Assembly and Regulation of the Membrane Attack Complex Based on Structures of C5b6 and sC5b9

Michael A. Hadders,1,7,8 Doryen Bubeck,2,7 Pietro Roversi,3 Svetlana Hakobyan,4 Federico Forneris,1 B. Paul Morgan,4 Michael K. Pangburn,5 Oscar Llorca,6,* Susan M. Lea,3,* and Piet Gros1,*

1Crystal and Structural Chemistry, Bijvoet Center for Biomolecular Research, Department of Chemistry, Faculty of Science, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands
2Division of Structural Biology, Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford OX3 7BN, UK
3Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK
4Department of Medical Biochemistry and Immunology, School of Medicine, Cardiff University, Heath Park, Cardiff CF14 4XN, UK
5Department of Biochemistry, Center for Biomedical Research, University of Texas Science Center, 11937 US Highway 271, Tyler, TX 75708-3154, USA
6Centro de Investigaciones Biológicas (CIB), Spanish National Research Council (CSIC), Ramiro de Maeztu, 9. 28040 Madrid, Spain
7These authors contributed equally to this work
8Present address: Medical Research Council, Laboratory of Molecular Biology, Hills Road, Cambridge CB2 0QH, UK
*Correspondence: ollorca@cib.csic.es (O.L.), susan.lea@path.ox.ac.uk (S.M.L.), p.gros@uu.nl (P.G.)
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SUMMARY

Activation of the complement system results in formation of membrane attack complexes (MACs), pores that disrupt lipid bilayers and lyse bacteria and other pathogens. Here, we present the crystal structure of the first assembly intermediate, C5b6, together with a cryo-electron microscopy reconstruction of a soluble, regulated form of the pore, sC5b9. Cleavage of C5 to C5b results in marked conformational changes, distinct from those observed in the homologous C3-to-C3b transition. C6 captures this conformation, which is preserved in the larger sC5b9 assembly. Together with antibody labeling, these structures reveal that complement components associate through sideways alignment of the central MAC-perforin (MACPF) domains, resulting in a C5b6-C7-C8β-C8α-C9 arc. Soluble regulatory proteins below the arc indicate a potential dual mechanism in protection from pore formation. These results provide a structural framework for understanding MAC pore formation and regulation, processes important for fighting infections and preventing complement-mediated tissue damage.

INTRODUCTION

Proteins of the terminal pathway of complement provide immune protection by forming lytic pores, membrane attack complexes (MACs), in membranes (Esser, 1994). Genetic deficiencies of MAC components lead to recurrent infections (Botto et al., 2009); however, unregulated MAC formation causes tissue damage (Morgan, 1999). Formation of the MAC is a sequential process. Upon complement activation, C5 is cleaved into C5a and C5b by the C5 convertase. C6 then captures a labile binding site in C5b (half-life: 2 min) (Cooper and Müller-Eberhard, 1970), followed by C7 association that renders the complex lipophilic (Preissner et al., 1985; Stewart et al., 1987). Binding of heterotrimeric C8αβγ defines the initial membrane insertion event, with C8β mediating attachment to the assembly precursor (Brannen and Sodetz, 2007; Stewart et al., 1987) and C8α penetrating the bilayer (Steckel et al., 1983). Inserted C5b8 functions as a receptor for C9 and catalyzes its oligomerization, leading to membrane perforation and target cell lysis (Podack et al., 1982; Tschopp, 1984; Tschopp et al., 1985). Off-target assembly of the MAC in solution leads to binding of clusterin and vitronectin, yielding a soluble complex called sC5b9 or sMAC. Dissociation of these chaperones by detergents reconstitutes membrane binding (Bhakdi et al., 1979; Podack and Müller-Eberhard, 1980). In addition, sC5b9 and the lytic MAC share a neo-epitope present in C9 that is associated with pore formation (Mollnes et al., 1985), suggesting similarities in how the soluble and membrane-associated complexes are assembled.

