



Complement component C3: A structural perspective and potential therapeutic implications

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ABSTRACT

As the most abundant component of the complement system, C3 and its proteolytic derivatives serve essential roles in the function of all three complement pathways. Central to this is a network of protein-protein interactions made possible by the sequential proteolysis and far-reaching structural changes that accompany C3 activation. Beginning with the crystal structures of C3, C3b, and C3c nearly twenty years ago, the physical transformations underlying C3 function that had long been suspected were finally revealed. In the years that followed, a compendium of crystallographic information on C3 derivatives bound to various enzymes, regulators, receptors, and inhibitors generated new levels of insight into the structure and function of the C3 molecule. This Review provides a concise classification, summary, and interpretation of the more than 50 unique crystal structure determinations for human C3. It also highlights other salient features of C3 structure that were made possible through solution-based methods, including Hydrogen/Deuterium Exchange and Small Angle X-ray Scattering. At this pivotal time when the first C3-targeted therapeutics begin to see use in the clinic, some perspectives are also offered on how this continually growing body of structural information might be leveraged for future development of next-generation C3 inhibitors.

1. Introduction

The complement system serves essential roles in the recognition and elimination of pathogens, clearance of physiological debris, coordination of immune responses, and numerous other aspects of homeostasis [1,2]. However, as an early acting sentinel that can rapidly initiate the inflammatory response, inappropriate or unregulated complement activity can also be major driving force behind many human diseases that differ in severity (i.e. ‘quality of life’ illness versus life-threatening), location (i.e. localized versus systemic), and duration (i.e. acute versus chronic) [3–5]. Throughout the last three decades, a broad and increasingly nuanced understanding of the pathophysiological contributions of complement to various inflammatory, autoimmune, hematologic, and nephrologic conditions has developed alongside more recent links to etiologically-diverse diseases, including cancer and neurodegenerative disorders [3–5]. Together, this has stimulated a renewed interest the complement system among basic scientists and clinicians alike.

As the central component of the complement system, the molecule

known as C3 has received considerable scientific attention since its initial isolation and characterization some 60 years ago by Müller-Eberhard and coworkers [6,7]. Throughout this time, understanding of C3 has grown to encompass most relevant aspects of basic molecular biology including identification of its naturally occurring genetic variants, appreciation of its relationship to other complement components, and description of the conformational changes that accompany its activation to C3b, to name but a few. Yet as causative links between the complement alternative pathway and human disease have become established with greater rigor [3–5], increasing attention has been given toward harnessing this wealth of basic information for development of therapeutic C3 inhibitors [8,9]. With recent approvals of the C3 inhibitor pegcetacoplan (Empaveli® and Aspaveli®) by the U.S. Food and Drug Administration and European Medical Agency for treatment of Paroxysmal Nocturnal Hemoglobinuria (PNH), what once seemed a far-off goal has now become reality.

The purpose of this review is to stimulate thought and discussion among those interested in developing the next generation of therapeutic C3 inhibitors. Needless to say, it would be a formidable task to compile

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all the relevant literature on C3 biochemistry, its function, and its role in human disease in one text. Fortunately, there are many authoritative and still current reviews on the complement system [1,2] and the function of C3 specifically [10], so these issues need not be covered here. By contrast, the literature lacks a unifying review that collects and contextualizes the breadth of structural information on C3 and its fragments, and their corresponding complexes with key enzymes, regulators, effectors, and inhibitors (Table 1). Since structural studies have had a major influence on understanding the molecular details underlying C3 function, it seems both appropriate and timely to revisit this topic with an eye toward inhibitor design, and to offer some perspectives on the challenges and opportunities and that lay ahead.

2. Structural features of complement component C3 and its major fragments

Whereas human C3 is synthesized as a 1663 amino acid pre-pro protein (UniProt Accession Number P01024), removal of its 22-residue signal peptide upon entry into the secretory pathway yields a 1641

residue pro-protein (Fig. 1A). Pro-C3 is oxidized during folding resulting in the formation of 13 pairs of disulfide bonds and is post-translationally modified with complex N-linked glycans at three distinct sites (Asn-85, Asn-939, and Asn-1617). Prior to export from the cell, cleavage of pro-C3 by furin proteases yields two distinct polypeptides conventionally known as the β -chain (residues 23–667; ~75 kDa) and α -chain (residues 672–1663; ~110 kDa) that remain covalently bound to one another through an intermolecular disulfide bond in native C3 (~185 kDa). Although the mature, native C3 protein consists of 13 distinct structural domains (Fig. 1A), a series of proteolytic reactions leads to removal of either small peptide segments (e.g. C3f) or entire domains (e.g. C3a). This gives rise to various fragments of C3 that differ not only in their compositions and three-dimensional structures, but in their functional properties as well [10]. In this manner, the relatively abundant, but otherwise inert C3 molecule can be cleaved sequentially into (i) both a small chemotactic fragment (i.e. C3a; ~9 kDa) and a surface-bound opsonin that serves as a platform for assembly of C3 convertases (i.e. C3b; ~176 kDa), (ii) an opsonin that no longer supports C3 convertase assembly (i.e. iC3b; ~174 kDa), and (iii) two terminal fragments (i.e.

Table 1

Compilation of X-ray Crystal Structures of Human C3 or Its Fragments, Either Free or Bound to Various Ligands Involved in Activation, Regulation, or Inhibition.

Class	C3 Protein	Ligand (s)	PDB ID ^{a,b}	Resolution (Å)	Authors	Citation
C3 Proper	C3	–	2A73	3.3	Janssen et al.	[12]
	C3a	–	4HW5	2.3	Bajic et al.	[92]
	C3b	–	2I07	4.0	Janssen et al.	[18]
	C3b	–	5F07	2.8	Forneris et al.	[46]
	iC3b1	hC3Nb1	6Y06	6.0	Jensen et al.	[93]
	C3c	–	2A74	2.4	Janssen et al.	[12]
	C3d	–	1C3D	1.8	Nagar et al.	[94]
	C3d dimer	–	6RMT	2.0	Wahid et al.	[95]
	C3 convertase	C3b	Factor B	2XWJ	4.0	Forneris et al.
C3b		Factor B	2XWB	3.5	Forneris et al.	[28]
		Factor D				
C3b		Factor Bb	2WIN	3.9	Rooijackers et al.	[37]
		<i>S. aureus</i> SCIN				
C3b		Factor Bb	6RUR	6.0	Pedersen et al.	[39]
Regulators	C345c	Properdin, monomeric form ^c				
		<i>S. aureus</i> SCIN				
	C345c	Properdin, monomeric form ^d	6S0B	2.3	van den Bos et al.	[40]
	C3b	MCP, CCP(1–4)	5F08	2.4	Forneris et al.	[46]
	C3b	CR1, CCP(15–17)	5F09	3.3	Forneris et al.	[46]
	C3b	DAF, CCP(2–4)	5FOA	4.2	Forneris et al.	[46]
	C3b	Factor H, CCP(1–4)	2WII	2.7	Wu et al.	[47]
	C3d	Factor H, CCP(19–20)	2XQW	2.3	Kajander et al.	[96]
			3OXU	2.1	Morgan et al.	[97]
			5O32	4.2	Xue et al.	[51]
Receptors	C3b	mini-FH ^e , CCP(1–4)+ (19–20)	5O35	4.2	Xue et al.	[51]
		Factor I				
	C3c	CR1g, Ig(1)	2ICE	3.1	Wiesmann et al.	[26]
	C3b	CR1g, Ig(1)	2ICF	4.1	Wiesmann et al.	[26]
	iC3b	CR3, I-domain	7AKK	3.4	Fernandez et al.	[64]
	C3d	CR2, CCP(1–2)	3OED	3.2	van den Elsen et al.	[61]
	C3d	CR3, I-domain	4M76	2.8	Bajic et al.	[63]
	C3b	<i>Variola</i> virus SPICE, CCP(1–4)	5FOB	2.6	Forneris et al.	[46]
	C3c	<i>S. aureus</i> SCIN	3OHX	3.5	Garcia et al.	[25]
	C3c	<i>S. aureus</i> SCIN-B	3T4A	3.4	Garcia et al.	[72]
Inhibitors	C3b	S77, Fab fragment	3G6J	3.1	Katschke et al.	[74]
	C3b	hC3Nb1	6EHG	2.7	Jensen et al.	[73]
	C3c	Compstatin, 4W9A	2QKI	2.4	Janssen et al.	[81]
	C3b	Compstatin, Cp40	7BAG	2.0	Lamers et al.	[80]
	C3d	<i>S. aureus</i> Efb-C	2GOX	2.2	Hammel et al.	[21]
	C3d	<i>S. aureus</i> Ehp	2NOJ	2.7	Hammel et al.	[22]
	C3d	<i>S. aureus</i> Sbi, Domain IV	2WY7	1.7	Clark et al.	[24]
			2WY8			
	C3d	Factor H, CCP(19–20)	5NBQ	3.2	Kolodziejczyk et al.	[98]
		<i>B. burgdorferi</i> OspE				

^aAs of this writing, there are 54 unique PDB depositions of crystal structures containing various forms of human C3.

^bIn general, only the first report of a structure determination is highlighted in this Table. However, certain structures representing particularly important features or significant improvements in either resolution or model quality are also included. Structures representing alternative crystal forms, site-directed mutants, or various ligands when unbound to C3 or its fragments are omitted in the interest of space.

^{c,d}Further information on the nature and characterization of the monomeric form of properdin may be found in the publication of Pedersen et al. [38].

^dFurther information on the nature and characterization of the mini-FH protein may be found in the original manuscript by Schmidt et al. [52].

