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Santiago Rodríguez de Córdoba, Claire L. Harris, B. Paul Morgan, Oscar Llorca. Lessons from functional and structural analyses of disease-associated genetic variants in the complement alternative pathway. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, 2010, 1812 (1), pp.12. 10.1016/j.bbadis.2010.09.002 . hal-00642422

HAL Id: hal-00642422

<https://hal.science/hal-00642422>

Submitted on 18 Nov 2011

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Accepted Manuscript

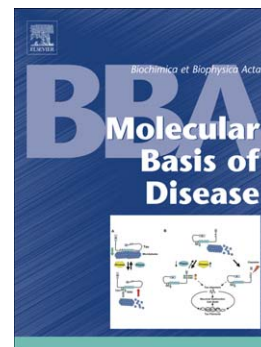
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PII: S0925-4439(10)00202-4
DOI: doi: [10.1016/j.bbadis.2010.09.002](https://doi.org/10.1016/j.bbadis.2010.09.002)
Reference: BBADIS 63160

To appear in: *BBA - Molecular Basis of Disease*

Received date: 23 July 2010
Revised date: 3 September 2010
Accepted date: 7 September 2010



Please cite this article as: Santiago Rodríguez de Córdoba, Claire L. Harris, B. Paul Morgan, Oscar Llorca, Lessons from functional and structural analyses of disease-associated genetic variants in the complement alternative pathway, *BBA - Molecular Basis of Disease* (2010), doi: [10.1016/j.bbadis.2010.09.002](https://doi.org/10.1016/j.bbadis.2010.09.002)

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Lessons from functional and structural analyses of disease-associated genetic variants in the complement alternative pathway.

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ABSTRACT

Complement is an essential component of innate immunity and a major trigger of inflammatory responses. A critical step in complement activation is the formation of the C3-convertase of the alternative pathway (AP), a labile bimolecular complex formed by activated fragments of the C3 and factor B components that is fundamental to provide exponential amplification of the initial complement trigger. Regulation of the AP C3-convertase is essential to maintain complement homeostasis in plasma and to protect host cells and tissues from damage by complement. During the last decade, several studies have associated genetic variations in components and regulators of the AP C3 convertase with a number of chronic inflammatory diseases and susceptibility to infection. The functional characterization of these protein variants has helped to decipher the critical pathogenic mechanisms involved in some of these complement related disorders. In addition, these functional data together with recent 3D structures of the AP C3-convertase have provided fundamental insights into the assembly, activation and regulation of the AP C3 convertase.

Keywords: atypical Hemolytic uremic syndrome (aHUS); Membranoproliferative glomerulonephritis Type II (MPGN2); Dense Deposit Disease (DDD); Age-related macular degeneration (AMD); Complement; alternative pathway; convertase

1. THE COMPLEMENT SYSTEM

Overview

Complement is a major component of innate immunity with crucial roles in microbial killing, apoptotic cell clearance, immune complex handling and modulation of adaptive immune responses [1, 2]. The complement cascade is triggered by three activation pathways, the classical pathway (CP), the lectin pathway (LP) and the alternative pathway (AP), which converge in the central and most important step of complement activation: the cleavage of C3 to generate the activated fragment, C3b. The critical step in these activation pathways is the formation of unstable protease complexes, named C3-convertases (C3bBb in the AP; C4b2a in the CP/LP). When C3b is generated, a reactive thioester is exposed which is attacked by nucleophiles (such as amine groups) on adjacent surfaces, resulting in covalent binding of C3b to the surface, targeting it for destruction and initiating inflammation. The efficacy of the complement cascade depends on the AP C3 convertase amplification loop in which the C3b generated by the convertase forms more AP C3-convertase and provides exponential amplification to the initial activation (Figure 1) [3].

In contrast to the CP and the LP, whose activation is triggered by immune complexes and bacterial mannose groups, respectively, the AP is intrinsically activated. Spontaneous activation of C3 in plasma occurs through the “tick-over” mechanism, which is initiated by the generation of a fluid phase C3b-like molecule, called C3i or C3(H₂O), by aqueous hydrolysis of the internal C3 thioester [4]. Activation of C3 may also occur by continuous low rate cleavage of C3 to C3b by plasma proteases [5]. The balance between the rate at which the initial trigger is amplified and the degree to which C3b and the AP C3-convertases are inactivated, determines the progression of the complement cascade to cell damage and death. Foreign substances on microbial pathogens (alternative pathway; AP), antibodies (classical pathway; CP), or mannan (lectin pathway; LP), tip the balance in favor of amplification, causing target opsonisation. The complement system includes other proteins, such as properdin,

which stabilize the AP C3-convertase and serve as a focal point for promoting local amplification of complement [6]. The lytic pathway fires when the AP C3-convertase incorporates one additional C3b molecule, which creates the C5-convertase and switches substrate specificity from C3 to C5, a C3 homologue. C5 cleavage triggers inflammation through production of C5a, leukocyte recruitment and initiation of membrane attack complex (MAC) formation. The MAC contains C5b plus the complement components C6, C7, C8 and C9, and lyses cells by punching holes through the membrane [7].

Complement and disease

In health, activation of C3 in the blood is kept at a low level and deposition of C3b and further activation of complement is limited to the surface of pathogens. Dysregulated complement activation in inflammatory diseases can damage self-cells and contribute to pathology. Complement is implicated in a long list of diseases [8], including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), glomerulonephritis, multiple sclerosis (MS), ischaemia/reperfusion injury and transplantation, in each of these diseases complement acts to sustain the 'vicious cycle' of inflammation and perpetuate tissue damage. In recent years, several reports have established that dense deposit disease (DDD) [9-12], hemolytic uremic syndrome (HUS) [13-22] and age-related macular degeneration (AMD) [23-32], are each associated with mutations or polymorphisms in the components and regulators of the AP C3 convertase (Table 1). These disorders are considered prototypic diseases caused by complement dysregulation.

Hemolytic uremic syndrome. HUS is a rare disease characterized by renal endothelial cell injury and thrombosis (thrombotic microangiopathy), resulting in hemolytic anaemia, thrombocytopenia and renal failure. Typically, HUS follows infections by enterohemorrhagic *E coli* (EHEC) and has a relatively good prognosis. In

contrast, atypical forms of HUS (aHUS), not associated with EHEC, affecting both adults and very young children, have a very poor long-term prognosis with more than 90% of the cases ending in end stage renal disease (ESRD) [33]. Recurrence of aHUS in the grafted kidneys is common with a mortality rate which approaches 30%. Mutations or polymorphisms in complement genes are the main genetic risk factors contributing to aHUS. In fact, analysis of the genes encoding the complement AP proteins factor H (fH), factor I (fI), membrane cofactor protein (MCP), C3 and factor B (fB) reveals a genetic defect in at least one of these in approximately 50% of the aHUS patients [34]. Functional characterization of many of these mutations has established that they cause complement dysregulation which affects primarily the protection of cellular surfaces from complement activation. When complement activation is triggered in carriers of these aHUS-associated complement mutations, deposition and amplification of C3b on the cellular surfaces in the microvasculature cannot be controlled, resulting in tissue damage and destruction. Importantly, complement dysregulation may result from either a defect in the regulatory proteins (i.e., loss-of-function or expression mutations in fH, fI or MCP) or the AP complement components (i.e., gain-of-function in C3 or fB). In addition to rare mutations, common polymorphisms in fH and MCP are also associated with aHUS and contribute to the genetic predisposition, either by increasing risk or conferring protection [35, 36].

