THE EMBO JOURNAL

Manuscript EMBO-2010-73687

Features critical for membrane binding revealed by DivIVA crystal structure

Maria A. Oliva, Sven Halbedel, Stefan M. Freund, Pavel Dutow, Thomas A. Leonard, Dmitry B. Veprintsev, Leendert W. Hamoen and Jan Löwe

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Review timeline:

Submission date:	15 January 2010
Editorial Decision:	05 February 2010
Revision received:	19 April 2010
Accepted:	22 April 2010

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 05 February 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments to the authors are shown below. As you will see the referees are generally positive about your work and would support its ultimate publication in The EMBO Journal after some revision. I would thus like to invite you to prepare a revised manuscript in which you need to address or respond to the referees' comments in an adequate manner. In particular it would be important to discuss your results and the publication by Stahlberg et al. (PMID: 15165232) on the (straight) "doggy bone" structures of a DivIVA mutant in more depth along the lines suggested.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

This paper presents a major advance in our understanding of the cell division protein DivIVA. It is based on separate crystal structures of the N- and C-terminal parts. The N-terminal domain is determined at high resolution. An in vitro analysis of membrane binding, including extensive mutagenesis, makes a strong case for the primary mechanism of membrane binding. It involves insertion of F17 into the lipid core, and interaction of R18 with the charged phosphates. The C terminus was more problematic, giving only low resolution crystals. These were, however, sufficient to visualize cylindrical structures corresponding to the individual helices of the coiled coils. The introduction of selenomethionine to form triangles was a clever way to localize the C-terminal aa's in the low resolution structure, and to pin down the overlap of two coiled coils at the center of the tetramer. There are still questions about how the structure recognizes the very slight curvature of the polar membrane, but the new structural information greatly advances the discussion. I enthusiastically recommend publication, and have only a few questions and suggestions for minor revisions.

1. In a previous study of DivIVA by EM, Stahlberg et al reported that they could not obtain expression of wild type DivIVA in E. coli, and had to rely on a mutant (which was functional in B. subtilis). The present authors apparently had no problem with expression, purification and storage. Readers might be interested in how the present study avoided the problems.

2. The previous EM structure is remarkably similar to that deduced here, with one exception. The "doggy bones" seen by EM are straight, while the x-ray structure is decidedly curved. The authors suggest that the "doggy bones" might be tetramers, but it does not seem that putting two of the curved x-ray structures together could produce the very thin and straight "doggy bone." One possible resolution is that the curvature might be induced by the crystal structure (see also point 6 below). Could the coiled coils of DivIVA be designed to have a directional flexibility that would permit the normally straight molecule to bend to accommodate the slight curvature of the polar membrane, and the more extreme curvature at the forming septum?

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Referee #2 (Remarks to the Author):

DivIVA is a conserved bacterial protein that plays an important role in localizing other proteins to the cell poles and to the division site, presumably through direct sensing of membrane curvature.

Here, Oliva at al. used a combination of X-ray crystallography and site directed mutagenesis to elucidate the structural mechanism by which DivIVA interacts with membranes and senses negative membrane curvature. The results show that the N-terminal domain of DivIVA forms a parallel coiled-coil structure, which is capped with two unique knotted loops. The exposed hydrophobic and positively charged residues are proposed to be essential for membrane binding. A low-resolution crystal structure of the C-terminal domain was also determined and a model of the full-length DivIVA protein is proposed. The work is of high technical quality and the findings presented in the manuscript are of significant general interest. However, there are several points that should be addressed before this manuscript is acceptable for publication.

1.The authors propose that the hydrophobic residues located at the tips of the DivIVA dimer (F17) insert into membrane while the positively charged residues bind to the membrane surface. However, additional biochemical experiments are required to confirm the mechanism of membrane interaction. The authors should use DPH anisotropy (see e.g. Saarikangas et al., 2009) to examine whether DivIVA indeed inserts into the lipid bilayer. If the DPH anisotropy experiments show that DivIVA indeed inserts into the membrane, the authors should also use the same assay to examine whether F17 is critical for the membrane insertion as now proposed in the manuscript.

