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Structure of the Legionella effector AnkX reveals the mechanism of phosphocholine transfer by the FIC domain

Valérie Campanacci, Shaeri Mukherjee, Craig R. Roy and Jacqueline Cherfils

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Editor: David del Alamo

1st Editorial Decision 17 September 2012

Thank you for the submission of your research manuscript to our editorial office and please accept my apologies for the delay in responding. I have read and considered your study on the background of the related literature and discussed its suitability from the scope of The EMBO Journal within our editorial team.

We certainly appreciate that your study describes for the first time the structure of the FIC domain of AnkX, both in its apo form and bound to its substrate and its reaction products. This leads you to propose the enzymatic mechanism by which AnkX transfers phosphocholine to hydroxyl groups in Rab proteins and itself. This being said, it has been very well established, that in contrast to other FIC-containing proteins. AnkX transfers phosphocholine instead of other substrates. Furthermore, structural analyses for other FIC domains exist and the overall structure of the domain, the configuration of the active site and the enzymatic mechanism proposed are essentially identical to those of AnkX. With these precedents in mind, I am afraid that your manuscript does not provide the kind of major conceptual advance that would justify its publication in The EMBO Journal and we have decided not to proceed with the review process.

Please note that we subject to external review only a small percentage of the manuscripts that we receive. I am sorry to disappoint you on this occasion and I hope for the rapid publication of your study somewhere else.

Additional Correspondence 24 September 2012

I'm writing to request that you kindly reconsider sending our manuscript to external reviews, for the following reasons.

We agree that there has been previous reports showing that AnkX is a FIC motif protein and that it acts as a phosphocholinator. However, the two major published papers (in Nature 2011 and in the EMBO Journal 2012) raised the issue that it may be working according to another mechanism, such that what the FIC motif is really doing was becoming unclear. This is because there are very few FIC proteins with known functions, and none of them has been crystallized with its unprocessed diphosphate substrate.

We think our contribution is therefore important for different reasons.

By solving the crystal structures of AnkX with CDP-choline, CMP and CMP phosphocholine, and analyzing its mutants, we establish of course how this important Legionalla enzyme works. We also show for the first time how the eukaryotic ankyrin motif found in Ank proteins has been co-opted by bacteria to be used as an intra-molecular scaffold rather as an inter-molecular scaffold as could have been expected. These findings are important for microbiologists working on Legionella and other pathogens.

Another important aspect of our work is that it establishes that the FIC motif is purely catalytic but carries no element of specificity. To strengthen this finding, we provide important data that refine the catalytic mechanism and clarify controversial issues. The mechanism we propose is significantly more elaborate than proposed in previous publications and should be general to all FIC enzymes, hence very useful to reasearchers investigating enzymes of this family.

Finally, I would like to emphasize that our structures show for the first time how diphosphate-containing substrates are recognized by FIC enzymes. This involves two well separated binding sites, one of which, which recognizes the leaving group, had been completely overlooked in previous work. With our series of structures, it becomes clear that the high level of variations seen in the active sites of FIC-motif enzymes reflects that they can recognize and process various substrates. Investigating what these substrates are, hence what the function of FIC enzymes is, should greatly benefit from information about the binding sites provided by our structural data.

For all these reasons, we believe that the structural and biochemical information that we report in this manuscript should provide a strong background for researchers working in the very active field of FIC enzymes from both prokaryotes and eukaryotes.

I would be be happy to discuss this in more detail over the phone if you wish, and I am open to trying to emphasize these aspects more strongly in our manuscript.

Additional Correspondence 02 October 2012

Thank you for your e-mail and my apologies for the delay in responding as I was out of the office last week.

I have considered the arguments presented in your rebuttal letter and have also sought external expert advice to further inform my decision and I am glad to say that I have decided to re-consider your study and send it out for peer-review.

I will contact you again as soon as I receive the comments from the referees.

Thank you very much for your patience and for giving us the opportunity to evaluate your work.

2nd Editorial Decision 19 October 2012

Thank you for your patience while your manuscript has been reviewed. We have just now received the reports from the two referees that were asked to evaluate your study, which I copy below. As you will see from their reports, they agree in the high interest of your study and their comments are therefore rather positive. However, some major -mainly technical- concerns will need to be addressed before the manuscript can be accepted for publication.