Dense deposit disease. DDD is a rare form of glomerulonephritis which affects both children and young adults and frequently results in ESRD [37]. The morphologic hallmark of DDD is the presence of dense deposits within the glomerular basement membrane (GBM), as resolved by electron microscopy. The chemical composition of the dense deposits is largely unknown. Notably IgG is absent from them and other regions of the glomerulus, which excludes a role for immune complexes in dense deposit formation. DDD is associated with complement abnormalities which lead to intense deposition of C3 in GBM and persistent reduction of C3 serum levels. Familial

cases of DDD are exceptional but extremely informative. They have revealed mutations in fH and C3 associated with DDD which demonstrated that control of complement activation is impaired in DDD patients. As with aHUS, dysregulation of the complement system may result from loss of activity of the complement regulatory protein fH or may also be a consequence of mutations that increase the activity of the AP C3 convertase. The functional characterization of human DDD-associated fH and C3 mutations [11, 38] and previous studies in animal models [39-41] have provided conclusive evidence that fluid phase complement dysregulation, resulting in the continuous generation of C3b in plasma, plays a major role in DDD pathogenesis.

Age-related macular degeneration. AMD is the most common causes of visual disability in the elderly in developed countries. The hallmark of early-stage disease is the development of drusen, lipoproteinaceous deposits localized between the retinal pigment epithelium (RPE) and Bruch's membrane. Later, an extensive atrophy of the RPE and overlying photoreceptor cells (geographic atrophy, GA) or aberrant choroidal angiogenesis is observed. This choroidal neovascularization (CNV) under the macular area is the leading cause for blindness. Although the pathogenesis of AMD is still unclear, it has been proposed that the inflammatory response plays an important role in its development [42]. The contribution of genetics to the development of AMD is considerable, representing as much as of 70% of the total predisposing factors. Two major AMD susceptibility loci (1q31, *CFH*, and 10q26, *LOC387715/HTRA1*) which independently contribute to AMD disease risk have been identified by candidate region linkage studies and whole genome association analyses [23, 26, 27, 29, 43]. The first of these susceptibility loci is in the coding region of the fluid phase complement control protein, fH, implicating complement in disease etiology. Indeed, these initial findings have been followed by a number of reports establishing additional genetic associations between a number of common polymorphisms in other complement genes and AMD [24, 25, 28, 30-32]. Interestingly, many of these complement polymorphisms are also

associated with aHUS and DDD, demonstrating that complement dysregulation is a common link between these pathologies. However, in contrast to aHUS and DDD, the precise functional alterations in complement which lead to AMD remain unclear.

In the following sections, we will describe how the functional characterization of the complement genetic variations associated with disease has provided insights into the underlying pathogenic mechanisms of disease. Importantly, these data also provide important insights into structural aspects of assembly and regulation of the AP C3 convertase.

2. THE AP C3 CONVERTASE

The components of the AP C3 convertase

The generation of the AP C3-convertase begins *in vivo* with the interaction between C3b (or the C3b-like molecule C3(H₂O)) and fB, in a Mg²⁺-dependent manner, to form the pro-convertase C3bB (or C3(H₂O)B). In the presence of factor D (fD), fB is cleaved and the N-terminal Ba fragment is released from the C3bB complex, resulting in the active AP C3-convertase, C3bBb (or C3(H₂O)Bb).

C3 is a large protein of 190 kDa composed of two polypeptides chains of 115 and 75 kDa, respectively. The crystal structure of native C3 has been resolved at atomic resolution using X-ray crystallography [44], and reveals an intricate arrangement of 13 domains, including an anaphylatoxin domain (ANA; C3a), a small link (LNK) domain, a core of eight homologous macroglobulin domains (MG1-8) forming a ring (MG-ring), a CUB domain connecting this ring with a TED domain that contains the reactive thioester, and the C345C domain (Figure 2). As indicated above, the reactive thioester is shielded in native C3 so that it is not accessible. Activation of C3 into C3b (or C3(H₂O)) induces a huge conformational displacement of the TED domain exposing the reactive group to nucleophilic reagents such as hydroxyl or amine groups [45-47]. Release of C3a (ANA domain) from the alpha chain of C3 generates a novel C3 domain termed α' NT.

The α 'NT and C345C domains in C3b include binding sites for fB required for the AP C3 convertase formation [48-50]. These domains are located in a part of the C3 molecule which undergoes large rearrangement upon activation of C3 into C3b [45], which explains why C3 does not interact with fB. Similarly, structural analyses have suggested that formation of the AP C3-convertase probably depends on the structure and orientation of the CUB domain of C3b and that the interaction between C3b and fB is independent of the TED domain [51].

fB (90 kDa) circulates as an inactive pro-enzyme in plasma. It is composed of five structural domains. Three short consensus repeats (SCRs) at the N-terminus comprise the Ba fragment, whereas the large Bb fragment at the C-terminus is comprised of a von Willebrand type A (VWA) domain followed by a serine-protease (SP) domain (Figure 2). Mutagenesis analyses of fB have revealed a number of residues near the Mg^{2+} -dependent metal ion-dependent adhesion site (MIDAS) and in the α 1 helix in the VWA domain of the Bb fragment which mediate the initial recognition of C3b by fB [52-54]. This initial interaction also involves contacts between C3b and the SCR1-3 domain (Ba propeptide segment) [55-58].

Activation of the AP C3 convertase

The crystal structure of human fB, recently resolved at 2.3Å resolution, demonstrated that the Ba domain is not extended but folded back onto the Bb domain (Figure 2) [59]. These structural data also indicated that the first SCR likely hinders access of the ligand C3b to the MIDAS of the VWA domain and that the triad of SCR domains is probably only weakly associated with the VWA and SP domains. Most interestingly, comparison of the fB pro-enzyme [59] and the Bb fragment [60] structures, led to the suggestion that fB must undergo conformational changes upon binding to C3b, so that the long linker domain between SCR3 and the VWA domains of fB, which contains the scissile bond, becomes accessible to fD [59, 60].

Very recently, better insight into the mechanisms leading to C3-convertase assembly and activation have been obtained thanks to the combination of high resolution studies by X-ray crystallography and medium resolution electron microscopy (EM) studies [59, 61-64]. These analyses have shown that fB binds to C3b in the closed conformation of the pro-enzyme through interactions involving both the VWA and the SCR domains (Figure 3). The VWA domain binds C345C whereas residues located in the SCR1-3 domain contact the α' NT region, MG2, MG3, MG7 and CUB domains. The SP domain does not contact C3b. After this initial loading, a large conformational change occurs in fB, opening its structure and generating an “activated” conformation where the scissile site is likely accessible to fD (Figure 3). Upon cleavage by fD and release of the Ba fragment, the active AP C3-convertase C3bBb is maintained by interactions between the C345C and VWA domains of C3b and Bb, respectively. The Bb fragment projects outwards from the complex so that the SP domain is far from contacting C3b [59, 61, 63]. Additionally, the EM structure of the C3 convertase suggests that some conformational flexibility of the C345C-VWA-SP arrangement permits the accommodation of the C3 convertase to the substrate so that the SP domain can reach and cleave C3 [63] (Figure 5B).

Disease-associated mutations and polymorphisms in the AP C3 convertase components

Factor B mutations are characteristic of a subgroup of aHUS patients showing persistent activation of the AP [16, 65]. Four of these mutations, D279G, F286L, K323E and K350N (all amino acids are numbered from the initial Met), have been functionally characterized and are gain-of-function mutations that result in enhanced formation of the C3bBb convertase or increased resistance to inactivation by complement regulators [16, 65] (Figure 4). Proconvertase (C3bB) formation by mutant fB-286L is much enhanced, producing more active enzyme in vivo than the normal fB-286F allele. In contrast, generation and natural decay of the AP C3 convertase formed by fB-323E

is normal; however, it is more resistant to accelerated decay by the complement regulators DAF and fH, which also results in enzyme dysregulation and increased C3-cleaving activity in vivo. fB-279G and fB-350N increase enormously the affinity between fB and C3b, which results in both enhanced enzyme formation and the generation of a AP C3 convertase which is very stable [16, 65] and resistant to accelerated decay by the complement regulators DAF and fH [16](Figure 4).

