THE EMBO JOURNAL

Manuscript EMBO-2009-71057

Insights into complement convertase formation based on the structure of the factor B-CVF complex

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Review timeline:

Submission date:	08 April 2009
Editorial Decision:	07 May 2009
Revision received:	04 June 2009
Accepted:	08 June 2009

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 07 May 2009

Thank you very much for submitting your research manuscript for consideration to The EMBO Journal editorial office. I received the comments of three scientists working on this topic that you will find enclosed for your information. Two of these clearly appreciate novelty and insight provided from the complex structure reported and have only minor comments on necessary modifications before publication. In contrast, ref#3 raises major issues related to experimental verification (we believe that stronger emphasizing published mutagenesis results might solve this issue), relationship to the recently published EM-structure of the FB/C3b complex and proper citation of precedent work. All in all, we would like to offer you the chance to carefully attend to these issues before returning a thoroughly revised version of your work. I also have to remind you that it is EMBO_J policy to allow a single round of revisions only, which means that the final decision on acceptance or rejection will depend on the content of the final version of your manuscript.

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The authors should be congratulated on a tour de force. A fascinating set of structures and analyses which should be rapidly published. The manuscript is clear, the experiments are elegant and the figures attractive.
One small note - it would nice to comment on how the face of the VWA that is buried in this complex relates to the face(s) earlier authors have implicated in interactions with decay acclerating factor.

Referee #2 (Remarks to the Author):

In their manuscript "Structure of factor B in complex with CVF gives insight into complement convertase formation" by Janssen et al., the authors report the crystal structure of the CVF in complex with factor B complemented by solution SAXS and negative stain EM studies. This work presents the first crystal structure of CCP containing proteins such as FB, FH and CR1 in complex with CVF, a molecule closely related to C3. The study provides insights into the formation of the pro-convertase complex, and the interactions between FB and CVF by analogy to C3bB explain the binding advantage of factor B towards C3b over C3, iC3b, and C3c. The authors compare their structure with earlier published EM structures of the C3bB complex and propose two functional states of the complex, namely the "loading" and "activation" state. Only in the "activation state" is the scissile bond of factor B exposed and susceptible to cleavage by Factor D.

The data presented are interesting and an important extension to already existing structural and biochemical work. The paper is well written, concise, and should be of interest to many researchers.

Minor comments:

-Abstract: "FB is loaded onto CVF through its pro-peptide Ba segment by specific contacts, discriminating C3b from native C3 and inactive products iC3b and C3c."
This sentence needs to be changed. FB is loaded onto CVF and not C3 - the statement here may only be correct by analogy.

-Section "CVF structurally resembles C3b": The crystal structure of CVF has been published recently; the authors need to reference this paper and should comment on differences/similarities between the two structures.

Krishnan et al. The Crystal Structure of Cobra Venom Factor, a Cofactor for C3- and C5-Convertase CVFBb, Structure, Volume 17, Issue 4, 611-619, 15 April 2009

-Figure 5B, legend: the color scheme does not match the colors used in the figure, please correct.

Referee #3 (Remarks to the Author):

Structure of factor B in complex with CVF gives insight into complement convertase formation, by Janssen et al

Overall comments
The manuscript describes structural determinations by crystallography, SAXS and EM of the complex between FB and CVF. My understanding is that mutagenesis studies are required to verify the key details of molecular interactions seen in the crystal structure. See the crystal structure of SCR-19-20 from complement factor H published by Jokiranta et al. in EMBO J. Mutagenesis is not reported here, thus it is not clear that the work is of sufficient quality for EMBO J. In addition, the authors have not referenced major prior work on FB, which by inference reduces the novelty of this manuscript.

The FB-CVF results are discussed in terms of the FB-C3b complex, which is indeed an important complex to have. However this is presented using arguments by analogy and not directly on experimental data on FB-C3b. See the quote page 7: "It therefore seems likely that the structure of CVFB resembles that of C3bB." There are differences with the 2009 PNAS paper (page 7). What is then the major impact or novelty of this work? How does knowledge of FB-CVF structure inform on FB-C3b, AMD, aHUS or transplant rejection? This was not so clear.

Major comments
The authors use SAXS and EM to justify their crystal structure (page 4, page 6). There are major issues with this. The SAXS fit in Figure 2B is 75% noise and proves nothing. No controls are reported to show that 100% complexes are formed for SAXS (the Kd of 1 uM is insufficiently low and predicts maybe 50-70% complex formation) and that any surplus of either FB or CVF was

removed. No linear Guinier plots are shown to prove absence of aggregation. No checks were performed for absence of radiation damage. How do the authors know that their EM structure is based on an intact complex that has not dissociated in vacuum or by staining? SAXS and EM are not high resolution methods - is mutagenesis not usually employed to verify crystal structures?