In brief, both referees agree in the interest and quality of your structural work, but are less impressed by the complementary biochemistry experiments that support some of your conclusions. In this regard, although referee #1 considers that your proposed new role for Ankyrin repeats could constitute a "paradigm-setting" finding, s/he also considers that the evidence supporting your conclusions is not sufficient and suggests several ideas to address this issue. Referee #2, in turn, also believes that your study describes an excellent structural work, but s/he points out to several major concerns regarding your functional assays that will also need to be addressed.

Given the referees' recommendations, I would like to invite you to submit a revised version of the manuscript. Please be aware that your revised manuscript must address the referees' concerns. I would also like to remind you that it is 'The EMBO Journal' policy to allow a single round of revision only and that, therefore, acceptance or rejection 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 iniciative, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

Please, do no hesitate to contact me in case you have any further question, need further input or any problem arises during the revision process.

REFEREE REPORTS

Referee #1

In the manuscript by Campanacci, et al., the authors describe the crystal structure of a 480 amino acid fragment of the L. pneumophila AnkX protein which catalyzes the phosphocholination of Rab1. The protein has a FIC domain, which is primarily associated with adenylation of targets, and a series of ankyrin repeats, normally associated with protein-protein interactions. The structure allows explanation for how the FIC homology sequence can promote the phosphocholination reaction, because the authors were able to determine structures for both the CDP-Choline substrate-bound forms as well as proteins having the hydrolysis products.

The structure is beautiful and the identification of what may be the transition state allows a good explanation for why phosphocholine is transferred, with the nucleotide in an inverted position relative to the adenyltransferase structure. Of course in retrospect this makes perfect sense (it always does, even when things are not obvious before data is obtained), since the transferred groups are in similar places in both adenylation enzymes and phosphocholination enzymes. The results here are unequivocal, and there is experimental evidence that backs up some of the most important points regarding the transfer reaction.

I do have real issues, however, with their treatment of the ankyrin repeats and ad hoc declarations that they act to scaffold the FIC, CMP and insert domains. This is a fairly radical model for how ankyrin repeats work and is unprecedented in the literature, which of course potentially makes this a paradigm-setting result. However, in spite of several declarations of this point, they don't provide structural evidence for this, and they don't provide experimental evidence either. First, the manuscript should state what the surface area is between the ankyrin repeats and these other domains. Secondly, there should be a panel devoted to showing what they believe are the critical interface residues in the Ank repeats that scaffold the other domains. Third, they should state why in the absence of the Ank repeats they think the structure should be destabilized. For instance, are hydrophobic interfaces being exposed? Finally, it seems certain that the authors must have tried to construct a derivative that is missing the 4 ank repeats. Is it unstable? If it is stable, is it protease sensitive, or has it lost either autocatalytic activity or the transferase activity? These are the minimal bits of data needed to support this interesting model.

Two minor points:

1) The authors give no rationale for why they chose the residues to mutate that they did. This should be explained.
2) Page 8: the statement that the "results unequivocally establish that AnkX autophosphocholination occurs in trans," is hyperbole. They have demonstrated that it "can" act in trans, but I don't see the evidence that it cannot act in cis.

Referee #2

Campanacci and colleagues present very interesting structures of the Fic protein AnkX, a phosphocolinator of the Rab1 GTPase. AnkX contains a Fic domain and multiple ankyrin repeats, both domains found in eukaryotes. Thus far, Fic proteins have been shown to use ATP (VopS, IbpA, others), UTP (AvrAC), and CDP-choline (AnkX) substrates. The structures presented in this paper offer insight into why AnkX uses CDP-choline rather than ATP for a substrate.
The paper presents three important structures: apo-AnkX, Anx captured with the substrate CDP-choline, and active AnkX with the cleavage products CMP and phosphocholine. Using these structures, the authors are able to offer several structural insights about Fic enzymatic activity by AnkX compared to other Fic proteins. Insights about the potential for the ankyrin repeats to constrain the intramolecular interactions of the Fic domain, the importance of the "CMP" and "insert" domains in giving the protein its specificity for CDP-choline, the roles of individual residues in catalyzing the phospho cleavage, and the differences between Fic active sites are all well presented in figures 1, 3, 4 and 5. Each of these figures provides novel information about Fic domain catalyzed reactions and substrate specificity.
Unfortunately, the attempts to validate the information gained from the structures with biochemistry in Figure 2 and S3 are poorly controlled and either misinterpreted or uninterpretable. This leaves the paper somewhat lopsided and theoretical, with a great structure and analysis, but little practical data supporting hypotheses derived from the structural data.

