

# Insights into complement convertase formation based on the structure of the factor B-cobra venom factor complex

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**Immune protection by the complement system critically depends on assembly of C3 convertases on the surface of pathogens and altered host cells. These short-lived protease complexes are formed through pro-convertases, which for the alternative pathway consist of the complement component C3b and the pro-enzyme factor B (FB). Here, we present the crystal structure at 2.2-Å resolution, small-angle X-ray scattering and electron microscopy (EM) data of the pro-convertase formed by human FB and cobra venom factor (CVF), a potent homologue of C3b that generates more stable convertases. FB is loaded onto CVF through its pro-peptide Ba segment by specific contacts, which explain the specificity for the homologue C3b over the native C3 and inactive products iC3b and C3c. The protease segment Bb binds the carboxy terminus of CVF through the metal-ion dependent adhesion site of the Von Willebrand factor A-type domain. A possible dynamic equilibrium between a 'loading' and 'activation' state of the pro-convertase may explain the observed difference between the crystal structure of CVFB and the EM structure of C3bB. These insights into formation of convertases provide a basis for further development of complement therapeutics.**

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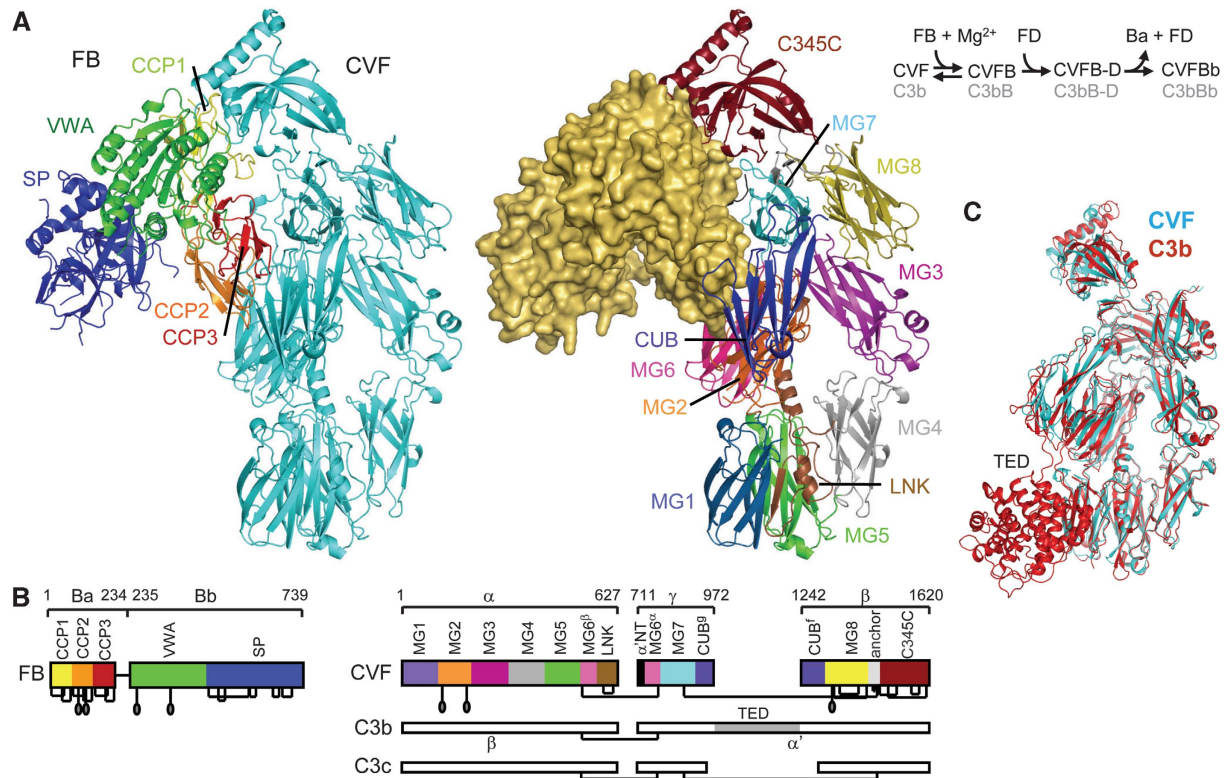
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## Introduction

The complement system is a key part of the innate and adaptive immune system and is critical for the resistance to infection and clearance of altered host cells. This intricate host defence system consists of over 30 plasma and cell-surface proteins that enables the host to recognize pathogens or immunogenic particles and eliminates them from the host's system (Muller-Eberhard, 1988; Walport, 2001). In the central step of the proteolytic cascade of the complement system, cells are covalently labelled, or opsonized, for B-cell stimulation, clearance by phagocytosis and cell lysis. On activation of the recognition pathways, protease complexes called C3 convertases form on the target cell surface that cleave and activate C3 into the large fragment C3b and a small fragment C3a that mediates inflammation (Walport, 2001). C3b molecules react indiscriminately with hydroxyls and hence bind covalently to the targeted surface, in which they act as labels for recognition by macrophages and B-cells (Muller-Eberhard, 1988). Two homologous surface-bound C3 convertases are formed. One through the antibody-mediated classical and lectin-binding pathways; and, one through the alternative pathway formed by C3b and pro-enzyme factor B (FB) that is used in the central amplification step of the complement response (Muller-Eberhard, 1988) (see Figure 1A).

Control over the activity of the complement system is of critical importance to the homeostasis of the organism and depends on formation and dissociation of the central convertases. Uncontrolled complement activity may lead to host tissue damage and is associated with several pathological conditions such as age-related macular degeneration, atypical haemolytic uraemic syndrome (aHUS) and rejection of transplants (Ricklin and Lambris, 2007). Recently, mutations in both C3 and FB have been associated with aHUS (Goicoechea de Jorge *et al*, 2007; Fremaux-Bacchi *et al*, 2008). On the other hand, lack of function, due to deficiencies or mutations in complement proteins, may predispose individuals to infectious diseases. Formation of the convertase complexes depends on a proteolytic assembly process, which starts with proteolytic activation of C3 into C3b. Next, FB binds surface-bound C3b forming the pro-convertase C3bB. When bound to C3b, FB becomes susceptible to proteolysis by factor D (FD). Cleavage by FD removes the pro-peptide fragment Ba (residues 1–234) and yields the active and labile convertase C3bBb (consisting of C3b and the protease fragment Bb (residues 235–739)), which amplifies the complement response by cleaving C3 into C3b (Muller-Eberhard, 1988). Similarly, the venom of the Indian cobra contains a C3 homologue called cobra venom factor (CVF) (49% identical in sequence to C3) (Fritzing *et al*, 1994), which is processed by proteases in the venom gland into a three-chain molecule, which has C3b-like activity and forms soluble convertases



**Figure 1** Structure of the CVFB complex at 2.2-Å resolution. **(A)** Ribbon representation of CVFB with FB coloured by domain and CVF coloured cyan (left) and of CVF coloured by domain with FB in wheat surface representation (right). The proteolytic assembly process of the C3 convertase is shown schematically. **(B)** Domain compositions, including disulphide bridges and glycan positions, of FB and CVF are indicated, together with the topology of C3b and C3c for clarity. **(C)** Comparison of CVF (cyan) with C3b (Janssen *et al*, 2006) (red) (see also Supplementary Table IIA).