The authors have consistently not cited key prior references. There is a mass spec study in JMB in 1999 that already revealed the C3b binding site to be on VFA, so the authors are not the first to report this (middle page 5). Allostericism has already been demonstrated by NMR in JMB in 2000 by that same group, not first by Mildner et al 2007 or Harris et al 2005 (cf: top page 8), and this needs proper placing in context. Metal binding by that same group is also reported in JMB in 2000, but again not referenced (cf: bottom page 5). There is a well-known third crystal structure for C3b by Ajees et al 2006 which is different from the authors' structure but also shows a rearrangement and it is not clear which C3b structure is the more correct. It is more ethical to mention both and simply state that there is a controversy (top page 7).

Minor comments
SCR is a common abbreviation for what the authors call CCP and needs to be mentioned properly in the text for general readers.

1st Revision - authors' response 04 June 2009

Ref#3 raises major issues related to experimental verification (we believe that stronger emphasizing published mutagenesis results might solve this issue), relationship to the recently published EM-structure of the FB/C3b complex and proper citation of precedent work.

Published mutagenesis results have been more strongly emphasized; please see our response to Referee #3 point 1.

Precedent work of Hinshelwood and Perkins (1999, 2000a and 2000b) was added; see response to Referee #3 point 5.

The EM structure of C3bB was already discussed at several places in the text and in detail in the Discussion sections 3 and 4. To address this further we have added "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." to the abstract. We have now also added the comparison to the structure of free CVF that was published after submission of the current manuscript; see response to Referee #2 point 2.

Please check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

We rephrased the title for clarity to the non-specialist:
"Insights into complement convertase formation based on the structure of the factor BñCVF complex"
To clarify the abstract further the sentence
"The MIDAS adopts the canonical high-affinity ligand-binding state, whereas the helices L- 7 remain in the locked position with the scissile loop partially occluded as observed in free FB"
was replaced with
"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."

Responses to Referee #1:

1. One small note - it would nice to comment on how the face of the VWA that is buried in this complex relates to the face(s) earlier authors have implicated in interactions with decay acclerating factor.

To address this we have added to the second section of the discussion (page 7):
"The proposed binding sited 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 to the individual components C3b and B (Harris et al., 2005; Pangburn, 1986)."

Responses to Referee #2:

1. Abstract: "FB is loaded onto CVF through its pro-peptide Ba segment by specific contacts, discriminating C3b from native C3 and inactive products iC3b and C3c."
This sentence needs to be changed. FB is loaded onto CVF and not C3 - the statement here may only be correct by analogy.

Sentence was changed to:
"FB is loaded onto CVF through its pro-peptide Ba segment by specific contacts, which explain the specificity for the homologous C3b over the native C3 and inactive products iC3b and C3c."

2. Section "CVF structurally resembles C3b": The crystal structure of CVF has been published recently; the authors need to reference this paper and should comment on differences/similarities between the two structures. Krishnan et al. The Crystal Structure of Cobra Venom Factor, a Cofactor for C3- and C5-Convertase CVFBb, Structure, Volume 17, Issue 4, 611-619, 15 April 2009

We could not have referred to the paper by Krishnan et al. as it was only published while our manuscript was under review at EMBO Journal. We now have compared our data in light of this recently published structure. This analysis shows that the structure of free CVF fully supports our structure of CVF bound to FB, which further supports our hypothesis that complex formation does not induce large structural rearrangements in CVF.

We have included a detailed comparison between the two CVF structures in Supplementary Table IIA and changed the legend accordingly. To the section "CVF structurally resembles C3b" (page 4) we added:
"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"

3. Figure 5B, legend: the color scheme does not match the colors used in the figure, please correct.

In the legend to Fig 5B "to CVF (cyan) and free" was corrected to "to CVF (orange) and free"

Responses to Referee #3:

1. My understanding is that mutagenesis studies are required to verify the key details of molecular interactions seen in the crystal structure. See the crystal structure of SCR-19-20 from complement factor H published by Jokiranta et al. in EMBO J. Mutagenesis is not reported here, thus it is not clear that the work is of sufficient quality for EMBO J.

Throughout the manuscript it is pointed out how the crystal structure explains the results of previous work involving mutagenesis, C3/CVF hybrids, the identification of the binding sites (see also our response to Referee 3 point 3), and last but not least, the explanation of functional properties such as the discrimination of binding to C3b and CVF vs. C3, iC3b, and C3c, as well as the inability of Bb to re-associate with CVF or C3b.