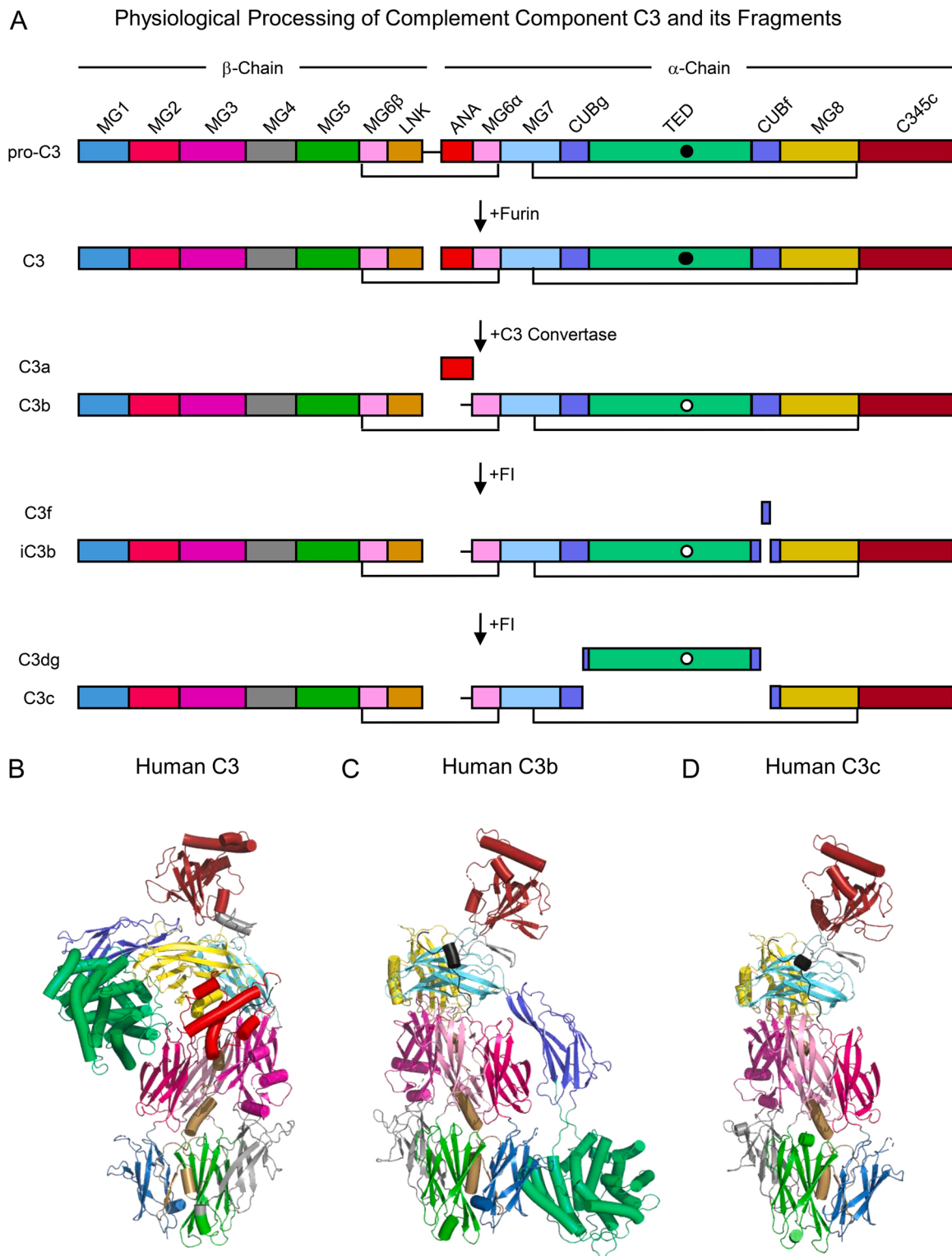


Fig. 1. Domain Organization and Three-Dimensional Structures of Complement Component C3 and its Fragments. (A) Domain structure of human C3 and its fragments. The names of each fragment are shown at the left-hand side of the panel, while the names of each individual domain are presented atop the schematic that represents pro-C3. Domain name abbreviations are as follows: MG, macroglobulin-like domain; LNK, linker domain; ANA, anaphylatoxin domain; CUB, complement C1r/C1s, Uegf, Bmp1 domain; TED, thioester-containing domain; C345c, C-terminal domain, alternatively referred to in the literature as the CTC domain. The identities of various proteases that catalyze processing of C3 are shown by the arrows that signify each transformation. Black lines represent key disulfide bonds that covalently link the C3 polypeptide chains together throughout proteolytic processing. The intramolecular thioester found in the TED domain is represented by a filled circle in its native state and by a clear circle in its hydrolyzed state. (B) Structure of native human C3 as drawn from the PDB entry 2A73 [12]. Individual domains are colored identically to panel A, with α -helices represented as cylinders and β -strands as arrows. (C) Structure of human C3b as drawn from the PDB entry 2I07 [18]. (D) Structure of human C3c as drawn from the PDB entry 2A74 [12]. Note that the orientation of the structures shown in panels C and D are fixed about the so-called keyring core of the molecule comprised of domains MG1-MG6.

C3c; ~139 kDa and C3d; ~34 kDa), only the latter of which retains biological function as a opsonin [10]. All of these proteolytic reactions result in changes to the α -chain while the β -chain remains intact throughout the complex lifecycle of the C3 molecule (Fig. 1A).

Throughout the last two decades, the structures of essentially all major fragments of C3 have been solved by X-ray crystallography. Collectively, this work provides important insight into the physical changes that underlie the distinct functions associated with C3 and its fragments. Some of the most fundamental features of C3 biochemistry can be understood by examining the structures of C3, C3b, and C3c, including (i) how the α and β chains relate to one another in the fully-matured molecule, (ii) how the intramolecular thioester required for opsonization remains protected in native C3, yet becomes accessible upon activation to C3b, and finally (iii) the nature of the conformational changes that occur upon activation, thereby allowing C3b to act as a hub for protein-protein interactions within the complement system. The structure of C3 reveals that its 13 individual domains fold into a highly asymmetrical shape, wherein 6 domains are derived from the α and β chain, respectively (Fig. 1 A, B). Although there are many aspects of C3 structure deserving of commentary but must be omitted in the interest of brevity, two features stand out insofar as protein architecture is concerned. The first of these is the MG6 domain, which is comprised of residues from both the α and β chain and which includes the lone interchain disulfide bond in native C3. The second of these is the CUB domain of the α -chain, which contains a large insertion that separates the CUBg and CUBf segments in the polypeptide sequence and which encodes the thioester-containing TED domain. Indeed, this entire CUB-TED region plays a crucial role in both preserving then exposing the functionality of the intramolecular thioester during activation of C3.

The intramolecular thioester is found within the eponymous TED domain and conveys the unusual ability of the protein to covalently opsonize surfaces and other biomaterial upon activation to C3b [10]. A necessarily labile moiety, the thioester arises from an isoglutamyl-cysteine bond between the sidechains of Cys-1010 and Gln-1013 [11]. The thioester is susceptible to reaction with small nucleophiles (e.g. methylamine) and is therefore prone to hydrolysis, particularly during purification and storage of C3 in laboratory settings. However, its access to bulk solvent is masked in the structure of native C3 wherein the thioester lies at a hydrophobic interface of the TED domain and MG8 (Fig. 1B) [12]. Upon activation through spontaneous tick-over to C3(H₂O) or following proteolytic cleavage to C3b, the exposed thioester is converted to a free thiolate anion on Cys-1010 and an acyl-imidazole intermediate between the sidechains of Gln-1013 and His-1126 [13,14]. This secondary step allows for preferential reaction of C3b with hydroxyl groups of nearby proteins but especially with carbohydrates found on biological surfaces (e.g. bacterial and fungal cells), thus facilitating their opsonization with C3b. In addition, C3b covalently linked to these molecules and surfaces sets the stage for amplification of complement activity by serving as a platform molecule for assembly of the C3 and C5 convertases. These latter events will be described in detail at a later point in the text.

It has been known for many years that hydrolysis of native C3 either via tick-over or through reaction with nucleophiles results in conformational changes to the molecule that impart C3b-like activities [15, 16]. While there is no crystal structure available for C3(H₂O), quantitative cross-linking and mass spectrometry studies show that C3 hydrolysis results in a large translocation of the TED domain within the molecule [17]. The likely nature of this translocation can be inferred by comparing the structure of native C3 with that of C3b [18], which reveals a striking example of protein conformational change (Fig. 1B, C). Whereas the keyring-like core of the molecule comprised of domains MG1-MG6 is more or less unchanged during activation, most other domains experience structural rearrangements to varying degrees [18]. These range from minor repositioning, as typified by domains MG7 and MG8, to large scale movements, such as those seen for the CUB and TED domains [18]. It is noteworthy that the TED domain undergoes the

greatest changes of all, which results in an ~85 Å translation and full exposure of the intramolecular thioester away from its position in native C3 [18]. Although little is known about the speed with which these changes occur upon C3 activation, such reorientation of the TED domain has been suggested to bring the highly reactive acyl-imidazole intermediate [13,14] into immediate proximity of hydroxyl groups borne by the target surface while minimizing its quenching by water [18]. It is also fitting that those regions of the C3 molecule that experience obvious structural changes are also those that undergo proteolytic processing during the stepwise activation and degradation of the protein (Fig. 1A). This feature can be appreciated most readily by examining the structure of C3c [12], which represents the structural scaffold of the C3 molecule absent those domains most directly involved in its key effector functions of promoting inflammation (i.e. C3a) and opsonization (i.e. TED) (Fig. 1D).

One of most enigmatic features of the C3 protein in physiological settings is its paucity of ligands prior to its activation. Aside from molecules specifically selected for their C3-binding activity, such as the peptidomimetic Compstatin [19] and various anti-C3 monoclonal antibodies [20], the panel of C3 interactors is limited to a handful of *S. aureus* immune evasion proteins (i.e. Efb-C [21], Ehp [22], and Sbi [23]), the C3 convertases themselves, and little else. By contrast, C3b is a hub of interactions within the complement system and has well over two dozen endogenous ligands alone [10]. The physical basis for this property is apparent from comparing the structure of native C3 with that of C3b. In this regard, all binding sites for known interactors, including the alternative pathway pro-protease Factor B (FB), regulatory proteins like Factor H (FH), and various complement receptors, are either masked or otherwise unavailable in native C3 [18]. The exceptions to this rule not surprisingly include each of the immune evasion proteins listed above, all of which bind a site on the TED domain that is freely accessible in native C3 [21,22,24], as well as the scissile bond that is cleaved by C3 convertases to simultaneously release C3a and generate C3b. Even ligands like the *S. aureus* immune evasion protein SCIN [25] and the Complement Receptor of the Ig Superfamily (CRIg) [26], whose binding sites are retained in the C3c fragment, require conformational rearrangements from the native C3 structure to assemble or unmask their sites. Thus, the structural changes that occur upon C3 activation to C3b not only serve to expose the thioester with maximal effect, they also manifest an array of protein-protein interaction sites that drive a majority of downstream C3b functions.

3. Structural features of C3 convertase formation, stabilization, and function

C3 is known to spontaneously activate through the process known colloquially as tick-over, yet this activation route is comparatively inefficient to the enzyme catalyzed route involving C3 convertases [1, 10,15,27]. The C3 convertases themselves are intrinsically labile, multi-subunit complexes that assemble in a step-wise manner [1]. Although fluid phase convertases can form to some extent as a consequence of tick-over, in the most typical of situations they form on target surfaces [1]. In the first step, a scaffold molecule covalently linked to the target surface, a pro-protease to provide the eventual catalytic functionality, and a divalent cation to facilitate the interaction between the two protein components all come together to form a structure known as the pro-C3 convertase (or pro-convertase for short). In the second step, a separate enzyme activates the pro-convertase, thereby releasing a fragment of the pro-protease and generating the corresponding C3 convertase with full catalytic activity. Despite the fact that they recognize the same native C3 substrate, C3 convertases come in two varieties that reflect at the molecular level whether their formation was initiated by either the classical or lectin pathways (i.e. C4b2a via C4 and C2) or by the alternative pathway (i.e. C3bBb via C3 and FB) [1]. Since far more is known at the structural level regarding the formation and function of the alternative pathway C3 convertase (Table 1), this topic will be examined

in greater detail below.