(Vogt *et al*, 1974) (see Figure 1B). The CVF-containing convertases are far more stable (with a half lifetime of ~7 h) (Vogel and Muller-Eberhard, 1982) than C3bBb convertases (half lifetime of ~90 s) (Fishelson *et al*, 1984) and cleave C3 and in some instances also C5 to consume complement components of the prey (von Zabern *et al*, 1980). This prolonged convertase activity underpins the putative therapeutic use of humanized CVF in pathological conditions in which tissue damage may be prevented by complement depletion (Vogel and Fritzing, 2007).

Like C3bBb, the CVFB convertase assembles in two steps, which are (i) Mg<sup>2+</sup>-dependent binding of FB to CVF ( $K_d$  of 1 μM) (Hensley *et al*, 1986) and (ii) subsequent cleavage of FB by FD (Figure 1A). In recent years, crystal structures have been reported of C3b (Janssen *et al*, 2006; Wiesmann *et al*, 2006), pro-enzyme FB (Milder *et al*, 2007) and of the isolated fragment Bb (Ponnuraj *et al*, 2004). C3b consists of 12 domains (see Figure 1B). The structure of FB is formed by five domains, three N-terminal complement-control protein domains (CCP1–3; also called short consensus repeat or SCR), a Von Willebrand factor A-type (VWA) domain and a C-terminal serine protease (SP) domain. Mutagenesis and binding studies located putative binding sites for FB on the C345C domain and the α' chain N-terminal tail (α'NT) of C3b (Taniguchi-Sidle and Isenman, 1994; Kolln *et al*, 2005; Fritzing *et al*, 2009) and for C3b or CVF at or near the metal-ion dependent adhesion site (MIDAS) of the VWA domain and on the CCP domains of FB (Hourcade *et al*, 1995, 1999; Tuckwell *et al*, 1997; Hinshelwood *et al*, 1999;

Thurman *et al*, 2005). An allosteric model for the activation of the pro-enzyme FB was proposed based on <sup>1</sup>H NMR spectroscopy studies (Hinshelwood and Perkins, 2000a,b). The crystal structure of the pro-enzyme FB (Milder *et al*, 2007) allowed a more detailed hypothesis for FB binding to C3b or CVF and exposure of the scissile loop in FB for cleavage by FD. Putatively, binding of C3b or CVF to the Mg<sup>2+</sup> ion of the MIDAS in the VWA domain of FB relocates the CCP1–3 domains and the linker helix αL (which together form the Ba pro-peptide segment). Dislocation of helix αL putatively allows docking of the activation helix α7 of the VWA domain into its canonical groove as observed in the structure of Bb and related integrin inserted domains. In the pro-enzyme FB, the scissile bond (Arg234–Lys235) is partially occluded with the Arg234 (the P1 residue of the scissile bond) interacting with the activation helix α7. Alterations in the αL–α7 arrangement may disrupt this interaction leading to exposure of the scissile loop for FD cleavage. A very recent electron microscopy (EM) model of C3bBb at ~27-Å resolution is consistent with the predicted C3b–Bb binding sites and supports the rearrangement of the CCP1–3 domains (Torreira *et al*, 2009). However, details of the C3b–FB or CVF–FB interactions and possible induced structural changes are unknown. Here, we study the CVFB complex at 2.2-Å resolution to determine the CVF–FB interaction sites, the conformational state of the MIDAS and the associated allosteric changes, which addresses the composite roles of the multiple domains of CVF and FB that underlie convertase formation and activation.

## Results

### Structure determination of the CVFB complex

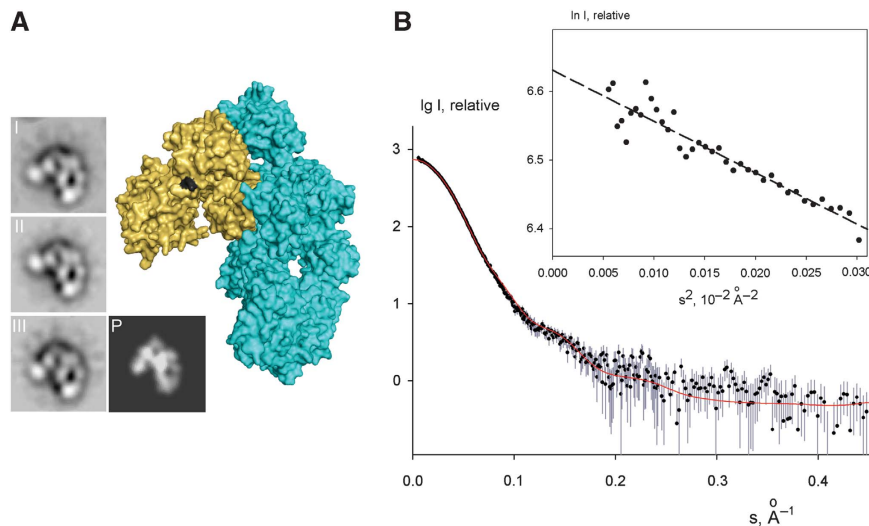
We determined the crystal structure of the pro-convertase complex using CVF (1266 residues) purified from cobra venom and recombinant human FB (739 residues). To enhance complex formation and increase the chances of successful crystallization, we used a double gain-of-function mutation (D254G/N260D) that increases stability of the pro-convertase (Hourcade *et al*, 1999) and eliminates the glycan moiety on N260 (see Materials and methods and Supplementary Figure 1; all amino acids are numbered according to mature, secreted protein thus excluding the 22 and 25 residue long signal peptides of CVF and FB, respectively). First, we solved the structure successfully at 8.5-Å resolution using glycosylated CVF; and, later at 3.0 and 2.2-Å resolution using deglycosylated CVF (Figure 1; Supplementary Figure 2; Supplementary Table I). Crystal structures were validated at low resolution by small-angle X-ray scattering (SAXS) and negative stain EM. SAXS data were collected on the CVFB complex consisting of glycosylated CVF and wild-type FB in solution (see Materials and methods and Figure 2). The experimental molecular mass of the solute ( $220 \pm 20$  kDa) agreed with the expected molecular mass of the complex (230 kDa), proving homogeneous complex formation in the samples. The radius of gyration of the crystal structure of CVFB accounting for the hydration shell (45.4 Å) matched the experimental SAXS value ( $45.8 \pm 0.5$  Å). Moreover, the computed SAXS curve from the crystal structure neatly fitted the measured scattering of CVFB indicating that the quaternary crystal structure is preserved in solution up to a resolution of ca 25 Å (Figure 2B). Furthermore, single-particle reconstruction using EM negative stained images of CVFB also correlated well with the crystal structures of CVFB (Figure 2A). Overall, the structure of the pro-convertase CVFB is characterized by an extensive interface that involves four out of five domains of pro-enzyme FB and five out of 11 domains of CVF.