Major Concers:
1. Figure 2A, S3. Cannot draw conclusions about important residues for Rab1 labeling, as labeling of Rab1 is abrogated in EVERY mutation. Authors must show negative controls with proteins that have nearby mutations that do NOT abrogate Rab1 labeling. Also, ssumptions are made about the mutants with a loss of auto-phosphocolination in that they are folded properly and that only the mutation alters activity.
2. Figure 2A, S3. Some of the bars shown in 2A do not match the eye test when looking at the blot in S3 (for example F107G band on top gel in S3 is nonexistent, but is supposed to be nearly as strong as the F107G band in bottom gel when looking at bars in 2A). Authors need error bars for this data if they want to display it as a histogram. Also, how was 2A calculated? Densitometry is assumed, but detail must be given in methods. Was this experiment done in triplicate? If so, where are the statistics and error bars? Were R237E, D265A, F311G, and the d110-179 mutants in S3 a separate exposure? Where is the WT band that they were compared to for the densitometry? Surely they were not compared over different exposures. FigS3 is lacking molecular weight markers and either a stain or blot to show loading. There is reduced autoactivity with F107 and D265 but not with

E226. This data was grouped together and misdescribed. Separate into panels with each gel having positive and negative controls (in triplicate).
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4. The title is somewhat awkward. Why not "Mechanism of phoshpocholine transfer by Ankx, the ankyrin repeats and FIC domain containing Legionella effector"
5. Heading on page 5: CDP-choline binding region explains substrate preference, not transfer of phosphocholine.
6. Fig S1 legend title ?? 1-484 not 1-184.
7. Add cyan to Hairpin in FigS1D so there is a common focal point in Fig S1B, C, D.
8. Page 7 change " ... were impaired in the absence of Mg2+." to ".. were impaired by the addition of EDTA."

1st Revision - authors' response 17 February 2013

Referee #1

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The structure is beautiful and the identification of what may be the transition state allows a good explanation for why phosphocholine is transferred, with the nucleotide in an inverted position relative to the adenyltransferase structure. Of course in retrospect this makes perfect sense (it always does, even when things are not obvious before data is obtained), since the transferred groups are in similar places in both adenylation enzymes and phosphocholination enzymes. The results here are unequivocal, and there is experimental evidence that backs up some of the most important points regarding the transfer reaction.

We thank the review for these encouraging and positive comments. This was not an easy structure to solve, and we were also struck by the clarity of how the structure explained why this FIC-domain protein functions as a phosphocholine transferase rather than a nucleotide transferase.

I do have real issues, however, with their treatment of the ankyrin repeats and ad hoc declarations that they act to scaffold the FIC, CMP and insert domains. This is a fairly radical model for how ankyrin repeats work and is unprecedented in the literature, which of course potentially makes this a paradigm-setting result. However, in spite of several declarations of this point, they don't provide structural evidence for this, and they don't provide experimental evidence either. First, the manuscript should state what the surface area is between the ankyrin repeats and these other domains.

We thank the reviewer for this recommendation. As stated above, the surface area is now included in the analysis.

Secondly, there should be a panel devoted to showing what they believe are the critical interface residues in the Ank repeats that scaffold the other domains.

This panel has been included in a new supplementary figure (Figure S3), in which the intramolecular contacts between the ankyrin repeats and the FIC sub-domains are listed.

Third, they should state why in the absence of the Ank repeats they think the structure should be destabilized. For instance, are hydrophobic interfaces being exposed? Finally, it seems certain that the authors must have tried to construct a derivative that is missing the 4 ank repeats. Is it unstable? If it is stable, is it protease sensitive, or has it lost either autocatalytic activity or the transferase activity? These are the minimal bits of data needed to support this interesting model.

