Structural insight into proteolytic activation and regulation of the complement system

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Keywords:

Complement, structural biology, proteolytic cascade, complement regulation, immune disorders

Summary

The complement system is a highly complex and carefully regulated proteolytic cascade activated through three different pathways depending on the activator recognized. The structural knowledge regarding the intricate proteolytic enzymes that activate and control complement has increased dramatically over the last decade. This development has been pivotal for understanding how mutations within complement proteins might contribute to pathogenesis and has spurred new strategies for development of complement

therapeutics. Here we describe and discuss the complement system from a structural perspective and integrate the most recent findings obtained by crystallography, small angle X-ray scattering and electron microscopy. In particular, we focus on the proteolytic enzymes governing activation and their products carrying the biological effector functions. Additionally we present the structural basis for some of the best known complement inhibitors. The large number of accumulated molecular structures enables us to visualize the relative size, position, and overall orientation of many of the most interesting complement proteins and assembled complexes on activator surfaces and in membranes.

Introduction

The complement system is an important effector of innate immunity and plays a crucial role in the detection, phagocytosis and killing of invading pathogens and clearance of immune complexes.

Complement is also increasingly recognized for its role in maintenance of host homeostasis through clearance of apoptotic and necrotic cells. Furthermore, increasing evidence supports the hypothesis of complement as a key player during the process of development (1). Activation of the complement system relies on pattern recognition molecules (PRMs) recognizing pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) on the activator. There are three well characterized activation pathways for complement, the classical pathway (CP), lectin pathway (LP) and the alternative pathway (AP). Upon activation, all three pathways converge via AP amplification into a common terminal pathway (TP) in which the final proteolysis event elicits a potent proinflammatory response and triggers the assembly of a sophisticated membrane penetrating complex capable of killing specific pathogens.

Structural biology has during the last decade, mostly through crystallographic studies, provided a rapidly expanding platform for understanding the details of the complicated proteolytic reactions occurring during activation and regulation of these pathways. Recent technical improvements in electron microscopy (EM) now makes it possible to gain structural insights into large activator bound complement complexes such as

the C1 complex from the CP (2) and the membrane attack complex (MAC) formed in the TP (3, 4). In the following we provide an overview of recent important developments in structural biology of the complement system with focus on assembly and regulation of the large and intricate proteolytic enzymes formed upon complement activation. We also describe how these enzymes recognize and cleave their substrates. Finally, recent breakthroughs within structural studies of MAC assembly and the therapeutic inhibition of the TP are discussed.

The classical and lectin pathways

The lectin pathway pattern recognition molecules and MASP proteases

Initiation of the LP takes place when PRMs bind to an activator presenting carbohydrate or acetyl groups on pathogens or abnormal host tissue and cells. The five LP PRMs possess the same overall architecture and their subunits are constructed from three chains. In each chain a short N-terminal region engages in disulfide bridges with the two other chains and other subunits, a central region assembles into a trimeric collagen stem and a C-terminal region folds into a globular domain, which interact strongly non-covalently with the equivalent domain of the two other chains. A variable number of such three-chain subunits then associate to form the functional oligomer. As an example, mannan binding lectin (MBL) is organized as oligomers with 2-8 subunits, but in serum the most abundant species are tetramers and trimers (5). The second lectin PRM CL-LK and the three ficolins (H-ficolin, L-ficolin and M-ficolin) form oligomers in the same way (6), but whereas MBL and the ficolins contains only one type of chains, the CL-LK subunits are most likely made from two chains of CL-K1 and one CL-L1 chain (7). The PRMs have two spatially separated functions. At one end the globular trimeric domain known as the carbohydrate recognition domain (CRD) in the lectins and the fibrinogen-like domain (FBG) in ficolins forms multivalent interaction with specific molecular patterns on the activator (8).

The second function of the LP PRMs located in middle of the collagen stem is to bind dimers of mannan-binding lectin associated serine proteases (MASPs). These share a conserved domain structure with two

CUB domains separated by an EGF domain, followed by two CCP domains and the catalytic serine protease (SP) domain. It is the PRM-MASP complexes which perform the proteolytic reactions in the LP (Fig 1A). Recent work has clarified the contributions of MASP-1 and MASP-2 in the cascade (9, 10). MASP-1 initiates the LP through auto activation and subsequent proteolytic activation of MASP-2, which can then cleave C4. The resulting nascent C4b becomes linked to the activator through a nucleophilic attack on the C4b thioester. Complex formation with the zymogen C2 results in formation of the proconvertase C4b2 which can be converted to the active C3 convertase C4b2a by either MASP-1 or MASP-2 (Fig 1A). The MASP CUB domains harbor a tightly bound Ca^{2+} ion which organizes aspartate and glutamate side chains around them. Structural studies indicate that lysine side chains from the PRM collagen stems are engaged in electrostatic interactions with the negative side chains in the CUB site (11), and mutational studies have precisely identified the responsible lysines in MBL and ficolins (12, 13). Structures of the CCP1-CCP2-SP fragment of the MASP proteases have revealed an architecture in which the two CCP domains form a rigid extended unit connected to the globular SP domain resulting in the overall appearance of a cudgel (14-16). The structure of MAp44 - a truncated version of MASP-1 – suggested how the CUB1-EGF-CUB2 fragment is connected to the CCP1-CCP2-SP in the intact MASP proteases (17) and allowed us propose a general model for PRM-MASP complexes featuring a rather flat and highly extended MASP dimer extending from the center of the PRM (8).

Activation of PRM-MASP complexes

Our PRM-MASP model was in opposition to the traditional model for MASP activation claiming that non-activated PRM-MASP complexes would adopt a closed compact conformation with the zymogen non-activated SP domains tucked inside a void delimited by the MASP CUB1-EGF domains together with the PRM collagen stem and CRD/FBG domains. According to this model activation of PRM:MASP-1 would be an intramolecular reaction occurring between two SP domains in the same MASP-1 dimer. Activation was assumed to require a conformational change driven by PRM binding to the activator, auto-activation of the associated MASP-1 dimer followed by the exposure of the SP domains in the activated MASP-1 dimer and

subsequent proteolytic activation of the substrates PRM:MASP-2 and C4b2 (18, 19). However, compelling biochemical evidence showing that the initial activation of the LP occurred through intermolecular proteolytic cleavage between SP domain from different MASP dimers and that binding to a glycan as such was not needed contradicted the model (5). This instead suggested that the activator glycan trigger LP initiation by creating a local high concentration of the PRM-MASP complexes and by orientating them correctly relative to each other. We further substantiated this alternative model by studying a complex between a predominantly tetrameric MBL in complex with a non-activated zymogen MASP-1 dimer by small angle X-ray scattering (SAXS) and negative stain EM (20). Both approaches unequivocally showed that the MASP-1 SP domains were highly accessible and separated by more than 200 Å (Fig 1B). These results strongly supported the alternative intermolecular activation model and appear to rule out the traditional activation model with MASP activation occurring within a dimer bound to the same PRM. Importantly, we still do not know the conformational difference between fluid phase non-activated PRM-MASP and activator bound non-activated or activated PRM:MASP complexes. Furthermore, our results do not exclude that conformational changes does occur upon activator association which might alter the accessibility of the MASP SP domains and thereby promote intermolecular activation.