### CVF structurally resembles C3b

The domain composition of mature, three-chain CVF falls in between that of the biologically active C3b (12 domains) and inactive C3c (10 domains) molecules. Mature CVF contains macroglobulin (MG) domains 1–8, a linker domain, a ‘complement C1r/C1s, Uegf, Bmp1’ (CUB) domain and a C-terminal C345C domain, but lacks a thioester-containing domain (TED). Overall, the 11 domains of CVF are arranged very similar to the corresponding domains in C3b (Janssen *et al*, 2006) and C3c (Janssen *et al*, 2005) (see Figure 1C). After submission of this study, the crystal structure of free CVF was published (Krishnan *et al*, 2009). The structures of free CVF and CVF bound to FB are very similar, with domain arrangements and conformations more similar between the two CVF structures than between CVF and C3b (Supplementary Table IIA). An exception is formed by the C345C domain, which has adopted an orientation closer to the CUB domain in free CVF (Krishnan *et al*, 2009). In the structure of FB bound CVF, this domain has an orientation more similar to that observed in structures of C3b. Reorientations of the CUB and C345C domains (Supplementary Table IIA) likely reflect inherent flexibility of the molecules (Janssen *et al*, 2007; Krishnan *et al*, 2009).

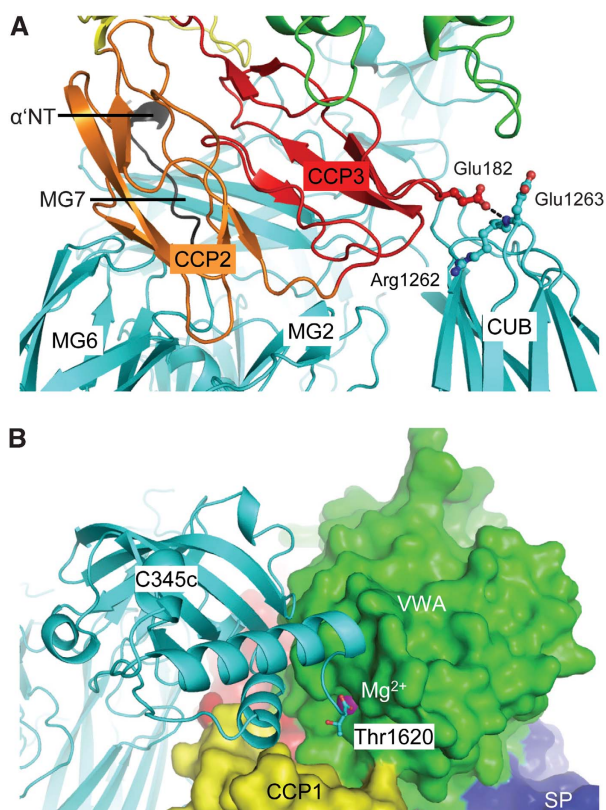
### Five domains of CVF contact FB

FB binds to the ‘top’ part of CVF, away from the cell-surface attachment site in C3b. Domains MG2, MG6, MG7, CUB, C345C and the  $\alpha$ NT region (using C3 chain names) of CVF form a concave ‘clasp’ that grabs around domains CCP1–3 and VWA of FB (Figure 1). This observation is supported by C3/CVF chimera studies in which more stable C3bBb complexes were generated by replacing the C345C domain of C3b with that of CVF (Kolln *et al*, 2005; Fritzing *et al*, 2009). In addition, mutagenesis studies in the  $\alpha$ NT of C3b that affect the ability of FB to bind to C3b underscore the role for the central  $\alpha$ NT in this interaction (Taniguchi-Sidle and Isenman, 1994). Proteolytic activation of C3 into C3b induces large



**Figure 2** The CVFB crystal structures correlate well with EM and SAXS of CVFB in solution. **(A)** EM class averages of the pro-convertase CVFB (I–III) correlate well with the crystal structure of CVFB, shown in surface representation (cyan and wheat, respectively, with the FB scissile bond black) and as a low-resolution projection (P). **(B)** The computed scattering curve of the 2.2-Å crystal structure of CVFB (red line) fitted with CRYSOLOG (Svergun *et al*, 1995) to the measured scattering data of 2 mg/ml CVFB (black dots with experimental errors) with good correlation ( $\chi^2$  of 1.2). The computed scattering curves of the 3.0-Å crystal structures of CVFB gave good correlation (both  $\chi^2$  of 1.2) with the measured scattering data as well (not shown). Inset; Guinier plot for CVFB from X-ray scattering.





