EMBO
reports

Manuscript EMBOR-2014-38841

The Crystal Structure of the Phosphatidylinositol 4-kinase IIα

Adriana Baumlova, Dominika Chalupska, Bartosz Rozycki, Marko Jovic, Eva Wisniewski, Martin Klima, Anna Dubankova, Daniel Paul Kloer, Radim Nencka, Tamas Balla and Evzen Boura

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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.)

Editor: Barbara Pauly

1st Editorial Decision 22 April 2014

Thank you for the submission of your research manuscript to our editorial office. Please find below the referees' comments that we have now received on your manuscript.

As you will see, all referees agree on the potential interest of the findings and, in principle, support publication of the study in EMBO reports. However, they also agree that functional data for the significance of the second ATP/PI binding site and the proposed open conformation is needed to strengthen the conclusions drawn from the structural work.

Overall, given these evaluations, the reviewers constructive comments and the potential interest of the study, I would like to give you the opportunity to revise your manuscript, with the understanding that the referee concerns must be addressed and their suggestions (as detailed above and in their reports) taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports 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.

Revised manuscripts should be submitted within three months of a request for revision; they will

otherwise be treated as new submissions, also with regard to the novelty of the findings at the time of the submission. Since you know of a competing manuscript that is under consideration somewhere else, I would recommend conducting the necessary experiments as soon as possible. The length of the revised manuscript should not exceed roughly 29,000 characters (including spaces). If you feel that the additional data requested by the reviewers would make the manuscript too long you may consider including some peripheral data in the form of Supplementary information. However, materials and methods essential for the repetition of the key experiments should be described in the main body of the text and may not be displayed as supplemental information only.

I look forward to seeing a revised form of your manuscript when it is ready. Should you in the meantime have any questions, please do not hesitate to contact me.

REFEREE REPORTS:

Referee #1:

Baumlova et al. determine the structure of phosphatidyl inositol 4-kinase IIalpha. Using Monte Carlo simulations, they suggest a model how the kinase interacts with membranes.
The structure of PI4K IIalpha is interesting, noteworthy and of general interest. However, the lack of any biochemical and cell-based data to support the structure and to obtain some mechanistic insights is not acceptable in my view. Furthermore, the new structural features should be worked out more thoroughly.

Introduction, p3 (starting with the title page):
'The new structure, which revealed both a novel fold ...'
Is this really a novel fold (for example, according to the definition in the SCOP database)? Or a non-typical kinase fold? Please make a qualified statement, e.g. by proper comparisons.

Figure 1, Structure:
It is very difficult from Figure 1A to recognize the fold of the structure and the relation to other published kinase structures. Please add a topology plot showing schematically the domain architectures of both domains and their relation to each other. Are the ATP-binding pockets the only features of this structure worth highlighting (I guess not)? For example, what about the linker regions between the domains? Some comparisons (schematically or superpositions) to other PI kinase or protein kinase structures would greatly help to appreciate the value of the new structure. Please improve the figures and put more work into this part!

Figure 2a:
Please label all residues and atoms shown in the figure (it is not very clear). The color scheme of the residues is also not very helpful (all in green), maybe color carbons of amino acid residues according to domain assignment? Is the organization of the first ATP-binding site similar to other kinases?

Figure 3: Monte-Carlo simulations.
The simulations are neither introduced nor discussed in detail. What one can take out of it, maybe the membrane interaction which is not surprising given the location of the lipid anchor. The second suggested conformation looks interesting, but without some experimental data confirming the physiological significance, this is too speculative. That the second confirmation is related to nucleotide exchange, is not evidenced by any data in the manuscript.

Functional data:
At a minimum, the authors need to probe the functional significance of the two ATP-binding pockets, using biochemical and cell-based assays. What is the suggested mechanism of PI phosphorylation? For example, is D308 part of a pocket accommodating the PI headgroup? Can this be supported by mutagenesis and functional data? Is the function of the second ATP binding pocket indeed related to cholesterol / inositol head group binding? Test by ITC, biacore or other methods! Are other kinetic factors influenced by mutations in this pocket?