The location of the D279G, F286L and K350N mutations in the VWA domain of the Bb fragment, close to the MIDAS site at the VWA-C345C interface provides a simple explanation for the functional consequences of these gain-of-function mutations [16, 65]. In contrast, the K323E mutation is more difficult to explain. It is located in the VWA domain, on the opposite side to the α 1 helix and far away from the MIDAS site which mediates the initial recognition of C3b by fB, this explains why this mutation does not affect formation and spontaneous decay of the AP C3 convertase. A recent description of another aHUS associated fB mutation, also altering the 323K amino acid residue (K323Q) [66], further supports that this residue of fB defines a critical site for the accelerated decay of the AP convertase by DAF and fH (Figure 4). Point mutagenesis studies of fB confirm that this region of Bb, distal to the MIDAS site, influences binding of DAF lending support to the hypothesis that this region is critical for efficient accelerated decay [67].

From a pathogenic point of view it is also intriguing that these fB gain-of-function mutations, causing profound complement dysregulation in plasma, exclusively associate with aHUS and not with DDD or AMD. Perhaps this apparent aHUS specificity, and the incomplete penetrance of the disease that is also observed among fB mutation carriers, illustrate the need of a second hit to manifest the potential pathogenicity of the fB mutations. For example, increased complement activation caused by gain-of-function mutations in fB or by infection, may also require impaired surface protection to trigger aHUS. In this respect, it is interesting that all aHUS patients with fB mutations in our cohort were also carriers of a common polymorphism

in the *MCP* gene (the *MCPggaac* allele) associated with decreased expression and increased risk of developing aHUS [16, 36].

Two common polymorphisms in the N-terminal region of fB, L9H and R32Q, strongly associate with protection to AMD [25]. L9H is located in the signal peptide and was suggested to modulate secretion of fB. The R32Q variation reduces the haemolytic activity of fB by decreasing the interaction between the Ba fragment and C3b [68]. These data provide an explanation for the association; the observed protection in each case was ascribed to a reduced activity of the complement alternative pathway. In addition these analyses illustrate that the 8-aa long unstructured fragment at the amino terminus of the Ba fragment plays an important role in the initial interaction between fB and C3b. Similarly it has been reported that the C3 polymorphism, R102G, increases risk to AMD [32]. Our preliminary data suggest that this association is a consequence of increased AP activity of the C3-102G variant compared to the C3-102R (our unpublished results).

Mutations in C3 are associated with aHUS [15, 66, 69] and DDD [38]. Different experimental approaches have shown that these mutations alter the sensitivity of C3b to inactivation by fH and MCP, and change the resistance of the AP C3 convertase to accelerated decay by fH and DAF [15, 38, 65], our unpublished results]. Importantly, these C3 mutations seem to affect differentially the sensitivity to regulation by fH and the membrane-associated regulators DAF and MCP. Thus, whilst the C3 mutation associated with DDD affects only regulation by fH [38], the aHUS-associated C3 mutations affect primarily the cell surface regulation [38, 65], our unpublished results], further supporting the hypothesis that the site of complement dysregulation (surface vs fluid phase) is critical in the pathogenesis of aHUS and DDD. The differential effects of the C3 mutations on fH, DAF and MCP regulatory activities illustrate the complexity of the interactions between C3b and the complement regulators. We discuss below the data generated from the functional characterization of these disease-associated C3

mutations which also suggest that regulation by fH, DAF or MCP has distinct structural requirements in C3b.

Substrate recognition by the AP C3 convertase

Understanding how the AP C3 convertase recognizes its substrate has been a challenge for many years. Both novel structural data and disease associated C3 mutations are now providing important insights into the regions of C3 and C3b relevant to substrate recognition by the AP C3 convertase [38, 65, 70].

Dimeric complexes of the AP C3 convertase (C3bBb) were observed in the crystals stabilized with the staphylococcal inhibitor, SCIN, strongly suggesting that the interface between the two C3b monomers in the crystal represented the natural binding interface between C3 and C3b molecules during C3 substrate recognition by the AP C3 convertase [61]. This binding interface is a large surface that involves an area of C3 (or C3b) including the domains MG3, MG4-5 and MG6-8 (Figure 5). The contribution of these C3 regions to substrate recognition is further supported by the fact that the binding sites for the inhibitors compstatin [71], CRIg [47] and antibody S77 [72], all of which block substrate binding to the AP C3 convertase, map within this area.

Very recently, a remarkable C3 mutation associated with DDD has been shown to render C3 resistant to cleavage by the AP C3 convertase [38]. The mutation is a deletion of two amino acids (Asp923, Gly924; 923delDG) in the MG7 domain of C3 that does not alter the normal expression and overall structure of C3 (Figure 5A). A deletion of two amino acids, however, will displace amino acid residues on either side from their positions in the wild-type protein structure. N-terminal of the deletion is a surface-exposed loop (residues Val916 to Ser922) located within the postulated region of interaction between C3 and C3b molecules during C3 substrate recognition by the AP C3 convertase (Figure 5). Based on these structural data and the functional data generated from the 923delDG C3 mutant we suggest that this particular loop N-terminal of the 923delDG mutation is an important interaction site between C3 and

C3b. The mutation data indeed suggest that C3 and C3b contact at this region and that it is the presence of the SCIN inhibitor that separates them in the crystal structure (Figure 5C). This region in the surface of the MG7 domain may represent a novel and important therapeutic target for inhibition of C3 activation.

3. REGULATION OF THE AP C3-CONVERTASE

The regulators of the AP C3 convertase

Following cleavage of C3 to nascent C3b, nucleophilic attack on the internal thioester bond results in covalent binding of C3b to the activating surface, thus focusing further activation on the target cell (Figure 1). Complement is a double-edged sword because nascent C3b can bind not only to pathogens, but also to “innocent” adjacent host cells, initiating inappropriate foci of complement activation. To prevent damage to self and to avoid wasteful consumption of components, complement is under the control of multiple regulatory proteins which limit complement activation, either by inactivating C3b or by dissociating the AP C3/C5 convertases (Figure 1, Figure 6). Foreign surfaces, such as those of pathogens, are usually devoid of membrane regulatory proteins, which inactivate the AP C3 convertase. This lack of regulation thus leads to amplification of complement.

fH (155 kDa) is a single polypeptide composed of 20 SCRs arranged in a contiguous fashion. fH is the prototypic AP regulator, essential in order to maintain complement homeostasis and to restrict the action of complement to activating/pathogen surfaces. fH binds to C3b, competing with fB for binding to C3b in proconvertase formation and accelerating the decay of the AP C3/C5-convertase enzymes. It also acts as a cofactor for the factor I-mediated proteolytic inactivation of C3b. fH regulates complement both in the fluid phase and on cellular surfaces. However, while fH binds and inactivates C3b promptly in the fluid phase, the inactivation of surface-bound C3b by fH is dependent on the chemical composition of the surface to which C3b is bound. The fH molecule includes different interaction sites

for C3b and polyanions, which delineate distinct functional domains at the N and C-termini. The C3b binding site in SCR1-4 is the only site essential for the decay and factor I cofactor activities of fH. Similarly, the C3b/polyanion-binding site located within SCR19-20 is the most important site for preventing alternative pathway activation on host cells. In the presence of polyanions, such as sialic acids, glycosaminoglycans or sulphated polysaccharides (heparins), the affinity of fH for surface-bound C3b increases as a consequence of the simultaneous recognition of both polyanionic molecules and surface-bound C3b by the same fH molecule (Figure 6; Figure 7) [73].

