THE EMBO JOURNAL

Manuscript EMBO-2009-71299

Structural basis for the preferential recognition of immature flaviviruses by a fusion-loop antibody

Mickael Cherrier, Barbel Kaufmann, Grant Nybakken, Shee-Mei Lok, Julia Warren, Christopher Nelson, Victor Kostyuchenko, Heather Holdaway, Paul Chipman, Beverly Chen, Richard J. Kuhn, Michael Diamond, Daved Fremont

Corresponding author: Michael Rossmann, Purdue University

Review timeline:

Submission date:	06 May 2009
Editorial Decision:	04 June 2009
Revision received:	13 July 2009
Accepted:	30 July 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 04 June 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three reviewers, whose comments are attached below. As you will see, all of them consider - to varying degrees - your structural insights into flavivirus binding to a weakly neutralizing antibody interesting and potentially important as a contribution to our understanding of antibody-mediated flavivirus neutralization. There are nevertheless also several concerns, regarding especially aspects of presentation and interpretation that would need to be addressed before eventual publication. In this respect, referee 3 particularly criticizes that the structural information has not been further exploited to derive and functionally test hypotheses on the mechanism of neutralization.

In light of the positive overall assessment, I would like to invite you to prepare a revised version of the manuscript in the spirit of the reviewers' comments and suggestions. Should you be able to adequately address the various points raised, we should be happy to consider a revised manuscript for publication. Please be however reminded that it is EMBO Journal policy to allow a single round of major revision only, and that it is therefore essential that you diligently answer to all the points raised at this stage if you wish the manuscript ultimately to be accepted. In any case, please do not hesitate to get back to us should you need feedback on any issue regarding your revision.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

———

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Cherrier et al. have determined the crystal structure of a complex made of the envelope protein E of West Nile virus (WNV) with the Fab fragment of a monoclonal antibody (E53) that had previously been shown to be broadly flavivirus cross-reactive and to recognize the fusion peptide loop at the tip of domain II of E. Furthermore, it had been found that the neutralizing activity of this antibody is dependent on the maturation state of virus particles. The crystal structure of this E-Fab complex allowed the identification of the precise contact site of E53 in E which involves several residues in the fusion loop and an adjacent loop that is linked by a disulfide bridge. The authors further determined cryo EM structures of immature WN and dengue viruses in complex with Fab E53, after discovering that this Fab was not able to form complexes with fully mature virions. Fitting the structures of E53-Fab and E into the cryo EM densities led to the important conclusion that only two of the three E molecules in the asymmetric unit of immature virions were able to bind the antibody. Similar fitting experiments revealed that the E53 binding site was completely inaccessible in mature virions, opening an interesting discussion as to how such an antibody could mediate virus neutralization.

Based on their data the authors propose that weakly neutralizing antibodies binding partially immature (and infectious) virions - but are unable to bind fully mature virions - could be involved in antibody dependent enhancement of infection, a phenomenon that is believed to contribute to severe disease forms (hemorrhagic fever and shock syndrome) in the course of secondary dengue virus infections. This is a new and interesting aspect related to flavivirus pathogenesis that also has a significant impact on the design of flavivirus vaccines. The study in general contributes to a structural understanding of the interplay of viruses with the immune system and highlights a previously unrecognized facet relating to the role of immature virions in viral immunopathogenesis.

Minor comments:

1. Fig.2: The designation AB loop is confusing to me. Does this conform to the generally used nomenclature for structures in the flavivirus E protein? Is this not part of the bc-loop?

2. The legend to Fig. 3 mentions dashed circles marking the positions where the E53 Fab is bound to the fusion loop. Such circles are visible in the Supplemental Fig. 1, but not in Fig. 3.

3. In the context of the inability of E53 to bind to mature virions, reference should be made to published literature which already demonstrated that flavivirus cross-reactive sites involving the fusion loop are inaccessible in mature virions.

Referee #2 (Remarks to the Author):

This manuscript describes structural studies on antibody interactions with flavivirus. Flaviruses such as West Nile (WNV), Dengue (DENV), yellow fever viruses are important human pathogens. These are enveloped RNA viruses. Although vaccines are available for some of the pathogenic flaviviruses such as yellow fever virus, tick-borne encephalitis virus, and Japanese encephalitis virus, none of these vaccines is approved for DENV and WNV. Vaccine development for the latter viruses is hampered because of the possibility of cross-reactive antibodies generated during primary infection, which constitute a significant portion of the humoral response and primarily recognize the conserved fusion loop of the capsid protein, enhance replication of heterologous strains. This manuscript addresses the structural basis of how a cross-reactive antibody (E53) interacts with flavivirus using both X-ray crystallographic and cryo-EM techniques. By determining the X-ray structure of the WNV E protein complexed with E53 Fab, authors provide atomic details of antibody-antigen interactions. Using cryo-EM studies on both WNV and DENV, they show that E53 antibody specifically interacts with immature flavirions. Based on the structural observations authors suggest:

1) In addition to inhibiting viral attachment E53 antibody may also be involved in preventing the structural transition from immature to mature virion. 2) During infection each virions may have variable distribution of the structural characteristics corresponding to mature and immature virions and that could be a mechanism for evading recognition by antibodies. 3) This also could be the reason for weak neutralizing activity of the cross-reactive antibodies such as E53 and the antibody-dependent enhancement (ADE) of infection.