MAC proteins and the homologous perforin are proposed to form β-barrel pores on the basis of the structural resemblance between MAC-perforin (MACPF) domains and bacterial cholesterol-dependent cytolysins (CDCs) (Hadders et al., 2007; Law et al., 2010; Lovelace et al., 2011; Rosado et al., 2007; Rossjohn et al., 1997; Shatursky et al., 1999; Slade et al., 2008). Modeling and labeling of the perforin pore based on a cryo-EM reconstruction indicated, however, an inside-out arrangement of the perforin core relative to the proposed pore model for CDCs (Law et al., 2010). Though a recent crystal structure of the heterotrimeric C8αβγ suggested a CDC-like arrangement of the MAC ring (Lovelace et al., 2011), it lacked the context of the larger MAC assembly. To understand how complement proteins come together to form a lytic pore important for immune defense, we combined crystallographic analysis of C5b6 with electron microscopy (EM) to determine the structure of the sC5b9 complex.
RESULTS

Crystal Structure of C5b6

C5b6 was assembled in vitro, purified, and crystallized. Crystals diffracted anisotropically to a resolution between 3.5 and 4.2 Å, and the structure was solved by molecular replacement (Table S1). The final model, consisting of all 12 domains of C5b and all 10 domains of C6, was refined to a final Rwork/Rfree of 25.6/27.0 and displayed good geometry (Figures 1A–1C, Figure S1A, and Table S1). Because of the limited resolution, we restrict our discussion of the structure to the level of individual domains.

C5 undergoes marked domain rearrangements upon cleavage into C5b and formation of the C5b6 complex. C5 consists of two peptide chains, denoted β (residues [res.] 1–674) and α (res. 678–1676), that form 13 domains (Fredslund et al., 2008) (Figure 1B). Similar to the conversion of homologous C3 to C3b (Janssen et al., 2006; Janssen et al., 2005), the domains of the α chain undergo major relocations, while most of the β-chain forms a stable ring-like structure (Figure 1E, Table S2, Movie S1). Removal of the anaphylatoxin (ANA/C5a) domain results in extensive movements of the macroglobulin (MG) 3, MG7, MG8, and “complement C1r/C1s, Uegf, Bmp1” (CUB) domains and the thioester-like domain (TED/C5d); C5 lacks the prototypical thioester present in this domain in other members of the C3/α2-macroglobulin protein family. Although the concerted movement of MG7 and CUB resembles that observed for the C3-C3b transition, the position of the connected TED differs dramatically (Figures 1D and 1E). A requirement for this conformation to be caught and stabilized by C6 may explain the short half-life of the C6 binding site in C5b (Cooper and Müller-Eberhard, 1970). Without C6 binding, C5b will irreversibly decay to a state incapable of binding C6 (Cooper and Müller-Eberhard, 1970).

The structure of C6 in the C5b6 complex reveals that its ten domains can be divided into two functional parts. The first consists of the six N-terminal domains and includes the “core” region common to all MACPF proteins in the MAC; these six domains are thrombospondin (TSP) domain 1, TSP2, low-density lipoprotein receptor class A domain (LDLRa), MACPF, epidermal-growth factor (EGF) domain, and TSP3. The second, C-terminal part consists of two complement-control-protein (CCP) and two factor I/MAC (FIMAC) domains (Figures 1A, 1C, and 1F). These regions are separated by a long flexible linker. A comparison with free C6 (Aleshin et al., 2012) reveals that whereas the N-terminal region is highly similar, the C-terminal region has a strikingly different arrangement (Figures 1A and 1F, Table S3) and forms the major interface with C5b.

The C5b-C6 interface buries ± 3100 Å² of solvent accessible surface area. The core of C6 binds to the “bottom” of C5b, in between MG1, MG4, and TED, to a highly conserved patch (Figure S2A). A second, major interface is formed by the linker and the CCP1-2 domains of C6 that wrap around the TED domain of C5b (Figure 2A). The CCP1 domain is wedged in between TED, CUB, and MG2 of C5b, where it seems to stabilize the observed position of TED (Figures 1 and 2). The importance of this interaction is supported by data showing that the CCP domains are essential for C6 activity (DiScipio et al., 1999). The linker preceding CCP1 also makes extensive contacts with TED (Figures 2A and 2C), where it interacts in part with a distinct β-hairpin that forms a unique insertion in TED (Figures 2C, 2D and S1B–S1D). We tested the importance of the linker region by mutating several conserved residues, all showing a reduced activity in a hemolytic assay (Figure 2B).