On cell membranes the complement regulators MCP and DAF inhibit the C3/C5 convertase enzymes. DAF accelerates decay of the convertases, whereas MCP binds to C3b on the surface and acts as a cofactor for its irreversible proteolytic inactivation by the plasma protease factor I. Both DAF and MCP are composed of four SCR at the amino terminus followed by a short 'stalk' which is heavily glycosylated and either a glycosyl phosphatidyl inositol (GPI) anchor (DAF) or a transmembrane domain and cytoplasmic tail with signaling properties (MCP) (Figure 6) [74].

AP C3-convertase decay by fH and DAF

The crystal structure of the complex between C3b and a truncated form of fH has shown that the first four SCRs of fH, responsible for its decay accelerating and factor-I cofactor activities, bind C3b in an extended configuration [70]. This results in an elongated contact interface with multiple domains of C3b, which partially overlaps that involved in the interaction with fB in the initial steps of the AP convertase assembly. These structural data support previous mutagenesis experiments and functional analyses of disease-associated mutations which indicate that fH competes with fB in the formation of the C3bB pro-convertase and may interfere with the positioning of the Bb fragment, destabilizing the active AP C3 convertase (C3bBb) [70]. Accordingly, it has been suggested that binding of fH SCR1-2 to C3b is sufficient for fH to displace Bb and account for the decay accelerating activity of this regulator [70]. Although binding

of fH to Bb is postulated not necessary for the decay accelerating activity of fH, some degree of interaction between fH and Bb may help to explain why the aHUS-associated mutation altering the K323 residue in the VWA specifically alters the decay accelerating activity of fH and not the formation or the spontaneous decay of the AP C3 convertase [16].

Based on the conserved location of functional residues in the homologous SCR domains of fH and DAF, it has been suggested that similar displacement effects also play a role in the case of the decay accelerating activity of DAF [70]. However, it has been previously shown that whilst DAF-SCR2 interacts with Bb, DAF-SCR4 interacts with C3b in the C3bBb complex and alone can mediate accelerated decay [75]. Furthermore, mutagenesis data indicate that DAF and fH have non-identical structural requirements for their decay activity [67], supporting two different mechanisms for the dissociation of the AP convertase. This is also supported by the DDD-associated C3 923delDG mutation described below [38], where deletion of two amino acids in the MG7 domain of C3b alters decay by fH, but not by DAF.

Cofactor activity of fH and MCP for the fI-mediated inactivation of C3b

fH and MCP are necessary cofactors for factor I, enabling cleavage of C3b in the CUB domain, yielding the inactive iC3b species. The crystal structure of the C3b-fH SCR1-4 complex revealed that fH SCR2-3 bound to C3b adjacent to the CUB domain where the fI cleavage sites are located [70]. fH SCR1-3 exhibits several conserved and charged patches on its surface which may be involved in the binding of fI. These and other data suggest that fI binds the C3b:fH complex at the area formed by SCR1-3 of fH, and C345C and CUB of C3b. The current model states that besides providing a platform for fI binding, fH may have a second role in cofactor activity, maintaining the position of TED while the connecting CUB domain undergoes cleavage. This suggested function is supported by disease-related mutations in either fH or TED of C3b, which indicate an important interaction of complement regulators with TED [15,

65, 69]. As with fH and DAF, which have different structural requirements for decay accelerating activity, analysis of the DDD-associated 923delDG C3 mutant suggests that fH and MCP, despite their common evolutionary origins, also have different structural requirements for their cofactor activity in the fI-mediated inactivation of C3b [38]. MCP can act as a cofactor for fI inactivation of 923delDG in the fluid phase, but fH cannot.

Disease associated mutations and polymorphisms in complement regulatory proteins

Mis-sense mutations in the C-terminal region of fH are the most prevalent genetic alterations amongst aHUS patients. In contrast to fH mutations associated with DDD, fH mutations associated with aHUS rarely result in hypocomplementemia or decreased fH plasma levels. Most aHUS-associated fH mutant proteins express normally and have normal cofactor activity for the factor I-mediated proteolytic inactivation of C3b in plasma [19, 76]. As indicated, aHUS-associated fH mutations cluster in the C-terminus of the protein, a region that is critical for control of complement activation on cell surfaces by binding both polyanions and deposited C3b (Figure 7). aHUS carriers of carboxy-terminal fH mutations express fH which has normal regulatory activity in plasma, but a limited capacity to protect cells from complement lysis due to a lack of binding avidity [76-78]. These findings fit well with the identification of aHUS-associated loss-of-function mutations in MCP and fI; mutations in both these proteins also lead to decreased protection of host cells from complement lysis without significantly affecting complement homeostasis in plasma [79] (Figure 6). The combination of an active complement system in plasma and defective protection of cellular membranes illustrates that aHUS is a disease caused by uncontrolled activation of complement on cell surfaces and damage to self. The same consequence is brought about by mutations which decrease concentrations of fH or fI in plasma, or MCP density on cell surfaces. In these cases, deposition and amplification of C3b on

cells in the microvasculature cannot be controlled and tissue damage and destruction ensues. This is clearly distinct from insufficient control of complement in plasma, which leads to complete C3 consumption and severe hypocomplementemia characteristic of DDD patients carrying either fH deficiencies [37] or fH missense mutations which knock-out the fl-cofactor and decay accelerating activities (fH-del224K mutation) [11]. It is most revealing that this distinction between surface and fluid phase regulation, illustrated by mutations in fH which cause different kinds of pathology, is also a characteristic of mutations in C3. Functional characterization of the C3 923delDG mutation associated with DDD reveals that the convertase is resistant to the decay and cofactor activities of fH in the fluid phase, but can be regulated by the membrane associated regulators DAF and MCP [38]. In this case, insufficient control in plasma, in the face of adequate control on cell surfaces, leads to DDD. In contrast, C3 mutant proteins associated with aHUS were resistant to inactivation by both fH and MCP [15], our unpublished results].

Association of mutations in complement proteins with pathology is understandable. It is more surprising that common polymorphisms in complement proteins are also linked to disease. The most studied polymorphism at the *CFH* locus is rs1061170 which causes a Tyr402His amino acid substitution in fH. Several independent studies have shown that the allele 402His confers a significantly increased risk to AMD in many different populations, with an odds ratio (OR) between 2.1 and 7.4 [23, 26, 27, 29]. Interestingly, the frequency of the 402His allele varies greatly between populations, which may contribute to the observed differences in the incidence of AMD among different ethnic groups [80].

The Tyr402His polymorphism lies in SCR7 of fH, within the cluster of positively charged amino acids implicated in the binding of heparin, and C3b (Figure 7). The substitution occurs towards the centre of this module and structural analyses of short, recombinant constructs containing this region indicate that whilst the presence of either 402His or 402Tyr has little effect on overall conformation, the substitution may affect

binding to polyanions [81]. *In vitro* functional studies with fH recombinant fragments confirm that the substitution of Tyr for His at position 402 alters the binding specificity of SCR7 for different glycosaminoglycans [82, 83] and decreases its binding to retinal pigment epithelial cells [84], although the physiological relevance of these observations is still unclear. It has also been reported that the Tyr402His polymorphism influences the binding of fH to C-reactive protein [84-86], but the difficulties in replicating these data cast doubt on their significance [87].