When C3b is generated and covalently attaches to a surface, it acts as a platform to recruit the pro-protease FB out of circulation and thereby form the pro-convertase, C3bB. The structure of C3bB shows that the FB binding site is derived primarily from the C345C and CUB domains of C3b, and to a lesser extent its MG2, MG3, MG7 domains and the nascent

N-terminus of the α' -chain (Fig. 2A) [28]. This site is not exposed in the structure of native C3, which provides an explanation for how native C3 and FB can circulate at high concentrations in the bloodstream without leading to inadvertent generation of the pro-convertase. Conversely, the residues on FB that form contacts with C3b are derived from all five structural domains of FB [28]. Among these, the von Willebrand factor

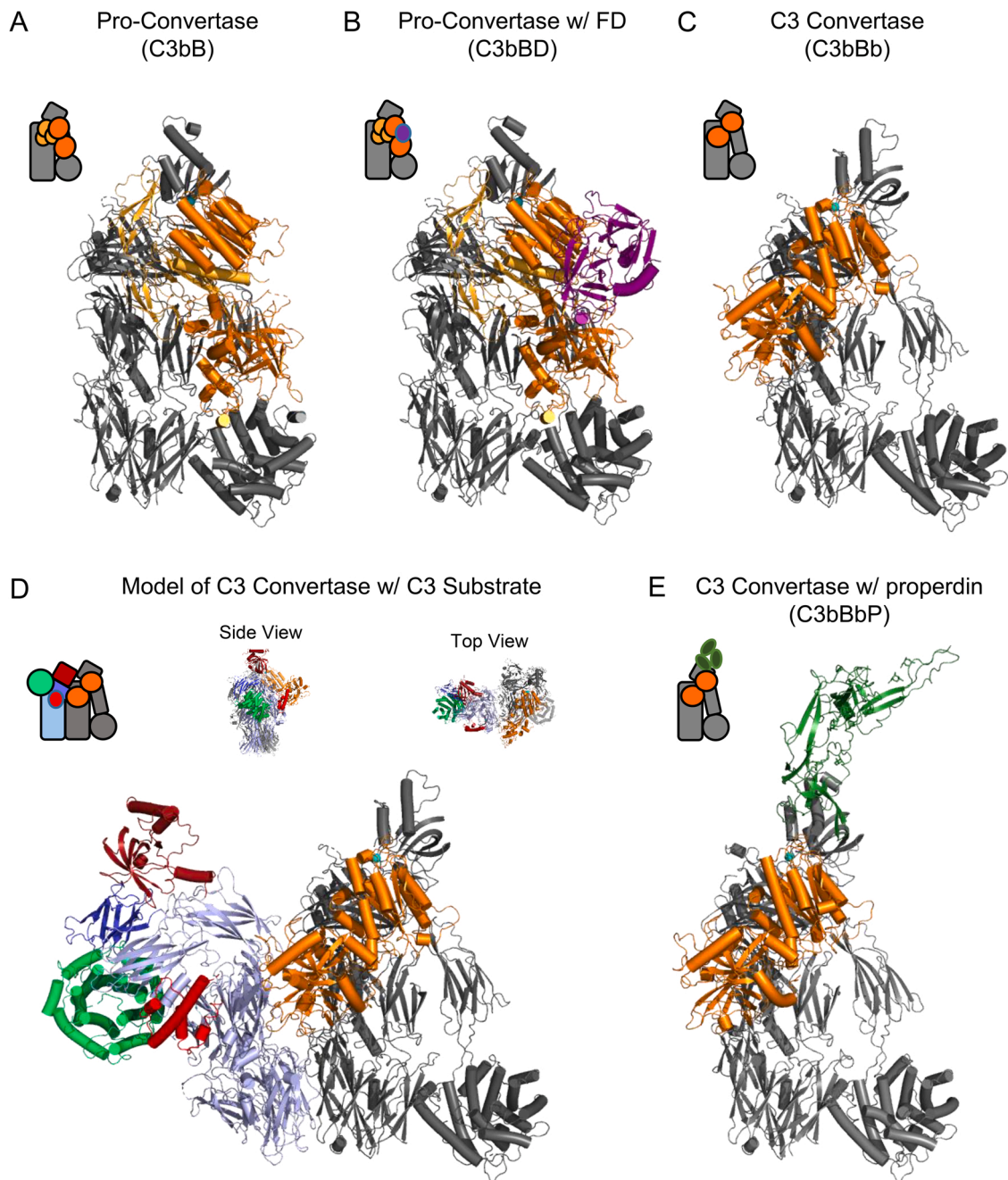


Fig. 2. Structural Features of the Alternative Pathway C3 Convertase. (A) Structure of the alternative pathway C3 pro-convertase as drawn from the PDB entry 2XWJ [28]. C3b is colored grey while FB is colored in shades of orange, with its Ba fragment in light orange and the Bb fragment in dark orange. A divalent cation, which in physiological conditions is Mg^{2+} , is represented as a blue sphere. (B) Structure of the alternative pathway C3 pro-convertase bound to FD as drawn from the PDB entry 2XWB [28]. C3b and FB are colored as in panel A, while FD is colored purple. (C) Structure of the alternative pathway C3 convertase as drawn from the PDB entry 2WIN [37]. Proteins are colored identically to panel A. Note the loss of the Ba domain of FB that occurs following FD cleavage. The *S. aureus* immune evasion protein SCIN [35,36,99] is omitted from this image for clarity, although it was used to facilitate crystallization of this otherwise labile complex [37]. (D) Model of the alternative pathway C3 convertase bound to its native C3 substrate. C3b and Bb are colored identically to panel C, while native C3 appears with color scheme like that used in Fig. 1. Both side and top-down views of the model are shown above the primary image. (E) Structure of the alternative pathway C3 convertase bound to a fragment of properdin as drawn from the PDB entry 6RUR [39]. C3b and Bb are colored identically to panel C, while the properdin fragment is colored green. The *S. aureus* immune evasion protein SCIN has likewise been omitted from this image for clarity. Simplified representations of each structure are shown in the top left corner of each panel.

type-A (VWA) domain within the Bb region of FB stands out as it contributes the so-called metal-ion dependent adhesion site (MIDAS). The MIDAS site lies at the interface between the VWA domain of FB and the C345C domain of C3b and is responsible for the Mg^{2+} -dependence of the alternative pathway C3 convertase in physiological systems. In the case of the C3bB crystal structure, Ni^{2+} was substituted for Mg^{2+} as it leads to a marked stabilization of the complex that facilitates structural and functional studies [29].

The pro-convertase C3bB has no enzymatic activity, and instead must be cleaved by the protease, Factor D (FD), to generate the fully active C3 convertase, C3bBb. FD is found in the bloodstream in a fully-matured, yet auto-inhibited state, which presumably contributes to the specificity of FD for the C3b-bound form of FB [30,31]. The structure of the Ni^{2+} -stabilized C3bB complex bound to a catalytic mutant of FD provides insight into how this occurs (Fig. 2B) [28]. Whereas the structure of FD on its own is characterized by binding of a self-inhibitory motif that distorts its active site residues from their expected positions [30,31], the structure of C3bB-bound FD reveals that this inhibitory loop is displaced and that a catalytically-competent active site is present [28]. There are several other features of this structure worth noting. First, although FD binding to its C3bB substrate displaces its self-inhibitory loop, the primary interaction between FD and C3bB occurs at an exosite that is relatively far removed from the FD active site proper. This FD exosite appears to be a crucial regulatory element that limits formation of the fully-active convertase [28]. Second, the scissile loop of FB that is cleaved by FD remains disordered even following FD binding. This is consistent with the poor catalytic profile of FD and its relatively inefficient processing of idealized substrates [32]. Finally, although FD is specific for the C3b-bound form of FB, FD itself forms no direct interactions with C3b. This element of specificity arises from binding of FB in a conformation seen only in the pro-convertase, and not in the structures of the unbound proteins [33].

The alternative pathway C3 convertase is notoriously challenging to study, since it is intrinsically labile and dissociates irreversibly within minutes of formation *in vitro* [34]. However, inclusion of the *S. aureus* immune evasion protein, SCIN, which stabilizes convertases in an enzymatically inactive but kinetically stabilized form [35,36], allows for formation of dimeric C3bBbSCIN complexes (i.e. (C3bBbSCIN)₂) amenable to crystallographic analysis [37]. New understanding of the C3bBb convertase was obtained through this approach (Fig. 2C) [37]. Most significantly, loss of the Ba fragment upon its proteolysis by FD results in rearrangement of the catalytic Bb fragment relative to its position in either C3bB or C3bBD (Fig. 2A-C). This involves elimination of all C3b contacts arising from the Ba fragment (i.e. to MG2, MG3, MG7, and the α -chain N-terminus) in addition to others from the Bb fragment (i.e. to MG2 and CUB) that are seen in the pro-convertase structures [28]. Nevertheless, the interactions between the VWA domain of the Bb fragment and the C345C domain of C3b are maintained through the MIDAS site and accessory protein-protein contacts [37]. This arrangement appears to allow the proteolytic Bb fragment to rotate in conjunction with the C345C domain, which itself exhibits inherent flexibility relative to the remainder of the C3b molecule. Presumably, this relative freedom of rotation permits the Bb protease active site to come into contact with the scissile bond of the C3 substrate [37]. Although native C3 is not a component of this structure, the location of the C3 binding site within the convertase can be extrapolated from the dimerization interface between the two copies of C3b found in each (C3bBbSCIN)₂ complex. In this regard, superposition of the native C3 structure onto the second copy of C3b shows good agreement between the rigid keyring core of the proteins. This model strongly suggests that C3b domains MG4 and MG5, and very likely MG3 and MG6–8 are critical determinants of C3 substrate recognition by C3bBb (Fig. 2D) [37]. At the present time, confirmation of these predictions awaits further structural studies of the C3 convertase bound to its *bona fide* substrate, C3.

The labile nature of the C3 convertase provides an intrinsic means of

regulating its enzymatic activity, yet this feature can be offset to some extent by action of the protein known as properdin. Properdin is the only known positive regulator of the alternative pathway. Although properdin has been ascribed pattern recognition receptor-like properties, its best understood function lies in stabilization of the C3bBb convertase [1]. Unfortunately, structural studies into its function are complicated by the fact that properdin is highly post-translationally modified and exists as polydisperse multimers, not to mention all the previously stated challenges associated with studying C3 convertases. These issues can be circumvented in part through extensive recombinant protein engineering and use of stabilizing nanobodies [38–40], thereby permitting crystallographic and functional studies. Structures of monomeric properdin analogues bound to the SCIN-stabilized C3bBb convertase show that properdin interacts primarily with the C345C domain of C3b, and to a lesser extent the Bb fragment (Fig. 2E) [39,40]. Significantly, this binding site lies in close enough proximity to contact the Bb domain and promote its retention on C3b, while not appearing to impede binding of the C3 substrate to the convertase (Fig. 2D). As properdin naturally exists as planar, rigid oligomers of extended conformations [41], the combination of these properties has been proposed to facilitate clustering alternative pathway convertases on susceptible surfaces. Given the self-amplifying nature of the alternative pathway, it is easier to understand how these peculiar structural features of properdin foster high-level alternative pathway activity in physiological systems.

4. Structural considerations underlying regulation of C3b function

The alternative pathway is capable of self-amplification and is responsible for a large majority of downstream complement activity in physiological settings [27,42]. Thus, it is not surprising that unregulated alternative pathway activity can become a pathophysiological driving force in many human diseases [3–5]. Nevertheless, the fact that most of these diseases are relatively uncommon in the general population indicates that alternative pathway activity is precisely regulated *in vivo*. Unraveling the many layers of regulation that control alternative pathway activity has required biochemical and functional studies spanning more than 50 years [43]. It has also befitting from more recent crystallographic studies on the convertase itself and a dedicated suite of regulatory molecules. Points of interest arising from both approaches will be summarized in the paragraphs that follow.