The manuscript is written well with adequate experimental details and illustrations. The structural results are appropriately discussed in the context of the neutralizing activity of the cross-reactive flavivirus antibodies involved in ADE. The results are interesting with possible implications for vaccine development for flaviviruses.

Referee #3 (Remarks to the Author):

This manuscript describes a crystal structure of the FAb of the cross-reactive, weakly neutralizing antibody E53 in complex with the West Nile virus E glycoprotein. It also describes electron cryomicroscopy image reconstructions this FAb in complex with West Nile virus and dengue virus immature virions (virions with uncleaved prM). No image reconstructions of the FAb in complex with mature virions could be obtained.

Previous work (Nelson et al., PLOS Pathogens, 4 e1000060, 2008 and Oliphant et al., J. Virol. 80:12149, 2006) has demonstrated that E53 has interesting properties. It recognizes the E fusion loop and binds partly and fully mature flavivirus particles with equivalent affinity and, on a suitable cell substrate, can enhance the infectivity of either partly or fully mature particles. However, on other cell substrates, E53 neutralizes particles with a mixture of mature (cleaved) and immature (uncleaved) prM more efficiently than it neutralizes fully mature particles. (Fully immature particles are not infectious.)

The data in the current paper confirm that the antibody binds the fusion loop. They show that the E53 epitope is more solvent exposed on two of the three quasi-equivalent E molecules of immature flaviviruses than on any of the E glycoproteins of mature flaviviruses. Binding to E of mature flaviviruses in its known pre-fusion conformational state appears to be prevented by steric clashes.

The authors argue that neutralization by E53 must, therefore, be due to its binding to immature E on partially mature particles and thereby indirectly inhibiting fusion mediated by mature E.

The data are convincing that E53 only binds immature E in its known conformations and could not bind mature E in the conformation observed in currently available pre-fusion structures. However, the proposed mechanism of neutralization is only one of several possibilities and is not consistent with all known data on the properties of this antibody. Moreover, the inability to obtain electron cryomicroscopy evidence of E53 binding to mature particles does not prove that such complexes can not be formed.

The techniques used in this paper, electron cryomicroscopy and X-ray crystallography, provide static, averaged reconstructions of molecular structures. The contention in the manuscript that E53 cannot bind mature particles contradicts previously published data (Nelson et al., 2008). Flaviviruses almost certainly have dynamic structures with components that at least transiently break symmetry. Therefore, other mechanisms of neutralization, such as
(1) E53 neutralizing by binding transient, unobserved conformations of mature E that form more readily on partially mature particle or
(2) E53 binding a transient conformation of mature E that is present on both mature and partially immature particles but selectively neutralizing partially mature particle because fully mature particles are more efficient in entry
are not excluded by the structural data presented in the manuscript.

In summary, the data in this manuscript provide a significant contribution to our understanding of some fascinating observations on neutralization of flaviviruses by fusion loop-specific antibodies. However, a broader interpretation of the results, taking into account the limitations of the experimental techniques, is merited by the data.

Specific comments:

Fig. 1. The E53 epitope includes resides in the AB loop of domain II and in the fusion loop. The figure 1 legend indicates that residues in the epitope are depicted as green balls and also that the fusion loop is green but the rest of domain II is yellow. Because all of the residues in the epitope are depicted as green balls, it is difficult to distinguish which epitope residues are in the fusion loop and which are in loop AB. It would be helpful to maintain the color coding of the structure in the epitope residues indicated by balls.

Fig. 3a. The legend mentions that dashed circles mark the position of FAbs bound to molecules A and B. No dashed circles are apparent on the figure.

A table of crystallographic data collection and refinement statistics should be included in the Supplementary Information.

A PDB accession code is needed.