The common fH polymorphism Val62Ile has been associated with lower risk for aHUS, AMD and DDD [26, 88]. Val62Ile lies within the N-terminal region that is essential for fH regulatory activities. Consistent with the role of complement dysregulation in these pathologies, it has been shown that the substitution of Val for Ile at position 62 increases the complement regulatory function of fH, thereby reducing AP activation [89]. This is the result of an increase in the affinity for fH Ile62 for C3b, leading to more effective competition with fB for C3b binding in proconvertase formation and enhanced cofactor activity for the factor-I mediated inactivation of C3b. Importantly, the Val62Ile substitution affects binding to C3b but not decay accelerating activity suggesting that different regions in fH are involved in binding C3b/cofactor activity and in decay accelerating activity [89].

fH Ile62 most likely protects from disease caused by complement dysregulation because it performs better as a complement regulator than fH Val62. One important conclusion from the functional characterization of the fH Val62Ile polymorphism, and also from the fB Arg32Gln polymorphism described earlier, is that whilst the functional alterations caused by these amino acid substitutions are subtle, the very nature of the complement system will amplify these small effects. Most importantly, these subtle effects are additive. Indeed, it has been shown that particular combinations of these fH and fB polymorphisms result in very different AP characteristics, markedly affecting formation and regulation of the AP C3 convertase in plasma and on cell surfaces [89]. We anticipate that identification of individuals carrying combinations of polymorphisms

(‘complotypes’) in complement components and regulators, which result in high or low AP activity, will be of great importance for prediction of disease risk and may also help in diagnosis and choice of treatment for diseases involving complement dysregulation.

4. CONCLUDING REMARKS

We have summarized our current understanding of the formation, activation and regulation of the AP C3-convertase and have reviewed how the analysis of genetic variants associated with different diseases has helped to determine the molecular events which are critical in the pathogenesis of various disorders. A clear conclusion from these analyses is that, despite a common link involving complement dysregulation, there are distinct functional alterations caused by these small changes in complement proteins which are essential in disease pathogenesis. It is now well established that mutations or polymorphisms altering the C3b/polyanion-binding site located at the C-terminal region of factor H, or that decrease the levels of fI or MCP, are strongly associated with aHUS because they specifically impair the capacity to protect host cell membranes from complement activation. Consistent with this idea that aHUS results from insufficient protection of the host cellular surfaces, aHUS-associated C3 mutants generate activated C3b molecules and AP C3 convertase enzymes which are more resistant to inactivation by surface-associated regulators. Gain-of-function fB mutations collaborate with additional risk factors in MCP, focusing damage by enhanced complement activity on to cell surfaces. On the other hand, mutations which disrupt regulatory activities in plasma cause failure of fluid phase complement control, resulting in massive activation of C3 which causes DDD.

AMD strongly and specifically associates with a common extended *CFH* haplotype carrying the 402His polymorphism, but the molecular bases of this association are controversial and still unclear. However, functional characterization of other common polymorphisms in components and regulators of the AP pathway has been more rewarding, demonstrating important differences in complement activities for

some combinations, which may have a significant role in determining individual predisposition to a number of common disorders. In particular, understanding how subtle alterations, which specifically associate with lower risk to disease, such as those in fB and fH, may guide future developments in complement therapeutics.

Recent advances in structure-function analyses of complement proteins by crystallography, electron microscopy and surface plasmon resonance have permitted a deeper understanding of the mechanistic basis of disease-associated genetic variations. In turn, the functional analysis of these genetic variations has aided our understanding of the significance of the structures and interactions solved. We anticipate that a multidisciplinary approach involving genetics, functional analyses and structural determination will be instrumental in achieving a complete understanding of the assembly, activation and regulation of the AP C3 convertase. This knowledge will have important implications in therapeutic developments.

ACCEPTED

ACKNOWLEDGEMENTS

This work was funded by the Spanish Ministerio de Educación y Cultura (grants: SAF2008-00226, SAF2008-00451 and SAF2006-02948), the Ciber de Enfermedades Raras (INTRA/08/738.2), the Red Temática de Investigación Cooperativa en Cáncer (RD06/0020/1001), the Fundación Renal Iñigo Alvarez de Toledo; the Fundación Ramón Areces, the Human Frontiers Science Program (RGP39/2008), and the MRC UK Project Grant Ref 84908.

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FIGURE LEGENDS**Figure 1.****Complement activation and regulation.****Figure 2.****Three-dimensional structure and domain organization of fB, C3 and C3b.**

Left panels outline the domain organization of fB and C3 in their primary structure whereas right panels show different views of the three-dimensional structures of fB, C3 and C3b. In all panels each domain has been colored differently. The structure of fB was obtained from Milder et al. [59] (PDB ID 2OK5), C3 was obtained from Janssen et al. [44] (PDB ID 2A73) and C3b was obtained from Janssen et al. [45] (PDB ID 2I07). The difference in domain composition between C3 and C3b is the ANA domain that is cleaved when C3 is activated to C3b. For representation, atomic structures of fB, C3 and C3b were low-pass filtered to a resolution of 2.5 nm to highlight the overall “low-resolution” structural organization of the molecules and the large-scale conformational changes taken place during C3 activation. fB is composed of the Bb fragment comprising the serine-protease (SP) and von Willebrand factor type A domain (VWA), packed against the Ba segment containing SCR1, SCR2 and SCR3. C3 is composed of a ring comprising 8 macroglobulin domains (MG-ring), crowned by the C345C domain, as well as CUB and TED domains that undergo a large displacement after the ANA domain is released during C3 activation.

Figure 3.**Assembly and activation of the AP C3 convertase.**

The figure describes the sequence of events that take place during the assembly and activation of the AP C3 convertase as described in the text. C3 is an

inactive protein not capable of interacting with fB. Activation of C3 to C3b induces a large conformational change including a large displacement of the TED domain and the exposure of new surfaces that are now competent for fB recognition. fB initially binds to C3b in a closed conformation. Subsequently, fB undergoes a large conformational change that leads to an open conformation of fB likely exposing the factor D (fD) cleavage site between the SCR1-3 (Ba propeptide segment) and VWA domains. Cleavage by fD removes the Ba fragment to generate an active AP C3 convertase with the serine-protease (SP) domain projecting outwards.

Models for C3, C3b, the closed conformation of the AP C3 proconvertase and the AP C3 convertase have been obtained after low-pass filtering of the atomic coordinates of C3 (obtained from Janssen et al. [44]; PDB ID 2A73), C3b (Janssen et al.[45]; PDB ID 2I07), the C3 convertase (Rooijackers et al. [61]; PDB ID 2WIN), and the conformation of the C3 proconvertase described by Janssen et al. [64]; PDB ID 3HRZ, Torreira et al., [62] and Torreira et al., [63]. The model for the open conformation of the AP C3 proconvertase shows the experimental 3D reconstruction at medium resolution obtained using electron microscopy (EMD ID EMD-1583, Torreira et al., [62]). The density corresponding to C3b is coloured in orange and that of fB in gray. A hypothetical model for the open conformation of fB bound to C3b has been proposed based on the fitting of atomic structures into the medium-resolution EM density [62], However, the precise location of the fB domains in the open conformation of the AP C3 proconvertase waits for an experimental structure at high-resolution.

Figure 4.

Disease-associated mutations in fB.

A) For simplicity only two representative aHUS-associated fB mutations, D279G and K323E are identified within the three-dimensional structure of the C3 convertase (Rooijackers et al. [61]; PDB ID 2WIN). The magnesium ion in the MIDAS site of the VWA domain has been represented as a yellow dot. The D279G mutation (red) is at

the interface between the VWA and the C345C domains, whereas the K323E fB mutation (orange) is located on top of the VWA domain of Bb, away from the MIDAS site and the site of interaction between the Bb and C3b.

B) Surface plasmon resonance (Biacore) analysis of the D279G and K323E fB mutations. The functional effects of the D279G and K323E mutations, located on opposing sides of the MIDAS site, were investigated by flowing recombinant (r) fB and native fD over a C3b-coated chip to form convertase. Formation of C3bBb-323E convertase (orange line) was normal and the enzyme decayed with a half-life of ~319 seconds, comparable to that formed by wild type r fB (black line). However, whereas the wild type convertase was rapidly and completely dissociated by soluble DAF, the fb-323E mutant convertase demonstrated resistance to accelerated decay. The C3bBb-279G convertase (red line) was formed at abnormally high levels, likely due to high affinity interaction, and was very stable. Similarly to the fb-323E mutant convertase, the enzyme was highly resistant to accelerated decay.

Figure 5.