Function and architecture of the C1 complex

Complement activation through the classical pathway (CP) is governed solely by the C1 complex containing the PRM C1q associated with a defined heterotetramer encompassing two subunits of both C1r and C1s, in the following denoted C1r₂S₂. Multivalent complexes of antigens and IgG and IgM antibodies are the best known CP activators, but the list of C1q binding patterns now comprises more than 100 targets including surface bound pentraxins and Aβ oligomers occurring in patients with Alzheimer's disease (21, 22).

Activation of CP supports the elimination of soluble immune complexes and antibody tagged pathogens but also participates in maintenance of homeostasis through clearance of apoptotic and necrotic cells (23).

Recent studies in mouse models have indicated a major role of C1 with respect to synaptic pruning in the nervous system during development but also in connection with pathogenesis in Alzheimer's disease and

frontotemporal dementia. In either case the actual C1 binding molecular pattern on the synapse to be eliminated remains to be identified (24-26).

The CP pattern recognition molecule C1q shares its overall structure with the lectin pathway PRMs in that it is based on a central collagen stem carrying C1r₂S₂, a globular C-terminal domain responsible for recognition of the activator called the gC1q domain and a short N-terminal region containing disulfide bridges. C1q has a defined stoichiometry and is based on three chains C1qA, C1qB and C1qC forming one C1q subunit, and six such subunits are assembled in C1q. The N-terminal of such six stems associate tightly in a cylinder shaped structure after which they diverge into separate collagen stems. At the junction a kink is observed, probably as a consequence of an imperfect GXY triplet pattern with an additional residue in C1qA and the presence of an alanine instead of glycine in C1qC (27). The crystal structure of gC1q revealed a compact sphere-like heterotrimer with a diameter of 50 Å in which the three subunits are tightly associated through non-covalent interactions (28).

In the C1r₂S₂ tetramer, the two C1rs heterodimers associate via head-to-tail C1s-C1s CUB1-EGF contacts in a manner highly resembling that observed for MASP proteases (29). Within the heterodimer C1r and C1s have been proposed to interact via similar head-to-tail contacts between their CUB1-EGF domains (29, 30). The CCP1-CCP2-SP fragments of both C1r and C1s have the same architecture as found in the MASP proteases (16, 31), and in C1s the CUB2 and the CCP1 domains are located in plane like in MAP44 but with a rather different angle between the two domains (29). The C1r₂S₂ tetramer uses a total of six CUB Ca²⁺ sites (four in C1r and two in C1s) to associate with C1q by contacts bridging specific lysines in C1qB and C1qC with the C1r and C1s CUB Ca²⁺ sites (32, 33).

The structure of non-activated C1

The well-defined composition of the C1 complex as compared to the PRM-MASP complexes raises the expectation that detailed structural insight is available. Unfortunately the structural knowledge regarding the intact C1 complex is currently only of low-resolution nature. Negative stain EM pictures have for many

years represented state of the art with respect to the structure of non-activated C1. These were interpreted to suggest that the central part of C1r₂s₂ was located inside the collagen stem cone of C1q while the remaining parts of C1rs were wrapped around the collagen stems (34). This model has subsequently been refined to a model which incorporates the partial crystal structures of C1r and C1s and the CUB-collagen interactions linking the C1q collagen stems with the C1r₂s₂ tetramer as described above. An essential feature of the model is that the CCP1-CCP2-SP domains of both C1r and C1s are tucked inside the C1q cone between the C1r₂s₂ CUB1-EGF domains and the gC1q domains (30, 33). A crystal packing interaction occurring in crystals of the C1r CCP1-CCP2-SP fragment between the C1r SP domain from one molecule and the CCP1 domain from a second molecule has been suggested to reflect an interaction between the two C1r molecules in the C1r₂s₂ tetramer (31). It is hypothesized that activator binding induces a conformational rearrangement in C1q collagen stems that is translated into a reorganization of C1r₂s₂ that brings the two C1r SP domains close enough to activate each other (31, 35). Activation of C1r and subsequent activation of C1s in the same tetramer is then proposed to be followed by exposure of the C1s SP domain in order to allow its binding and cleavage of its substrates C4 and C4b2. In a very recent model it is suggested that activated C1s remains within the C1q cone and cleaves its substrates in this location (29). Compared to the new and rather simple activation model for PRM:MASP complexes, the existing C1 activation models appear rather elaborate. Further validation by experimental data and in particular more detailed structural studies are needed to confirm the existing model or develop a new CP activation model.

The structure of an activator bound C1 complex

With respect to activator bound C1 a recent ground-breaking study conducted with cryo EM tomography has provided the first true structural insight into how PRM-protease complexes are bound to an activator. In this study a liposome was decorated with dinitrophenyl hapten and a hapten-specific IgG antibody containing mutations strongly enhancing the formation of Fc hexamers (2). Subsequently C1 was added and due to the hexameric Fc platform, which provide strong avidity to the C1-Fc interaction, well defined

complexes between C1 and the hexameric Fc platform could be visualized (Fig. 1D). The resulting tomogram confirmed the presence of a cylindrical C1 stalk containing the N-terminal part of the six collagen stems in a parallel arrangement and a central horizontal layer presumably containing C1r₂S₂. However, the CCP1-CCP2-SP parts of either protease were not visible but suggested to protrude out from the center of the molecule to an exposed location (2) similar to the organization of the MBL:MASP-1 complex observed by SAXS and EM. In line with that, the C1 tomogram indeed compare very well with our SAXS based MBL:MASP-1 structure (Fig 1B and 1D). The Fc hexamer plane was readily recognizable and parallel to the central C1r₂S₂ layer in C1 and contacts between the Fc hexamer and four of the six gC1q domains were observed (2). It should be noticed that the tomography study described the activator bound C1 with a relatively low resolution of 6 nm which might explain why the catalytic domains or C1r₂S₂ and single collagen stems were not visible, but flexibility of especially the C1r and C1s EGF-CUB2 and CUB2-CCP1 junctions may also contribute.

C4 and its complex with MASP-2

Whereas an impressive number of crystal structures involving C3 and C3b have been determined since 2005, it was only in 2012 we presented the first structure of C4. One reason is perhaps that C4 is the most heterogeneous protein of the complement system. In addition to the two isotypes of the C4 gene, C4A and C4B, a total of 23 allotypes of the genes have been reported. The two isotypes differ within a short sequence of six residues that determines whether the thioester in nascent C4b preferentially reacts with amino (C4A) or alcohol (C4B) containing nucleophiles from the activator (36). Most individuals express both isoforms and very recently the relative expression of the C4A isoform have been linked to the risk of developing schizophrenia (37). Our structural studies on the various functional states of the human protein have all been based on C4 purified from outdated plasma likely to contain a mixture of the C4A and C4B isotypes. The structure of C4 follows the principles first established for C3 (Fig 1E). Eight MG domains forms the basic building blocks, six of these (MG1-MG6) are located in a helical arrangement often referred to as the MG ring. The thioester (TE) domain, the CUB domain and the MG8 domain form a tightly packed