2. The analytical ultracentrifugation experiments presented in the manuscript demonstrate that full-length DivIVA has a tendency to aggregate into higher-order structures than tetramers. Also, previous work by Stahlberg et al. suggest that full-length DivIVA forms large oligomers. Because the affinity of both N-terminal and C-terminal fragments as well as the full-length protein to membranes are quite low, it is possible that binding of DivIVA to membranes is facilitated by protein oligomerization to high-order complexes. Therefore, if F17 does not insert into the membrane bilayer (see above) the authors should test possible effects of the F17V mutation on DivIVA higher-order oligomerization by analytical ultra centrifugation.

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The manuscript by the Löwe group describes a thorough structure-function study of Bacillus subtilis DivIVA, a conserved protein in bacteria that localizes to the cell poles and septum. The authors present the crystal structures of the isolated N- and C-terminal domains of the protein. While the N-terminal domain was solved at high resolution and reveals a unique knotted loop structure important for membrane binding, the structure of the C-terminal domain forms a predominantly helical oligomer with intrinsic curvature. Although the resolution of the latter structure is extremely low, the authors benefited from the high solvent content in the crystals and from experimentally phased maps. Functional assays (in vitro and cell-based) corroborate the structural data, leading to a plausible model for DivIVA membrane interaction that deviates from a previously proposed mechanism. The studies are of high quality and technically sound.

Minor points:
1. In the low-resolution structure of the C-terminal domain of DivIVA, most of the methionine residues appear to be solvent exposed. This is a bit unusual for hydrophobic residues. Is it possible that the methionine side chains are involved in interactions with a membrane leaflet as well? Please comment.

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3. Page 12/13: In the discussion referring to an independent study in which a DivIVA mutant (G162K) was used, the authors speculate that the mutation could cause the "bone-like" structures of DivIVA observed in cryo-EM images. It should be straightforward to test this hypothesis by comparing the quaternary structure of the wild-type protein and the mutant in assays described in the present manuscript.

1st Revision - authors' response 19 April 2010

Reply to reviewers comments (19th April 2010)

EMBOJ-2010-73687
Oliva et al., "Features critical for membrane binding revealed by DivIVA crystal structure"

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To be honest, we have had no real problems with the expression of the full-length and mutant proteins, as described in the manuscript. In fact, in our endeavor to crystallise DivIVA, we have expressed many dozens of DivIVAs from other species with equally little problems. The proteins have been electrospray masspec'ed and give deviations form predicted masses (minus Met1) of less than 2 Daltons, repeatedly, so mutations can pretty much be excluded (and of course all plasmids have been sequenced). The expressing cells grow fine and show a very minor filamentous phenotype when induced (as is normal for T7 systems). The only problem we encountered is proteolysis, if not careful during purification, and this can actually be seen in Figure 1C as a slight double band for the full-length protein. In the smaller band, due to proteolysis, the protein loses its last 4 amino acids, but is not affected in any of the assays presented. This is presumably because those residues are not part of the ordered structure as reported in the manuscript. No changes.

2. The previous EM structure is remarkably similar to that deduced here, with one exception. The "doggy bones" seen by EM are straight, while the x-ray structure is decidedly curved. The authors suggest that the "doggy bones" might be tetramers, but it does not seem that putting two of the curved x-ray structures together could produce the very thin and straight "doggy bone." One possible resolution is that the curvature might be induced by the crystal structure (see also point 6 below). Could the coiled coils of DivIVA be designed to have a directional flexibility that would

permit the normally straight molecule to bend to accommodate the slight curvature of the polar membrane, and the more extreme curvature at the forming septum?

We agree that flexibility of the structure reported here is a distinct possibility and this is now better reflected in the manuscript in several places. The whole curvature might just come from crystal packing, although coiled coils are actually quite stiff since bending would require sliding of the residues against each other. We did try to image the tetramers using EM ourselves in search for an independent answer but the molecule is very small and without additional contrast (for example using cryo-negative staining as used in Stahlberg et al, which has its own problems) it is almost impossible to see. Shadowing is another option but again, DivIVA is only 30 nm long so this would be a very difficult task indeed. Several changes, including figure legends and discussion.

3. The sequence alignment in Fig. 1b is far too small to be legible. This should be moved to SI and made large enough that it can be read, maybe in landscape format.