There are two intrinsic regulatory mechanisms that act at the level of the convertase components themselves [1]. First, the complex sequence of events required to form the fully active alternative pathway C3 convertase represents a key point of regulation (Fig. 2). It is this requirement of not one, but two activation steps prior to convertase formation that allows the precursor proteins (i.e. C3 and FB) to exist quiescently in the same environment prior to initiation of complement activity via a specific biochemical cue. Once it is formed, though, the C3 convertase is subject to a second point of intrinsic regulation. In the absence of other factors (e.g. substitution of Ni^{2+} for Mg^{2+}), the C3 convertase is intrinsically labile and decays with a half-life of approximately one minute in physiological settings [34]. This step is also irreversible, as the Bb fragment released cannot reform an active convertase. Although these latter properties are influenced to some degree by the action of properdin, together they provide important contextual and temporal restrictions on alternative pathway activity in both *in vitro* and *in vivo* settings.

There are likewise extrinsic control mechanisms that rely on the action of a dedicated suite of proteins known as the Regulators of Complement Activation (RCA; [44,45]). RCA proteins share a common modular architecture based upon repeating complement control protein (CCP) domains. Although up to 30 CCP domains may be found in an RCA molecule, their regulatory functions localize to a discrete and contiguous stretch of repeats through which they bind directly to C3b [44,45]. RCA proteins inhibit alternative pathway activity through two distinct

mechanisms [1,44,45]. On one hand, decay accelerating activity not only promotes the irreversible dissociation of the Bb fragment from C3b, it also blocks pro-convertase formation by competing with FB binding to C3b. The RCA proteins Decay-Accelerating Factor (DAF) and Complement Receptor 1 (CR1) are localized at the cellular surface and protect self-cells via their decay-accelerating activity; complement Factor H (FH) and its splice variant FHL-1 do likewise following their capture out of circulation via interactions with self-cell associated carbohydrates and other polyanionic structures, although these regulators can also exert their activities on fluid-phase convertases. On the other hand, cofactor activity arises when RCA proteins bind C3b and facilitate recruitment of the degradative protease known as Complement Factor I (FI). Although the RCA proteins themselves are not altered in this process, cleavage of C3b by FI generates iC3b, which no longer supports FB binding or downstream convertase formation. The RCA proteins Membrane Cofactor Protein (MCP) and CR1 exhibit cofactor activity at the self-cell surface, while both surface-adsorbed and fluid-phase FH and

FHL-1 do as well. As a group, this collection of RCA proteins provides an important self-protective mechanism against the alternative pathway by diminishing C3 convertase stability and effectively reducing the levels of C3b available for convertase formation.

Although they differ in distribution (i.e. cell surface-retained versus soluble) and manifestation of inhibitory mechanism, all RCA proteins share important structural features beyond that of their repeating CCP domain architecture. In this regard, structural studies of C3b bound to the minimal regulatory fragments of MCP (domains CCP(1–4); [46]), CR1 (domains CCP(15–17); [46]), and DAF (domains CCP(2–4); [46]), as well that of C3b bound to FH (domains CCP(1–4); [47]) reveal that all RCA proteins employ a common C3b-binding mode (Fig. 3A-D, G). At the most fundamental level, each of these regulators binds in an elongated fashion and in an identical orientation relative to C3b [46]. A deeper interpretation of these structures reveals four apparently generic RCA-binding sites on the C3b surface, denoted sites CCPi-iv; these are derived from the nascent N-terminus of the α -chain and MG7 (CCPi),

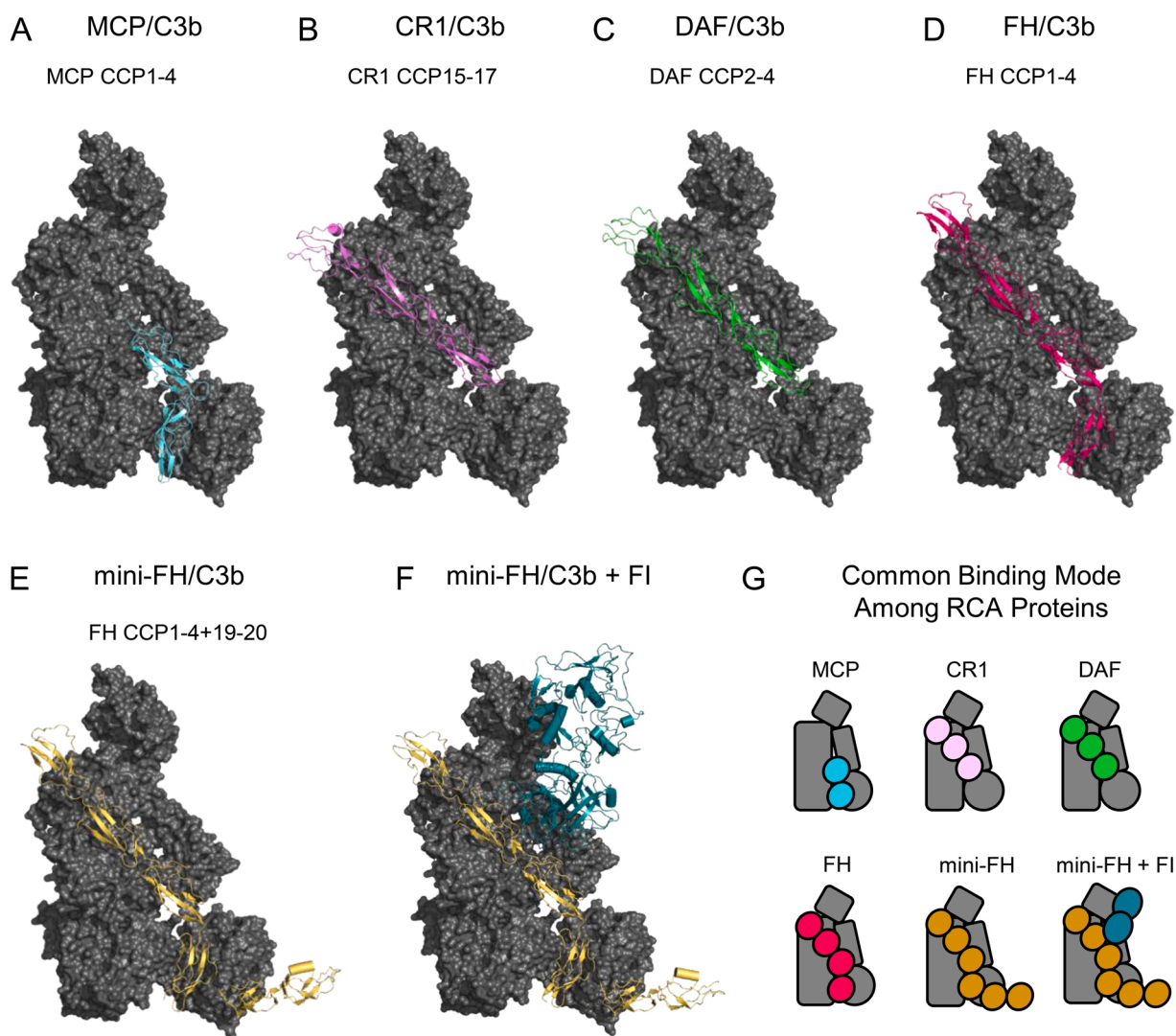


Fig. 3. Structural Features of C3b-binding Complement Regulatory Molecules. (A) Structure of C3b bound to CCP domains 1–4 of Membrane Cofactor Protein (MCP) as drawn from PDB entry 5FO8 [46]. C3b is colored as a grey surface, while MCP is colored light blue. (B) Structure of C3b bound to CCP domains 15–17 of Complement Receptor 1 (CR1) as drawn from PDB entry 5FO9 [46]. C3b is colored as a grey surface, while CR1 is colored pink. (C) Structure of C3b bound to CCP domains 2–4 of Decay Accelerating Factor (DAF) as drawn from PDB entry 5FOA [46]. C3b is colored as a grey surface, while DAF is colored green. (D) Structure of C3b bound to CCP domains 1–4 of Complement Factor H (FH) as drawn from PDB entry 2WII [47]. C3b is colored as a grey surface, while FH is colored magenta. (E) Structure of C3b bound to CCP domains 1–4 and 19–20 of mini-FH as drawn from PDB entry 5O32 [51]. C3b is colored as a grey surface, while mini-FH is colored gold. (F) Structure of C3b bound to mini-FH and Complement Factor I (FI) as drawn from PDB entry 5O35 [51]. C3b and mini-FH appear identically to panel E, while FI is colored dark blue. (G) Simplified representations of the structures shown in panels A-F. Note the common binding surface recognized by each of these C3b-binding regulatory molecules.

MG6 and MG7 (CCPii), MG2 and CUB (CCPiii), and MG1 and TED (CCPiv), respectively [46]. Regardless of where the minimal regulatory fragment lies in any given RCA protein, the N-terminal most domain visible in its C3b-bound structure occupies the site closest to the generic CCPi site [46]. Indeed, the common binding mode shared among RCA proteins that exhibit decay accelerating activity only (i.e. DAF), cofactor activity only (i.e. MCP), or those with both (i.e. FH and CR1) suggests that these proteins may have diverged from a common evolutionary precursor [46].

In addition to identifying their conserved mode of C3b binding, structural investigations of RCA proteins also illustrate the physical basis for their function. Recognizing that the presence of additional domains that increase the affinity for C3b will also enhance apparent regulatory activity [48], correlating this portfolio of structural information with pre-existing functional studies on the same RCA fragments leads to a model wherein binding to sites CCPi-iii imparts decay accelerating activity while binding to sites CCPi-iv imparts cofactor activity. For example, the RCA binding sites CCPi-ii are occupied by domains CCP (2–3) of DAF [46], which are also known to interact with Bb [49]. It is not entirely certain how this latter interaction promotes dissociation of Bb from C3b; however, a model has been proposed whereby regulator binding to the complex may result in changes in conformation or dynamics that promote dissociation of the proteolytic component from C3b [50]. Adjacent to this, RCA binding sites CCPi-iii share significant overlap with the binding site of FB seen in the C3bB pro-convertase structure (c.f. Fig. 2 A, B and Fig. 3B-D) [28]. Presumably, this leads to direct competition between any regulators binding to this region and FB, thereby diminishing further convertase formation. Finally, the roles of RCA binding sites CCPi-iv in promoting cofactor activity can be inferred from the structure of a designer RCA protein, mini-FH, bound to both C3b and FI [51]. Mini-FH is a fusion protein consisting of the regulatory domains of FH (i.e. CCP(1–4)) linked to the FH cell surface targeting domains (i.e. CCP(19–20)), but exhibits enhanced affinity and regulatory potency when compared to native FH [52]. The structure of mini-FH bound to C3b alone (Fig. 3E) is almost indistinguishable from that of FH domains CCP(1–4) bound to C3b (Fig. 3D, G) [47,51], illustrating that its regulatory domains occupy RCA binding sites CCPi-iv. However, the structure of the ternary complex shows extensive interactions between mini-FH CCP(2–3) and the serine protease domain of FI (Fig. 3 F, G) [51]. As mini-FH CCP(2–3) occupy RCA binding sites CCPi-iii, it appears these domains help recruit FI to C3b and properly orient the FI active site toward the scissile bonds found within the C3b CUB domain [51]. The role of mini-FH CCP(4), which occupies RCA site CCPiv, therefore seems to rest in binding TED and restricting its translation away from the keyring core. It is worth noting that multiple studies indicate flexibility between the CUB-TED region of C3b and the remainder of the molecule [53–55]. Moreover, separate work on the viral RCA homolog Kaposica has suggested that restricting flexibility in the CUB-TED region is crucial for efficient cofactor activity [56]. Therefore, in some respects the CCPiv interaction can also be viewed as properly orienting the scissile bonds within CUB to the FI active site, culminating in the cleavage of C3b to its degradation product, iC3b.