**Figure 3** The CVFB interface consists of two patches. **(A)** Ribbon representation of CCP2–3 (coloured orange and red, respectively) of the Ba segment interacting with MG2, MG6, MG7, CUB (all coloured cyan) and  $\alpha'$ NT (coloured black) of CVF. Glu182 of FB interacts with Arg1262 and Glu1263 of CVF (ball-and-stick representation), which correspond to the first FI-cleavage site in C3b. **(B)** VWA of the Bb segment, shown in green surface representation, interacts with C345C of CVF shown in cyan ribbon representation. The C-terminus (Thr1620) of CVF binds to the  $Mg^{2+}$  ion (purple sphere) in FB.

rearrangements of  $\alpha'$ NT, MG7 and CUB (Janssen *et al*, 2005, 2006) that are required to form the observed FB-binding site, which explains that FB binds to C3b and not to native C3. Inactivation of C3b is caused by cleavages in the CUB domain by factor I (FI) yielding iC3b and finally C3c, which do not bind FB and cannot form convertases (Ross *et al*, 1983). The FB-binding site is virtually present in C3c except for the CUB domain, which is missing in C3c. Furthermore, FB contacts Arg1262–Glu1263 of the CUB domain in CVF, which correspond to Arg1281–Ser1282 in C3b that is the first scissile bond cleaved by FI when forming iC3b (Figure 3A). The structural data, therefore, indicate that pro-convertase formation depends on an arrangement of five domains in CVF or C3b with a critical role for an intact CUB domain, which is used in the regulation of complement activity.

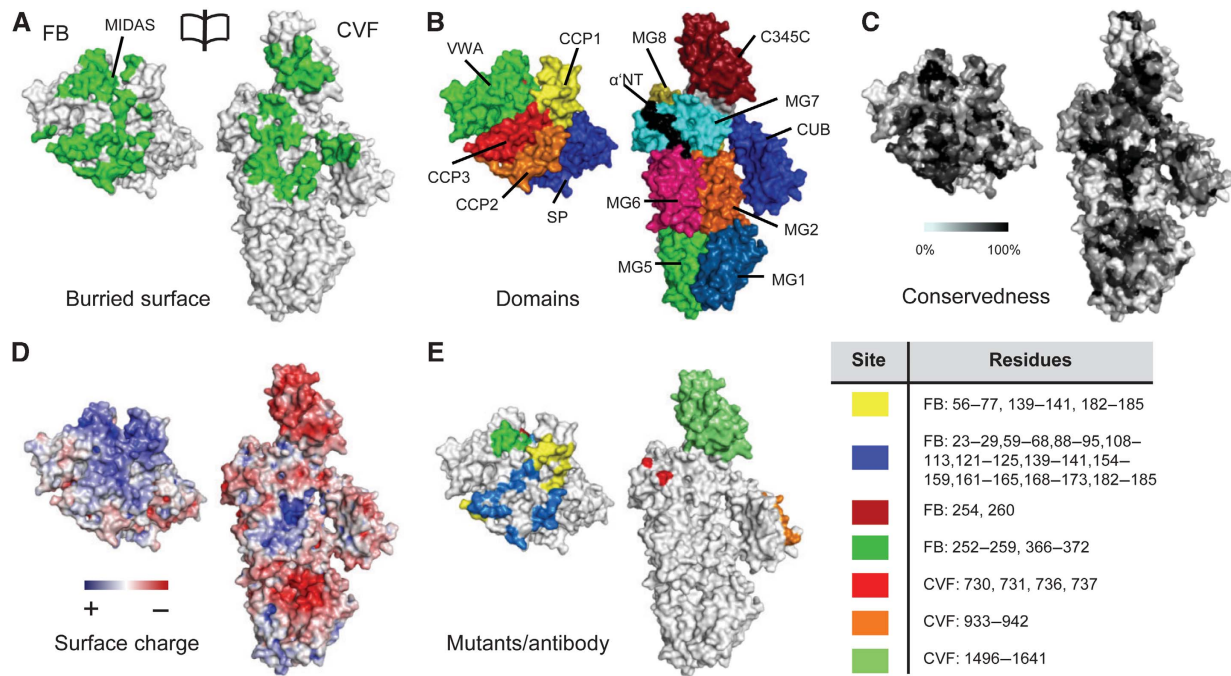
#### The FB interface consists of two distinct functional patches

The FB interface is divided into a large contact site formed by the pro-peptide segment Ba and a small contact site formed by the protease segment Bb ( $\sim 3600$  and  $\sim 1300$  Å<sup>2</sup> buried surface areas, respectively). The anti-parallel arranged CCP2–3 domains of the Ba segment contact  $\alpha'$ NT and MG2, MG6, MG7 and CUB domains of CVF (see Figures 3 and 4). This binding site includes epitopes of antibodies that block pro-

convertase formation (Hourcade *et al*, 1995; Thurman *et al*, 2005), and explains the effects of FB/C2 chimeras in which replacement of several short parts in Ba of FB with those of C2 increased the binding of FB to C3b (Hourcade *et al*, 1995) (Figure 4E). The orientation of CCP1 is variable in the structures determined at 3.0- and 2.2-Å; CCP1 contributes only 30–600 Å<sup>2</sup> buried surface area, respectively, to the CVF–B interface (Supplementary Figures 2 and 3). The Bb segment contacts the C345C domain of CVF through its VWA domain (Figure 3B). This is supported by previous biochemical and mutagenesis studies in the VWA domain of FB that identified the VWA domain to be involved in pro-convertase formation (Tuckwell *et al*, 1997; Hinshelwood *et al*, 1999; Hourcade *et al*, 1999). In contrast, no contacts are made to CVF by the SP domain of FB, as predicted earlier (Smith *et al*, 1982; Prydzial and Isenman, 1987). A positive charged patch on FB, centred on VWA, complements a negative charged patch on C345C of CVF, in an otherwise largely neutral interface (Figure 4D). In conclusion, the CCP domains of the Ba segment and the VWA domain of the Bb segment form two distinct functional interfaces with CVF in which Ba makes specific contacts that discriminate C3b from native C3 and inactive cleavage products iC3b and C3c and in which the VWA–C345C interface is likely important for the activity of the active convertase.