structural entity called the α -chain superdomain with the thioester buried between the TE and MG8 domains. The MG7 and the C-terminal C345c domains are quite solvent exposed domains located next to and on top of the α -chain superdomain, respectively. The C4a domain released upon C4 cleavage acts like a wedge between the α -chain superdomain and the MG-ring (38). Our structure of C4 in complex with a MASP-2 fragment encompassing the two CCP and the SP domains revealed an unexpected binding mode for the protease. The enzyme and the substrate interact through two major interfaces. First, the C4 scissile bond region is inserted in the catalytic site and in particular the P1 residue Arg756 is accommodated in the MASP-2 S1 pocket confirming that our structure represents a Michaelis complex. Prior biochemical experiments provided evidence for an important role of the CCP domains in both C1s and MASP-2 for C4 cleavage, and our structure confirms this by revealing a second major point of intermolecular interaction formed by the insertion of the C4 C345c domain into a cleft between the two MASP-2 CCP domains. We showed that mutation of MASP-2 residues from this interface led to strongly decreased C4 deposition, and the isolated C4 C345c domain was functional as a competitive inhibitor of C4 cleavage by C1s pointing to the relevance of this interaction for the CP as well (38). A third minor interaction involves the C4 specific sulfotyrosine region, which appeared to be trapped by electrostatic interactions with the MASP-2 SP domain and the C4a domain. This interaction was later found to occur between the C4 sulfotyrosine region and the C1s SP domain as well (39) further supporting that C4 is recognized in the same manner by MASP-2 and C1s. The substrate C4 undergoes a significant conformational change as a result of binding MASP-2 which significantly reorders the C4a domain and the scissile bond region due to tighter contacts with the neighboring MG3 domain and the presence of the MASP-2 SP domain (38).

Structural basis for C4b function

Our structure of the C4b product revealed a conformation that very closely resembled that of C3b (40) in agreement with their common function as the non-catalytic subunit of the convertases (see below) and their shared mechanism of regulation by FI degradation. The thioester in C4b is fully exposed and has moved by 80 Å as compared to its location in C4 due to a major rearrangement involving the TE, CUB and

MG8 domains (Fig 1G). In the crystal structure of C4b the TE domain is associated with the MG1 domain, but our SAXS studies indicated that in solution the TE domain may be separated from the MG1 domain, a phenomenon also inferred from SAXS studies of C3b (40). Very recently, we have also determined the solution structures of the CP proconvertase C4b2 and the CP C3 convertase C4b2a (Fig 1H-I). In crystal structures involving FB from the AP two conformations – open and closed – are known, differing by a rotation of FB SP domain, see further below for a detailed description of these. Within the CP C4b2 we clearly observed a closed conformation of C2 (41). Our SAXS studies of CP C3 convertase was challenging due to the rapid dissociation of C2a from C4b, but by introducing stabilizing mutations in C2 and by conducting a rapid analysis using in-line SAXS that separates intact C4b2a from its dissociation products we were able to conduct the first solution study of a convertase. The resulting C4b2a conformation resembled that known from the AP C3 convertase trapped by a bacterial protein called SCIN (see below) and the C4b2a apparently does not need to undergo major conformational changes to bind and cleave its substrate C3 (41).

The alternative pathway

Initiation and amplification

With the first C3b molecule formed by the CP C3 convertase, the AP takes over and strongly amplifies the initial pattern recognition events in the CP and LP. It has been estimated that up to 90% of the deposited C3b molecules are generated through the AP regardless of whether CP or LP generated C3b initiate the AP (42, 43). The AP may however initiate without prior activation of the two other pathways. Spontaneous hydrolysis of the thioester bond in C3 – also known as tick-over – is believed to cause similar conformational change as those occurring in nascent C3b. The product C3(H₂O) cannot covalently associate with the activator, but may be recruited to a properdin binding surface (see below). Upon association with C3(H₂O), FB can be cleaved by FD and the resulting C3(H₂O)Bb complex is a proteolytically active fluid-phase C3 convertase (44). This may cleave C3 into C3a and C3b, of which some of the C3b molecules will attach

covalently to hydroxyl or amino groups on surface carbohydrates and proteins in close proximity. C3b generated in by this AP initiation pathway is no different from C3b produced by the CP C3 convertase. The AP C3 convertases C3(H₂O)Bb and C3bBb are inherently labile and dissociate irreversibly with half-lives in the range of two minutes at 37 °C (45). However, both complexes can be stabilized up to 10 fold upon binding of properdin (44, 46). In vivo, the short convertase life-time probably represents a safety mechanism that prohibits long-term complement activation and consequently excessive generation of the strong pro-inflammatory signal molecules C3a and C5a.

Structures of the AP C3 convertase

Considerable structural information has been generated concerning the AP proconvertase. In particular, a combination of EM and crystal structures of C3bB, C3bBD, and of FB in complex with the C3b homolog cobra-venom factor (CVF) have provided valuable insight into the assembly and activation of the AP proconvertase. The transition from an AP proconvertase to a proteolytically active C3-convertase involves two major events: initial binding of FB in the “closed” conformation to C3b, followed by a transition of FB into the “open” conformation cleavable by FD (47). The structure of CVFB represents the closed state, whereas those of C3bB and C3bBD represent the open state (Fig 2B-C). In both conformations, FB interactions with C3b/CVF are mediated by the vWA and the CCP domains. The primary interaction occurs between the carboxylate group in the C-terminal residue of the C3b/CVF C345c domain and the divalent cation bound to the metal ion-dependent adhesion site (MIDAS) of the FB vWA domain. In addition, residues located in the three FB CCP domains form contacts with the C3b/CVF Nt-α' region and the CUB domain (48-51). The main difference between the open and closed conformations is the position of the FB SP domain. In the closed conformation, this domain only forms contacts with the vWA domain of FB, whereas in the open conformation the SP domain has rotated by roughly 90° thereby making additional contacts with the C3b MG2 and CUB domains possible. Importantly, it is only in the open conformation of FB that the scissile bond Arg259–Lys260 is exposed and accessible to FD (48). The 24 kDa FD is composed of single SP domain, and circulates in plasma in a mature but self-inhibited form. FD binds with nano-molar affinity to the vWA-

SP interface in the open conformation of FB primarily through an exosite located 25 Å from the catalytic center. This high-affinity binding of FD to C3bB induces a conformational rearrangement in the self-inhibitory loop (Thr203-Arg207) of FD resulting in an active configuration of the catalytic triad (48). The open and the closed conformation of C3bB and CVFB are in equilibrium. EM studies performed under physiological Mg^{2+} concentrations show that CVFB strongly favors the closed conformation, while for C3bB the equilibrium is more evenly distributed between the two conformations (47). By substituting Mg^{2+} with Ni^{2+} it is possible to shift the equilibrium towards the open confirmation of C3bB. Un-bound FB exists exclusively in the closed conformation in which the scissile bond is protected from FD cleavage (52). Our recent SAXS structure of the CP homologue indicates that C2 adopts a conformation that is in between the open and closed conformation before binding to C4b and then switch to the closed conformation upon binding to C4b (41). Furthermore, the scissile bond is likely to be accessible in both C2 and C4b2 as these are cleaved with quite comparable kinetics by MASP-2, and C1s also readily turns over both substrates (53).