As mentioned above, before attempting to crystalize AnkX, we did limited proteolysis experiments on full-length AnkX and found the smallest stable domain containing the FIC domain always included the ankyrin repeats. These data were essential in determining the minimal AnkX construct that could be purified and crystallized. We have attempted to express a smaller derivative lacking the ankyrin domains in *E. coli,* and no product could be detected after induction, which supports the notion that the ankyrin repeats are needed for stability.

Two minor points:

1) The authors give no rationale for why they chose the residues to mutate that they did. This should be explained.

We appreciate these comments and have modified the manuscript to try and to clarify our reason for selecting mutants and what we are testing using these mutants.

We now focus on residues predicted to be involved in enzyme catalysis based on the mechanism of phosphoryl transfer by other FIC proteins, and demonstrate that these same residues are needed for phosphocholine transfer by AnkX. Thus, there is a conserved mechanism of phosphoryl transfer by FIC motifs. This is now shown in Figure 3B.

We dedicate another section to investigating other residues that would be predicted to facilitate substrate binding, and show that these mutants also display phosphocholine transfer defects. This is now shown in Figure 3A.

2) Page 8: the statement that the "results unequivocally establish that AnkX autophosphocholination occurs in trans," is hyperbole. They have demonstrated that it "can" act in trans, but I don't see the evidence that it cannot act in cis.

The question we were trying to address in these experiments was whether auto-phosphocholinated AnkX represented an intermediate in the enzymatic reaction. Although we believe we had evidence that auto-phosphocholination could occur in trans, we agree that our data did not rule out the possibility that it can also occur in cis. In light of the reviewer comments, we realized our original experiments could be improved and the approach we used to address this question was changed.

Specifically, we wanted to design an experiment that better addressed whether the auto-phosphocholinated AnkX protein represents an intermediate in the phosphocholine transfer reaction, where the phosphocholine is attached to the catalytic histidine residue. Toward this end we used the purified $AnkX_{1-484}$ protein, and removed all epitope tags so that the protein being assayed does not have any other domains attached. Our data using this construct show clearly that this protein is enzymatically active, and can transfer phosphocholine onto Rab1 in vitro. However, the $AnkX_{1-484}$ protein is not auto-phosphocholinated. The data are shown in Figure 3D.

Thus, the autophosphocholinated species detected when using larger constructs or constructs that still have large tags such as MBP or GST attached is clearly not an intermediate in the phosphocholine transfer reaction, which means that auto-phosphocholination likely represents transfer of the phosphocholine group to another domain in the protein via a reaction that requires a functional FIC motif, and this can occur either in trans or in cis.

Referee #2

Campanacci and colleagues present very interesting structures of the Fic protein AnkX, a phosphocolinator of the Rab1 GTPase. AnkX contains a Fic domain and multiple ankyrin repeats, both domains found in eukaryotes. Thus far, Fic proteins have been shown to use ATP (VopS, IbpA, others), UTP (AvrAC), and CDP-choline (AnkX) substrates. The structures presented in this paper offer insight into why AnkX uses CDP-choline rather than ATP for a substrate.
The paper presents three important structures: apo-AnkX, Anx captured with the substrate CDP-choline, and active AnkX with the cleavage products CMP and phosphocholine. Using these structures, the authors are able to offer several structural insights about Fic enzymatic activity by AnkX compared to other Fic proteins. Insights about the potential for the ankyrin repeats to constrain the intramolecular interactions of the Fic domain, the importance of the "CMP" and "insert" domains in giving the protein its specificity for CDP-choline, the roles of individual residues in catalyzing the phospho cleavage, and the differences between Fic active sites are all well presented in figures 1, 3, 4 and 5. Each of these figures provides novel information about Fic domain catalyzed reactions and substrate specificity.
Unfortunately, the attempts to validate the information gained from the structures with biochemistry in Figure 2 and S3 are poorly controlled and either misinterpreted or uninterpretable. This leaves the paper somewhat lopsided and theoretical, with a great structure and analysis, but little practical data supporting hypotheses derived from the structural data.

We thank the reviewer for the constructive comments and fully agree with the reviewer that the biochemical studies needed improvement. In response to the reviewer's concerns, we have improved what we believe are the experiments central to studying the mechanism of phosphoryl transfer by the FIC motif in AnkX, which for the most part were made apparent by the structure and conform with other studies examining the key catalytic residues in FIC motifs that would mediate phosphoryl transfer. Thus, we believe the biochemistry provided now validates the key predictions drawn from the structure.