The title does not take into account conclusions that can be made from previously published work. The previous finding (Nelson et al., 2008) that E53 neutralizes partially mature flavivirus virions more efficiently than it neutralizes fully mature virions indicates that partially mature virions are infectious and have unique biological characteristics. The structural data in the current manuscript increase our understanding of these results but do not establish the biological role of partially mature particles - their role was established by the previous publicatioin. An alternative title could be:

"Structure of complexes between immature flaviviruses and an FAb that selectively neutralizes partially mature virions"

1st Revision - authors' response 13 July 2009

Referee #1 (Remarks to the Author):

The study in general contributes to a structural understanding of the interplay of viruses with the immune system and highlights a previously unrecognized facet relating to the role of immature virions in viral immunopathogenesis.

Minor comments:

1. Fig.2: The designation AB loop is confusing to me. Does this conform to the generally used nomenclature for structures in the flavivirus E protein? Is this not part of the bc-loop?

Indeed the labeling in the figure is incorrect. It should have been bc as pointed out by the referee. This has now been fixed both in the figure and in the text.

2. The legend to Fig. 3 mentions dashed circles marking the positions where the E53 Fab is bound to the fusion loop. Such circles are visible in the Supplemental Fig. 1, but not in Fig. 3.

The referee is correct. We had forgotten to add the dashed circles and the "A" and "B" molecular labels to this figure, although we did do this for the DENV equivalent figure in the Supplementary Figure 1. This has now been corrected.

3. In the context of the inability of E53 to bind to mature virions, reference should be made to published literature which already demonstrated that flavivirus cross-reactive sites involving the fusion loop are inaccessible in mature virions.

Presumably, the referee is referring to the paper by Nelson et al (PloS Pathology 4, e1000060). This is an excellent paper. We have added additional references to Nelson et al in the final paragraph of the introduction and in the final paragraph of the results section. The paper also refers to this paper in three other appropriate places. One of these references, in the second sentence of the discussion, explicitly refers to the point made by the referee. Thus, it is difficult to understand why the referee has made this comment.

Referee #2 (Remarks to the Author):

The manuscript is written well with adequate experimental details and illustrations. The structural results are appropriately discussed in the context of the neutralizing activity of the cross-reactive flavivirus antibodies involved in ADE. The results are interesting with possible implications for vaccine development for flaviviruses.

The referee does not have any specific comments or questions.

Referee #3 (Remarks to the Author):

The data are convincing that E53 only binds immature E in its known conformations and could not bind mature E in the conformation observed in currently available pre-fusion structures. However, the proposed mechanism of neutralization is only one of several possibilities and is not consistent with all known data on the properties of this antibody.

To the best of our understanding the proposed mechanism by which E can be poorly neutralizing is consistent both with our data and with Nelson et al 2008. Nelson et al (2008) write "..one potential mechanism for an increased stoichiometry of binding to prM-containing viruses is an increase in the accessability of epitopes on the E proteins when arranged as trimers associated with prM, relative to their accessability in the pseudo-icosahedral mature virus". That is exactly what we have now shown!
Our data directly demonstrate E53 binding to immature virus, consistent with Nelson et al who indirectly showed that E53 binds to immature virus.

Moreover, the inability to obtain electron cryomicroscopy evidence of E53 binding to mature particles does not prove that such complexes can not be formed.

Maybe the referee means that perhaps only a few of the theoretical possible E53 binding sites might be occupied, which would mean that the icosahedral averaging process used in the cryo EM reconstruction would essentially obscure evidence for the presence of occasional bound E53 molecules. We have modified the paper to indicate this possibility. However, we know from the structure that, were E53 to bind to the epitope as established crystallographically, the specific E protein would need to change its conformation to that found in the immature virus. This possibility, although improbable, was hinted at by Nelson et al who wrote "Alternatively, changes in E protein epitope accessibility associated with maturation may reflect dynamic aspects of virion structureÖ". Indeed we have shown that mature dengue virus can be quite dynamic in briefly exposing epitopes for the 1A1D-2 neutralizing antibody (Lok et al. Nat. Struct. Mol. Biol. 2008, 15, 312-317). If this were the case, then in essence E53 would be forcing the virus to return to a partially immature conformation, albeit lacking pr. In order to accommodate the referee we have now clarified that there is no way that E53 can bind to the mature conformation of the virus.

The techniques used in this paper, electron cryomicroscopy and X-ray crystallography, provide static, averaged reconstructions of molecular structures. The contention in the manuscript that E53 cannot bind mature particles contradicts previously published data (Nelson et al., 2008).

Nelson et al write that "…it remains uncertain how prM increases the accessibility of epitopes that are poorly accessible on the mature virus". In other words Nelson et al have not

established that E53 binds to the mature virus. Nevertheless, we have now put various caveats into the text (as stated above) concerning the possibility that some E53 Fab molecules might have bound to the virus but in so doing would have had to cause parts of the virus to return to the immature conformation.

Flaviviruses almost certainly have dynamic structures with components that at least transiently break symmetry.

