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# Human Complement Protein C8: The "Hole" Story

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As the recipient of the 2011 SC Governor's Award for Excellence in Scientific Research, I've been asked to describe my research and some of the accomplishments of my laboratory while at the University of South Carolina. My research is focused on understanding the structure and function of the pore-forming proteins of the human "complement system", and in particular complement protein "C8". The complement system is a group of blood proteins that have a key role in immune defense. Much of what is known today about the structure and function of human C8 can be attributed to work performed over many years by my graduate students and postdoctoral fellows.

## Introduction -

The human "complement system" is composed of approximately 35 different proteins, enzymes, receptors and regulatory molecules found primarily in blood. The system is referred to as complement because it "complements" or enhances the ability of the immune system to defend against pathogens, e.g. bacteria, viruses, etc. The system is "activated" either by the interaction of complement proteins with antibody-antigen complexes (classical pathway) or by interaction with the unusual carbohydrate found on the surface of pathogenic (non-self) organisms (lectin and alternative pathways). Each activation pathway leads to the "terminal pathway" and formation of the end-product of complement activation. This end-product is the "membrane attack complex" of complement or "MAC", a large (~10<sup>6</sup> kDa) complex composed of complement proteins C5b, C6, C7, C8 and multiple copies of C9 (1-4). The MAC kills bacteria by forming a pore or "hole" in the bacterial cell membrane.

The sequence of interactions leading to MAC formation is well defined; however, the mechanism by which the MAC disrupts membrane organization is poorly understood. MAC assembly begins with local production of C5b by activated complement (Fig. 1). This is followed by binding of C6 to form a soluble, noncovalently-linked C5b-6 complex. Subsequent binding of C7 produces an amphiphilic complex (C5b-7) that has an affinity for cell membranes. C5b-7 binds to the outer portion of membrane bilayers and only minimally penetrates the interior. Once on the membrane, C5b-7 binds C8 and forms a tetrameric C5b-8 complex. The ultrastructure of C5b-8 has no pore-like features; however, this complex causes leakage of synthetic lipid vesicles, increases ion-conductance in planar lipid bilayers, and promotes the slow osmotic lysis of simple cells such as heterologous erythrocytes. Photolabeling studies using membrane-restricted probes identified the C8 $\alpha$  subunit as the major C5b-8 component inserted in the membrane (5). In the final step of MAC formation, C5b-8 binds and initiates the self-polymerization of C9 to form a cylindrical transmembrane pore composed of 12-18 C9 molecules (poly C9) (3,6). On bacteria, the MAC increases outer membrane permeability, which in turn induces lethal changes in the inner membrane. Other than proteolytic cleavage of C5, all steps leading to MAC formation are nonenzymatic and the protein interactions

are all noncovalent.

Individually, the five MAC components circulate in blood as hydrophilic proteins, but when combined they form an amphiphilic complex capable of intercalating into cell membranes. The MAC does not degrade membrane lipid but instead produces a disruptive rearrangement that causes osmotic lysis of simple cells such as erythrocytes, initiates intracellular signaling events in nucleated cells, and disrupts the outer membrane of bacteria. Our own cells contain CD59, a membrane-anchored protein that protects us from complement-mediated damage by preventing assembly of a functional MAC.

The MAC components interact in a highly specific and sequential manner once C5b is formed. As each intermediate complex is formed, binding specificity changes and is directed towards the next component incorporated. Once associated, the affinity between components is high despite the noncovalent nature of their interactions. Dissociation can only be accomplished by solubilization of the membrane and denaturation of the MAC. The goal of our program has been to identify structural features that specify the order in which these proteins bind, and the mechanism by which they undergo a hydrophilic to amphiphilic transition that enables them to bind to lipid. Our strategy has been to focus on identifying structure-function relationships in one component, namely human C8, and thereby gain insight into how the other MAC components function. Understanding how the MAC is assembled and its function may lead to the development of therapeutic pore-forming analogues that could be used to attack undesirable human cells, e.g. cancer cells.

## The MAC Family Proteins

Human C5b contains two disulfide-linked subunits and is the largest MAC protein (180 kDa). C6, C7 and C9 are single chain proteins of approximately 105 kDa, 92 kDa and 72 kDa, respectively. C8 (151 kDa) is the most complex in that it is composed of three nonidentical, genetically distinct subunits ( $\alpha$  = 64 kDa;  $\beta$  = 64 kDa and  $\gamma$  = 22 kDa) (7). These subunits are arranged as a disulfide-linked C8 $\alpha$ - $\gamma$  heterodimer that is noncovalently associated with C8 $\beta$ . The affinity between C8 $\alpha$ - $\gamma$  and C8 $\beta$  is high. Dissociation can only be achieved using denaturing agents or high ionic strength buffers.