We would like to point out that we do not address the mechanism by which AnkX engages Rab1, which would require solving the structure of a co-crystal of Rab1 in a complex with AnkX. We can speculate from the structure of AnkX and the structures of other FIC-domain proteins bound to small GTPases, how this might occur and what residues might be important for binding to Rab1; however, we have removed reference to potential Rab1-binding determinants from the results section given that we have no biochemical Rab1-binding data or structural data to support direct interactions between these residues and Rab1 at this time.

Major Concerns:
1. Figure 2A, S3. Cannot draw conclusions about important residues for Rab1 labeling, as labeling of Rab1 is abrogated in EVERY mutation. Authors must show negative controls with proteins that have nearby mutations that do NOT abrogate Rab1 labeling. Also, assumptions are made about the mutants with a loss of auto-phosphocolination in that they are folded properly and that only the mutation alters activity.

We agree with the reviewer that these data were not sufficient to suggest that there were specific residues involved in the Rab1 interaction and this has been corrected. However, we feel that these data fully support that phosphoryl transfer by AnkX requires the key catalytic residues in the FIC motif, which were predicted from the structure and from other studies showing these residues participate in phosphoryl transfer by other FIC-domain enzymes. We have no indications to suggest that the mutations affecting active site residues in the FIC-motif have affected folding, and our data are consistent with other studies indicating these residues are fundamental in the phosphoryl transfer reaction. Thus, we feel the conclusion drawn from the mutant analysis, which is that the AnkX FIC motif mediates phosphoryl transfer by a catalytic mechanism that is shared by other FIC-domain enzymes, is accurate.

2. Figure 2A, S3. Some of the bars shown in 2A do not match the eye test when looking at the blot in S3 (for example F107G band on top gel in S3 is nonexistent, but is supposed to be nearly as strong as the F107G band in bottom gel when looking at bars in 2A). Authors need error bars for this data if they want to display it as a histogram. Also, how was 2A calculated? Densitometry is assumed, but detail must be given in methods. Was this experiment done in triplicate? If so, where are the

statistics and error bars? Were R237E, D265A, F311G, and the d110-179 mutants in S3 a separate exposure? Where is the WT band that they were compared to for the densitometry? Surely they were not compared over different exposures. FigS3 is lacking molecular weight markers and either a stain or blot to show loading. There is reduced autoactivity with F107 and D265 but not with E226. This data was grouped together and misdescribed. Separate into panels with each gel having positive and negative controls (in triplicate).

We agree with the reviewer that these experiments needed to be improved and better displayed. We now show the phosphocholination experiments in 4 different panels (now Figure 3 instead of Figure 2), each addressing a specific issue.

Experiments shown in Figures 3A and 3B address whether residues that bind CDP-choline are important for AnkX enzymatic activity. Panels 3A and 3B show the densitometry analysis of immunoblot experiments which analyze Rab1 phosphocholination by AnkX constructs carrying mutations of choline- and cytosine-binding residues (3A) and or FIC motif residues (3B). Densitometry was carried out using the GE Healthcare ImageQuant LAS 4000 gel doc system, which captures chemiluminescence data in the linear stage of the reaction providing accurate measurements, and was not done by simply scanning film blots. The data were obtained from a minimum of 3 independent experiments and are shown with error bars, and are expressed as the percentage of phosphocholination relative to wild-type AnkX used as a control. Representative blots are shown in supplementary Figure 5, along with auto-phosphocholination experiments carried out with the same set of mutants. As indicated above, AnkX constructs carrying mutations in the FIC motif did not show auto-phosphocholination activity, as would be expected if they are involved in the catalytic reaction (S5B). The AnkX fusion proteins carrying a mutation in the CDP-choline binding site outside the FIC motif did not show any evidence of aggregation or instability, and retained the ability to mediate limited auto-phosphocholination. Thus, it seems unlikely that they have folding defects that would severely affect their ability to modify Rab1 in vitro and the defects would be more consistent with impaired substrate binding, as predicted. Thus, we feel our statement that these mutations are consistent with the structural data predicting that these residues would be important for function are accurate.