We have more strongly emphasized published mutagenesis results:

- *To the section "Five domains of CVF contact FB" in Results was added "by C3/CVF chimera studies in which more stable C3bBb complexes were generated by replacing the C345C domain of C3b with that of CVF (Fritzinger et al., 2009; Kolln et al., 2005).*
- *In addition, mutagenesis studies in the iNT of C3b that affect the ability of FB to bind to C3b underscore the role for the central iNT in this interaction (Taniguchi-Sidle and Isenman, 1994)"*
- *to the section "The FB interface consists of two distinct functional patches" in Results was added "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)"*
- *to the section "The FB interface consists of two distinct functional patches" in Results was added "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 (Hinshelwood et al., 1999; Hourcade et al., 1999; Tuckwell et al., 1997)"*
- *to the section "The FB MIDAS adopts a high affinity state" in Results was added "which has previously been shown 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)."*
- *to the second section of the discussion was added "The proposed binding sited 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 to the individual components C3b and B (Harris et al., 2005; Pangburn, 1986)." See also referee #1 point1.*

2. In addition, the authors have not referenced major prior work on FB, which by inference reduces the novelty of this manuscript.

References to prior work on FB by Hinshelwood and Perkins (1999, 2000a and 2000b) have been added; see page 3, referee #3 point 5.

We do not think that these studies reduce the novelty of the current manuscript. The presented crystal structure of the CVF-FB complex provided detailed and comprehensive insights that could not be achieved by 1H NMR, infrared or CD measurements or modelling.

3. The FB-CVF results are discussed in terms of the FB-C3b complex, which is indeed an important complex to have. However this is presented using arguments by analogy and not directly on experimental data on FB-C3b. See the quote page 7: "It therefore seems likely that the structure of CVFB resembles that of C3bB." There are differences with the 2009 PNAS paper (page 7). What is then the major impact or novelty of this work? How does knowledge of FB-CVF structure inform on FB-C3b, AMD, aHUS or transplant rejection? This was not so clear.

Knowledge of the CVFB structure directly informs on C3bB as illustrated by the many functional mutations on C3b and FB that can be explained by the CVFB structure. This is already mentioned at several occasions in the text, for example:

- *Results section "Five domains of CVF contact FB": "This observation is supported by biochemical studies that identified the central iNT and the C345C domain as important sites for binding FB (Fritzinger et al., 2009; Kolln et al., 2005; Taniguchi-Sidle and Isenman, 1994)".*
- *Results section "The FB interface consists of two distinct functional patches": "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 (Hourcade et al., 1995) (Figure 4E)." and "In contrast no contacts are made to CVF by the serine protease (SP) domain of FB, as predicted previously (Pryzdial and Isenman, 1987; Smith et al., 1982)."

- *Results section "The FB MIDAS adopts a high affinity state": "These details confirm the prominent role for Mg2+-dependent MIDAS-mediated complex formation (Hinshelwood and Perkins, 2000b; Hourcade et al., 1999; Tuckwell et al., 1997); and, C3/CVF chimera studies underscore the role for the C345C domain in this interaction (Fritzinger et al., 2009; Kolln et al., 2005)." and "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 1 that is coupled to the MIDAS loop rearrangements (Figure 5A)".*
- *Discussion second section "The iNT has also been implied in binding complement regulators factor H (FH) and CR1 (CD35) to C3b (Pryzdial and Isenman, 1987; Weiler et al., 1976). This overlapping binding site for FB, FH and CR1 results in steric hindrance, which explains the observed competitive binding (Pryzdial and Isenman, 1987; Weiler et al., 1976)" ; and,*
- *Figure 4E and legend "Previously proposed sites involved in complex formation. The yellow colored 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 (Fritzinger et al., 2009; Kolln et al., 2005) (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 Isenman, 1994) (red)."*

To address this further published mutagenesis results have been stronger emphasized; please see our response to Referee #3 point 1.

The CVFB structure has direct relevance to AMD/aHUS as we described in results section "The FB MIDAS adopts a high affinity state" where a possible explanation for the aHUS related mutation F261L is given: "Similarly, mutation F261L, which is located in the refolding region of helix 1, may favour this rearrangement and hence enhance pro-convertase formation causing atypical haemolytic uremic syndrome (Goicoechea de Jorge et al., 2007)"

4. The authors use SAXS and EM to justify their crystal structure (page 4, page 6). There are major issues with this. The SAXS fit in Figure 2B is 75% noise and proves nothing. No controls are reported to show that 100% complexes are formed for SAXS (the Kd of 1 uM is insufficiently low and predicts maybe 50-70% complex formation) and that any surplus of either FB or CVF was removed. No linear Guinier plots are shown to prove absence of aggregation. No checks were performed for absence of radiation damage. How do the authors know that their EM structure is based on an intact complex that has not dissociated in vacuum or by staining? SAXS and EM are not high resolution methods - is mutagenesis not usually employed to verify crystal structures?