We are anxious to applaud the referee that he has recognized that flaviviruses appear to oscillate widely about a mean. We established this fact (Lok at al NSMB 2008) clearly, although whether the virus loses its icosahedral symmetry during the dynamic breathing we are not sure.

Therefore, other mechanisms of neutralization, such as
(1) E53 neutralizing by binding transient, unobserved conformations of mature E that form more readily on partially mature particle or
(2) E53 binding a transient conformation of mature E that is present on both mature and partially immature particles but selectively neutralizing partially mature particle because fully mature particles are more efficient in entry are not excluded by the structural data presented in the manuscript.

While we admire the referee for his thorough exploration of possibilities, we do not fully understand either of his hypotheses. For instance, what does he mean by "transient". Maybe he thinks of the antibody binding and unbinding in rapid succession to a conformation that occurs in the mature virus for only a short time? Or does he mean that, once the antibody has captured one of the transient conformations, this structure is then locked and stable? Whichever might be the case, it is difficult to know what to do with these concepts as far as the available data is concerned. Nevertheless, we have now discussed the impact of dynamic motion on the binding of E53 to the mature conformation of the virus.

In summary, the data in this manuscript provide a significant contribution to our understanding of some fascinating observations on neutralization of flaviviruses by fusion loop-specific antibodies. However, a broader interpretation of the results, taking into account the limitations of the experimental techniques, is merited by the data.

As far as is possible we have added conditional phrases that mention more complex explanations, consistent with our observations.

Specific comments:

Fig. 1. The E53 epitope includes resides in the AB loop of domain II and in the fusion loop. The figure 1 legend indicates that residues in the epitope are depicted as green balls and also that the fusion loop is green but the rest of domain II is yellow. Because all of the residues in the epitope are depicted as green balls, it is difficult to distinguish which epitope residues are in the fusion loop and which are in loop AB. It would be helpful to maintain the color coding of the structure in the epitope residues indicated by balls.

We have modified the figure to differentiate the residues in the fusion loop and in the AB loop. Additionally, the "AB loop" reference to this figure in the text has been changed to "bc loop".

Fig. 3a. The legend mentions that dashed circles mark the position of FAbs bound to molecules A and B. No dashed circles are apparent on the figure.

Unfortunately we had forgotten to insert the dashed circles, although they were in the corresponding DENV results shown in the supplemental data. This has been corrected.

A table of crystallographic data collection and refinement statistics should be included in the

Supplementary Information

That is absolutely correct. A table has been included, provided as Supplementary Table I. We have also provided a contact table as Supplementary Table II.

A PDB accession code is needed.

That is also correct. The accession number has now been provided in the revised manuscript.

The title does not take into account conclusions that can be made from previously published work. The previous finding (Nelson et al., 2008) that E53 neutralizes partially mature flavivirus virions more efficiently than it neutralizes fully mature virions indicates that partially mature virions are infectious and have unique biological characteristics. The structural data in the current manuscript increase our understanding of these results but do not establish the biological role of partially mature particles - their role was established by the previous publicatioin.
An alternative title could be:
"Structure of complexes between immature flaviviruses and an FAb that selectively neutralizes partially mature virions"
We have modified the title slightly to "Structural confirmation of the biological role of partially immature flavivirions for infection".

Additional modifications:

(1) We have added one additional author (Beverley R. Chen) who was inadvertently omitted in the original submission.
(2) We have made some changes in the methods section describing the crystallographic refinement.
(3) We slightly modified the abstract to reflect the additional crystallographic analysis and to provide more mechanistic insight as to why the fusion-loop binding antibodies might enhance disease and affect vaccine development.

Decision letter 30 July 2009

Thank you for submitting your revised manuscript for our consideration. We have now received referee 3's comments on it (attached below), and I am happy to inform you that as there are no further objections from his/her side, we shall be pleased to accept your manuscript for publication in The EMBO Journal!

You will receive a formal acceptance letter shortly.

Yours sincerely,

Editor
The EMBO Journal

Referee 3 (comments to authors):

The original manuscript presented high quality and interesting structural data on the binding of a weakly neutralizing mAb to immature components of flavivirus virions. The data supported a model of neutralization of flaviviruses by binding to partly mature virions. In the original manuscript, alternative hypotheses that took into account the dynamic nature of viral particles were not fully considered. The revised manuscript addresses these concerns by briefly discussing alternative

hypotheses and modifying the title to better reflect the new contribution contained in the manuscript. Practical comments on figure labeling, inclusion of crystallographic statistics, and inclusion of database deposition information have also been addressed in the revised manuscript. The revised manuscript is a rigorous and substantive contribution to the literature on flaviviruses and mechanisms of viral neutralization.