Model for the substrate C3 recognition by the AP C3 convertase.

A) The medium resolution three-dimensional structure of the purified C3-923delDG mutant determined by electron microscopy (EM) illustrates that this C3 mutant, despite being resistant to cleavage by the AP C3 convertase, shows no major structural changes compared to normal C3 [38]. The structure is represented as a grey transparent density with the atomic structure of wild type C3 (Janssen et al. [44]; PDB ID 2A73) integrated within it. The position of the 923delDG C3 mutation is indicated with a circle. Note that upstream of the mutation there is a peptide loop exposed on the adjacent surface in the wild type MG7 domain of C3 (right panel) which will likely be modified by the 923delDG mutation.

B) The atomic structure of the C3 convertase stabilized with the bacterial inhibitor SCIN, revealed a dimeric interface between two C3b molecules contacting each other through the MG rings. This prompted Gros and collaborators to suggest that a similar interaction could take place during the recognition of the C3 substrate by the AP C3 convertase. The figure shows a model of such a putative complex based on their atomic structures (Rooijackers et al [61]; PDB ID 2WIN). In this model, the contact between the two MG rings is sustained by interactions at the bottom of the MG ring, locating the SP domain close to its target sequence in C3. The figure illustrates that the 923delDG mutation is located at the top of the MG ring, which suggests that this region is also relevant for the formation of the complex between C3 and the AP C3 convertase. Since the 923delDG mutation completely blocks cleavage by the convertase, we speculate that the presence of the SCIN inhibitor in the crystal structure of the C3 convertase (Rooijackers et al. [61]; PDB ID 2WIN) may prevent contact between the MG rings at the site of the 923delDG mutation which would otherwise be present in the active complex. The model also reveals that there is a substantial distance between the SP domain of the AP convertase and the C3 substrate. However, the structure of the AP C3 convertase in solution obtained by electron microscopy [62], revealed that the Bb fragment, which project outwards, displays considerable conformational flexibility, which is probably important for the SP domain to reach its target sequence.

Figure 6

Activation and regulation of the complement AP in plasma and on cellular surfaces.

Cartoon illustrating the role of fH, fI, MCP and DAF in the regulation of the AP of complement in plasma and on the surface of endothelial cells or pathogens. This illustrates the crucial role of the interaction between the C-terminal region of fH and negatively-charged heparins or glycosaminoglycans (GAGs) on endothelial cells which

discriminates between self cells and pathogens, devoid of these negatively-charged components. When heparins and GAGs are present, the avidity of fH for C3b deposited on cell surfaces increases and fH efficiently controls amplification of the AP and damage to self-tissues, both by dissociating the AP C3 convertase and inactivating C3b. On surfaces lacking negatively charged molecules, like pathogens, there is no interaction through the C-terminal region of fH, which decreases binding avidity and allows amplification of C3b deposition, triggering opsonophagocytosis and cell lysis. Mutations that disrupt the function of the C-terminus of fH, like those associated with aHUS, interfere the capacity of fH to protect self components from complement-mediated damage. Note the similarities in the functional activities between fH and the membrane associated regulators, MCP and DAF. These proteins work together with fl to prevent damage to self and to avoid wasteful consumption of complement components.

Figure 7

Disease-associated mutations in fH.

Figure depicts the structure of fH indicating the distinct functional domains and sites of interaction with other molecules (Top). The bottom diagram illustrates the differential distribution of aHUS- and DDD-associated mutations found in factor H.