Our discovery in the 1980's that each C8 subunit was

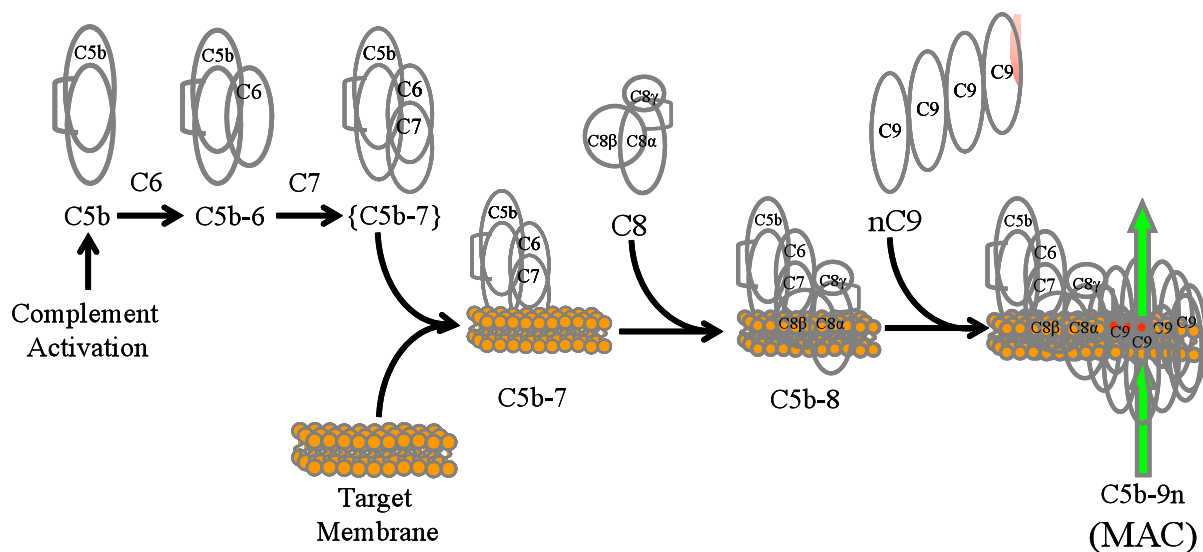


Fig. 1 Assembly of the Membrane Attack Complex (MAC). The components and sequence of interactions are described in the text. The inset shows an EM of a typical membrane pore formed by poly C9 within the MAC.

encoded in a different gene was significant because C8 was thought to be a single gene product, i.e. synthesized as a single-chain precursor that was posttranslationally cleaved. This discovery came as we were characterizing human liver cDNA clones to determine the amino acid sequence of C8 (8). The results established that C8 was composed of three different proteins rather than one. Our efforts to determine the sequences and genomic organization of C8 together with the work of others provided the first evidence of a structural and evolutionary relationship between the MAC proteins.

Human C6, C7, C8α, C8β, and C9 are homologous and together form the "MAC family" of proteins. Family members exhibit sequence similarity and have a highly conserved modular organization (Fig. 2) (4,9). Their genomic structures are also similar with respect to exon length and boundaries. A distinctive feature of each is the presence of cysteine-rich N- and C-terminal modules that are ~40–80 amino acids in length. The modules exhibit sequence similarity to those found in a variety of proteins unrelated to complement. Although not considered a module, the central portion of each protein (~40 kDa) is designated MACPF to emphasize its conservation among the MAC proteins and its sequence similarity to perforin, a 70 kDa pore-forming protein released from secretory granules of cytotoxic T lymphocytes.

The conserved organization of the MAC proteins initially suggested the modules themselves may mediate protein-protein interactions during MAC assembly. This was consistent with the view that the MAC can be thought of as a "heteropolymer" formed from structurally similar but distinct monomeric units. Also noteworthy was the fact these modules mediate protein-protein interactions in other systems. MAC assembly requires binding interactions that are highly ordered and the question of whether specificity is determined by

differences in fine-structure of the modules or sequence variations within the MACPF domains was important to answer.

## Properties of Human C8

Our focus on C8 has provided valuable insight into the specificity of interactions between the MAC components and their ability to undergo a hydrophilic to amphiphilic transition. Unlike the other MAC proteins, C8 occurs in blood as a complex of C8α-γ and C8β. Our ability to purify C8 in large quantities from blood, dissociate C8α-γ from C8β, and separate C8α from C8γ has allowed the assignment of individual roles for these subunits in MAC formation and function. Results indicate each subunit performs multiple binding functions. Extending these findings to the next level of detail, i.e. localizing the binding regions in each subunit, has been particularly challenging. The large size and number of disulfide linkages within C8α and C8β has limited our ability to produce and manipulate these proteins using recombinant DNA technology, i.e. bacterial or eukaryotic expression systems. Nevertheless, we succeeded in narrowing the location of sites in each subunit that are involved in several key binding interactions.