The observed association of C6 to C5b positions the MACPF domain with its putative β-barrel forming transmembrane segments (TMS) at the periphery of the complex (Figure 1A). The two TMS regions in C5b6 (C6 res. 255–312 and res. 381–438) adopt helical conformations loosely folded onto the central curved four-stranded β sheet of the C6 MACPF; a similar arrangement is observed in structures of free C6 (Aleshin et al., 2012), C8 (Hadders et al., 2007; Lovelace et al., 2011; Slade et al., 2008), perifin (Law et al., 2010), and CDCs (Rossjohn et al., 1997). Importantly, we do not observe a structural rearrangement of these regions compared to free C6, suggesting that binding to C5b is not the trigger for unfolding of these segments. Thus, we interpret the MACPF fold of C6 in C5b6 as a premembrane attack state, which is in agreement with the soluble nature of C5b6.

Cryo-EM of the Soluble Regulated Pore, sC5b9

To understand how the MAC proteins arrange to form a pore, we examined by EM sC5b9 purified from activated serum (Figure S2). Broadly, sC5b9 is a thin, square-shaped complex with a single protrusion at one corner, as suggested by two-dimensional (2D) images (Figure 3, Figures S2A–S2E). Three-dimensional structures of both negatively stained and cryo-EM sC5b9 (Figure S2F) further define two prominent features of the square-like central region. The protrusion (Figure 3A, indicated by a brown arrow) connects to the core at one corner of an arc-shaped crescent, while large connected densities form a butterfly arrangement below the arc (Figure 3A, indicated by a gray surface).

Docking of the C5b6 crystal structure into the 24 Å cryo-EM molecular envelope clearly defined the identity of the protrusion as C5b6. Strikingly, the labile conformation of C5b trapped by C6 in the C5b6 crystal structure is preserved in the larger complex (Figures 1A and 3E). The orientations of the C-terminal FIMAC domains of C6 and the C345C domain of C5b, likely affected by crystal packing, did not fit the density of the sC5b9 reconstruction (Figures S1E–S1G). In addition, the C6 TSP1 domain, thought to regulate assembly of MAC precursors (Aleshin et al., 2012), was also out of density. Our map could accommodate these domains (Figure 3E, indicated by asterisks); however, low resolution precluded modeling their orientations in the sC5b9 reconstruction.

Next, we generated a model of multiple MAC proteins that contain the conserved TSP-LDLRa-MACPF-EGF domain architecture (Figure S3) by duplicating the MACPF-MACPF orientation of C8α (Lovelace et al., 2011). Five MAC proteins fit the arc below the protrusion (Figure 3E). Labeling with a monoclonal anti-C9 antibody, recognizing a neo-epitope present in both sC5b9 and the lytic pore, identified C9 as the MAC protein in the arc furthest from the C5b protrusion (Figures 3C–3E and S4). Previous biochemical data showed that C9 binds to C8α...
Figure 1. The Structure of C5b6
(A) A cartoon and surface representation of C5b6 in two orientations. C5b is colored in cyan, and C6 is colored by domain boundaries.
(B) A schematic representation of the domain architecture of C5b.
(C) A schematic representation of the domain architecture of C6.
(D) A cartoon representation of C5b (cyan) superimposed onto C5 (blue; PDB code 3CU7). C5a is colored red.
(E) A cartoon representation of C5b (cyan) superimposed onto C3b (purple; PDB code 2I07).
(F) A cartoon representation of C6 from the C5b-C6 complex (brown) superimposed onto free C6 (green; PDB code 3T5O), based on their MACPF domains.
(Slade et al., 2006) and that C8β binds C5b7 (Brannen and Sodetz, 2007; Stewart et al., 1987), indicating C8β as the two MAC components preceding C9. Independent docking of the five-MAC model and C5b6 into the EM map superimposes the core of C6 onto the first MAC position (Figure S3A), suggesting that C6 is the first MAC protein and C7 is in the remaining unoccupied position, the second position between C6 and C8β. Models involving a six-MAC protein arc correlated less well with the EM density (correlation coefficients of 0.88 and 0.84 for five- and six-membered arcs, respectively). It is noteworthy that models involving the C6-C7-C8β-C8α-C9 arrangement, in which the position of C6 was defined by docking C5b6 as a rigid body, correlated 7% better with the map than those ordered C7-C6-C8β-C8α-C9 as previously proposed (Aleshin et al., 2012).

Density in the center of the arc accommodates the lipocalin fold of C8γ (Figure 3E, indicated as a solid gray surface), a MAC component that is flexibly, but covalently, attached to C8α (Lovelace et al., 2011; Slade et al., 2008) (Figure S3C). C8γ enhances lysis, but it is not essential (Parker and Sodetz, 2002) for MAC activity, and its orientation in sC5b9 suggests a role in stabilizing the MACPF-MACPF interactions before closure of the ring. Finally, density present at a ridge along C5b (Figure 3E, indicated with a dashed orange line) could account in part for the unmodeled CCP1-2 and FIMAC1-2 domains of C7, known to interact with the C345C domain of C5b (Thai and Ogata, 2004).