#### The FB MIDAS adopts a high-affinity state

On binding to CVF, the distorted MIDAS in free FB has rearranged into a canonical high-affinity ligand-bound state, as in fragment Bb (Ponnuraj *et al*, 2004) and the isolated VWA domain (Bhattacharya *et al*, 2004) (Figure 5). MIDAS residues Ser255 and Asp364 move up to 7.4 Å and together with residues Asp251, Ser253, Thr328 and two water molecules coordinate the  $Mg^{2+}$  ion. The COO<sup>−</sup> terminus (Thr1620) of CVF is the sixth chelating ligand of the  $Mg^{2+}$  ion (Figure 5A). Thus, the Bb segment binds CVF through its MIDAS, in which the carboxy terminus of CVF completes the coordination sphere of the  $Mg^{2+}$  ion. These details confirm the prominent role for  $Mg^{2+}$ -dependent MIDAS-mediated complex formation, which has been shown earlier by mutagenesis studies in which replacement of two MIDAS loops (252–259 and 366–372) of FB with those of C2 decreased the binding of FB to CVF (Tuckwell *et al*, 1997), by a combined affinity and mass spectrometry approach that identified two segments that contain the MIDAS (229–265 and 355–381) to be involved in pro-convertase formation (Hinshelwood *et al*, 1999), and by gain-of-function mutations (D254G and N260D) near the MIDAS of FB that increased stability of the pro-convertase (Hourcade *et al*, 1999) (Figure 4E). Furthermore, C3/CVF chimera studies underscore the role for the C345C domain in this interaction (Kolln *et al*, 2005; Fritzing *et al*, 2009) (Figure 4E). Reduction of steric hindrance explains the D254G gain-of-function mutation in FB. Deletion of the glycan in the N260D gain-of-function mutant possibly facilitates rotation by 163° and elongation of VWA helix  $\alpha 1$  that is coupled to the MIDAS loop rearrangements (Figure 5A). Similarly, mutation F261L, which is located in the refolding region of helix  $\alpha 1$ , may favour this rearrangement and hence enhance pro-convertase formation causing atypical haemolytic uremic syndrome (Goicoechea de Jorge *et al*, 2007). In conclusion, FB binding to CVF induces a local but functionally important rearrangement in the MIDAS and



**Figure 4** Surface representation of the CVFB interface coloured functionally. **(A)** An opened view of the 4900 Å<sup>2</sup> footprint of the FB–CVF interface is highlighted in green. **(B)** Domains of FB and CVF coloured according to Figure 1. **(C)** FB and CVF colour-coded to residue conservation; from non-conserved (white) to conserved (black). Figure is produced using CONSURF (Glaser *et al*, 2005). **(D)** FB and CVF coloured by electrostatic potential from red ( $-10 k_b T/e_c$ ) to blue ( $-10 k_b T/e_c$ ). The VWA:C345C interface consists of conserved complementary electrostatic patches. **(E)** Previously proposed sites involved in complex formation. The yellow coloured patches are epitopes to which antibody binding decreases complex formation (Hourcade *et al*, 1995; Thurman *et al*, 2005). The other patches are based on FB to C2 chimeras that increase binding of FB to C3b > 150% (Hourcade *et al*, 1995) (blue) or decrease binding < 10% (Tuckwell *et al*, 1997) (green); on C3 to CVF chimeras that increase C3bBb complex stability (Kolln *et al*, 2005; Fritzing *et al*, 2009) (lime); on an alternative proteolytic product of C3, that supports activation of FB (O’Keefe *et al*, 1988) (orange) or on single site mutants (single numbers) that increase complex formation (Hourcade *et al*, 1999) (dark red) or decrease complex formation (Taniguchi-Sidle and Iseman, 1994) (red). Legend for colour-coding and residue numbers are presented in the table. CVF residue numbering is according to human C3.

surrounding loops from a distorted to a high-affinity ligand-binding state.

#### CVF does not induce domain rearrangements in FB

We observe no further large conformational changes in FB on binding to CVF neither in the crystal structures (Figure 5B; Supplementary Table IIB), SAXS data or EM classes (Figure 2). Apparently, FB provides sufficient space for the C-terminal tail of CVF to access the MIDAS without dislocating the CCP1 domain. Correspondingly, helix  $\alpha L$  and  $\alpha 7$  do not relocate and the scissile bond 234–235 remains partially occluded, when FB binds CVF (Figure 5C). Detailed comparison of the VWA domain in CVFB and in free Bb (Ponnuraj *et al*, 2004) shows that activation of FB into Bb repositions helix  $\alpha 7$  into the groove previously occupied by helix  $\alpha L$ , away from the elongated helix  $\alpha 1$ . This additional rearrangement may further stabilize the high-affinity MIDAS configuration that is observed in Bb (and the isolated VWA domain (Bhattacharya *et al*, 2004)). Possibly the VWA domain of FB adopts this conformation in the active convertase CVFBb and C3bBb. This rearrangement may prevent release of Mg<sup>2+</sup> from C3bBb (in which the MIDAS is locked into a stable configuration) and allows Mg<sup>2+</sup> release and dissociation of C3bB or CVFB, when treated with EDTA (Harris *et al*, 2005). We observe no structural changes in the active site of the SP domain between free FB, the pro-convertase and free Bb (Supplementary Figure 4), indicating that enzyme activity is possibly controlled by quaternary changes in the enzyme

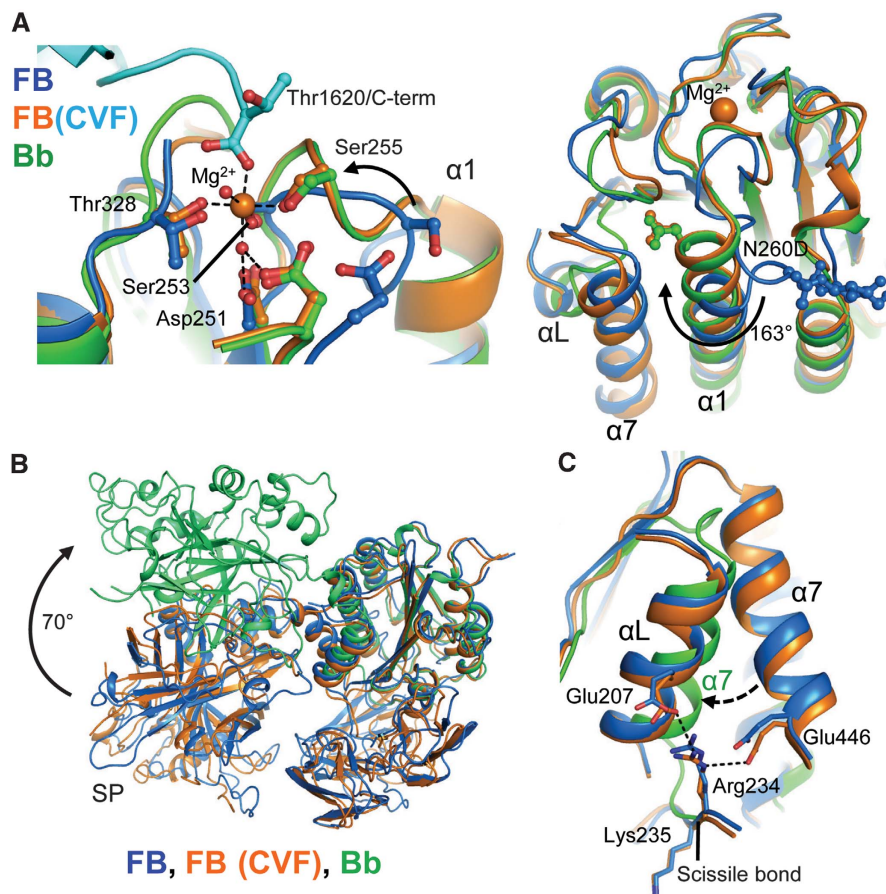
complex; that is by substrate (C3) binding to the convertase. Thus, except for the MIDAS and surrounding loops, FB binding to CVF does not induce major conformational changes in either FB or CVF.