## Binding Sites on C8α

C8α contains several binding sites (Fig. 3). One mediates interaction between C8α-γ and C8β to form C8. C8α also has a site that recognizes C8γ and facilitates the biosynthetic assembly of C8α-γ. Within C5b-8, C8α functions to bind and direct C9 into the MAC. C8α is also recognized by CD59, a complement regulatory protein that protects human cells by binding C8α and C9 and inhibiting formation of the MAC.

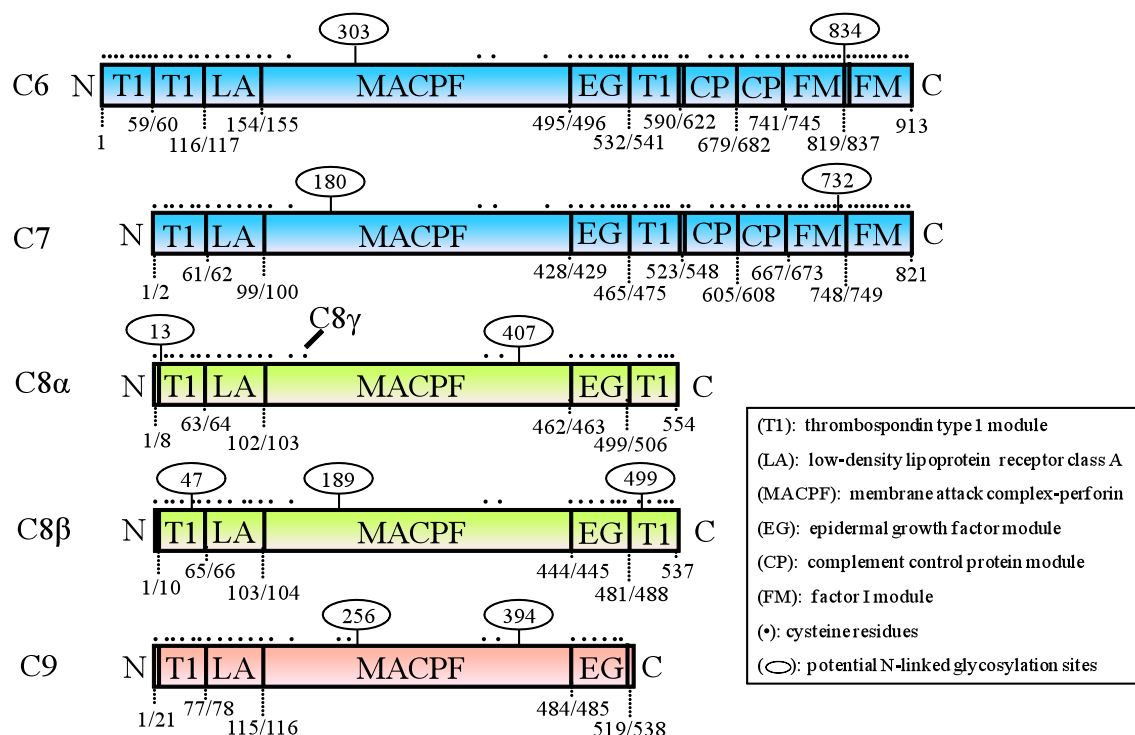


Fig. 2 The MAC Family Proteins. Modules and other structural features of each protein are described in the inset. All cysteines form intrachain disulfide bonds except C164 in C8 $\alpha$  that is linked to C40 in C8 $\gamma$ . Residue numbers identify module boundaries.

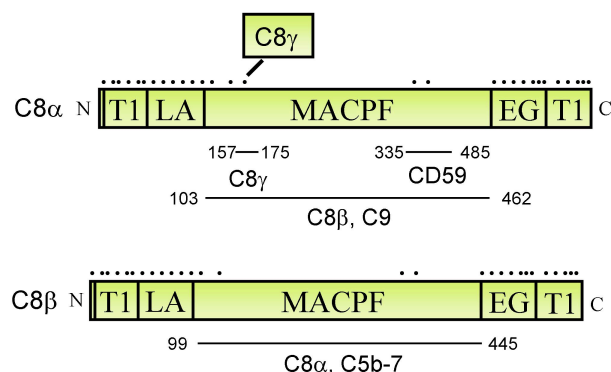


Fig. 3 Location of Binding sites on C8 $\alpha$  and C8 $\beta$ . Residue numbers identify segments containing binding sites for the indicated proteins.

C8 $\alpha$  also contributes to the cytolytic activity of the MAC by interacting directly with membrane lipid. These interactions occur simultaneously during MAC formation; therefore they were assumed to involve physically distinct, nonoverlapping sites. The following summarizes our progress towards assigning these binding functions to specific regions of C8 $\alpha$ .

#### C8 $\beta$ Binding Site

Binding between C8 $\alpha$  and C8 $\beta$  was initially observed using C8 $\alpha$  that was purified after cleavage of the interchain disulfide in C8 $\alpha$ - $\gamma$  (10). C8 $\alpha$  bound C8 $\beta$  to form a 1:1 complex in solution. Subsequent studies used recombinant full-length C8 $\alpha$  produced in baculovirus to demonstrate

noncovalent binding to C8 $\beta$  in the absence of C8 $\gamma$  (11). Together, these results suggested that binding of C8 $\alpha$ - $\gamma$  to C8 $\beta$  is mediated strictly by C8 $\alpha$ .