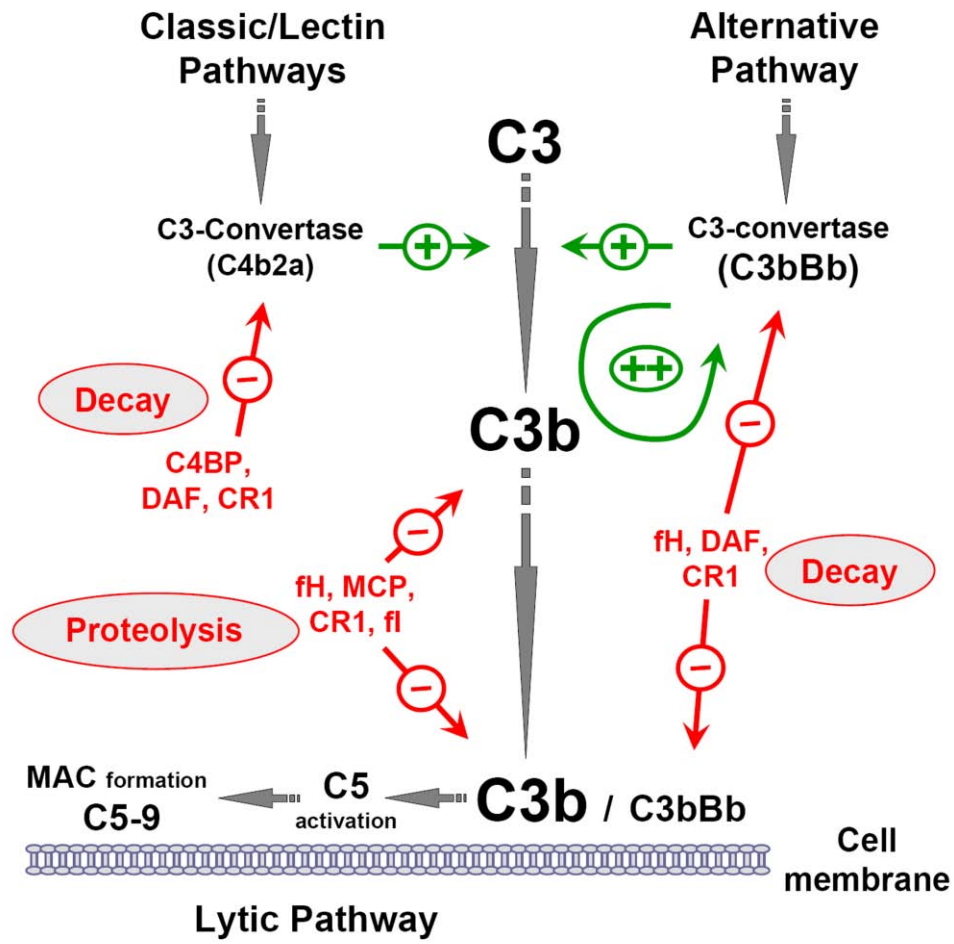


Figure 1

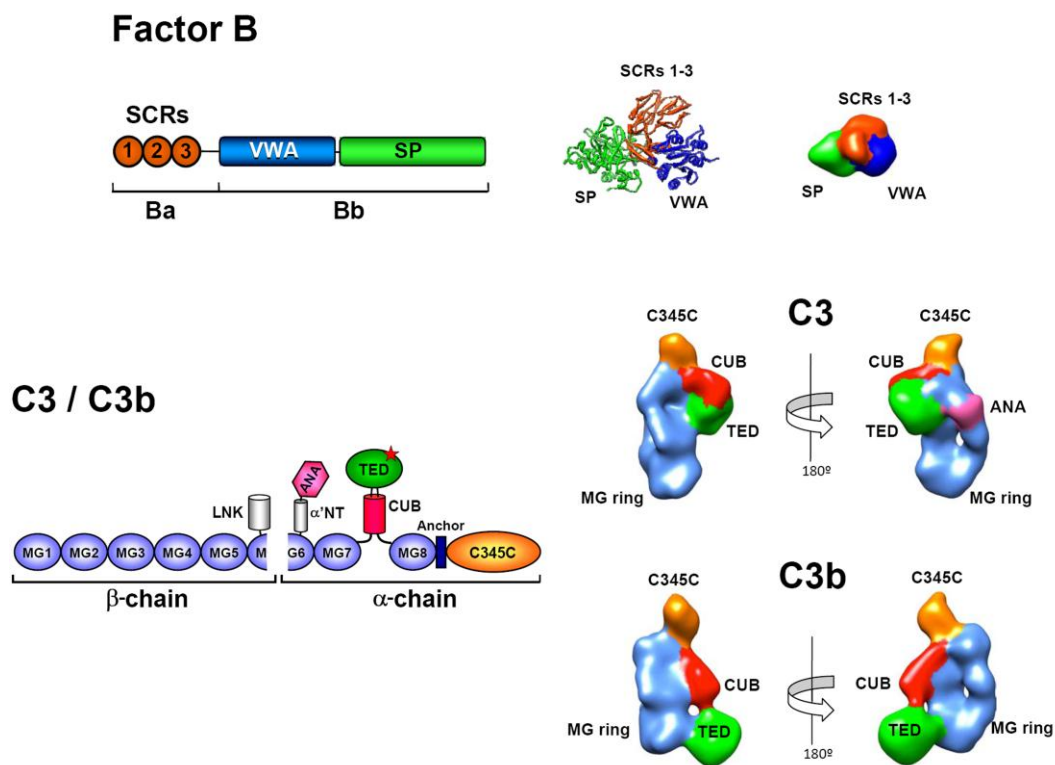


Figure 2

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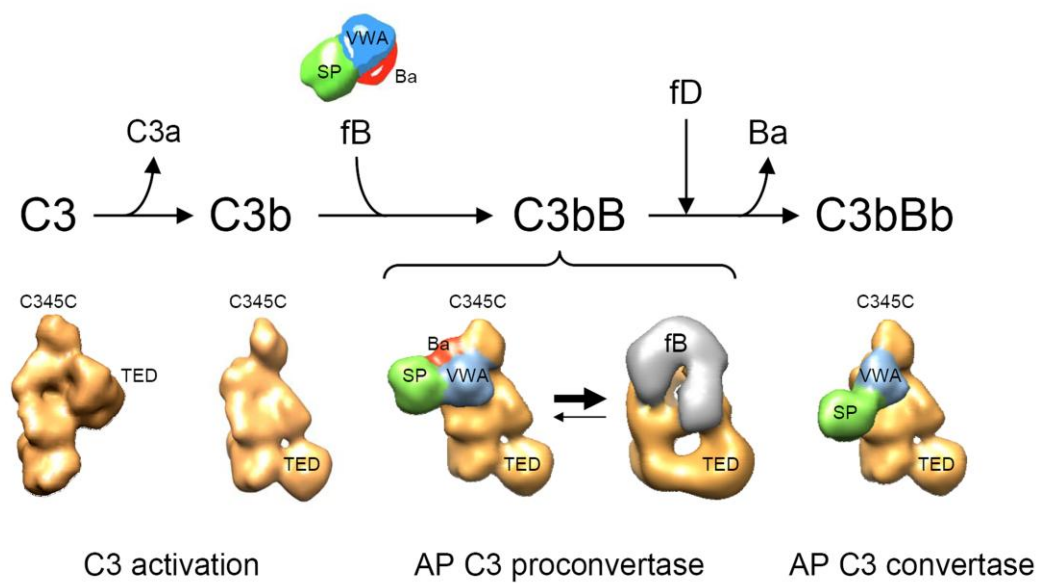


Figure 3

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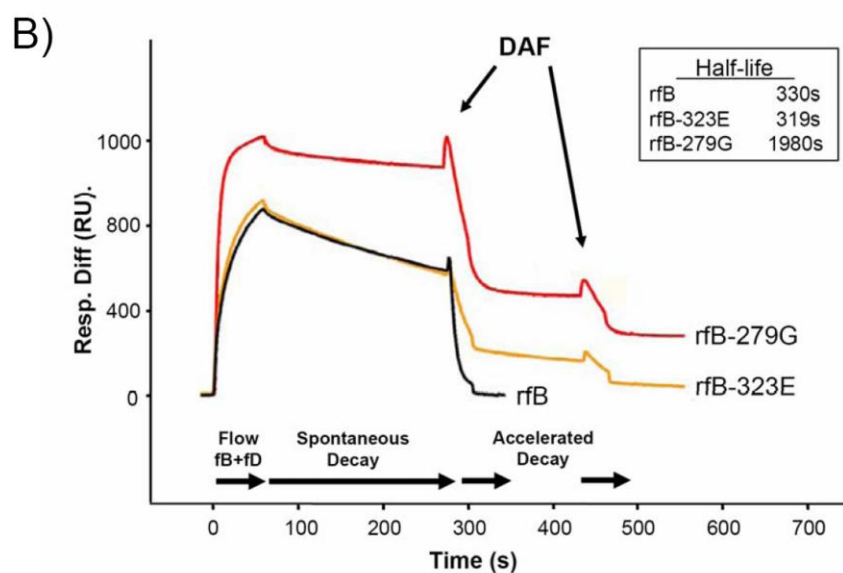
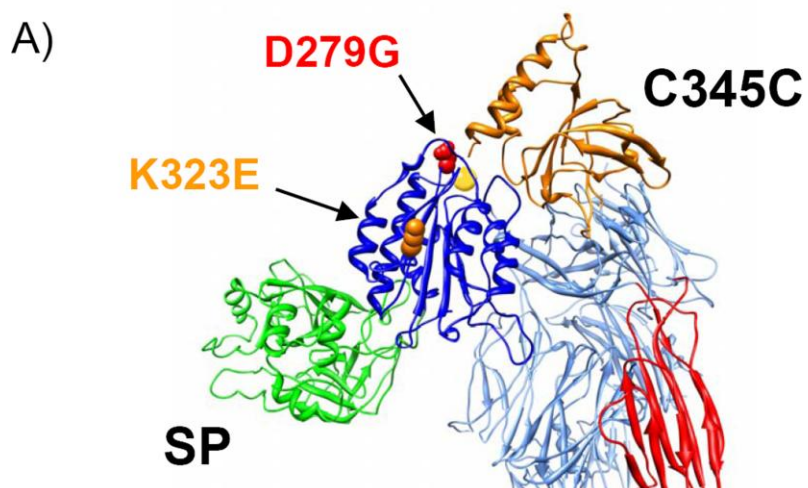