Upon FD cleavage the active convertase C3bBb irreversibly dissociates within minutes making structure determination of the AP C3-convertase and its substrate complex very challenging. As a response to the function of complement to clear invading pathogens, numerous of these have evolved mechanisms to evade especially the AP and the TP in order to spread to the tissue of interest (54, 55). Perhaps the most prominent example of how structural studies can benefit from these evasion proteins is the structure of the C3bBb complex determined using the staphylococcal complement inhibitor (SCIN). This 10 kDa protein exhibits a triple α -helical bundle structure and traps the convertase through dimerization in a very stable $(C3bBbSCIN)_2$ complex (56). This in turn blocks the substrate binding site and locks the SP domain of Bb and prevents convertase dissociation. Within the SCIN stabilized convertase the only C3b-Bb contact is centered on the Mg^{2+} dependent interaction of the C3b C345c carboxylate and the MIDAS within the Bb vWA domain (Fig. 2D). Moreover, the catalytic SP domain of Bb extends away from C3b in contrast to its location in contact with the MG2 and CUB domains in C3bB (56). Modelling and comparison with the C5-CVF structure

(see below) suggests that the Bb subunit has to swing closer towards the C3b MG7 domain in order to reach the substrate.

Properdin is a convertase stabilizing PRM

The 53 kDa basic glycoprotein properdin (FP) is a positive regulator of complement capable of stabilizing the AP C3 convertase (57). The protein is produced predominantly by monocytes, T-cells and granulocytes in humans (58). In hemolytic assays C3bBb appears to have a half-life of approximately 2 minutes at 37 °C, but this may be extended to 11 minutes when Ni^{2+} is used instead of Mg^{2+} . The presence of FP resulting in formation of the C3bBbP complex increases convertase half-life to 18 and 76 min at 37 °C in Mg^{2+} and Ni^{2+} buffers, respectively (45). Beside N- and O-linked glycosylations FP is post-translationally modified with 14 mannoses each attached via a C-glycosidic bond to the indole moiety of Trp residues in Trp-Xaa-Xaa-Trp motifs and mannosylation has been proposed to promote FP binding on bacterial surfaces (59). Hereby FP may provide a platform for AP convertase assembly and amplification and properdin has been shown to bind directly to the surface of several pathogens and effectively function as PRM. However, in contrast to the LP and CP PRMs, FP does not directly bind a zymogen protease which is activated upon PRM association with the activator, but does so indirectly by binding C3b that again may associate with zymogen FB. In this way the AP can be activated by the presence of a pathogen alone (60). FP has been reported to bind *E.coli* (LPS mutants lacking O-Ag), *Neisseria gonorrhoeae*, *Neisseria meningitidis*, yeast cell wall (zymosan), *Chlamydia pneumoniae* (61, 62) and host epithelial cells, apoptotic and necrotic T-cells, and activated platelets (63, 64). In solid phase assays FP has been observed to bind lipooligosaccharide, lipopolysaccharide, myeloperoxidase, and neutrophil extracellular traps (65-70). To what extent these observations are artifacts of high-order oligomers and aggregates of properdin is still up for discussion (71). FP oligomerizes and is primarily found as dimers, trimers and tetramers in plasma at a concentration of approximately 5-25 µg/ml (59, 72). However, even higher oligomers may be formed during purification and

storage, and the properties of FP depend on its oligomeric state, complicating performance of functional studies of the protein (71). No high-resolution structure of FP is available but based on sequence homology it is predicted to contain seven thrombospondin repeats (TSRs). Studies involving recombinant FP showed that variants lacking TSR6 are unable to form oligomers and that both TSR4 and TSR5 are important for binding to C3b and stabilization of C3bBb, whereas TSR3 is dispensable (73). Based on EM and SAXS studies, FP is thought to oligomerize in a head-to-tail fashion (74, 75). A recent EM study clearly showed that FP contains one vertex per monomer, which was suggested to contain four TSR domains from two separate monomers leaving three domains to form the elongated connections between vertexes (75). This study also suggested a structure of the FP-stabilized AP C3 convertase C3bBbP. The vertices of triangular or square FP trimers/tetramers were found to contact the C3b C345c domain and the Bb vWA domain offering an explanation for its stabilizing effect on the AP C3 convertase (75). In Figure 3D we present the FP vertex envelope from the EM study in the correct scale on top of the SCIN stabilized AP convertase C3bBb.

Decay acceleration and FI cofactors

Healthy host cells may become tagged by C3b due to the generation of C3(H₂O) in the AP or through the “bystander” effect whereby C3b generated from any of the three pathways in the vicinity becomes attached to a host cell. The AP must therefore be tightly regulated, and the lifetime of convertases on host cells is short due to regulators with decay acceleration activity. Moreover C3b is irreversibly degraded by factor I (FI) assisted by cofactors, to iC3b and C3dg which do not support convertase assembly. In humans the AP regulators are factor H (FH), decay acceleration factor (DAF/CD55), membrane cofactor (MCP/CD46) and complement receptor 1 (CR1). FH is a soluble 150 kDa protein and the major regulator of human complement. It binds fluid-phase and surface-bound C3b/C3(H₂O) thus preventing further convertase formation as it serves as cofactor for FI cleavage of C3b/C3(H₂O), but FH also accelerates dissociation of AP C3 convertases. By binding to host specific glycans such as sialic acid, heparin and sulfated glycosaminoglycans, FH is able to distinguish self from non-self and prevent complement activation on host surfaces (76).

FH consists of 20 repeating CCP modules arranged as beads on a string which can adopt multiple folded-back conformations in solution (77). The protein contains at least two C3b-binding regions; one located at the N-terminal CCPs 1–4 containing the regulatory activities (78), and another located at the C-terminal CCPs 19–20 which is able to interact with both C3b and host surface glycans simultaneously and even bridge activator bound C3b with a neighboring C3b molecule (79-81). FH carries at least two additional glycan-binding sites located in CCP7 and CCPs 9-15 which may cooperate for binding to host surfaces (82, 83).

Both decay-accelerating and FI cofactor activities are located in FH CCP 1-4. The binding of these FH domains to C3b is likely to confer cofactor activity by inducing a C3b conformation ready for FI binding and subsequent FI cleavage of the C3b CUB domain (84). A structure based comprehension of these FH functions were founded with the crystal structure of the C3b:FH CCP1-4 complex (78) and lately made general with four structures of C3b bound to active fragments of DAF, MCP, CR1 and the viral cofactor SPICE (85). In FH the four CCP domains adopt an overall L-shape with a kink between the CCP3 and CCP4 domains, but the orientation of CCP4 relative to CCP3 is different for C3b bound SPICE and MCP although the overall L-shape is preserved (85). FH contacts the C3b Nt- α' region, the MG domains 1, 2, 6, 7, and the TE domain (Fig 2E) and this overall binding scheme is also observed for the other four regulators and will most likely also apply to regulators binding C4b as well (85). A comparison with the structure of C3bBb (Fig 2D) complexes readily reveals that Bb and the first two CCP domains of FH competes for C3b thus offering an explanation for the decay accelerating activity exerted by FH CCP1-4 and equivalent CCP domains in the other cofactors. In the structure of the C3b-MCP complex the equivalent of these CCP domains are not stably bound to C3b but instead mobile, which provides a rationale for its lack of decay acceleration activity (85). FH CCP domains 2-3 and their equivalent CCP domain in the other regulators bind next to the CUB domain and may together with the C3b C345c domain provide the binding platform for FI allowing it to cleave twice in the CUB domain (78). The crystal structure of FI (Fig 2E) revealed a zymogen like

conformation of its SP domain although it had undergone maturation. Presumably a ternary complex between C3b, the cofactor and FI has to be formed to induce the catalytically competent state of FI (78) .