This was confirmed when the C8 $\alpha$  MACPF domain was shown to contain the binding site for C8 $\beta$ . The MACPF segment of C8 $\alpha$  ( $\alpha$ MACPF) was coexpressed with C8 $\gamma$  in COS cells and formed a disulfide-linked  $\alpha$ MACPF- $\gamma$  dimer (12). Assays showed the secreted dimer had the ability to bind C8 $\beta$ . Subsequently, we succeeded in producing the C8 $\alpha$  MACPF domain alone in large quantities in *E.coli* and showed that it also can bind C8 $\beta$  with high affinity (13). This established unequivocally that the principal binding site for C8 $\beta$  lies within the C8 $\alpha$  MACPF domain.

#### C8 $\gamma$ Binding Site

Although C8 $\alpha$  occurs as a disulfide-linked heterodimer with C8 $\gamma$ , it retains the ability to interact noncovalently with C8 $\gamma$  after cleavage of the interchain disulfide bond. Such binding has also been observed with recombinant forms of C8 $\alpha$  and C8 $\gamma$  in which the linking Cys residues have been deleted (12). These observations and the fact that biosynthetic processing in the liver hepatocyte requires noncovalent association prior to disulfide bond formation indicates C8 $\alpha$  and C8 $\gamma$  contain mutually recognizable binding sites.

The location of the C8 $\gamma$  binding site within the MACPF of C8 $\alpha$  was suggested from a comparative analysis of MACPF sequences in C6, C7, C8 $\alpha$ , C8 $\beta$ , and C9. The C8 $\alpha$  MACPF sequence is distinctive in that it contains a unique indel (insertion/deletion) between residues 159-175, which includes C164 that forms the disulfide bond to C40 in C8 $\gamma$ . Indels in

homologous proteins frequently correspond to loops and are often regions of functional significance; therefore this segment of C8 $\alpha$  was considered a possible C8 $\gamma$  binding site. The indel was examined for its ability to mediate binding of C8 $\gamma$  by using a chimeric form of C8 $\beta$  as a substitute for C8 $\alpha$  (14). Insertion of the C8 $\alpha$  indel sequence into C8 $\beta$  and coexpression of this chimera with C8 $\gamma$  in COS cells produced an atypical disulfide-linked C8 $\beta$ - $\gamma$  dimer. These results unequivocally established that intracellular binding of C8 $\gamma$  to C8 $\alpha$  is mediated solely by residues within the C8 $\alpha$  indel.

### C9 Binding Site

One function of C8 is to mediate incorporation of the first C9 into the MAC. C9 has little affinity for C5b-6 or C5b-7 but readily binds to C5b-8. Such binding induces an apparent conformational change in the first C9 that initiates self-polymerization and formation of poly C9. Studies using purified C8 and C9 have shown that these proteins can form a 1:1 complex in solution (15,16).

Binding between C8 and C9 is mediated principally by C8 $\alpha$ . Interaction between C9 and C8 $\alpha$ - $\gamma$  or C8 $\alpha$  can be demonstrated over a range of ionic strengths whereas binding to C8 $\beta$  or C8 $\gamma$  is negligible. C8 $\alpha$  when combined with C8 $\beta$  exhibits both hemolytic and bactericidal activity in the presence of C9 (11,17). Thus, C8 $\alpha$  is the key component for binding and incorporation of C9 into the MAC.

In an effort to locate the C9 binding site on C8 $\alpha$ , truncated and chimeric C8 $\alpha$  in which modules were deleted or exchanged for those in C8 $\beta$  were expressed independently or as heterodimers with C8 $\gamma$  and combined with C8 $\beta$ . Initial studies used COS cells for low level expression and then later used *E. coli* to express larger quantities of  $\alpha$ MACPF or  $\alpha$ MACPF- $\gamma$  for purification and characterization (13). These were tested for their ability to bind C9 in solution and express hemolytic activity. Collectively, the results showed that binding between C8 and C9 is dependent on a site located within the C8 $\alpha$  MACPF domain.

### CD59 Binding Site

MAC formation is regulated in part by the binding of CD59 to C8. Human CD59 is a 20-kDa membrane-bound complement regulatory protein that protects human blood and vascular cells from damage by complement. Analysis of the physical association of CD59 with components of the MAC indicated C8 $\alpha$  contains a binding site for CD59 (18). A series of studies using recombinant human-rabbit C8 chimeric proteins established that CD59 binds to a conformationally sensitive site on C8 $\alpha$  that is centered on residues 334-385 (19). This binding interferes with the normal assembly of the MAC and thereby protects our own cells from random MAC formation and damage.