The concerns of the referee on the SAXS data have been addressed by adding more detail to the SAXS description. Specifically:

- *We present a revised version of Figure 2B which better demonstrates that the portion of the SAXS data up to s=0.25 (i.e. up to the resolution of 25 Angstroms, which contains information about the quaternary structure) is recorded with sufficient accuracy to reveal the absence of systematic deviations in the fit. This does prove that the overall crystal structure is conserved in solution. The legend of Figure 2B has been changed accordingly.*
- *To Results section "Structure determination of the CVFB complex" was added "The experimental molecular mass of the solute (220±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 ≈) matched the experimental SAXS value (45.8±0.5 ≈). 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 ≈ (Figure 2B)."

- *The SAXS data revealed the molecular mass of the solute compatible with a 100% complex formation indicating that no noticeable dissociation took place.*
- *A Guinier plot is added as insert in Figure 2B.*
- *Monitoring for radiation damage belongs to standard procedures of any synchrotron SAXS experiment and in our experiment no radiation damage was observed. To address this "To monitor for radiation damage, four successive 30 sec exposures on the same sample were compared, and no changes were detected." was added to the Materials and Methods section Small angle X-ray scattering.*

In the EM data the most populous and prominent classes are of the CVFB complex. This is shown in figure 2A where both the FB and CVF component are clearly visible in our EM classes of the CVFB complex. Our method of sample preparation for EM purposes is widely used and very suitable for imaging of complexes as described in Ohi et al. which is referred to in the section "Electron microscopy and image classification" in Materials and Methods.

Indeed SAXS and EM are not high resolution methods. To emphasise this, the sentence "Crystal structures were validated at low resolution by small-angle X-ray scattering (SAXS) and negative stain electron microscopy (EM)." was added to Results section "Structure determination of the CVFB complex". However in contrast to SAXS and EM our x-ray diffraction data of the CVFB crystal structure is of high resolution (2.2-≈). The structure of CVFB is in good agreement with published mutagenesis results which is extensively referred to throughout the manuscript; see referee #3 point 3 and additional emphasis on published mutagenesis results; see referee #3 point 1.

5. The authors have consistently not cited key prior references. There is a mass spec study in JMB in 1999 that already revealed the C3b binding site to be on VFA, so the authors are not the first to report this (middle page 5). Allostericism has already been demonstrated by NMR in JMB in 2000 by that same group, not first by Mildner et al 2007 or Harris et al 2005 (cf: top page 8), and this needs proper placing in context. Metal binding by that same group is also reported in JMB in 2000, but again not referenced (cf: bottom page 5).

We do not claim to be the first to observe the C3b binding site to be on the VWA domain of FB. Instead, these previously published data from mutagenesis and biophysical experiments support the observed crystal structure of the complex. Moreover, all these prior observations and models could now be placed into context of this high-resolution structure of a pro-convertase complex.

We added the reference to the mass spec study in JMB 1999 (Hinshelwood et al. 1999) to the third section of the introduction and the section "The FB interface consists of two distinct functional patches" in results (page 6). To further emphasize this point, we added the sentence "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 (Hinshelwood et al., 1999; Hourcade et al., 1999; Tuckwell et al., 1997)" to the section "The FB interface consists of two distinct functional patches" in results (page 5-6).

In Results section "The FB MIDAS adopts a high affinity state" (page 6) was added "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)"

We added "An allosteric model for the activation of the pro-enzyme FB was proposed based on 1H NMR spectroscopy studies (Hinshelwood and Perkins, 2000a; Hinshelwood and Perkins, 2000b)" to the third section of the introduction (page 3), and changed the following sentence to "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".

6. There is a well-known third crystal structure for C3b by Ajees et al 2006 which is different from the authors' structure but also shows a rearrangement and it is not clear which C3b structure is the

more correct. It is more ethical to mention both and simply state that there is a controversy (top page 7).

We have raised very serious concerns with respect to the data published by Ajees et al. in Nature 2006. Nature has published our concerns in their "Brief Communications Arising" section (Janssen et al Nature, 2007).

Narayana and Volanakis are co-authors of the Ajees paper. They are also authors of the new publication on the structure of free CVF (Krishnan et al, 2009). In that paper they do not refer to the Ajees paper. We prefer not to refer to the Ajees paper either.

7. SCR is a common abbreviation for what the authors call CCP and needs to be mentioned properly in the text for general readers.

We added "also called short consensus repeats or SCR" to section three of the introduction (page 3).