This figure is meant to be schematic and the individual sequences are not to be read. The figure shows that the alignment breaks DivIVA into blocks, interrupted by occasional insertions and one such insertion was used to define the domain boundaries between the N-terminal and C-terminal domain, in conjunction with the coiled-coil prediction. No change to the figure, but improved legend.

4. The Nt dimer is described as "a knotted dimer." It doesn't look like a knot to me, since if you pulled on the two c-c's they would just slip apart. I believe the DNA field has pretty specific terminology for knots. I have seen a protein structure where the polypeptide had an overhand knot, but this is rare. I would suggest that the authors reconsider the use of the term knot.

We concede. We used 'knot' according to the looser, everyday definition. The stricter, topological definition does not allow the use of the term 'knot' if two open ends are involved (as there are here) since pulling on the two ends will always get them apart if friction is not considered. We have replaced 'knot' with 'crossed loops' in the text.

5. In Fig. 5 the colors are confusing. In 5D the colors change completely for the 45 deg rotation. I would strongly suggest keeping them the same. Also, the colors should be the same in 5A (they seem to correspond to those in the top 5D).

Apologies for the confusion. The two colour schemes are meant to show two things: the 5D, top (and 5A) rainbow colour scheme shows the parallel nature of the coiled coil, the 5D, lower chain-based scheme shows the tetramer nature of the whole assembly. Kept as is, but added an explanation to the figure and legend.

6. It seems there must be a substantial overlap of the N-termini of the coiled coils to make the circles, since six molecules are too long to fit in the 880 Å; circumference. Some comment on the structure of this overlap would be welcome (although recognizing that this is a crystal packing, probably not possible in the full length molecule).

The extent of the overlap can be seen in figure 5A, right. Overlap starts appox. at the beginning of the coiled coil (around residue 72) and ends around residue 90, hence 20 amino acids overlap. We think it is unlikely that this is one of the interactions of the full-length protein with itself when it oligomerises since the N-terminal domain is missing from the construct. It cannot be excluded, though.

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Apologies, it is Figure 6B. Changed.

8. I would suggest putting the crystallography data (Table 1) as SI.

We would normally agree to this but because of the low-resolution data and phasing we would like to keep it in the main text. The 7 Å phasing worked so beautifully that we would like to keep this evidence in the main part. No change.

Referee #2:

DivIVA is a conserved bacterial protein that plays an important role in localizing other proteins to the cell poles and to the division site, presumably through direct sensing of membrane curvature. Here, Oliva at al. used a combination of X-ray crystallography and site directed mutagenesis to elucidate the structural mechanism by which DivIVA interacts with membranes and senses negative membrane curvature. The results show that the N-terminal domain of DivIVA forms a parallel coiled-coil structure, which is capped with two unique knotted loops. The exposed hydrophobic and positively charged residues are proposed to be essential for membrane binding. A low-resolution crystal structure of the C-terminal domain was also determined and a model of the full-length DivIVA protein is proposed. The work is of high technical quality and the findings presented in the manuscript are of significant general interest. However, there are several points that should be addressed before this manuscript is acceptable for publication.

1. The authors propose that the hydrophobic residues located at the tips of the DivIVA dimer (F17) insert into membrane while the positively charged residues bind to the membrane surface. However, additional biochemical experiments are required to confirm the mechanism of membrane interaction. The authors should use DPH anisotropy (see e.g. Saarikangas et al., 2009) to examine whether DivIVA indeed inserts into the lipid bilayer. If the DPH anisotropy experiments show that DivIVA indeed inserts into the membrane, the authors should also use the same assay to examine whether F17 is critical for the membrane insertion as now proposed in the manuscript.