## Discussion

The central amplification step of the complement system is crucial for the defence against invading pathogens and the homeostasis of the host. Lack of activity may result in recurrent bacterial infections, whereas unregulated activation may lead to tissue damage. Convertase formation and activity is, therefore, a tightly regulated process and reduced control, for example caused by mutations or deficiencies in proteins that form the convertase (C3b and FB) or in complement regulators that dissociate convertases, have been associated with several immune-related diseases (Ricklin and Lambris, 2007). Detailed understanding of the formation, activity and regulation of the convertase will be instrumental in the development of therapeutics to control complement related diseases. Here, we show for the first time in atomic detail the interactions that underlie pro-convertase formation.

Restricting the assembly of the convertase in place and time is an important regulatory mechanism of the complement system. FB only binds to the activated form C3b and not to C3, iC3b or C3c. The data presented here reveal that the Ba segment of FB determines this specificity. Segment Ba has specific interactions with  $\alpha'NT$ , MG7 and CUB, which

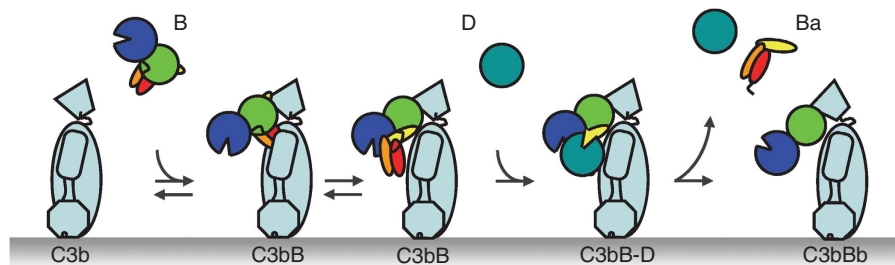




**Figure 5** Conformational rearrangements in the VWA domain of FB. (A) The MIDAS site in VWA rearranges from distorted in free FB (Milder *et al*, 2007) (blue) to a high-affinity  $Mg^{2+}$ -bound conformation in CVFB (cyan and orange) similar to free Bb (Ponnuraj *et al*, 2004) (green) (left panel). Helix  $\alpha 1$  elongates and glycan-linked Asn260 (mutated to Asp in CVFB) rotates  $163^\circ$  (right panel). (B) Comparison of free FB (blue), FB bound to CVF (orange) and free Bb (lime). (C) Helices  $\alpha L$  and  $\alpha 7$  and the Arg234–Lys235 scissile bond do not rearrange on FB binding to CVF. Arg234 remains hydrogen bonded to Glu207 and Glu446. In Bb  $\alpha 7$  has swapped with  $\alpha L$  that is removed. Colour scheme as in (A).

undergo conformational rearrangements in the activation of C3 to C3b (Janssen *et al*, 2005, 2006; Wiesmann *et al*, 2006). Complex formation depends on an intact CUB domain, which is degraded in the conversion of C3b to iC3b and C3c as shown structurally by EM (Nishida *et al*, 2006). The  $\alpha'$ NT has also been implied in binding complement regulators factor H (FH) and CR1 (CD35) to C3b (Weiler *et al*, 1976; Pryzdial and Isenman, 1987). This overlapping binding site for FB, FH and CR1 results in steric hindrance, which explains the observed competitive binding (Weiler *et al*, 1976; Pryzdial and Isenman, 1987). The proposed binding site for decay accelerating factor (DAF/CD55), identified by mutagenesis studies on helix 4 and 5 (Hourcade *et al*, 2002) and at aHUS-related residue K298 (Goicoechea de Jorge *et al*, 2007) located on the VWA domain of FB, is exposed in the complex. This is in line with the over 10-fold higher affinity of DAF for the C3bB complex compared with the individual components C3b and B (Pangburn, 1986; Harris *et al*, 2005). Both segments Ba and Bb contribute to the binding interface, but segment Ba provides 73% of the total  $\sim 4900 \text{ \AA}^2$  buried surface area and is apparently essential to load Bb onto CVF or C3b, as the Bb fragment itself cannot bind to either. Thus, although segment Ba itself is not part of the active convertase, it has a crucial function in its assembly and regulation.

Several studies have indicated that the CVFB and C3bB complexes are functionally similar (Vogt *et al*, 1974; Smith *et al*, 1982; Vogel and Muller-Eberhard, 1982; Vogel *et al*, 1984; Hensley *et al*, 1986). And although similarity was inferred from sequence homology (Fritzinger *et al*, 1994) we and others (Krishnan *et al*, 2009) also show in structural detail that CVF is very similar to C3b. It, therefore, seems likely that the structure of CVFB resembles that of C3bB. After completion of this study, a low-resolution ( $\sim 27 \text{ \AA}$ ) structure of negative stained C3bB was published (Torreira *et al*, 2009). In agreement with our high-resolution data, these EM data indicated that FB binds to the top part of C3b involving CUB, C345C and  $\alpha'$ NT of C3b and that C3b does not change conformation on binding of FB; however, the details of the C3b–FB interaction could not be resolved due to the low resolution of the EM images. In contrast to our observations on FB binding to CVF, Torreira *et al* show that FB undergoes a large conformational change on binding to C3b involving a relocation of the Ba segment towards CUB, although it was not possible to reveal the details of the conformational change nor could it be resolved whether the Bb part of FB undergoes a conformational change on binding to C3b (Torreira *et al*, 2009). Therefore, the X-ray and EM data of CVFB and C3bB indicate that there is a difference in



**Figure 6** Convertase formation and activation model. Schematic representation of the assembly of the pro-convertase complex and its subsequent activation by FD into the active C3 convertase (colour scheme as in Figure 1A, left panel).

conformation of FB when bound to CVF and bound to C3b. This difference in conformation is unlikely to arise from crystal packing events as the CVFB crystal structure is very similar to the structure determined by solution SAXS and EM studies. Possibly, this difference illustrates two functional different states of the pro-convertase structure, which may exist in a dynamic equilibrium.