Density of the butterfly-shaped region of the sC5b9 map can be attributed to regulatory proteins, vitronectin and clusterin, known to bind exposed lipophilic regions of MAC precursors. This is supported by previous EM analysis of gold-labeled vitronectin localizing oligomers to this region (Preissner et al., 1989). Moreover, the location of regulatory proteins is consistent with the positioning of predicted transmembrane segments below the MACPFs in the arc and the interpretation that these segments are either disordered or flapped out into their β-hairpin conformation (Figure 4). The structural data, therefore, indicate a potential dual mechanism in protection from pore formation: the two “wings” of the butterfly-shaped regulatory region cap the ends of the MAC arc and thereby block C9 oligomerization, and they enwrap the lipophilic segments to prevent membrane interaction.

DISCUSSION

Pore formation for MACPF-containing proteins involves a dramatic conformational change in which helical bundles transform into a membrane-spanning β-barrel. In contrast to

Figure 2. Interface of the C5b6 Complex
(A) The interface between C5b and C6 with the complex “spread” apart. C5b is cyan, with the C6 footprint colored according to the contacting C6 domains. C6 is colored as in Figure 1, and the footprint of C5b is cyan.
(B) Hemolytic activity of C6 linker mutants expressed as a percentage relative to recombinant wild-type C6. Each sample was tested at six different concentrations, and standard errors were determined with the use of a nonlinear fitting program (GraFit 5.0).
(C) A close-up of the C5b-C6 interaction shows the extensive interface between TED and the C6 linker. Mutated residues tested in (B) are shown as spheres. The unique β-hairpin of C5 (TED) that interacts with the linker is highlighted in red.
(D) Structure-based alignment of C5 and C3; the unique insertion in C5 is boxed.

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the current model for immune pore formation, which is based on perforin (Law et al., 2010), our structural analysis of C5b6 and the sC5b9 complex supports a model for the MAC that resembles bacterial CDC pores (Figure 4). Docking of C5b6 into the complex, the curvature of the arc, and density for C8γ inside the pore are all in concordance with the CDC orientation of MACPF proteins in the membrane. Despite its similarity to simpler bacterial pores, the complement-mediated immune response has evolved complex assembly and regulatory mechanisms that are likely required to prevent host tissue damage yet effectively clear pathogen infections. Our model suggests that the ability of C6 to capture a labile binding site in C5b to form an assembly competent state provides the first checkpoint in MAC formation. Next, C7 binds the C5b6 complex, making extensive contacts to C5b through its C-terminal CCP and FIMAC domains, thereby aligning MACPF domains of C6 and C7. Binding of C7 then drives rearrangement of the TMS regions, making C5b7 lipophilic and creating the novel hybrid-binding site for C8. Subsequent association of C8αβγ through alignment of the C8β and C8γ MACPF domains relocates C8γ, which in the soluble C8αβγ complex may serve to inhibit C9 association before incorporation into the larger assembly. Finally, host regulatory proteins clusterin and vitronectin can prevent pore formation by blocking both hairpin insertion into the membrane and oligomerisation of C9. Together, our data show how the MAC is assembled and regulated in blood, providing a framework for understanding the role of complement in microbial infection and inflammatory disorders.

EXPERIMENTAL PROCEDURES

Purification of C5b6
C5b6 was isolated from a mixture of purified human C5 and C6 in which the C5 was activated by addition of a mixture of cobra venom factor (CVF), factor B, and factor D in the presence of 0.5 mM MgCl2. C5 and C6 were purified from pooled normal human serum as previously described (Kolb et al., 1982; Tack et al., 1980). The formed C5b6 complex was separated from the other components by ion exchange chromatography over a Mono Q column (GE Healthcare) and subsequently by gel filtration over BioGel A0.5 m (BioRad). Fractions were pooled on the basis of C5b6 functional activity (Rawal and Pangburn, 2000), concentrated to 0.7 mg/ml and dialyzed against 10 mM HEPES (pH 7.2), 120 mM NaCl, and 0.02% w/v NaN3.