5. Structural basis for recognition of C3b and its fragments by complement receptors

Opsonization of biomaterial with C3b sets the stage for C3 convertase formation and amplification of the complement response via the alternative pathway [1,10]. It likewise provides a route through which C3 fragment-opsonized material can be engulfed, destroyed, and in certain cases processed for antigen presentation by phagocytic cells [1, 10]. This essential effector function of complement is known as opsonophagocytosis and is predicated upon recognition of C3b and its degradation products by cell surface-retained complement receptors. Complement receptors cannot simply be high-affinity binders, though. They must also be selective for the activated forms of C3 since major

classes of phagocytic cells continually encounter the high concentrations of native C3 found in circulation. This all-important selectivity is achieved through two different mechanisms. First, the complement receptor binding sites are only revealed following the structural transitions that accompany C3 activation to C3b [1,10]. Second, the step-wise degradation of C3b to iC3b and ultimately C3d provides increased access of certain binding sites to the large, surface bound receptors that recognize them [1,10]. Not surprisingly, structural studies have contributed to understanding this process at the molecular level, as will be described below.

The Complement Receptor of the Ig superfamily (CRIg) is expressed on tissue resident phagocytes and acts as a high-affinity receptor for C3b and iC3b [57]. As its name suggests, the extracellular region of CRIg consists of either one or two Ig repeats depending on alternative splicing of the transcript [57]. The structure of C3b bound to an Ig-domain from CRIg shows that its binding site is comprised of MG3-MG6 as well as the LNK domains of the C3b β -chain (Fig. 4 A) [26]. While these domains are accessible in the structure of native C3 [12], a slight rotation of the MG3 domain along with translation of the LNK domain are needed to complete the CRIg-binding site. This explains the inability of CRIg to bind native C3 [57]. By contrast, CRIg maintains high-affinity binding to both iC3b and C3c [57]. Although the functional relevance of its interaction with C3c is unknown, the structure of the same CRIg Ig-domain bound to C3c is almost identical to that of C3b [26]; this suggests that there are no major changes to the CRIg binding site as C3b is processed sequentially to iC3b and then C3c. A final noteworthy feature of CRIg is that its C3b-binding domain is also a potent inhibitor of the alternative pathway [26]. Since CRIg does not display any RCA-like activity, the most likely explanation for this observation is that the CRIg binding site on C3b is essential for substrate binding to the C3 convertase [26]. Indeed, this is apparent from the model of the C3-bound convertase presented in an earlier section (Fig. 2D) [37].

Complement Receptor 1 (CR1) is more widely distributed than CRIg and is found on the surface of practically all types of peripheral blood cells [58]. The opsonin-binding profile of CR1 includes not only C3b and iC3b, but C4b as well [1,58]. CR1 is a large molecule that in its most common variant contains 30 CCP repeats in its extracellular region [58]. However, functionally redundant regions with nearly identical sequences have been identified within CR1 [58], allowing useful inferences to be gained from studies on the prototypic region of domains CCP(15–17). The structure of CR1 domains CCP(15–17) bound to C3b shows an extended binding site formed from the nascent N-terminus of the α -chain, along with the MG2, MG6, MG7, and CUB domains (Fig. 4B) [46]. As with many other C3b-binding molecules, this binding site is masked in native C3 and requires both proteolytic release of the C3a fragment and the accompanying conformational change to assemble [12,18]. Although the main features of this binding site must be retained upon conversion to iC3b, it is interesting to note that iC3b is a somewhat lower-affinity ligand for CR1 than C3b [51,58]; since the CUB domain contributes to the CR1 binding site, this reduction in affinity of CR1 for iC3b most likely comes from changes to the CUB domain that occur following its sequential cleavages by FI [55]. Nevertheless, the interaction of CR1 with iC3b is sufficient to be the only cofactor that supports all three proteolytic cleavages of C3b by FI that are needed to generate C3c and C3dg [51,59]. CR1 is thus unique among complement receptors, as it exhibits RCA properties as well [1,10,58].

In contrast to either CRIg or CR1, the remaining receptors for C3-derived opsonins do not bind C3b [10]. Instead, their binding sites only become fully accessible upon cleavage of C3b to iC3b. The physical basis for this long-standing observation can be understood from a series of non-crystallographic structural studies. Both electron microscopy and small angle X-ray scattering analyses of iC3b are consistent with a TED domain dislodged from its position against the keyring core [51] when compared to the crystal structures of C3b alone [18,46]. This suggests that a fundamental change in the flexibility of the CUB region must occur upon C3b cleavage to iC3b. Strong evidence regarding the nature

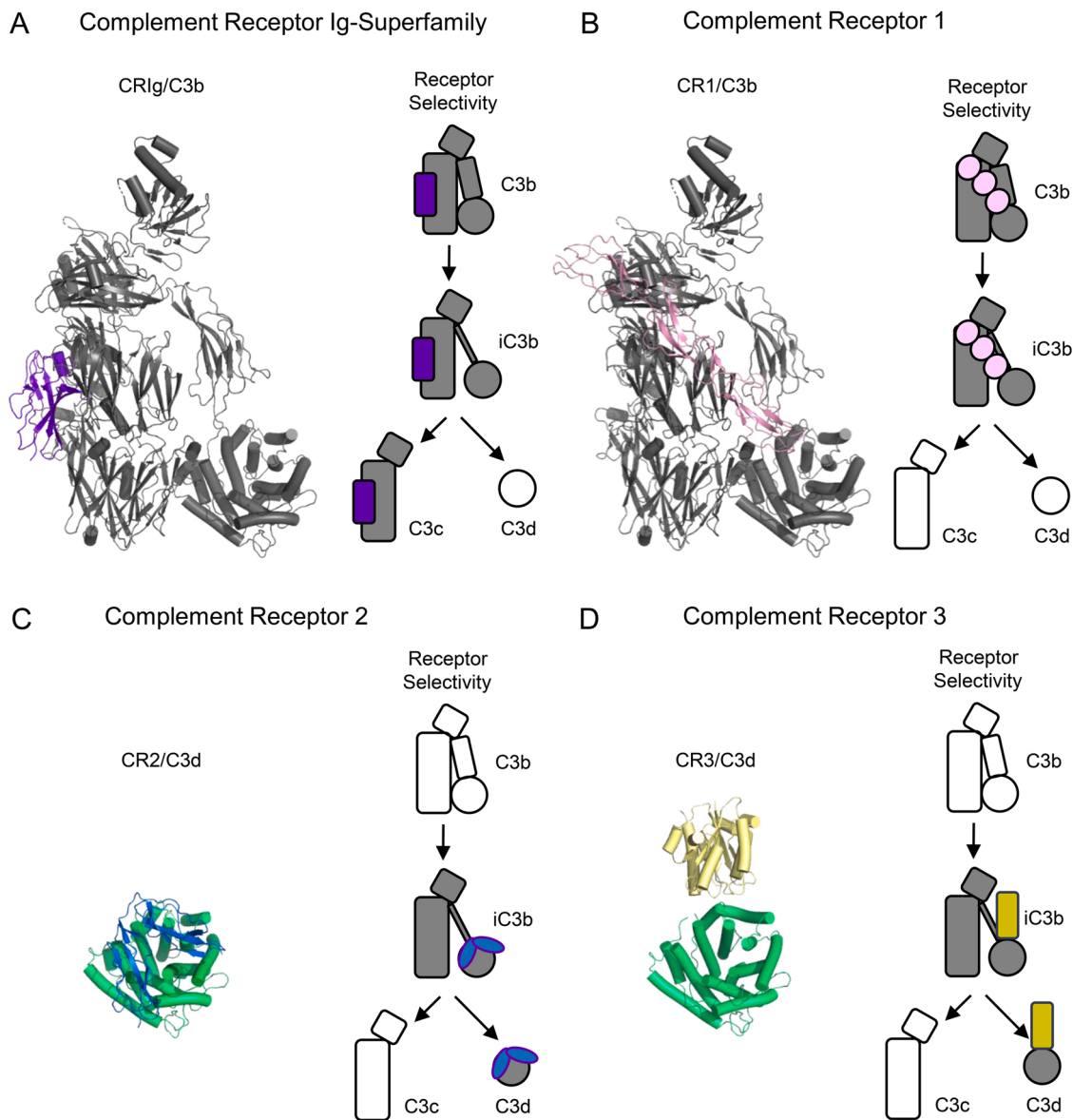


Fig. 4. Structural Features of Complement Receptors that Bind to C3-derived Oponins and Fragments. (A) Structure of C3b bound to a domain from the Complement Receptor of the Ig-superfamily (CRIg) as drawn from PDB entry 2ICF [26]. C3b is colored grey, while CRIg is colored purple. (B) Structure of C3b bound to CCP domains 15–17 from Complement Receptor 1 (CR1) as drawn from PDB entry 5FO9 [46]. C3b is colored grey, while CR1 is colored pink. (C) Structure of CCP domains 1–2 from Complement Receptor 2 (CR2) bound to C3d as drawn from PDB entry 3OED [61]. C3d is colored green, while CR2 is colored blue. (D) Structure of the I-domain from Complement Receptor 3 (CR3) bound to C3d as drawn from PDB entry 4M76 [63]. C3d is colored green, while CR3 is colored yellow. Simplified representations of each receptor and the various fragments of C3 that it recognizes are shown at the right of each panel. Shaded images signify interactions known to occur, while hollow images signify activated forms of C3 that are not recognized by the receptor in question.

and extent of this change comes from peptide-level resolution hydrogen-deuterium exchange studies comparing C3b to iC3b [55]. Whereas peptides within the CUB domain exhibit varying degrees of solvent protection in C3b, these same peptides are almost uniformly solvent-exposed in iC3b [55]. The simplest interpretation of these results is that site-specific cleavage by FI within the CUB domain causes it to unfold [55]. Ultimately, this leaves behind a flexible tether that connects what will become the C3c fragment to the covalently-bound opsonin fragment that will become C3d [55].

Although the complement receptors selective for iC3b and C3d share a common ligand-binding profile, they exhibit divergent structural features themselves. Complement Receptor 2 (CR2) is expressed on the surface of B lymphocytes and follicular dendritic cells [1,10]. Its extracellular region consists of 15 CCP repeats and therefore bares some architectural similarities with CR1. The iC3b and C3d binding activity of

CR2 resides in domains CCP(1–2) [60]. The structure of this CR2 fragment bound to C3d reveals that the CCP domains from the receptor fold back upon one another and recognize a binding site on the opposing face of the C3d molecule from its former thioester site (Fig. 4C) [61]. This ensures that CR2 binding to iC3b or C3d would not be impeded by their covalent linkage to a surface. Moreover, the selectivity of CR2 for iC3b and C3d versus C3b can be explained an obvious steric clash with MG1 when this CR2 binding site is inspected in C3b crystal structures [61]. This steric clash is presumably relieved by unfolding of the CUB domain upon cleavage of C3b to iC3b [55]. In contrast to CR2, Complement Receptor 3 (CR3) is expressed primarily by myeloid-lineage cells [62]. CR3 is an integrin and its iC3b and C3d binding activity resides within its I-domain. The structure of the CR3 I-domain bound to C3d shows that its binding site also lies opposite the former thioester (Fig. 4D) [63]. However, the CR3 binding site on C3d is slightly offset

from that of CR2 such that it would sterically clash with the intact CUB domain of C3b instead of MG1 [63]. But similarly to CR2, this steric clash would be removed as the CUB domain unfolds upon cleavage of C3b to iC3b [55], thus explaining its selectivity for these late-stage opsonins [1,10]. It also seems likely that binding of CR3 to this site would be facilitated by the conformational flexibility that a fully unfolded CUB domain provides, since it allows the C3c-like moiety to move away from the surface-bound C3d domain [51,55]. Indeed, a very recent crystal structure of iC3b bound to the CR3 I-domain shows examples of two different orientations of the C3c-like core relative to the C3d domain [64]. This further alludes to the need for increased structural flexibility in iC3b to support its recognition by cell surface-exposed complement receptors.