### Lipid Binding Site

C8 $\alpha$  contains one or more lipid binding sites that become exposed within the MAC. Evidence for this comes from photolabeling studies using membrane-restricted probes to identify components of the MAC that are inserted into the bilayer (5). In both C5b-8 and the MAC, extensive labeling of

C8 $\alpha$  suggests it is inserted into the lipid bilayer, and that it contributes significantly to membrane perturbation. In the MAC, C9 is also heavily labeled, consistent with its role in forming poly C9 and the pore-like structure of the MAC.

### Binding Sites on C8 $\beta$

C8 $\beta$  also contains several binding sites (Fig. 3). One site mediates binding to C8 $\alpha$  in C8 $\alpha$ - $\gamma$ . A second site mediates incorporation of C8 into the MAC. C8 $\beta$  alone has a high affinity for C5b-7 and carries the structural epitope(s) recognized by this complex. Because C8 $\beta$  can simultaneously interact with C8 $\alpha$ - $\gamma$  and C5b-7, the respective binding sites must be physically distinct. Within the MAC, C8 $\beta$  also has the capacity to associate with lipid. Photolabeling experiments indicate C8 $\beta$  is in contact with the bilayer, although to a lesser extent than C8 $\alpha$  or C9.

### C8 $\alpha$ Binding Site

To localize the C8 $\alpha$  binding region, C8 $\beta$  constructs were prepared in which the N- and/or C-terminal modules were deleted or exchanged with the corresponding modules in C8 $\alpha$ . These were expressed recombinantly and the products tested for binding to C8 $\alpha$ - $\gamma$ . Results initially suggested a role for the N-terminal T1 module and MACPF domain but later studies using recombinant C8 $\beta$  MACPF expressed in *E. coli* revealed that the binding site for C8 $\alpha$  resides entirely within the C8 $\beta$  MACPF domain (20,21).

### C5b-7 Binding Site

Binding of C8 to C5b-7 is essentially irreversible; dissociation can only be accomplished by denaturing the resulting C5b-8 complex. Binding studies using purified C8 subunits revealed that C8 $\beta$  and C8 have a comparable affinity for C5b-7 (22). Furthermore, C8 $\beta$  effectively blocks C8 incorporation into the MAC; therefore it must compete for the same binding site on C5b-7. To identify the portion of C8 $\beta$  recognized by C5b-7, truncated recombinant C8 $\beta$  constructs were combined with C8 $\alpha$ - $\gamma$  and assayed for their ability to associate with C5b-7 and express C8 hemolytic activity (20). Assays were limited to constructs that bind C8 $\alpha$ - $\gamma$  because C8 $\alpha$  is required for C9 binding. Constructs that contained only the C8 $\beta$  MACPF domain when combined with C8 $\alpha$ - $\gamma$  were found to be hemolytically active, thus confirming that the C5b-7 binding site in C8 $\beta$  lies within its MACPF domain (21).

### Properties and Structure of C8 $\gamma$

Although its structure is well characterized, the function of C8 $\gamma$  in the complement system remains elusive. Over the years, several possibilities have been considered but subsequently discounted by studies performed in our laboratory (reviewed in ref 23). C8 $\gamma$  is not required for the synthesis and secretion of C8 $\alpha$ ; C8 $\alpha$  can be expressed independently as a recombinant protein in mammalian and insect cells. Binding between C8 $\alpha$ - $\gamma$  and C8 $\beta$  is likewise not dependent on C8 $\gamma$ ; purified C8 $\alpha$  and C8 $\beta$  can form a 1:1 complex in the absence of C8 $\gamma$ . C8 $\gamma$  is also not essential for incorporation of C8 into the MAC nor is it required for MAC

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cytolytic activity. Although not as efficient, a complex of C8 $\alpha$  + C8 $\beta$  is an effective substitute for C8 in the MAC-mediated lysis of simple cells such as erythrocytes and in the killing of gram-negative bacteria (11,17).

C8 $\gamma$  is structurally unrelated to any complement protein; it is a member of the lipocalin family of proteins that have the common ability to bind small hydrophobic ligands, e.g. retinol, fatty acids, steroids, pheromones, etc (24-26). Lipocalins exhibit little sequence similarity yet have a highly conserved fold. The core structure consists of an eight-stranded antiparallel  $\beta$ -barrel that resembles a calyx or cup-shaped structure with a binding pocket for a small molecule. Residues lining the interior of the calyx and the size of the calyx vary among family members, thus lipocalins exhibit different ligand specificities.