Figure 4

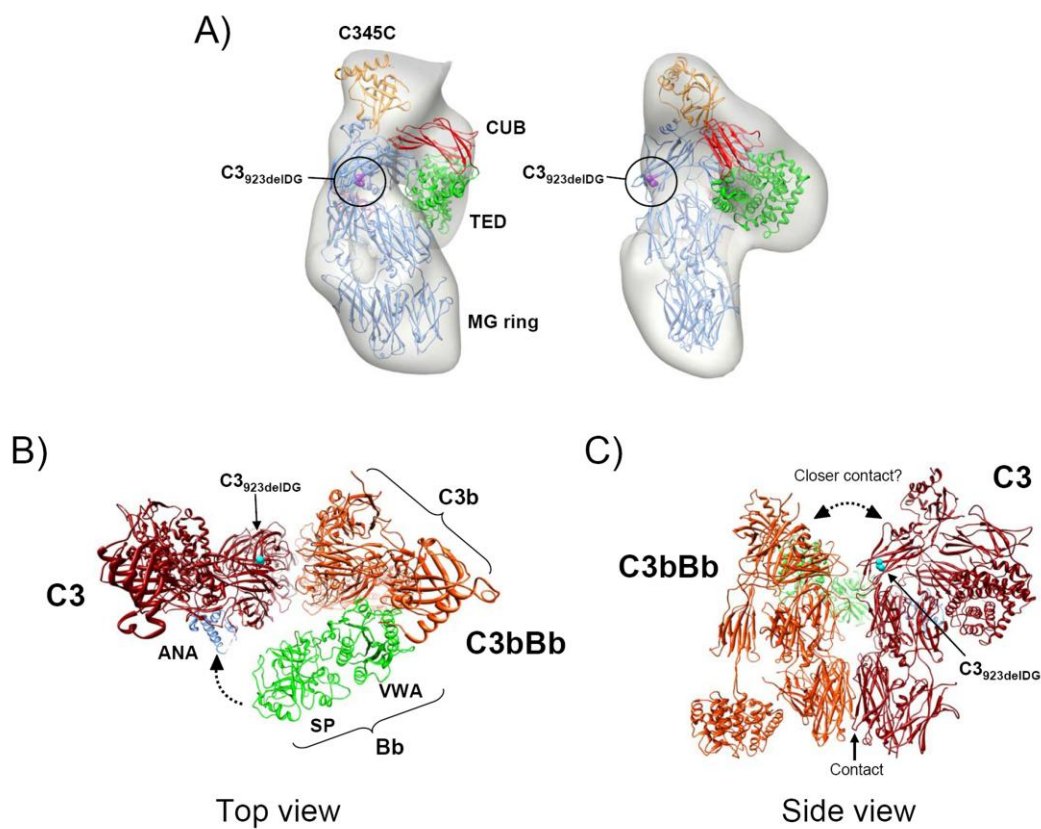


Figure 5

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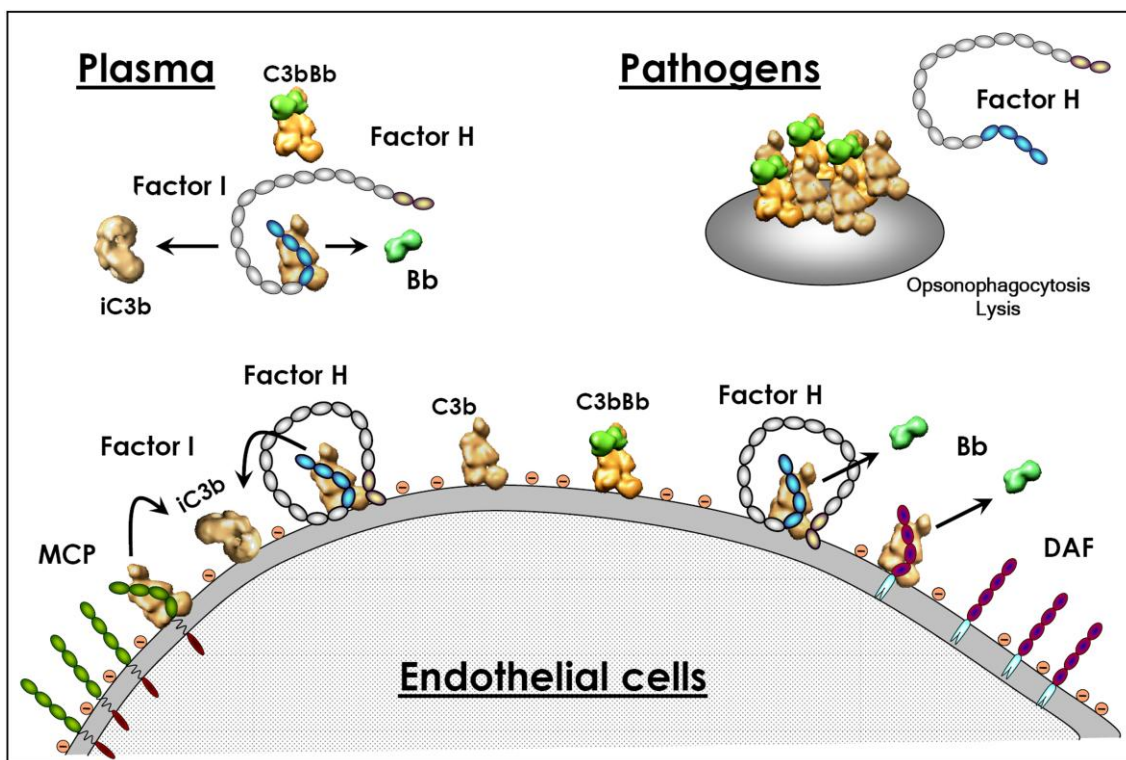


Figure 6

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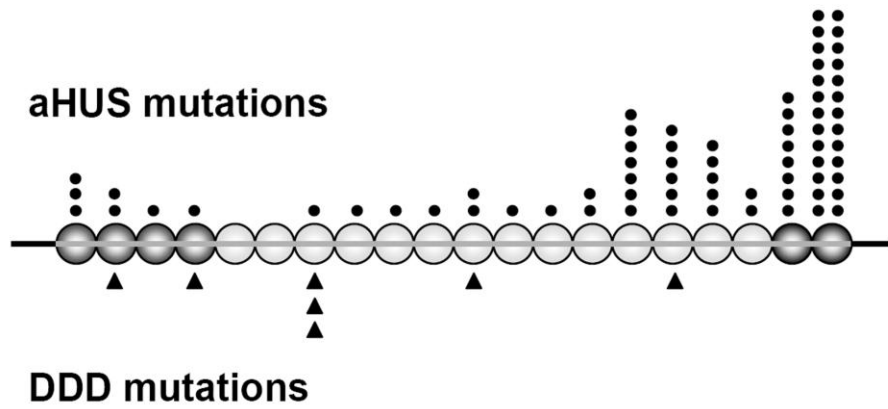
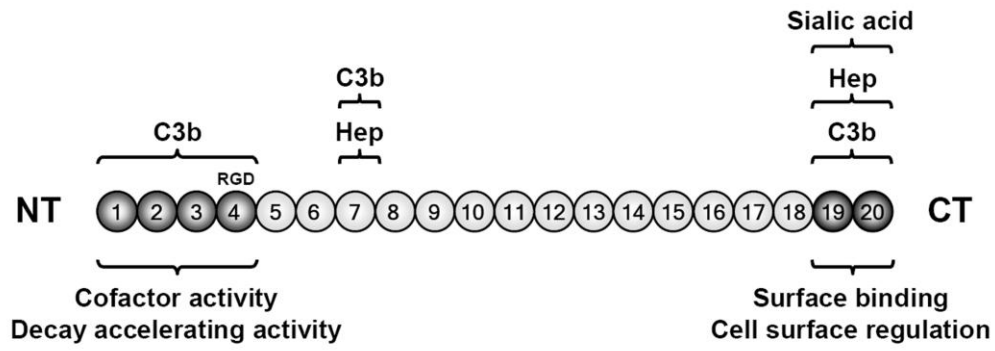


Figure 7

ACCEPTED

TABLE 1. Risk factors associated with aHUS, DDD and AMD

	aHUS	DDD	AMD
Genetic factors			
- Rare mutations	<i>CFH</i> <i>MCP</i> <i>CFI</i> <i>CFB</i> <i>C3</i> <i>THBD</i>	<i>CFH</i> <i>C3</i>	<i>CFH</i>
- Common polymorphisms	<i>CFH</i> <i>MCP</i> <i>CFHR1/CFHR3</i>	<i>CFH</i> <i>CFHR1/CFHR3</i> <i>CFHR5</i>	<i>CFH</i> <i>AMRS2</i> <i>(LOC387715)</i> <i>CFB</i> <i>C2</i> <i>C3</i> <i>CFHR1/CFHR3</i> <i>C1INH</i> <i>(SERPING1)</i> <i>TLR3</i>
Autoantibodies	Factor H	C3Nef, Factor H	
Environmental factors	Infection Immunosuppres. drugs Cancer therapies Oral contraceptives Pregnancy Childbirth, etc.	Infection (?)	Smoking Diet Exercise, etc.

- Complement dysregulation is a common link to several chronic and infection diseases.
- Both gain-of-function and loss-of-function mutations associate with pathology.
- Functional analyses of mutations revealed distinct pathogenic mechanisms.
- Mutations aided understanding activation and regulation of complement.

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