6. Structural themes arising from complement inhibitors targeting C3 or its fragments

The interconnected events of C3 activation, degradation, and effector function are tightly controlled spatially and temporally, and require over a dozen proteins in addition to C3 itself [1,10]. Although deciphering these intricate mechanisms at the structural and functional level required decades of experimentation, it also revealed many individual steps that could be attractive targets for inhibitor development in the process. Nevertheless, since high-level complement activity and its downstream effector events are predicated upon the activation of C3, any discussion of inhibitors acting at this level in the pathway must begin with molecules that block either the enzyme (i.e. convertase or its C3b scaffold) or the substrate (i.e. native C3) involved in this all-important reaction. In this regard, structural studies on numerous naturally occurring and manmade inhibitors have provided clues into the various ways that targeted inhibition at the C3 level can be achieved.

C3 activation can be potentially inhibited by molecules that interfere with convertase dynamics (i.e. its formation and/or stability) similarly to endogenous RCA proteins [1,44,45]. Two such examples are the *Vaccinia* virus complement control protein, typically referred to as VCP [65], and the *Variola* virus small-pox inhibitor of complement enzymes, typically referred to as SPICE in the literature [66]. Although both viral proteins are orthologs of human MCP and DAF, SPICE exhibits more potent decay-accelerating and FI-cofactor activities than does VCP [66,67]. The structure of SPICE bound to C3b shows that it occupies generic RCA-binding CCPi-iv sites on the C3b surface (Fig. 5A) [46]. Thus, its overall C3b-binding pose most closely resembles that of FH CCP(1–4) (Fig. 3D) [47] and its engineered variant mini-FH (Fig. 3E) [51,52]. In the specific case of mini-FH, however, the additional interactions with the TED domain contributed by FH CCP(19–20) result in enhanced affinity for C3b and inhibitory potency against the alternative pathway when compared to either FH CCP(1–4) or native FH [52]. Thus, the successful design of mini-FH using structure/function information as the primary guide suggests that a similar approach could be taken to produce novel classes of complement inhibitors.

Other well characterized inhibitors of C3 activation employ mechanisms distinct from RCA proteins. SCINs are a family of small (~10 kDa) immune evasion proteins secreted by *Staphylococcus aureus* that bind directly to C3b and inhibit multiple processes required for C3 cleavage by the convertase [35,36,68–71]. Structural studies of SCIN proteins bound to C3b and C3c show that their primary binding site is formed by the nascent N-terminus of the α' -chain, along with the MG6 and MG7 domains (Fig. 5B) [25,72]. Although this binding site largely overlaps with the generic RCA-binding CCPi-CCPii sites on C3b [46], SCINs do not exhibit canonical RCA-like activities. Instead, SCINs compete with FB and slightly diminish convertase formation [36]. They likewise compete with FH and block convertase decay [35,36]. While convertases bound by SCINs are more stable, they are catalytically inactive [35,36]. In addition to this, SCIN proteins also contain an N-terminal extension that mediates pseudo-dimerization of C3b via a binding site within the MG7 and MG8 domains of a second C3b molecule [25,37,72].

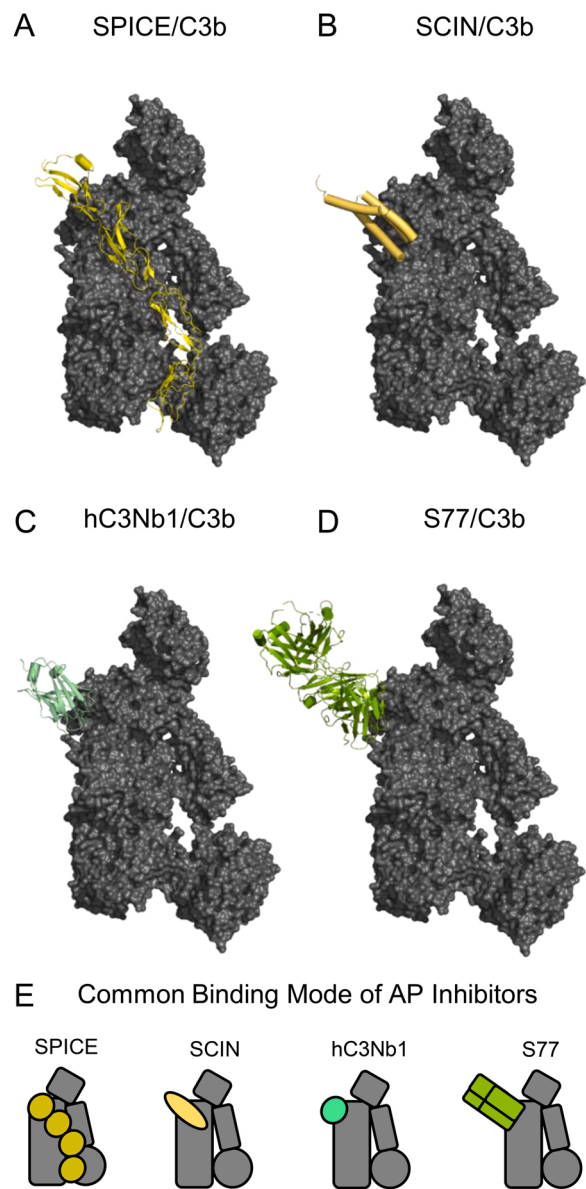


Fig. 5. Structural Features of C3b-binding Inhibitors of the Alternative Complement Pathway. (A) Structure of C3b bound to CCP domains 1–4 of the *Variola* virus complement inhibitor SPICE as drawn from PDB entry 5FOB [46]. C3b is colored as a grey surface, while SPICE is colored yellow. (B) Structure of C3b bound to the *S. aureus* immune evasion protein SCIN as drawn from PDB entry 3OHX [25]. C3b is colored as a grey surface, while SCIN is colored pale orange. (C) Structure of C3b bound to the nanobody hC3Nb1 as drawn from PDB entry 6EHG [73]. C3b is colored as a grey surface, while hC3Nb1 is colored light green. (D) Structure of C3b bound to the F(ab) fragment of monoclonal antibody S77 as drawn from PDB entry 3G6J [74]. C3b is colored as a grey surface, while S77 is colored olive green. (E) Simplified representations of the structures shown in panels A–D. Note the common binding surface recognized by each of these C3b-binding Alternative Pathway inhibitors.

Structural interpretations of the resulting SCIN-mediated pseudodimers give rise to the currently accepted model for substrate recognition by the C3 convertase (Fig. 2D) [10,37]. However, dimerization per se is not required for SCINs to inhibit C3 cleavage, as SCIN deletion mutants lacking the N-terminal extension still exhibit inhibitory activity [72]. Moreover, SCIN-inhibited convertases prepared under limiting C3b conditions also bind well to native C3 even though they cannot cleave it [36,70]. Collectively, these observations are consistent with the proposal that SCINs inhibit the alternative pathway C3 convertase by

blocking access of its proteolytic component to the scissile bond of C3 [37].

The primary SCIN binding site overlaps with not only FB and FH [25, 47], but those of other complement regulatory components such as CR1 and DAF [4]. This suggests that other molecules targeting this so-called functional hotspot on C3b may also exhibit inhibitory activities [36,71]. Indeed, two such inhibitors have been reported in the literature. The camelid-derived, anti-C3 nanobody known as hC3Nb1 binds with sub-nanomolar affinity to C3, C3b, and iC3b and inhibits the alternative pathway [73]. The structure of hC3Nb1 bound to C3b reveals that this nanobody recognition site is comprised of surface-exposed loops in MG7, and to a far lesser extent the nascent N-terminus of the α' -chain (Fig. 5 C) [73]. Consistent with the properties attributed to other molecules that bind MG7, hC3Nb1 blocks assembly of the pro-convertase, degradation of C3b by FI, and even C3 binding to pre-formed convertases to a degree [73]. Unlike hC3Nb1, the antigen-binding fragment of an antibody known as S77 does not bind to native C3, but binds with low-nanomolar affinity to C3b, iC3b, and C3c [74]. The structure of S77 bound to C3b shows that this inhibitor also recognizes MG7 (Fig. 5D), whereby it displays a similar series of functional properties to hC3Nb1 [74]. Since SPICE, SCIN, hC3Nb1, and S77 all share a similar binding site on C3b and they all inhibit C3 activation via the alternative pathway, it seems that targeting this specific area of C3b represents a generalizable strategy for developing potent C3 inhibitors (Fig. 5E).

Of all the inhibitors that act at the level of C3, perhaps the best understood is a family of molecules known as Compstatins [10,75–77]. Compstatins bind to native C3 and its C3c-containing fragments and inhibit C3 activation via all three complement pathways [10,75,76]. The parental compound of Compstatin was identified from a phage display screen for novel ligands of human C3 and consists of 13 residues held together in a cyclic structure [19]. A single chemical modification and two amino acid substitutions produced an analogue known as 4W9A/Cp01 and which has ~10-fold improved affinity ($K_D=1.2 \mu\text{M}$) over the parental compound [78]. Further improvements based on the Compstatin scaffold that incorporate non-natural amino acids and backbone methylation to limit conformational flexibility resulted an analogue with ~5000-fold enhanced affinity ($K_D=500 \text{ pM}$) over the initial molecule [79]. A recently reported structure of a prototypic third generation Compstatin known as Cp40 bound to C3b shows the same interaction site as that of 4W9A/Cp01 bound to C3c (Fig. 6A) [80]. However, it also reveals important differences accounting for the striking improvement in its potency (Fig. 6B) [80]. Chief among these are improved hydrophobic contact with the binding site mediated by incorporation of the N-terminally-extended D-Tyrosine, as well as shielding effects on structural solvent molecules [80]. Separately, both of these structures show that the Compstatin binding site lies at the interface of the MG4 and MG5 domains [81]. This site is fully accessible in native C3 and explains why Compstatins are not selective for activated forms of C3. Nevertheless, this binding site appears critical for interaction of the C3 substrate with the C3 convertase (Fig. 2D) [37]. Indeed, this likely explains functional studies which show that Compstatin inhibits C3 activation by binding to native C3 and blocking its interaction with the C3 convertase, as opposed to inhibiting the C3 convertase enzyme itself [80].