Although we feel that such detailed analysis goes beyond the scope of the current study, we have attempted the suggested experiment to use the fluorescent hydrophobic membrane dye DPH to investigate perturbations of the hydrophobic core of the membrane upon DivIVA binding. We re-purified BsDivIVA WT and F17A and added it in increasing amounts to lipid vesicles after the addition of DPH at a ratio of 0.002-0.0002 X of the lipid molarity, as suggested by the mentioned paper and other related reports. The resulting binding curves show K_D values around 15 µM for both WT and F17A. The surprising explanation is that the same binding curve is also measured without lipid, with essentially the same affinity of 15 µM! Our conclusion is that DPH binds quite tightly to the protein and this signal dominates the experiment. Unfortunately, this makes the suggested experiment useless, as much as we would have liked this to work because it would have been a nice addition. Nevertheless, we are confident that our interpretation given in the manuscript is strong without this additional data but we agree that ultimately direct prove for the membrane insertion of F17 is something that needs to be addressed in future research.

2. The analytical ultracentrifugation experiments presented in the manuscript demonstrate that full-length DivIVA has a tendency to aggregate into higher-order structures than tetramers. Also, previous work by Stahlberg et al. suggest that full-length DivIVA forms large oligomers. Because the affinity of both N-terminal and C-terminal fragments as well as the full-length protein to membranes are quite low, it is possible that binding of DivIVA to membranes is facilitated by protein oligomerization to high-order complexes. Therefore, if F17 does not insert into the membrane bilayer (see above) the authors should test possible effects of the F17V mutation on DivIVA higher-order oligomerization by analytical ultra centrifugation.

We found that the WT, full-length protein is predominantly a tetramer (as is stated in the manuscript). There is some irreversible formation of aggregates (in the low percent range) but we are not confident that this is functionally relevant (in the absence of lipid). We therefore believe the point raised is not very important. F17A shows exactly the same behaviour and is a tetramer. As a side note: D4A aggregation behaviour as seen in AUC is very salt dependent. Below 100 mM NaCl much more aggregation can be seen. It is currently unclear to us how relevant this is in cells and therefore this is not discussed in the text.

3. The data presented in Figs. 3B and 4D should be quantified to allow better interpretation of the results.

The images shown are truly representative of field views and we believe show very clearly the effects. Quantification may prompt the reader to put too much emphasis on the quantities when the assay is only used to indicate qualitative differences.

4. Page 14, line 27. As the BAR field has advanced tremendously in recent years, and thus a more recent review should be cited here (e.g. Frost et al., 2009).

Done. Many thanks!

5. Page 14, lines 29-30, "For BAR-like domains, lipid interactions can be single residue interactions". At least to my knowledge this is not the case, and the sentence should thus be modified.

We agree after some more discussions with local experts in the field. Modified.

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Modified, we hope it is clearer now.

Referee #3:

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Minor points:
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This is an interesting idea! Clearly, more mutagenesis is needed. We did perform some mutagenic analysis already, using the in vivo assays described here, selecting residues based on conservation (such as 130, 133, 136 and others) but the Met residues were not amongst them. We feel this needs much more work.

2. The authors comment on the observation that the GFP-tagged proteins may show stronger membrane binding than the untagged proteins, possibly due to the oligomerization behavior of GFP. Although feasible, an experimental confirmation would make this point stronger (by directly comparing the oligomerization state of the proteins and/or by using tags do not dimerize).

So far, we have not been able to get to the cause of the discrepancy. We do not think that a detailed analysis of the effect of GFP tags will help us much further in this case, but what is now desirable, and this is somewhat connected to the previous point, is the need for a thorough investigation of the oligomerisation and membrane binding behaviour of the full-length protein. However, this goes beyond the scope of the current study.

3. Page 12/13: In the discussion referring to an independent study in which a DivIVA mutant (G162K) was used, the authors speculate that the mutation could cause the "bone-like" structures of DivIVA observed in cryo-EM images. It should be straightforward to test this hypothesis by comparing the quaternary structure of the wild-type protein and the mutant in assays described in the present manuscript.

The previous work was not cryo-EM (no stain), it was cryo-negative stain. We have not performed the suggested experiment since we cannot really see why we would want to look at a randomly

mutated protein and its behaviour. The Stahlberg study finds the same tetrameric quarternary structure of the mutant protein using centrifugation as we find for the WT protein, so we do not think we can add very much to that. In terms of imaging, all sorts of things can happen when using negative stain since saturated ammonium molybdate solutions (!) were used to introduce contrast. I agree, a careful study without stain would be desirable but the protein is very/too small for that but this is another project in itself.