The terminal pathway

C5 cleavage and biological effector function of cleavage products

The terminal pathway is initiated once a certain density of C3b has been reached on the activator as this trigger a shift in substrate specificity for the C3 convertases, which will make them cleave C5 instead of C3 (Fig. 3A). Cleavage of the C5 scissile bond (R751-L752) results in formation of the effector molecules C5a and C5b. The anaphylatoxin C5a will through binding to C5aR1 and C5aR2 on host cells induce a number of inflammatory events including phagocytosis, leukocyte recruitment, stimulation of oxidative burst, histamine release, and vasodilation (reviewed in (86)). C5 does not feature the internal thioester making the C5b molecule incapable of binding covalently to the activator surface, instead nascent C5b captures C6. To the resulting C5b6 complex, C7, the C8 $\alpha\beta\gamma$ complex and 12-18 copies of C9 adds sequentially during the assembly of the membrane attack complex (MAC), also referred to as the C5b-9 complex (55). The complexes formed from C5b6 and onwards are also collectively referred to as terminal complement complexes (TCC). Parts of C6-C9 will refold into transmembrane β -hairpins (TMHs) that will insert into the lipidic bilayer of a membrane near the activator. This resulting insertion of MAC into pathogenic cell membranes or dysregulated host cell membranes may lyse the targeted cell (55).

C5 recognition by convertases

Although C3 convertases can cleave C5, it has long been known that the the formation of an efficient C5 convertase requires a certain density of C3b on the activator (87). To facilitate the following discussion we define C3b' as one or more C3b molecules that confer high affinity for C5 to the CP C4b2a or the AP C3bBb convertases. It is important to distinguish C3b' from the Bb-bearing C3b in the AP C3 convertase. We can now define the C5 convertases as C4b2a3b' or C3bBb3b', but the exact relationship between the C3b' and the two other subunits is controversial (see below). Kinetic studies found that the C5 convertases bind the substrate with a K_M of 5-180 nM depending on the surface/activator and the method employed to obtain

high C3b density (88, 89). These affinities are well below the physiological concentration of C5 (0.4 μM) suggesting that C5 convertases readily bind their substrate (89). However, the turnover number for cleavage is 1.44 and 0.33 C5 molecules min^{-1} for the CP and AP C5 convertase, respectively, making the C5 cleavage one of the slowest reactions experimentally investigated. Interestingly, whereas the presence of C3b' strongly improves the affinity for C5 as compared to the corresponding C3 convertase, the turn over number is more or less unchanged (89).

Due to the inherent instability of the C5 convertases and difficulty in fluid phase reconstitution, there is no detailed structural knowledge regarding these pivotal proteases. In order to clarify how convertases recognise their substrates we took advantage of CVF from *Naja naja*. CVF lacks the TE domain but otherwise structurally resembles C3b and forms a C3/C5 convertase upon complexation with host FB and subsequent cleavage by FD (87, 90, 91). The fluid phase convertase CVFBb has high affinity towards C5 (K_M 42 nM) and the turn over number for C5 cleavage (0.43 min^{-1}) is comparable to those of endogenous convertases. In contrast to CP and AP C5 convertases which have half-lives of a few minutes at 37 °C (88, 89), the half-life of the CVFBb C5 convertase is approximately 7 hours (91, 92). Both CVF and CVFBb bind C5 with affinities of 42-102 nM suggesting that CVF is the main contributor to substrate binding (87). Hence, our crystal structure of the C5:CVF complex (93) provides a good model for substrate recognition by the convertase although it does not describe C5 interactions with Bb. C5 binds to CVF with the long axis of the two proteins aligned roughly in parallel. Upon complex formation, C5 undergoes a significant conformational change as compared to unbound C5 (Fig 3B-C) which can be described as a coordinated rotation of the C5a, C5d, CUB, MG7 and MG8 domains by 18° relative to the MG1, MG4 and MG5 domains (94). The intermolecular interface between C5 and CVF consists of two components. The largest of these in terms of area involves the MG4 and MG5 domains of both proteins and is rather flat. The second component of the intermolecular interface is the set of contacts formed between the C5 MG7 domain and the MG6 and MG7 domains of CVF. Based on the C5:CVF complex we proposed a general model for how substrates are recognized by the non-catalytic subunits C3b/C4b of the endogenous C3 and C5 convertases

(94). This model is in accordance with previously published results describing how compstatin and CRIg interacting with the MG4 and MG5 domains of C3 and the S77 antibody interacting with the MG7 domain of C3 prevent substrate binding to the AP C3/C5 convertases but not CP/LP C5 convertases. The model furthermore accounts for mutations in C3b and C4b conferring weaker activity to the C5 convertases (93).

Assembly of the MAC

Nascent C5b undergoes a major conformational change, but in contrast to the TE domain in C3b and C4b, the C5d domain of C5b only travels halfway down from MG-ring due to its association with C6, that extensively interacts with this C5b domain as well as the MG1, MG2, MG4, C5d and CUB domains (Fig. 3D) (95, 96). A comparison with the structure of unbound C6 reveals that the C-terminal CCP and factor I-MAC (FIMAC) domains rotate and translate by 158° and $115 \text{ \AA}$, respectively, relative to the central MAC-perforin (MACPF) domain upon binding to C5b (97). It has been suggested that the C5b6 complex is able to stay associated with the C5 convertases (98, 99), and a structural alignment of the C5:CVF and C5b6 complexes suggests that most of the MG4-MG5 domain interface is retained and accessible in C5b whereas the orientation of the MG7 domain is altered. The putative contact between C5b6 and the activator bound C5 convertase may help to orient the C6 molecule correctly towards the extracellular membrane leaflet (Fig. 3D). The C5b6 complex functions as a receptor for C7 that confers lipophilicity to the resulting C5b-7 complex (100). Subsequently the C8 $\alpha\beta\gamma$ complex will associate with the C5b-7 complex through a C7:C8 β interaction (101). The TMHs of C8 α , C8 β and C9 facilitate the membrane penetration and induce the pore formation through C9 polymerisation. Recently published cryo-EM and cryo-ET structures of the entire MAC (Fig. 3E), either solubilized in detergents or formed on liposomes, respectively, describes a heterogeneous pore with a split-washer conformation (3, 4). The transmembrane region of the MAC does not form an idealized β -barrel as it might have been expected. The inner and outer diameters of the pore are around 11 and 25 nm, respectively, but the pore is not completely closed on the face where the initiating C6 molecule faces the terminating C9 molecule. The terminating C9 is located closer to the center compared to C6 and the initiating and terminating ends of the pore are twisted somewhat upwards and

downwards, respectively, as compared to the rest of the MAC pore. In the cryo-EM structure, the MAC consists of C5b-8 and 18 C9 molecules, whereas only 16 C9 molecules were placed in the cryo-ET structure (3, 4). From the cryo-ET studies it was moreover found that whereas half of the pores identified were single C5b-9 ring structures, the other half were multimeric pores formed from C5b-8 complexes associating with each other (4).