7. Exploring new targets: a case for conformation-specific ligands as inhibitors?

Although there are currently more than 50 PDB depositions describing crystallographic structure determinations of human C3, its fragments, and various complexes (Table 1), many relevant aspects of C3 function cannot be discerned through crystallography alone. Indeed, most questions related to protein dynamics, conformational flexibility, and changes therein in response to biochemical transformations or ligand binding can only be meaningfully addressed through solution-based methods. Obtaining solution structural information on

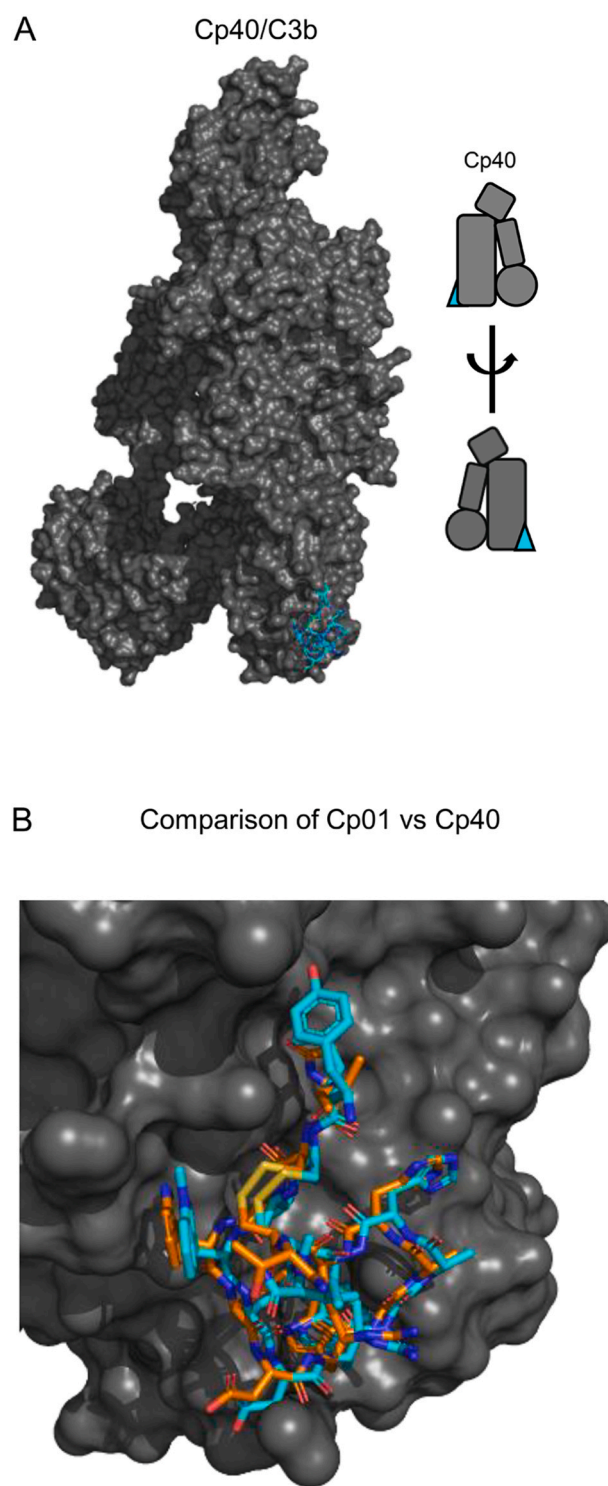


Fig. 6. Structural Features of Compstatin Class Complement Inhibitors. (A) Structure of C3 bound to the third-generation Compstatin Cp40 as drawn from the PDB entry 7BAG [80]. C3b is colored as a grey surface, while Cp40 is shown in stick convention and colored blue. A simplified representation of the structure is inset at the right of the panel. (B) Comparison of bound structures for the second-generation Compstatin Cp01 and Cp40 as drawn from the PDB entries 2QKI [81] and 7BAG [80], respectively. Both inhibitors are drawn in stick convention with the carbon atoms of Cp01 in orange and those of Cp40 in blue. Note that this image was constructed by superimposing the structure of Cp01 bound to C3c upon that of Cp40 bound to C3b using the keyring core of C3 as the basis for calculations.

molecules the size of C3 and its fragments is more feasible now since improvements in instrumentation, experimental workflow, and data analysis platforms have led to a rapid maturation of Hydrogen-Deuterium Exchange Mass Spectrometry (HDX-MS) and Small Angle X-ray Scattering (SAXS) approaches. However, it is deployment of these solution methods in combination with crystallography that has proven most useful for revealing unexplored structure/function features of C3 and its fragments. One such example identified discrete conformational substates in C3b that have divergent ligand-binding and functional properties [82]. Since selectively targeting one conformation over another may provide opportunities for future development of complement inhibitors, the studies related to this discovery will be described in the paragraphs that follow.

The immune evasion protein Efb is secreted from *Staphylococcus aureus* and consists of two functional domains [83,84]. Whereas its N-terminal domain is intrinsically disordered and binds to fibrinogen, the C-terminal domain (hereafter Efb-C) adopts a helical bundle fold, binds to C3, and is itself a potent inhibitor of the alternative pathway [21,84–86]. Efb-C recognizes a site within the TED domain that is freely accessible in the structure of native C3 [12,21], and forms high-affinity interactions with C3, C3b, iC3b, and C3d [21,55]. More recent surface plasmon resonance measurements of Efb-C binding to site-specifically immobilized C3b estimate the equilibrium dissociation constant in the low picomolar range ($K_D=110$ pM) (Fig. 7A); this extremely tight interaction is characterized by a very slow dissociation rate constant of $1.87 \times 10^{-4} \text{ s}^{-1}$ and an associated half-life of approximately 1 h. Despite this high-affinity interaction, the Efb-C binding site observed in its co-crystal structure with C3d is sterically occluded by the proximity of the MG1 and TED domains in the structure of C3b alone [18,21]. A potential explanation for this discrepancy is that the CUB-TED region of C3b rotates away from the keyring core, thus relieving the potential of steric clash with Efb-C by conformational change. This possibility is consistent with previous electron microscopy studies noting distinct conformational subsets in C3b [53], as well as with the observation that Efb-C binding leads to enhanced exposure of the epitope for the anti-C3b monoclonal antibody, C3-9 [21,87].

Differential HDX-MS studies provide a better understanding of the changes in C3b arising following Efb-C binding (Fig. 7B) [21,82]. In agreement with the Efb-C/C3d co-crystal structure, the exchange profiles for TED domain peptides corresponding to the Efb-C binding site show significant protection in the presence of Efb-C [82]. Oppositely, peptides representing the linker that connects the CUB and TED domains and within the CUB domain itself show significant enhancements in their exchange profiles [82]. Intriguingly, this property extends to two peptides within the MG8 domain that are far removed from the Efb-C binding site [18,82]. This suggests that Efb-C binding results in increased exposure of a region of C3b extending from the TED domain up through MG8. SAXS analyses performed on samples of either C3b alone or when bound to Efb-C illustrate how this may occur at the structural level [82]. Whereas C3b on its own appears to be distributed across two conformers distinguished by a rotation of the CUB-TED region (hereafter called “closed” and “open”), the Efb-C bound form is restricted to the open conformer whose CUB-TED region is displaced from the keyring core. Since Efb-C does not readily dissociate from C3b, these results indicate that Efb-C binding preferentially stabilizes an open conformer of C3b that normally exists within a population of C3b molecules (Fig. 7C).

Many peptides that display significant exchange differences in the Efb-C/C3b complex versus C3b alone map to known ligand binding sites [82]. This suggests that the open conformer of C3b might have altered ligand binding properties when compared to its closed counterpart. Although no experimental tools exist at present to selectively stabilize the closed conformation of C3b, the tightly binding Efb-C protein that does not readily dissociate provides a means to assess the functional attributes of the open C3b conformer alongside a normally distributed population of C3b molecules. Thus, surface plasmon resonance can be

used to investigate binding of several ligands to C3b alone or C3b pre-saturated with Efb-C [82]. Neither the *S. aureus* immune evasion protein SCIN (Fig. 8A), nor a fusion protein displaying the first-generation Compstatin sequence 4W9A show any changes in binding as a function of Efb-C (Fig. 8B) [88]. However, CCP domains 19–20 from FH show a marked increase in binding in the presence of Efb-C (Fig. 8C); since this region of FH recognizes a site within the TED domain of C3b, this observation suggests that the FH(19–20) binding site is more accessible in the open C3b conformer. By contrast, binding of FB is reduced by over 75% in the presence of Efb-C (Fig. 8D) [82]. This demonstrates that Efb-C inhibits the alternative pathway by blocking formation of the pro-convertase and therefore formation of the active convertase responsible for complement amplification [82].

Interestingly, a greater than 50% reduction in the interaction between C5 and C3b is also seen in the presence of Efb-C (Fig. 8E) [88]. Interpreting this observation is not as straightforward since the structure of neither the C5/C3b complex nor the C5 convertase has been reported. However, information on the nature of this interaction can be derived from the structure of the C3b analogue, Cobra Venom Factor, bound to C5 [89]. The C5/CVF structure bares many similarities to the model of the C3 convertase bound to C3 (Fig. 2D), including binding of the C3/C5 substrate on the side of the scaffolding molecule (i.e. C3b or CVF) opposite its CUB-TED region [37,89]. This side of C3b also harbors binding sites for CRIg and Compstatin, both of which inhibit substrate binding to C3b-containing convertases [26,80,81]. Curiously, there are minimal differences in the exchange profiles on this side of C3b in response to Efb-C binding (Fig. 7B) [82]. Although the peptide coverage map is not complete in this area and limits the information available, a single peptide within the MG3 domain does exhibit significant exchange enhancement and is oriented toward the putative C3/C5 substrate-binding interface [82]. While it is nearly certain that C3/C5 binding involves multiple points of contact with the C3b scaffold of the convertase [89], it is tempting to speculate that this peptide from MG3 and the region surrounding it might play an important role. Further investigation is clearly needed to fully understand the physical basis for C3/C5 substrate binding to the C3b-containing convertases. Nevertheless, these data suggest that substrate binding to the convertase is influenced by the conformational state of C3b. When considered together with the fact that the open conformer of C3b is inefficient at supporting C3 convertase assembly [71,82], selective targeting of conformational substates in C3b seems to be an attractive option for future inhibitor discovery.