The experiment shown in Figure 3C addresses the requirement of Mg^{2+} for AnkX phopshocholine transferase activity. Figure 3C shows an immunoblot analysis of auto-phosphocholination of full-length AnkX that has been dialyzed against a metal-free buffer prior to the experiment, without or with subsequent addition of 1mM Mg^{2+}. This experiment clearly shows that AnkX is essentially inactive in the absence of Mg^{2+} and recovers full phosphocholinating activity by addition of Mg^{2+}. We feel this is an improvement over the previous data showing EDTA can inhibit activity.

The experiment shown in Figure 3D addresses whether autophosphocholination represents a phosphohistidine intermediate in the catalytic reaction. Figure 3D shows that untagged $AnkX_{1-484}$ is not auto-phosphocholinated, thus indicating that the auto-phosphocholinated product is not a phosphohistidine intermediate. Molecular markers are shown, and protein loading was assessed by Ponceau staining (shown in supplementary Figure S5C). All experiments in Figure 3 were done at least in triplicate.

3. Figure 2B. The labeling of His AnkX H229A by GST-AnkX 1-484 is NOT robust, looking very weak compared to bands of either of the catalytically active proteins. If the same densitometry were performed here as in Figure 2A this would be very obvious. This figure appears to be entirely misinterpreted, and suggests to me the labeling is in "cis", whether true or not. Also, why does the GST-AnkX 1-484 band show up in lane 7, where only the His-AnkX is supposed to be? This figure is also lacking molecular weight markers and either a stain or blot to show loading. Also, GST-AnkX H229A is a necessary control for this experiment. Most disappointing is the attempt to add more substrate and see no consistent increase in the labeling. Please repeat the data and re-consider the interpretation of this data.

As described above, the question being addressed, but perhaps not stated clearly, was whether autophosphocholination represents a phosphocholine-histidine intermediate in the transfer reaction or if this represents transfer in cis or trans to another domain. We have improved these data using the untagged $AnkX_{1-484}$ protein and show clearly that this protein is not autophosphocholinated.

Thus, the autophosphocholinated product is not a phosphocholine-histidine intermediate. We believe these new data address this concern.

4. The title is somewhat awkward. Why not "Mechanism of phoshpocholine transfer by Ankx, the ankyrin repeats and FIC domain containing Legionella effector"

We agree with the reviewer and the title has been changed to, “Structure of the *Legionella* effector AnkX reveals the mechanism of phophocholine transfer by the FIC domain.”

5. Heading on page 5: CDP-choline binding region explains substrate preference, not transfer of phosphocholine.

The heading has been changed to: The orientation of CDP-choline binding to AnkX provides a mechanism for transfer of phosphocholine by the FIC motif.

6. Fig S1 legend title ?? 1-484 not 1-184.

This has been corrected.

7. Add cyan to Hairpin in FigS1D so there is a common focal point in Fig S1B, C, D.

This has been done

8. Page 7 change " ... were impaired in the absence of Mg2+." to ".. were impaired by the addition of EDTA."

The experiment has been redone by removing Mg^{2+} from the protein sample and the phosphocholinating buffer, as indicated in Figure 3C.

3rd Editorial Decision 28 February 2013

Thank you for your patience while your manuscript has been reviewed. Your study has been sent to former referees #1 and #2, who consider that the manuscript has been significantly improved, although some concerns remain.

I would like to draw your attention to referee #2 in particular, who in his/her own words considers that "beautiful structural data is complemented with marginal biochemistry". S/he believes that most biochemical concerns have been not properly addressed and this prevents publication of your study at this stage. Although it is our policy to allow a single round of revision, in this case, given the very positive consideration of your manuscript by the referees in terms of novelty and interest, I would like to give you the opportunity to address the remaining issues mentioned by the referees. Be aware, however, that all concerns raised by referee #2 must be addressed in the final version of your study before acceptance.

Do not hesitate to contact me in case you have any further questions.

Thank you again for your patience and the opportunity to consider your work for publication. I look forward to the final version of your manuscript.

REFEREE REPORTS

Referee #1

The structure is interesting and the authors answered my queries sufficiently well to satisfy my criticisms.

Referee #2

The paper is better, however most of criticisms for the biochemistry were answered by simply eliminating data.
The problem is that the beautiful structural data is complemented with marginal biochemistry.