To gain insight into the identity of a putative ligand for C8 $\gamma$ , we produced human recombinant C8 $\gamma$  in insect cells and solved the crystal structure to 1.2 Å resolution (27). C8 $\gamma$  displays a typical lipocalin  $\beta$ -barrel fold with a potential ligand binding site. Although it has a similar fold, C8 $\gamma$  differs from most lipocalins in that its binding pocket is divided into a hydrophilic upper portion and an unusually large, lower hydrophobic cavity. Access to the lower cavity is restricted by the close proximity of two tyrosine side chains. The entrance to the lower cavity is narrow; however it is accessible to a ligand if there is movement of the tyrosines. This was shown in crystallographic studies using lauric acid as a ligand; movement of the tyrosine side chains allowed the alkyl portion of laurate to penetrate into the lower cavity (28). The features of the binding pocket suggest that any natural ligand for C8 $\gamma$  will have a narrow hydrophobic moiety at one end and a negatively charged group at the other end.

Related studies indicate C8 $\gamma$  may not normally bind a small molecule ligand. C8 $\gamma$  was co-crystallized with a 19-residue synthetic peptide encoding the C8 $\alpha$  indel that binds C8 $\gamma$  (29). The crystal structure showed the indel peptide completely filling the upper portion of the C8 $\gamma$  ligand binding pocket and in contact with all four loops at the calyx entrance. Binding studies performed with the peptide in solution suggested the C8 $\gamma$  ligand binding site is also blocked by the indel segment of C8 $\alpha$  within C8. This was later confirmed when structures of  $\alpha$ MACPF- $\gamma$  and C8 (see below) showed that access to the C8 $\gamma$  ligand binding site would require a major conformational change to move the C8 $\alpha$  indel away from the calyx entrance.

### **$\alpha$ MACPF and the Pore-Forming Bacterial Cholesterol Dependent Cytolysins (CDCs)**

One of the most significant advances in our understanding of the MAC came about as a result of our ability to produce the ~40 kDa  $\alpha$ MACPF domain on a large scale in *E. coli*. Bacteria often do not form the correct disulfide bonds in recombinantly expressed proteins. We found that *E. coli* can not only produce a soluble, functional  $\alpha$ MACPF domain with the correct disulfide bonds but also a disulfide-linked dimer of  $\alpha$ MACPF and C8 $\gamma$  ( $\alpha$ MACPF- $\gamma$ ) when a dual expression vector is used to simultaneously express both proteins (13). Recombinant  $\alpha$ MACPF and  $\alpha$ MACPF- $\gamma$  were purified and

found to be functional. Our results showed unequivocally that the  $\alpha$ MACPF domain contains the binding sites for C8 $\beta$  and C9. Most importantly, production of  $\alpha$ MACPF and  $\alpha$ MACPF- $\gamma$  on a large scale enabled our laboratory and others to crystallize these fragments and obtain the first detailed structural information for a human MAC family protein.

Insight into how MAC proteins insert into membranes came when x-ray crystal structures were simultaneously reported for the MACPF-containing protein PluMACPF, and human recombinant  $\alpha$ MACPF that was generated by our methods *E. coli* (30,31). Importantly, these proteins were found to display a fold similar to the pore-forming bacterial CDCs. This was subsequently confirmed when we determined the crystal structure of  $\alpha$ MACPF- $\gamma$  (32). CDCs are virulent pore-forming proteins secreted from gram positive bacteria and used to invade eukaryotic cells (33). The mechanism of pore formation by CDC proteins is well understood. 30-50 CDC monomers self-polymerize on cholesterol-rich regions of the eukaryotic cell surface to form a circular "pre-pore", which upon completion inserts into the membrane as a functional pore (34). This process involves a concerted conformational change whereby two regions of each CDC monomer, which are initially in an  $\alpha$ -helical conformation, refold into an extended conformation and insert into the bilayer as two amphipathic  $\beta$ -hairpins (TMH). TMHs from neighboring molecules "share edges" of their  $\beta$ -hairpins to form a hydrogen-bonded transmembrane  $\beta$ -barrel.

Figure 4 compares the structure of  $\alpha$ MACPF- $\gamma$  to intermedilysin (ILY), a well-characterized CDC from *Streptococcus intermedius* (35). Key similarities in the core structures include the overall size, the presence of a central  $\beta$ -sheet, and two  $\alpha$ -helical bundles that in ILY refold into amphipathic  $\beta$ -hairpins. In the CDCs, refolding of the TMH1 and TMH2 helical bundles to form  $\beta$ -hairpins is accompanied by elongation of the core  $\beta$ -sheet and alignment with a neighboring monomer (36,37). The striking structural similarity between complement MACPF proteins and the CDCs suggests that complement uses a CDC-like mechanism for pore formation.

### **Structure of Human C8 and a Model of the MAC Pore**

A major milestone was reached in 2011 when we published the x-ray crystal structure of whole C8 purified from blood (38). This is the first complete structure of a MAC family protein and of a protein containing two tightly associated MACPF domains. The large size of C8 and its intrinsic flexibility made it particularly challenging to crystallize and solve the structure, a project that required many years to complete. Not surprisingly, C8 $\alpha$ - $\gamma$  and C8 $\beta$  have many of the same CDC-like features seen initially in  $\alpha$ MACPF- $\gamma$ . The sequence of C9 is 27% and 26% identical to the corresponding regions of C8 $\alpha$  and C8 $\beta$ , respectively, thus C9 likely has a fold similar to these two subunits and the CDCs.