8. Concluding remarks

The last decades have witnessed an increased appreciation for the role of complement as a pathophysiological driving force across the human inflammatory disease landscape [3–5]. Yet throughout most of this time, the only complement specific therapeutic approved by regulatory agencies for clinical use has been the anti-C5 monoclonal antibody eculizumab (Soliris®). While debate continues as to which point of inhibition is best for a given disease or indication, there is ample evidence to suggest that therapeutic inhibition of C3 may not only be desirable but preferable to C5 blockade in various situations [8,9,90,91]. Although the developmental timeline of the first C3-targeted inhibitors spanned 25 years from the initial discovery till the recent approval, the availability of these first-in-class drugs ushers in a new era in complement therapeutics. Indeed, with proof of efficacy established and approvals thus obtained, the future of C3 inhibitors may be as promising now as it has ever been. Despite this prevailing sense of optimism, there must also be understanding that no single type of inhibitor will be best for every indication where C3 inhibition appears warranted. Paradoxically, the very complexity of the C3 molecule from its biogenesis, to its activation, to its degradation and effector functions provides numerous opportunities for intervention (Fig. 1). With the encompassing collection of structural information on C3, its various

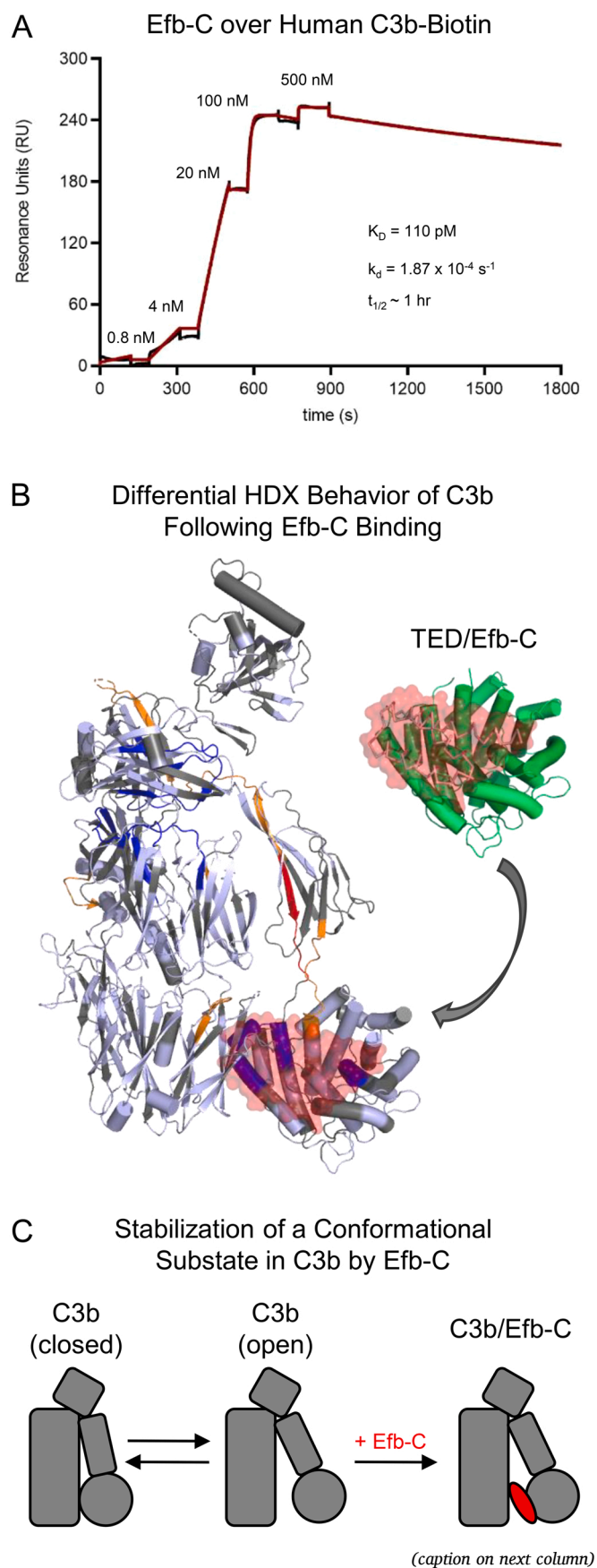


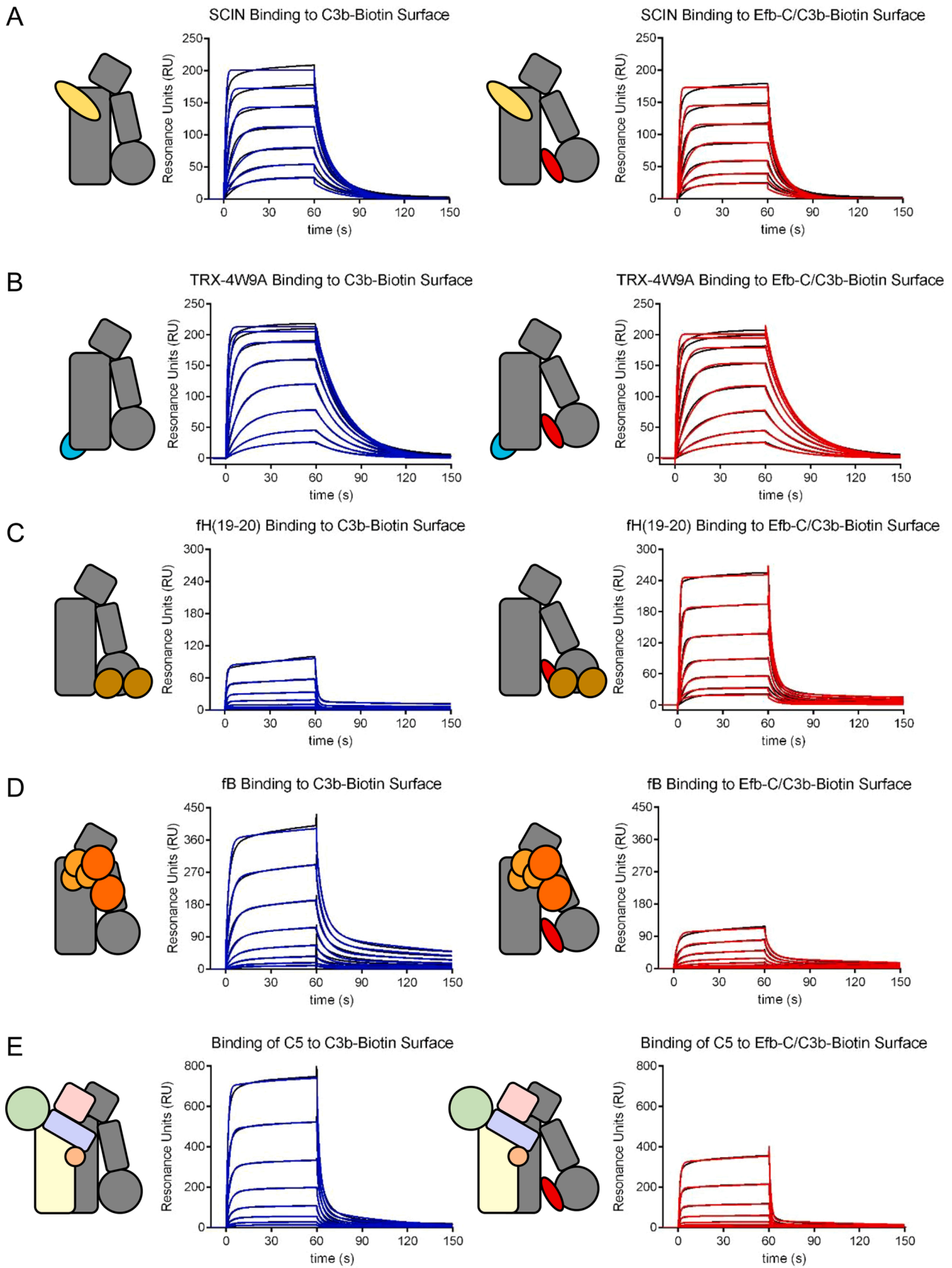
Fig. 7. Selective Stabilization of an Open Conformational State in C3b by the *S. aureus* Immune Evasion Protein Efb-C. (A) Characterization of Efb-C binding to site-specifically immobilized C3b as assessed by surface plasmon resonance. Human C3b that had been site-specifically biotinylated at its thioester cysteine [36,72] was captured on a streptavidin-coated biosensor surface prior to injecting increasing concentrations of Efb-C in a single-cycle kinetic study. The reference-corrected sensorgram is shown with a black line while the outcome of fitting to a Langmuir model is shown with a red line. Efb-C binding to C3b exhibits low-picomolar affinity ($K_D \sim 110 \text{ pM}$) and is kinetically stable as indicated by its dissociation rate constant ($\sim 10^{-4} \text{ s}^{-1}$) (inset). (B) Differential Hydrogen/Deuterium exchange mapping of C3b as a function of Efb-C. The Hydrogen/Deuterium Exchange (HDX) profile was characterized by Mass Spectrometry for samples of C3b in the presence of either Efb-C or a non-binding mutant [82]. Peptides from C3b that exhibited increased HDX are colored either orange (moderate increase) or red (highest increase), while those that exhibited decreased HDX are colored dark blue. Peptides from C3b that displayed no change are colored in pale blue, while peptides that were absent from the coverage map are colored grey. A full-scale representation of the structure of Efb-C bound to the TED domain from C3b [21] is inset at the top right of the panel, with Efb-C colored as a transparent red surface and TED colored green. Superposition of this structure onto the HDX map of C3b reveals that residues comprising the Efb-C binding site within the TED domain are highly exchange-protected, thereby demonstrating consistency between the crystallographic and in solution studies. (C) Simplified representation showing stabilization of an open conformational state in C3b upon Efb-C binding. As the interaction between Efb-C and C3b is ultra-high affinity and kinetically stable, Efb-C binding is depicted as an essentially irreversible step in this mechanism.

fragments, and their complexes described here (Table 1), the field seems well-positioned to leverage these insights not only for basic research purposes, but for investigating and developing new therapeutic concepts as well.

As always, charting the course forward will require careful considerations of the benefits and limitations of various approaches. Recurring structural themes, such as multiple types of inhibitors binding to the MG7 domain of C3b (Fig. 5), and the shared C3b-binding modes of naturally-occurring regulatory molecules (Fig. 3) [46], should definitely be taken into account. Future work should likewise consider information that can help identify those strategies likely to be well-tolerated from those that will not. In this regard, harnessing the endogenous regulators already found within the body seems prudent. Capitalizing on unexpected activities within these regulators, such as CR1g inhibiting the alternative pathway (Fig. 4) [26], or the molecular engineering of gain-of-function variants, such as mini-FH (Fig. 3) [52], or a recently described DAF-MCP chimera [48], are merely a handful of examples. Finally, the biological proof of concept provided by immune evasion proteins that have arisen through millennia of host-pathogen co-evolution is something that should not be dismissed. The observation that SCIN, Efb, and related proteins from *S. aureus* all target C3b-containing convertases is certainly not coincidental [71]. Any molecules that employ similar mechanisms and mimic their activities ought to be powerful complement inhibitors in their own right. Future work in this area seems particularly warranted, given the finding that Efb-C selects for a conformation of C3b that is deficient in both convertase formation and substrate recognition (Fig. 7 & 8). Although there are certain to be challenges ahead and there is no guarantee that any single approach will lead to success, the many opportunities highlighted above suggest that the next generation of C3-targeted therapeutics should not take nearly as long to develop as the first.

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(caption on next page)

Fig. 8. Certain Ligands Exhibit Conformational Dependence in Their Affinity for C3b. The binding of various ligands to either C3b alone (left panels) or C3b that had been presaturated with Efb-C and therefore trapped in the open state (right panels) was assessed by surface plasmon resonance. Reference-corrected sensorgram series are shown with black lines, while the outcome of fitting to kinetic models is shown with either blue (C3b alone) or red (Efb-C/C3b) lines. (A) Comparison of binding of the *S. aureus* immune evasion protein SCIN. (B) Comparison of binding of the fusion protein TRX-4W9A consisting of *E. coli* thioredoxin (TRX) and a minimal Compstatin-like sequence (4W9A) [88]. (C) Comparison of binding of CCP domains 19–20 of human FH. (D) Comparison of binding of human FB. (E) Comparison of binding of human C5. Whereas neither SCIN nor TRX-4W9A displayed sensitivity to the presence of Efb-C, FH(19–20) exhibited increased affinity for C3b in the presence of Efb-C, presumably due to increased exposure of its binding site within the TED domain. By contrast, the affinity of both FB and C5 were significantly diminished in the presence of Efb-C. This suggests that molecules which trap C3b in an open conformational state may be potent inhibitors of key aspects of complement function dependent upon FB/C3b and C5/C3b interactions.

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