) with bovine serum albumin (BSA) serving as a control. Low level labeling of C9 proteins was about 20% of that obtained with BSA.

Figure 4.

Functional activity of C9 glycan mutants.

Panel A: Hemolytic activity of C9 mutants expressed in COS-7 and Sf9 cells

a: Serum C9

b: rC9 expressed in COS-7 cells

c: C9-T258M (pYW30) expressed in COS-7 cells

d: C9-T396M (pYW31) expressed in COS-7 cells

e: C9-T258M/T396M (pYW33) expressed in Sf9 cells

f: C9-258M/396M – K311N/E313T (pYW34) expressed in Sf9 cells

g: C9-258M/396M – P26N (pVR11) expressed in Sf9 cells

Standard assays of C9 hemolytic activity (Sodetz and Esser, 1988) consisted of 5×10^8 sheep EAC1-8 and 50 μ L of sample dilution in a total volume of 0.1 mL isotonic buffer. Incubations were conducted for 45 min at 37°C followed by addition of ice-cold buffer and centrifugation. 100% lysis was achieved by addition of 1 mL H₂O in place of buffer after incubation. The amount of recombinant C9 used in all assays was approximately 5 ng/mL as measured by ELISA (Tomlinson et al., 1993).

Panel B: Killing of *E. coli* strain C600 by (a) C9-deficient serum reconstituted with 8 μ g of rC9 or (b) with 8 μ g aglycosyl-C9 (C9-258M/396M) per mL. (c) C9-deficient human serum. Error bars indicate means $\pm$ standard deviations (from 3-5 experiments).

Figure 5.

Lectin blotting of C9 and glycan mutant proteins after SDS-Page under non-reducing conditions.

Top Panel: Detection by Coomassie blue (CB) staining and *Sambucus nigra* agglutinin (SNA) blotting.

Lane 1: 5 µg serum C9

Lane 2: 0.5 µg serum C9

Lanes 3 & 5: Fetuin (positive control)

Lane 4: Carboxypeptidase Y (negative control)

Lane 6: C9-258M/396M- K311N/E313T expressed in COS-7 cells

Bottom Panel: Detection by Coomassie blue (CB) staining and *Galanthus nivalis* agglutinin (GNA) blotting

Lane 1: 5 µg serum C9

Lane 2: rC9 expressed in Sf9 cells

Lane 3: C9-258M/396M- P26N expressed in Sf9 cells

Lane 4: Carboxypeptidase Y (positive control)

Lane 5: rC9 expressed in COS-7 cells

Figure 6.

Recognition of membrane-bound C9 by anti-peptide antibody binding as measured by flow cytometry. The left trace (white peak in both panels) shows the autofluorescence of complement-lysed ghosts incubated with non-immune IgG. Incubation with anti-(305-319) IgG (top panel) or anti-(311-324) (bottom panel) shifts the fluorescence (middle trace, gray peak in both panels) to the right and maximum fluorescence is obtained after incubation with polyclonal anti-C9 (black peak in both panels).

Figure 7.

Comparison of the first putative transmembrane hairpin (TMH1) sequence in C9 from different species. The cartoon shows locations of two β -strands within the putative transmembrane hairpin regions. Amino acids are coded according to character: hydrophilic (white letters in gray box), hydrophobic (black letters) and those that do not fit the alternating hydrophilic/hydrophobic amphipathic pattern are shown in underlined bold black letters. Potential glycosylation sites [NXS/T] are placed within rectangles.

Figure 8.

Membrane anchoring of C9.

Panel A: Transmembrane orientation of C9 as proposed by Peitsch et al. (1990).

Panel B: Monotopic anchoring of C9.

The C9 sequence shown is the same in both panels and is homologous with helices D and E in the MACPF C8 α - γ structure of Slade et al. (2008).