The structure of C8 is shown in Fig. 5. The central part of the C8 $\alpha$  and C8 $\beta$  MACPF domains form large, four-stranded, antiparallel  $\beta$ -sheets with a bend and twist in the middle,

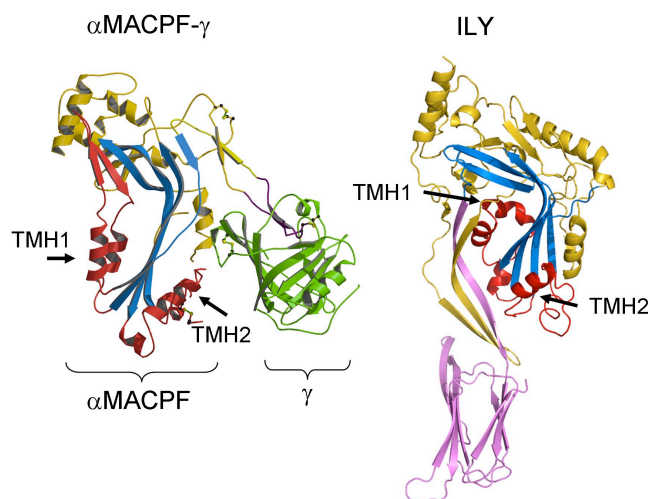


Fig. 4 Comparison of the  $\alpha$ MACPF- $\gamma$  and Intermedilysin (ILY) Structures. Shown are ribbon models of  $\alpha$ MACPF- $\gamma$  and ILY. The core  $\beta$ -sheet is in blue, the two  $\alpha$ -helical bundles (TMH1 and TMH2) known to re-fold into  $\beta$ -hairpins in ILY and predicted to do so in  $\alpha$ MACPF are in red. C8 $\gamma$  is green and the cholesterol-binding domain in ILY is lavender. Other structural elements are gold. As predicted from earlier studies, the entrance to the C8 $\gamma$   $\beta$ -barrel is occupied by a large loop containing the indel segment of  $\alpha$ MACPF.

which gives them a “T” shape. Their lower part is flanked by the TMH  $\alpha$ -helical bundles. Together with another helix they form the “lower” MACPF subdomain. The upper part of the sheet and helices surrounding it forms the “upper” subdomain, which makes contacts with the modules, and most likely with other MAC proteins. The relative positions of the C8 $\alpha$  and C8 $\beta$  MACPF subdomains differ as a result of a different bend and twist in their central  $\beta$ -sheets. The upper MACPF subdomains form very thin, tabular structures tightly packed together with the  $\beta$ -sheets facing one another in C8. These interactions are likely responsible for the strong binding between C8 $\alpha$ - $\gamma$  and C8 $\beta$ . The lower subdomains differ in that the sheets are not facing one another but are nearly coplanar; a small gap between them shows no edge sharing. Separation of the lower subdomains could facilitate refolding of the TMHs during insertion into membranes whereas the tightly packed upper subdomains are unlikely to change their relative position and may have a role in directing and maintaining the circular shape of the pore.

In CDCs, during transition from pre-pore to pore, the bend in the central  $\beta$ -sheet is reduced, the sheet changes from the “T” shape to a “□” shape, and the TMHs refold from an  $\alpha$ -helical to an extended conformation, thus elongating the central  $\beta$ -sheet and allowing insertion of THMs into the membrane. This transition requires a large movement between the upper and lower subdomains and is thought to be facilitated by four glycines located at the bend of the central  $\beta$ -sheets (37). These residues are conserved in C8 $\alpha$  and C8 $\beta$  and likely have a similar role in refolding of the MACPF domains.

The oligomeric C8 structure has provided valuable insight into how the MAC components interact to form a circular