In this equilibrium, the CVFB structure may represent the 'loading' state of FB to the cofactor, whereas the C3bB structure indicates the form that is activated for cleavage by FD. Activation of FB by FD occurs solely when FB forms a pro-convertase. In the CVFB structure, the scissile bond 234–235 has remained partially occluded similar to its position in free FB. In the C3bB structure, relocation of the Ba segment may have exposed the scissile bond and helix  $\alpha 7$  has possibly relocated to the position previously occupied by helix  $\alpha L$ . Unfortunately, these details could not be resolved due to the low resolution of the EM data (Torreira *et al*, 2009). Our data suggest that FB bound to CVF remains predominantly in the initial 'loading' state, whereas FB bound to C3b adopts more frequently an 'activation' state with an exposed scissile loop that can be cleaved by FD. In support of this hypothesis, we observe a much slower activation rate of FB by FD in CVFB than in C3bB (Supplementary Figure 5) and Harris *et al* demonstrate a conformation change in the C3bB complex in surface plasmon resonance experiments (Harris *et al*, 2005). Although a difference in the initial 'loading' of FB to CVF and C3b cannot be excluded at present, the data presented here and the observation that FB in the CVFB complex is very similar in domain arrangements to free FB indicate that FB is likely to bind initially in a similar manner to C3b as it binds to CVF. Thus, there may be two distinct, 'loading' and 'activation', states of the pro-convertase structure represented by the CVFB and C3bB structures. However, the details of the differences between the CVFB and C3bB structures can only be resolved with high-resolution data of the C3bB complex.

In conclusion, the CVFB complex indicates that the CCP domains of the Ba segment of FB provide a scaffold for the protease segment Bb to be loaded onto the C-terminal C345C of CVF or C3b in an  $Mg^{2+}$ -dependent manner (Figure 6). By binding to MG7, CUB and  $\alpha NT$ , the Ba segment determines the specificity of FB for CVF and C3b. Complex formation may be followed by a conformational change in FB in which the scissile bond is exposed and which enables FD to activate FB. Conversion of the pro-convertase into the active convertase releases the Ba fragment and affects the orientation of the SP domain (Torreira *et al*, 2009). Therefore, control over convertase activity is determined by pro-convertase complex formation and activation in which release of the Ba fragment

provides conformational freedom for C3 activation. These detailed structural insights into pro-convertase formation are instrumental to the development of novel therapeutic approaches that modulate this central step in the complement system.

## Materials and methods

### Protein expression and purification

CVF was purified from lyophilized Indian cobra (*Naja kaouthia*) venom as described (Vogel and Muller-Eberhard, 1984) including an additional final size-exclusion chromatography step. Purified CVF (7 mg/ml) was deglycosylated by incubation for 4 days at 37°C, in phosphate-buffered saline (PBS), 0.1% w/v azide, 10 mg/ml soybean trypsin inhibitor, 5 mM ethylenediaminetetraacetic acid, 5 mM benzamidine, 1 mM phenylmethylsulphonyl fluoride and 0.3 Units/ml endo-beta-N-acetylglucosaminidase F (Endo-F3). Deglycosylation of CVF does not have an effect on its activity (Gowda *et al*, 1994) but in our hands improves the quality of crystals considerably. As a final step, deglycosylated CVF was purified by size-exclusion chromatography. To promote a more stable pro-convertase and to enhance the rate of successful crystallization, we used a double gain-of-function mutant (D254G/N260D) of FB in which the glycan moiety on N260 is removed (Hourcade *et al*, 1999). The large and flexible glycan moieties present on glycoproteins are often detrimental to crystallization. In fact, deglycosylation of CVF was necessary to obtain crystals that diffracted to high resolution. The double gain-of-function mutant (D254G/N260D) (Hourcade *et al*, 1999) was fused to a N-terminal His-tag containing a TEV cleavage site, entirely sequenced to confirm a correct DNA sequence and expressed in N-acetylglucosaminyltransferase I (GnTI) deficient human embryonic kidney 293 cells that stably express EBNA1 (HEK293ES). The deficiency of GnTI results in production of homogeneous N-linked glycosylation (Reeves *et al*, 2002). FB was purified by metal-affinity chromatography as described (Milder *et al*, 2007). The His-tag was removed by TEV protease cleavage, followed by a second column passage on Ni-NTA superflow beads and a size-exclusion chromatography step. To verify the crystal structure of the CVFB complex and to validate that the (D254G/N260D) mutation in FB and the deglycosylation of CVF do not have an effect on the structure of the CVFB complex, we used wt FB and glycosylated CVF in solution SAXS and negative stain EM studies. Wild-type human FB was expressed in HEK293 cells stably expressing EBNA1 (HEK293E) (Durocher *et al*, 2002) and purified as described (Milder *et al*, 2007).

### Crystallization and data collection

Glycosylated CVF (14 mg/ml) and FB mutant D254G/N260D (11 mg/ml) were mixed at a molar ratio of 1:1 to a final concentration of 12 mg/ml (52  $\mu M$ ) and 10 mM Tris pH 7.4, 5 mM  $MgCl_2$  and 13 mM NaCl. Crystals were grown in sitting drops from mother liquor containing 12% w/v PEG-monomethylether 2000, 50 mM malic acid 2-(N-morpholino)ethanesulfonic acid tris (hydroxymethyl)aminomethane buffer (MMT) pH 6.8, at 30°C, to typical dimensions of  $80 \times 30 \times 20 \mu m$  within 2 weeks. For cryo-protection, 20% v/v glycerol was added to the mother liquor, and crystals were flash-cooled in liquid nitrogen. Crystals displayed space group  $P2_12_12_1$  ( $a = 129.8$ ,  $b = 134.0$ ,  $c = 291.8 \text{ \AA}$ ), contained two