Structure and therapeutic inhibition of C5a

Due to the high potency of C5a with respect to induce pro-inflammatory signaling, the activity of C5a is tightly regulated by carboxypeptidase cleavage of the C-terminal Arg751 resulting in C5a-desArg that retains up to 50% of its efficiency as compared to full-length C5a depending on the system used to evaluate activity (102-104). Both as a domain within C5 and as isolated protein C5a is comprised of four antiparallel helices stabilised by three disulphide bridges connecting helices 2, 3 and 4. In most structures helix 1 interacts with helices 2 and 4 (105-107) - a pattern also found in C3a (108, 109). However, structures of human C5a-desArg and an antagonistic version of C5a (A8C5a) revealed two conformations in which helix 1 was released from the disulphide bridged core encompassing helices 2-4 (103, 110). The flexible nature of helix 1 might be of importance in C5 biology. All known structures of C5 feature Arg751 as inaccessible. We suggested that detachment of helix 1 from its contacts with helix 2 and 4 within C5 would allow unfolding of the scissile bond region (93). Secondly, the distance from the C-terminal residue L673 in the LNK region to the N-terminal Thr678 of C5a is 58 Å, which is too far to span with the four residues found in the pro-C5. Hence, helix 1 may adopt the extended conformation prior to the furin processing step of pro-C5 (94). The C-terminus of free C5a is very flexible in most crystal structures, but in the NMR structure of human C5a, residues 746-751 forms an extra small helix pointing towards helix 1 (107). Interestingly, a helical conformation of residues 746-754 is also observed in our recent crystal structure of C5 in complex with an eculizumab Fab fragment (111) and in the C5:OmCl:RaCl complex (112).

Mutational studies suggest that C5a possess two surface patches mainly involved in C5aR1 binding and signalling, respectively. The first patch includes basic residues within the disulphide bridged three-helical

core and is essential for the high affinity binding towards C5aR1. The second patch consists of the C-terminus, of which the last eight residues is the minimal motif that may activate C5aR1. We have recently presented the structure of mouse C5a bound to the inhibitor NOX-D20, an L-RNA/L-DNA aptamer developed by NOXXON Pharma AG (113). Our structure revealed that NOX-D20 renders patch 1 of C5a inaccessible for receptor binding. This suggests that NOX-D20 works by inhibiting the high affinity binding of C5a to C5aR1 rather than directly preventing the receptor activating part of C5a in patch 2 from interacting with the receptor (Fig. 3F).

Therapeutic inhibition of C5 by eculizumab

The insertion of MAC in membranes of non-altered self-cells is normally prevented by either MAC specific regulators or by upstream control of convertases (reviewed in (92)). In some diseases, this regulation however is abnormal. The most prominent example is paroxysmal nocturnal hemoglobinuria (PNH) which is caused by a somatic mutation in the X-linked *PIGA* gene resulting in deficiency of the PIG-A protein involved in the addition of a glycosylphosphatidylinositol (GPI) anchor to proteins (114, 115). This modification is functionally essential as it targets the GPI carrying protein to the outer leaflet of plasma membranes. Two such proteins are DAF/CD55 and CD59. Whereas CD55 negatively regulates the AP through its dissociation of convertases, CD59 directly blocks MAC assembly. The *PIGA* mutation leads to an expansion of hematopoietic stem cells with a deficiency in the pathway for addition of GPI-linkages resulting in erythrocytes lacking these regulators. Uncontrolled C3 and C5 consumption combined with an ablated MAC-inhibitory mechanism confer constant formation of TCC complexes on these erythrocytes leading to severe hemolysis (116). Another example of a disease caused by insufficient complement regulation is atypical hemolytic uremic syndrome (aHUS), a rare and life-threatening disease involving hemolysis and dysfunctions of the kidney. AP dysregulation conferring over-activation of C3 on erythrocytes, platelets and endothelial cells results in excessive amounts of MAC formation and are characteristics of aHUS (117, 118). Mutations leading to loss-of-function in AP regulators such as FH or FI either alone or in combination with

gain-of-function mutations in C3 or FB result in C3 convertases with an apparent enhanced stability. Also FH autoantibodies have been found to increase the risk of aHUS development (119).

The monoclonal antibody eculizumab (also known as Soliris) manufactured by Alexion Pharmaceuticals has been used to treat PNH and aHUS in the clinic for almost a decade. Eculizumab is a humanized antibody selected in mice by immunization with human C5. The antibody binds human C5 with an affinity of 120 pM and is highly species specific. Most recently we have determined the crystal structure of C5 bound to a Fab fragment with a sequence identical to eculizumab (called ecuFab) (111). The structure reveals how all complementarity determining regions (CDRs) of ecuFab except light chain CDR2 are involved in C5 recognition. The C5 epitope is exclusively located within the MG7 domain and ecuFab binds orthogonal to the major axis of its antigen and is protruding out in a very distinct manner from the C5 molecule (Fig. 3G). Our crystal structure is in complete agreement with a low resolution negative stain EM envelope of C5 bound to OmCI, RaCI and ecuFab (112). The C5-ecuFab interface we observed was validated by a complete mutational scan of all CDR residues which identified 28 residues of importance for the binding to C5. Despite the limited resolution of 4.2 Å for the C5-ecuFab structure we were able to rationalize why these mutations altered C5 binding. We could further pinpoint C5 Trp917 as a major contributor to the epitope, which neatly explains the strict species specificity of eculizumab. Moreover we were able to suggest why PNH patients possessing C5 with the mutations Arg885His and Arg885Cys are non-responsive to eculizumab treatment (120). The ecuFab forms a large binding pocket for Arg885 that involves numerous residues within all heavy chain CDR regions (111). A histidine or a cysteine at the 885 position in C5 is too small to fill this pocket, and its presence will most likely destabilize the conformation of the pocket forming CDR loops. Comparison of the CVF and ecuFab interface on the C5 MG7 domain revealed massive overlap suggesting that eculizumab works by sterically hindering C5 binding to the convertases and not by preventing access to the scissile bond region or by inducing a C5 conformation incompatible with convertase binding and subsequent cleavage (111).