1- Figure 3A NEEDS a negative control; mutate an amino acid that is not predicted to affect auto-phosphocholination or phosphocholination of Rab.
2. For Figure 3A and B, the y-axis needs a label.
3. The text says Figure 3A is obtained from triplicates and a representative example is shown in Figure S5A. The example contain data for a lane (D265A) that should not be used in this assay as it appears to have smeared band. This type of data is not optimal for quantitation.
4. Please explain why the mutations in Figure 3A/S5A exhibit auto-phosphocholination. How does this fit with your model for the contribution of these amino acids with your biochemical mechanism for transfer of the substrate.

2nd Revision - authors' response 06 March 2013

Referee #2

The paper is better, however most of criticisms for the biochemistry were answered by simply eliminating data. The problem is that the beautiful structural data is complemented with marginal biochemistry.

In the point-by-point response of the first revision, we explained that the manuscript had been modified to make it clearer that we were not addressing the mechanism by which AnkX engages Rab1. For this reason, while we improved the quality of the presented experiments for the mutants involved in catalysis and CDP-choline binding , we removed reference to potential Rab1-binding determinants given that we have no biochemical Rab1-binding data or structural data to support direct interactions between these residues and Rab1 at this time. Besides the removal of these experiments, we designed and added a novel experiment that better addressed whether the auto-phosphocholinated AnkX species represents an intermediate in the phosphocholine transfer reaction. This experiment demonstrated that phosphoryl transfer to Rab1 by AnkX does not involve a phosphoryl-histidine intermediate, and that auto-phosphocholination is not required for robust Rab1 phosphocholination. We believe that these experiments provide evidence that phosphoryl transfer by AnkX requires the same catalytic residues in the FIC motif that are used in nucleotide transfer reactions by other FIC enzymes, and also identify key residues that are specific for the recognition of the CDP-choline substrate. For the most part these were made apparent by the structure and the experiments validate the key predictions drawn from the structure.

1- Figure 3A NEEDS a negative control; mutate an amino acid that is not predicted to affect auto-phosphocholination or phosphocholination of Rab.

We believe that the key control that is needed to analyze the phosphocholination activity of AnkX mutants is to show the activity of wild-type AnkX as a reference. This is given in 3A and 3B. We apologize to the reviewer that we fail to see how a mutant that would be unaffected in phosphocholination (hence would behave as wild-type AnkX) would validate further the quality or the interpretation of the presented data.

2. For Figure 3A and B, the y-axis needs a label.
In addition to the explanation provided in the legend (« Rab1 phosphocholination is expressed as the relative percentage of Rab1 phosphocholination by wild type AnkX"), we have added a label to the y axis in these figures (« % of phosphocholination by $AnkX^{WT}$ ».

3. *The text says Figure 3A is obtained from triplicates and a representative example is shown in Figure S5A. The example contain data for a lane (D265A) that should not be used in this assay as it appears to have smeared band. This type of data is not optimal for quantitation*.

We explained in the point-by-point letter that densitometry was carried out using the GE Healthcare ImageQuant LAS 4000 gel doc system, which captures chemiluminescence data in the linear stage of the reaction thus providing accurate measurements, and was not done by simply scanning film blots. Thus, the quantitation is independent of the shape of the quantified band, provided there is no leakage between adjacent lanes, which is clearly not the case as can be seen in Figures S5A and S5B showing representative blots. To make that point clearer, we have now added the details of the densitometry procedure in the legend of Figure S5A.

4. Please explain why the mutations in Figure 3A/S5A exhibit auto- phosphocholination. How does this fit with your model for the contribution of these amino acids with your biochemical mechanism for transfer of the substrate.

As the novel experiment shown in figure 3D and S5D reveals, auto-phosphocholination is not necessary for efficient Rab1 phosphocholination, and is probably due to non-specific modification of flexible parts of the protein such as those found in the tags used for protein purification. We therefore believe that the relevant phosphocholination reaction to consider is that of the small GTPase Rab1, which is the physiological substrate. Therefore, we surmise that differences in binding modalities between Rab1 and flexible regions within some of the AnkX constructs explain the differences between Rab1-phosphocholination and auto-phosphocholination than can be seen for some of the AnkX mutants in Figure S5A. We have now added this explanation in the legend of Figure S5A.