complexes per asymmetric unit and diffracted to 8.5-Å resolution at European synchrotron radiation facility (ESRF) beamline ID23-2. Purified deglycosylated CVF (12 mg/ml) and FB mutant D254G/N260D (15 mg/ml) were mixed to a molar ratio of 1:1 and dialyzed against 10 mM Tris pH 7.4, 5 mM MgCl<sub>2</sub> and 10 mM NaCl resulting in a final concentration of 12 mg/ml (50 µM). This sample yielded two different crystal forms. Crystals with space group C22<sub>2</sub><sub>1</sub> ( $a = 128.9$ ,  $b = 283.5$ ,  $c = 134.4$  Å) were grown in sitting drops from mother liquor containing 6.6% w/v PEG 3350, 16 mM Bis-Tris propane pH 6.5, 66 mM Na/K Phosphate, at 18°C, to typical dimensions of 400 × 100 × 80 µm within 1 week. For cryo-protection, 30% v/v PEG 400 was added to the mother liquor, and crystals were flash-cooled in liquid nitrogen. These crystals contained one complex per asymmetric unit and diffracted to 2.2-Å resolution at ESRF beamline ID23-1. Crystals with space group P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub> ( $a = 134.0$ ,  $b = 137.0$ ,  $c = 283.7$  Å) were grown in sitting drops from mother liquor containing 8.25% w/v PEG 1500, 33 mM MMT pH 9.0, at 18°C, to typical dimensions of 200 × 60 × 40 µm within 1 week. For cryo-protection, 12% v/v 2,3 butanediol was added to the mother liquor, and crystals were flash-cooled in liquid nitrogen. These crystals contained two complexes per asymmetric unit and diffracted to 3.0-Å resolution at ESRF beamline ID23-1. All diffraction data was integrated and scaled with MOSFLM (Leslie, 1992) and SCALA (Evans, 2006) in CCP4 (CCP4, 1994).

### Structure determination

Initially the 8.5-Å resolution structure was solved by molecular replacement with PHASER (McCoy *et al*, 2007) using the isolated structures of C3b (Janssen *et al*, 2006) (pdb code: 2I07) with the TED domain omitted and FB (Milder *et al*, 2007) (pdb code: 2OK5) as the search models. Owing to limited resolution, only rigid body refinement (using 18 groups) was performed in REFMAC (Murshudov *et al*, 1997) resulting in  $R_{\text{work}}/R_{\text{free}}$  values of 34.4/34.9%. At a later stage, the 2.2-Å resolution structure was solved by molecular replacement in PHASER (McCoy *et al*, 2007) starting with the β-chain and MG6 part of the α-chain from C3b followed step-by-step by SP, VWA and CCP2–3 from FB and α'NT-MG7-anchor, MG8, CUB and C345C from C3b and CCP1 from FB, respectively, using molecular graphics in COOT (Emsley and Cowtan, 2004) with rigid-body refinement in PHASER. This partial model was re-built automatically by ARP/wARP (Perrakis *et al*, 1999) and completed by several cycles of manual rebuilding in COOT and refinement in PHENIX (Adams *et al*, 2002) to a final  $R_{\text{work}}/R_{\text{free}}$  value of 18.0/22.6%. The 3.0-Å structure was solved by molecular replacement in PHASER using the refined 2.2-Å structure and was completed by several cycles of manual rebuilding in COOT and refinement in PHENIX to a final  $R_{\text{work}}/R_{\text{free}}$  value of 18.9/24.3%. All molecular graphics figures were generated with pymol (W Delano; <http://www.pymol.org/>).

### EM and image classification

FB (from HEK293E cells) and CVF were mixed to a molar ratio of 1.2:1 and a final concentration of 1 mg/ml in PBS, 5 mM MgCl<sub>2</sub> and 1 mM NiCl<sub>2</sub> and incubated for 15 min at room temperature. The sample was diluted to 5 µg/ml in water and immediately applied to a freshly glow-discharged carbon layer supported by an EM grid and negatively stained with 0.75% uranyl formate as described (Ohi *et al*, 2004). Micrographs were recorded under low-dose conditions on a 4k × 4k CCD camera with a FEI Tecnai 12 transmission electron microscope operating at 120 kV and a magnification of 39 000 × resulting in a pixel size of 3.8 Å. A total of 10 943 particles were selected from the micrographs, respectively, using the program 'Boxer' from the EMAN software package (Ludtke *et al*, 1999). The

contrast transfer function of the microscope for each micrograph was estimated and corrected using XMIPP (Sorzano *et al*, 2004). The extracted particles were classified into 20 classes with the maximum-likelihood multireference refinement protocol in XMIPP as described (Scheres *et al*, 2008). The three most populous or representative classes are shown in Figure 2. The projection was generated from the crystal structure of CVFB after low-pass filtering to 25-Å resolution in EMAN (Ludtke *et al*, 1999).

### Small angle X-ray scattering

FB (from HEK293E cells) and CVF were mixed to a molar ratio of 1:1 in Tris-buffered saline and 5 mM MgCl<sub>2</sub> and incubated for at least 15 min at room temperature. Synchrotron X-ray scattering data from a 1 mg/ml and a 2 mg/ml solution of the CVFB complex were collected following standard procedures at the European Molecular Biology Laboratory (EMBL) beamline X33 (Roessle *et al*, 2007) (storage ring DORIS-III, Deutsches Elektronen-Synchrotron (DESY), Hamburg) using a Pilatus 500K detector (DECTRIS, Switzerland). To monitor for radiation damage, four successive 30 s exposures on the same sample were compared, and no changes were detected. The data were processed using PRIMUS (Konarev *et al*, 2003). The molecular mass of the solute was calculated by normalization against the scattering from a reference solution of bovine serum albumin. The SAXS curves were computed from the 2.2- and 3.0-Å crystal structures of CVFB by CRYSOLO (Svergun *et al*, 1995).

### Accession codes

Coordinates and structure factors of the CVFB complex have been deposited in the Protein Data Bank with accession numbers 3HRZ (2.2-Å resolution) and 3HS0 (3.0-Å resolution).

### Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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**Author contributions:** DCF purified CVF. LG cloned, expressed and purified FB and deglycosylated CVF. BJCJ crystallized the complex, determined and analysed the structures. PG, LG and DIS collected, processed and analysed the SAXS data. RIK and BJCJ prepared and tested the EM grids. RIK collected the EM data under supervision of AJK. BJCJ processed the EM data. RIK and BJCJ analysed the EM data. BJCJ, C-WV and PG conceived the project. BJCJ and PG wrote the paper.

## Conflict of interest

The authors declare that they have no conflict of interest.

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