Complement evasion at the C5 level

The TP is frequently target for pathogen complement evasion strategies. Here we focus on structures of C5 bound to evasion proteins, which have recently provided considerable insight into the molecular mechanism of evasion and at the same time provided us with important clues regarding the conformational properties of C5 and possible ways to develop new TP inhibitors. The soft tick *Ornithodoros moubata* secretes a 16 kDa protein with a lipocalin fold known as OmCI which prevents cleavage by binding to the C5 molecule with an affinity of 19 nM (121, 122). Like ecuFab, OmCI binds the C5 molecule distant from the scissile bond region but also far from the MG4-5 and MG7 domains of C5. So how does OmCI prevent C5 cleavage then? The tick evasion protein interacts with both the C5d and the CUB domains and with an additional contact point in a loop from the C5 C345c domain (Fig. 3H). It was suggested that OmCI functions by locking the C5 molecule in a conformation that is non-cleavable by endogenous C5 convertases but still functional as substrate for the CVFBb convertase (123). The crystal structure of C5 in complex with OmCI also included the newly identified tick C5 inhibitor, *Rhipicephalus appendiculatus* C5 Inhibitor (RaCI) (123). Three members were identified in this novel inhibitor family. The 8 kDa RaCI proteins adopt a novel fold with a twisted four stranded antiparallel β -sheet stabilized by three conserved disulfide bridges. Two loops in the RaCI inhibitors are highly involved in contacting C5 at a binding site located in a cleft between the MG1, MG2 and C5d domains (Fig. 3H). Like for OmCI, this binding site is distant from the scissile bond region and the convertase recognition sites, and the RaCI inhibitors are also hypothesized to lock the C5 molecule in a non-cleavable conformation (123).

Turning to bacterial pathogens, one of the best characterized with respect to evasion is the gram-positive bacteria *Staphylococcus aureus* which has evolved strategies for evading complement activation, C3/C5 convertase substrate cleavage, and the downstream biological effector function of both C3 and C5 fragments (124). The bacterium secretes several Staphylococcus superantigen-like (SSL) molecules, one of them being the SSL7 molecule (125). SSL7 binds human C5 with an affinity of 5-20 nM through a β -grasp domain and the Fc region of IgA with an affinity of 1 nM via an OB-domain (126, 127). The SSL7 β -grasp

domain primarily interacts with the C5 MG5 domain supplemented with contacts to the MG1, MG2 and MG6 domains (Fig. 3I). The β -sheet of SSL7 and the interacting β -sheet from the C5 MG5 domain interact through pairing of two β -strands, resulting in an intermolecular continuous nine stranded β -sheet (126). SSL7 binds more than 70 Å from the C5 convertase cleavage site and does not induce large conformational changes in the C5 molecule allowing C5 recognition by the convertases. This agrees with data showing that in the absence of IgA, SSL7 does not significantly inhibit C5 cleavage (126, 127). *In vivo* SSL7 completely prevents C5 cleavage by simultaneously binding to the Fc-region of an IgA molecule and C5 resulting in a pentameric IgA-SSL7₂-C5₂ complex in which convertase binding is prevented by massive steric hindrance (94, 126).

The quaternary structure of C5 convertases

The clinical success of eculizumab has conclusively demonstrated that inhibition of the TP and in particular C5 cleavage may cure some complement related diseases without serious adverse effects (128). A detailed structure based understanding of the C5 convertase could strongly accelerate development of novel TP targeted therapeutics. To date it has not been possible to reconstitute a fluid phase C5 convertase containing C3b'. Early studies suggested that C3b' has to be activator bound in a specific manner whereas both the CP and the AP C3 convertase can be recruited from the fluid phase once C3b' is present (129). A recent study with C3b immobilised on streptavidin beads through a biotinylated thioester ensuring a 'native'-like orientation of both the C3b and the C3b' on the activator appears as a promising system for controlled reconstitution and functional analysis of C5 convertases that could pave the way for detailed structural insight. It was found that an average intermolecular distance of a maximum of 140 Å between the involved C3b molecules was needed to form an efficient C5 convertase suggesting that the C3b molecules actually have to be in physical contact (130). The C3b molecules must also be oriented specifically relative to the activator since randomly coupled C3b did not support C5 convertase activity. In the CP C5 convertase the function of C3b' is easier to analyze as it cannot be confused with the C2a-bearing C4b. Serine 1236 in human C4b is capable of exerting a nucleophilic attack on the thioester bond in nascent

C3b', resulting in a well-defined and specific covalent C4b3b' dimer (131, 132). This occurs with a remarkably high efficiency of more than 50% during self-amplification on red blood cells. The resulting C4b3b' binds C5 with high affinity in the absence of C2a, and the high-affinity site was inhibited by both C3 and C4 antibodies (131). The covalent C4b-C3b' linkage is however not a prerequisite for the CP C5 convertase, since C4b mutants of Ser1236 still forms a non-covalent complex with the metastable C3b' molecule resulting in an enzyme with activity comparable to the CP C5 convertases formed with wt C4b (132). Taken together these findings suggest that a specific non-covalent complex between C4b and nascent C3b' is formed on an activator and within this complex the covalent linkage may be formed. Similarly, it was found that two C3b molecules may be linked if a nucleophile from an activator bound C3b reacts with the thioester in a second nascent C3b. However, the identity of the C3b nucleophile, if specific, is still unknown (133, 134).

There are at least two ways in which C3b' might contribute to the high C5 affinity. In an allosteric model, C3b' induce a conformational change in C3bBb or C4b2a leading to high C5 affinity but does not interact directly with the substrate (Fig. 3J). This model requires well-defined C4b-C3b' and C3b-C3b' complexes and based on the crystal structure and SAXS studies of C4b and the well characterized C4b-C3b' crosslink we proposed that the C3b' molecule might associate with the CP C3 convertase such that the FH interface of C3b' is shielded by the interaction with the C4b molecule (135). An alternative mechanism is the sandwich model in which C3b' does not form a well-defined complex with C3bBb/C4b2a but instead contributes by recruiting C5 and perhaps primes its conformation for convertase binding and subsequent cleavage. The substrate may either bind simultaneously to C3b' and the C3 convertase (Fig. 3K) or first bind to C3b' and then diffuse to a nearby C3 convertase (Fig. 3L). The sandwich model was proposed already in the 70s (129) but the identification of especially the C4b-C3b' dimers led to the perception of a preformed C5 convertase with a defined stoichiometry in which C3b' is located in specific manner relative to the two other C3 convertase subunits (131). The sandwich/C5 priming model has recently been revitalized based on experiments indicating that at high concentrations the ecuFab:C5 complex may compete with C5 for

binding to the AP C5 convertase in apparent contradiction to our structure of the C5:ecuFab complex (112). The competition effect could therefore mirror C5 interaction with C3b' rather than with C3bBb. Alternative explanations could be that ecuFab:C5 may be able to form weak contacts with a preassembled C5 convertase through the MG4-MG5 interface known from the C5:CVF complex or that C5 cleavage is so slow (see above) that ecuFab can displace convertase bound C5 before the actual cleavage takes place. Further experiments with both CP and AP C5 convertases and in particular C3b binding inhibitors with a known binding site like compstatin, CRIg and the S77 Fab might be informative. Perhaps a structure of the C5 convertase with bound C5 on an activator model obtained with EM-tomography – as pioneered with the C1 complex and the MAC (2, 4) – will be needed to decide between the two models.