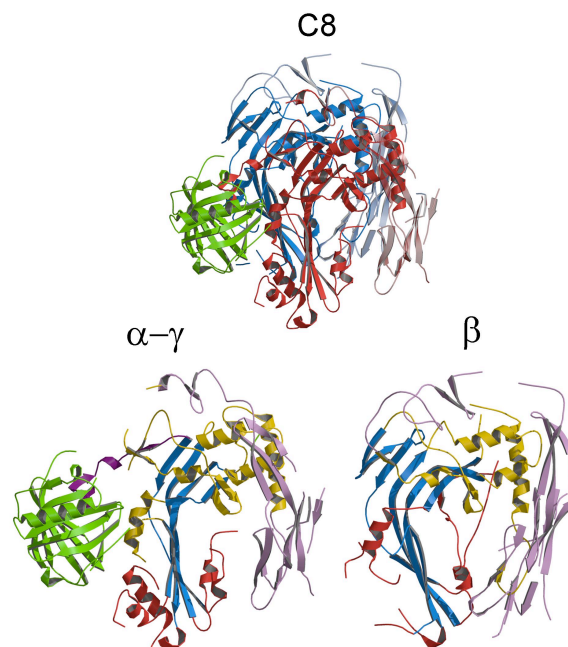


Fig. 5 Structure of Human C8. Upper panel is a ribbon model of C8 showing C8 $\alpha$  (red), C8 $\beta$  (blue), and C8 $\gamma$  (green). MACPF domains are in dark colors, modules in light. Lower panel shows separate views of C8 $\alpha$ - $\gamma$  and C8 $\beta$  oriented as in the upper panel. The central MACPF  $\beta$ -sheet is blue,  $\alpha$ -helices in TMH1 and TMH2 are red, and the remainder of the MACPF domain is gold. The modules are located on the periphery of C8 and are lavender.

pore. Although the structure is of a pair of MACPF proteins in a “non-pore” state, the unusual geometrical relationship between C8 $\alpha$  and C8 $\beta$  has important functional implications. Most frequently, homodimeric proteins are related by a 180° rotation. By contrast, C8 $\alpha$  and C8 $\beta$  are related by a rotation of 22°, while the translation between their centers is almost perpendicular to the rotation axis, resulting in only a small (1.6 Å) translational component parallel to the rotation axis. It is well established that C8 mediates the binding and incorporation of C9 into the MAC, and that this involves a specific C9 binding site within the C8 $\alpha$  MACPF domain. Assuming the modes of binding between C9 and C8 $\alpha$  and between C9 and C9 are similar to that between C8 $\alpha$  and C8 $\beta$ , with no translational component, we constructed a model for the C8-C9 complex (Fig. 6A). To do this, a homology model of C9 was generated based on C8 $\alpha$  and rotated (~22°) according to the geometrical relationship between C8 $\alpha$  and C8 $\beta$ . Rotations of the C9 model by multiplicities of 22° yielded the positions of further C9 molecules and resulted in a model for the circular MAC pore (Fig. 6B, 6C). The inner diameter of the ring is 110 Å, the outer diameter is ~220 Å, and the height is 90 Å. This agrees well with EM images of pores formed by poly C9, which generally have dimensions of ~100 Å and 210 Å for the inner and outer diameters, respectively. Also, our pore model contains 16 molecules per ring, in excellent agreement with low resolution EM studies of pores formed by MAC and poly C9 (3,6).



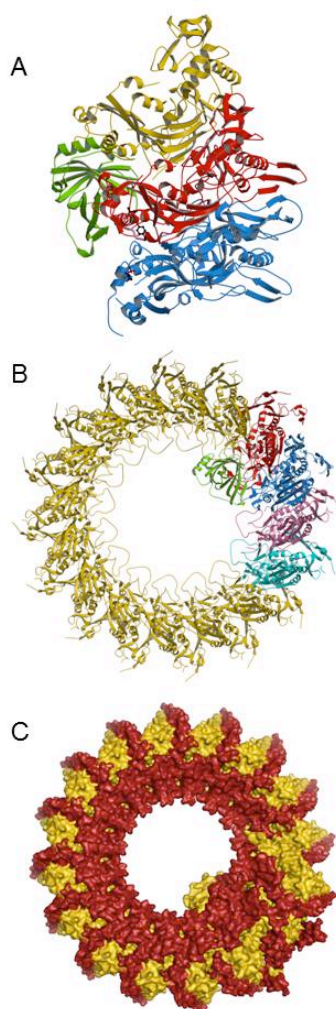


Fig. 6 Model of the MAC Pore. The model assumes that the binding mode between C8α and C8β MACPF domains is conserved for C6, C7 and C9. (A) View of the modeled C8-C9 complex. Colors correspond to C8α (red), C8β (blue), C8γ (green), and C9 (gold). (B) Partial model of the MAC. View is of the side facing the membrane. Proteins are colored C6 (cyan), C7 (pink), C8α (red), C8β (blue), C8γ (green), and C9 (gold). Absent are C5b, the CP and FM modules of C6 and C7, and the additional N-terminal T1 module in C6, the locations of which could not be predicted. It is assumed the missing components do not interfere with circular packing of the MACPF domains. (C) The molecular envelope of the model with modules in red and MACPF domains in gold. View is of the side facing away from the membrane. The modules account for a substantial fraction of the exposed surface, which is consistent with their peripheral location in C8.

## Conclusion

Over the years our research has used the tools of protein biochemistry, molecular biology and structural biology to study human C8 and obtain information that has significantly advanced our understanding of the formation and function of the MAC. As a result of our work and related work by others, it is now apparent that human complement proteins make "holes" in bacterial cells by a mechanism similar to that used

by bacteria and other lower organisms to make holes in our cells. What remains now is to decipher the details of that mechanism. This will require a detailed analysis of the structure and function of C6, C7 and C9, which will now be facilitated by the progress we have made on C8.

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## Notes and references

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