Crystallization and Data Collection
The C5b6 complex was crystallized by vapor diffusion in hanging drops consisting of 2.5 ml protein (0.7 mg/ml) mixed with 0.5 ml 1M HEPES-NaOH (pH 7.8). Drops were equilibrated at 18°C over 300 ml reservoir solution consisting of 0.1M HEPES-NaOH (pH 7.8) and 250 mM NaCl. Crystals grew to maximum dimensions of 800 × 80 × 20 μm in ~3 weeks and were cryoprotected by brief incubation in reservoir solution supplemented with 30% (v/v) ethylene glycol, followed by flash freezing in liquid N2. A complete data set was collected at ESRF beamline ID29 in seven wedges of 15° that were collected along the length of the crystal through the use of 1° oscillations. The diffraction data were integrated and scaled by XDS (Kabsch, 2010) and Aimless (Collaborative Computational Project, Number 4, 1994). The crystals belong to space group...
Figure 4. A Model for MAC Formation
The complement terminal pathway is initiated by the cleavage of C5 to C5b. C6 traps a labile conformation of the C5b TED domain to form C5b6, a platform for the stepwise assembly of components C7, C8, and C9. Regulatory proteins in the plasma block MAC assembly in solution by binding exposed hydrophobic regions and sterically inhibit C9 oligomerization. Binding of C5b8 to membranes recruits multiple C9 molecules whose MACPF domains arrange to form a β-barrel pore similar to that of CDCs.
Negative Stain Electron Microscopy

Immediately after gel filtration, a volume of 2.5 μl of sC5b9 (16 μg/ml) or anti-body-labeled sC5b-9 (9 μg/ml), were applied to glow-discharged carbon-coated copper grids. Grids were negatively stained with 0.75% uranyl formate. Images were taken under low-dose conditions (~10 e/Å² per exposure) at a nominal magnification of 72,500 on a JEOL JEM-1230 operated at 100 kV. Images were recorded on a 4k × 4k TemCam-F416 camera (TVIPS) and 2.28 Å/pixel. 11330 sC5b9 and 1104 immune-labeled windowed particles were each subject to reference-free alignment with the use of EMAN2 (Tang et al., 2007) and classified into 149 and 31 classes, respectively. The standard EMAN2 initial-model-generation program (e2initialmodel.py) was used to obtain an initial template for refinement. With the use of this methodology, several models were constructed from a series of randomly generated Gaussian blobs, masked according to the sC5b9 particle diameter, and used to initiate the angular assignment of reference-free-generated 2D class averages. The resulting models were ranked on the basis of the agreement of the projection with the class average. The top choice was used as template for the refinement of negatively stained sC5b9 single particles with the use of EMAN2. Handedness of the map was determined on the basis of an 8% difference in correlation coefficient used for measuring the agreement of the Cs6 β crystal structure with the reconstruction.

Cryo-Electron Microscopy

The identical preparation used in the negative stain EM experiments described above was also subjected to analysis by cryo-EM. Aliquots (4 μl) of purified sC5b9 (0.1 mg/ml) were applied to glow-discharged holey carbon grids (QUANTIFOIL R 1.2/1.3) and vitrified in liquid ethane with an FEI Vitrobot. Images were acquired on a JEM-2100FS FEG electron microscope (JEOL) operating at 200kV, equipped with an in-column energy filter (OMEGA). Images were recorded with a defocus range of −3.0 to −6.0 microns underfocus and at a magnification of 54,400 on a 4k × 4k ULTRASCAN 4000 SP CCD camera, corresponding to 2.76 Å/pixel. A total of 18,983 individual sC5b9 particles were windowed with e2boxer (EMAN2), corrected for the contrast transfer function (CTF) with Bsoft (Heymann and Belnap, 2007), and low-pass filtered to 11 Å. The sC5b9 negative stain reconstruction served as a reference for the refinement of cryo-EM data through the use of projection-matching algorithms in Xmipp (Scheres et al., 2008). All fitting and rigid-body refinement of pseudo-atomic models into the cryo-EM envelope were performed in Chimera (Pettersen et al., 2004). Figures were generated with V pymol (PyMOL Molecular Graphics System, version 1.3, Schrödinger).

ACCESSION NUMBERS

Coordinates and structure factors for the crystal structure of Cs6 β have been deposited in the Protein Data Bank under accession code 4A5W. The cryo-electron microscopy density map for sC5b9 has been deposited at the Electron Microscopy Data Bank under accession number EMD-1991.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, three tables, and one movie and can be found with this article online at doi:10.1016/j.celrep.2012.02.003.

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