Conclusions and outlook

Future increasingly reliable models for the large scale geometry and architecture of complement will form the basis for improving our mechanistic understanding of complement activation, convertase assembly and the regulation. We predict that this will significantly accelerate the generation of new complement therapeutics as exemplified by the development of mini-FH, a fusion protein with a linker connecting FH CCP1-4 and CCP19-20 (136). The recent technical improvements in EM and the successful demonstration that application of cryo-EM and cryo-ET can be used to structurally describe large complexes of the complement system (2-4) and pave the way for more structures of activator and membrane associated complement molecules in the coming years. This will hopefully result in higher resolution structures of the activating complexes giving a more detailed insight into the physiological relevance of intra- vs intermolecular activation of C1 and PRM:MASP complexes. Additionally, structures of activating complexes with C4 and C4b2 bound could further elaborate on the assembly and orientation of CP C3 convertase on the activator surface. And although we start to appreciate how FP stabilizes the AP convertases, a much more detailed understanding is needed to truly understand how this important AP regulator functions. Another pending question is the functional composition of the C5 convertases, and also in this regard the use of properdin for stabilization might be beneficial in reconstituting the AP C5 convertase. Finally, the

commercial interest in understanding anaphylatoxin signaling and activation due to the steadily accumulating data linking uncontrolled C3aR/C5aR signaling to the development of several disorders (137) combined with the expanding field of crystallography based GPCR structural biology (138) might result in structures of C3aR/C5aRs bound to agonists and antagonists. Such structures will form neat platforms for structure based engineering to improve therapeutics targeting anaphylatoxin receptors in terms of affinity and selectivity.

Acknowledgements

GRA was supported by the LUNA nanomedicine centre, the Lundbeck Foundation, and the Novo Nordisk Foundation through a Hallas-Møller Fellowship. GRA declares collaboration with Alexion Pharmaceuticals.

Figure legends

Fig. 1. C4 cleavage and downstream events in the lectin and the classical pathways. (A) Activation of complement through the classical and lectin pathways occurs upon PAMP/DAMP recognition by the CP C1 complex or the LP PRMs associated with MASPs. C4b deposited on the activator associates with C2 forming the proconvertase C4b2, which is cleaved resulting in formation of the C3 convertase C4b2a. (B) Structure determined by SAXS of MBL (brown) with a bound non-activated homodimeric MASP-1 dimer (SP domain colored orange, remaining domains red). (C) Crystal structure C4 (blue, C4a in yellow) in complex with a MASP-2 fragment placed roughly in parallel with MASP-1 in panel A to visualize how C4 may relate to PRM:MASP-2 and C1 complexes. (D) The C1 complex bound to a hexameric Ab deposited on hapten-coated liposomes as visualized by cryo EM tomography. Within C1, C1s is likely to bind C4 in the same manner as MASP-2. The activator surface roughly separates C1 from the hexameric IgG platform. Structures and EM tomogram in panels B-D are on the same scale. (E-F) Crystal structure of C4 and the C4:MASP-2 complex for comparison. (G) Structure of C4b, notice how the thioester (red sphere) is now bound to the activator. (H) SAXS structure of the C4b2 complex resembling the closed conformation of the proconvertase known from the AP. The C2 SP domain is colored orange, the vWA and CCP domains red. (I) The SAXS structure of the C4b2a complex suggests that the CP C3 convertase is structurally highly similar to the AP C3 convertase.

Fig. 2. Activation and regulation of the alternative pathway. (A) The AP may be initiated by C3b produced by the CP C3 convertase or by C3(H₂O) generated via thioester tick-over. Upon C3b complexation with FB and cleavage by FD, the AP C3 convertase C3bBb is formed that may be further stabilized by interaction with FP. At high C3b density on the activator, the AP C5 convertase is formed. (B) Crystal structure of the CVFB proconvertase in the closed conformation (CVF colored green, SP domain orange, the rest of FB in red). (C) Crystal structure of C3b (blue) in complex with FB and FD (purple) with the proconvertase FB in the open conformation. (D) Crystal structure of the SCIN stabilized C3bBb convertase (56). The EM envelope of a FP vertex is displayed to illustrate its suggested interaction with the C3 C345c domain and the Bb vWA domain. (E) Structural representation of the activator bound C3b:FB complex assembled from structures of

C3b:FH CCP1-4 (beige) and FH CCP19-20 (light blue) simultaneously bound to C3d and a sialylated trisaccharide (yellow). Two cleavage sites (Arg954 and Arg1303) within the C3b CUB domain are shown in green spheres, the third site (Arg1320) is hidden by FH. The structure of FI with the SP domain in orange and the remaining domains in pink is docked on top of the C3b:FH complex. All structures and EM envelopes are shown at the same scale. (F) Schematic model of a single C3b molecule (blue) in complex with full-length FH and FI. C3b is recognized by the FH CCP1-4 (beige) and CCP19-20 (cyan) as in panel E and with the CCP5-18 domains looping out. The interaction of the FH CCP19-20 glycan is indicated by the yellow star.

Fig. 3. The terminal pathway and its inhibitors. (A) The TP is initiated upon cleavage of C5 by the convertases C4b2a3b' and C3bBb3b'. C5a functions as a potent pro-inflammatory mediator while C5b together with C6, C7, the C8 $\alpha\beta\gamma$ complex and 12-18 copies of C9 form the MAC. (B) Crystal structure of unbound C5 (blue, with C5a in yellow). Notice that the C5 C345c domain is turned towards the MG7 domain. (C) A model of the C5:CVFBb complex based on the structure of C5 bound to CVF (green) and placement of Bb by comparison with the SCIN stabilized C3bBb complex. (D) Crystal structure of C5b (blue) in complex with C6 (salmon). The orientation of C5b is similar to panels B and C, illustrating how elements of the C6 MACPF domain might be oriented towards the extracellular membrane leaflet if nascent C5b6 remains associated with the activator bound C5 convertase. (E) Cryo-EM envelope of the MAC complex with 18 C9 molecules. The two planes approximating the membrane leaflets are separated by 40 Å. (F) Structure of mouse C5a (yellow) bound to the L-RNA/L-DNA aptamer NOX-D20. The putative C5aR1 interacting residues of C5a (red spheres) are shielded in the complex, explaining the inhibitory mechanism of NOX-D20. (G) Crystal structure of C5 in complex with ecuFab (green), the entire epitope resides in the C5 MG7 domain far from the scissile bond. (H) Crystal structure of C5 in complex with the two tick evasion proteins OmCI (pink) and RaCI (red). Both proteins bind distant from the scissile bond region and are suggested to lock C5 in a non-cleavable conformation. (I) Crystal structure of C5 bound to the *S. aureus* evasion protein SSL7 (orange). SSL7 exerts inhibition of C5 cleavage by simultaneously binding to the Fc-

part of an IgA molecule and to C5. All structures and the cryo-EM envelope in panels B-I except panel F are shown at the same scale. (J-L) C5 convertase models. The allosteric C5 convertase model (J) suggests that the substrate interacts with a pre-assembled C5 convertase consisting of a C3 convertase and an associated C3b' molecule. The catalytic subunit (Bb/C2a) is shown in orange. (K-L) The 'sandwich' C5 convertase models suggest that C5 is primed by the C3b' molecule prior to cleavage by a C3 convertase. This may happen either by C5 simultaneously interacting with C3b' and the C3 convertase (K) or by an initial priming by a C3b' followed by dissociation and cleavage by a nearby C3 convertase (L).

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