Methods:
What about the first 76 first amino acids? Anything known about the function of these?

Data collection statistics
Space group 18 should be conventionally described as P21 21 2 with a, b, c = 104.8, 79.5, 78.7 (this needs a molecular replacement in the correct space group and one more refinement run).

Referee #2:

BAUMLOVA ET AL
EMBOR-2014-38841V1

This interesting manuscript describes a series of new findings regarding the functional architecture of the PtdIns 4-OH kinase IIa enzyme responsible for synthesis of a significant pool of PI4P in yeast and mammalian systems. Because the authors describe a new structure for an enzyme of large interest to the lipid signaling and membrane trafficking communities, this MS promises to be of interest to a broad readership. It is generally written in a clean and scholarly manner. However, as it stands, the MS is limited to the report of a crystal structure of a non-canonical kinase fold and some coarse-grain simulations that address how the enzyme may associate with membranes. Some functional data need to be further developed to solidify what promise to be important conclusions.

Major Comments:

This MS totally rests on solution of the PtdIns 4-OH kinase IIa crystal structure and some crude molecular dynamics simulations to model how the enzyme docks onto a membrane surface. The progress is important because the work describes the first structure for a PtdIns 4-OH kinase, and the structure describes in detail what was expected to be (and is) an unusual kinase fold. The resolution of the structure is OK, and the statistics report a quality model. Two other interesting pieces of information are reported by the new structure. First, there is an important clarification regarding the defect in the previously assumed kinase dead mutant. Second, the authors find a second ATP binding site that they propose is actually a cholesterol (or other lipid) binding site filled by ATP in the crystallization process. Some important functional experiments flow from these new insights but none of these are developed in this MS. This is a significant weakness. Comments follow:

1. Can the second ATP binding site accommodate the headgroup of PtdIns? This is the obvious possibility for a lipid ligand and could be addressed by modeling. Does soaking crystals in inositol or inositol-1-P fill the site?

2. In a related vein, there are no mutagenesis data to show the second 'ATP-binding' site is of any relevance to in vitro or in vivo activity. If the site is important for activity, it is interesting. If not, then a potentially important new insight fades away.

3. Regarding the coarse-grain MDS runs, it is not clear to this reviewer exactly what the two configurations of the enzyme on the membrane surface mean. It seems these are two families of solutions, but the actual parameters for the MDS are inadequately described. If one initiates the MDS with either solution as a starting structure in an all-atom MDS, does one observe interconversion between the two? These points have to be made more clear in the MS. Interconversion during an MDS run would be interesting.

4. The relevance of the simulated open conformation is easily testable by mutating the 165-172 substructure. This should be done. The authors should also decide how to test docking predictions from the tight structure and do those experiments.

Referee #3:

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This study reports for the first time a structure for a family member of the novel family of PI4K. The study provides some insight into how this specific PI4K might function.
However the manuscript lacks any functional data that might be gleaned from the novel structure that the authors describe.
I suggest that the authors follow up on the observation of a novel hydrophobic pocket using mutational studies coupled with localisation and enzymatic function assays to assess the role of this hydrophobic pocket in the regulation/maintenance of activity.
this would strengthen the manuscript in terms of relating the structural data observed with functional enzymatic output.

1st Revision - authors' response 27 June 2014

We would like to thank the Reviewers for their quick evaluation and that they found our description of the structure of PI4K IIalpha of general interest. We also thank for the positive and constructive comments that helped us prepare a greatly improved manuscript. Below are our point by point reply to their comments and the way they have been dealt with.

Referee #1:

Baumlova et al. determine the structure of phosphatidyl inositol 4-kinase IIalpha. Using Monte Carlo simulations, they suggest a model how the kinase interacts with membranes. The structure of PI4K IIalpha is interesting, noteworthy and of general interest. However, the lack of any biochemical and cell-based data to support the structure and to obtain some mechanistic insights is not acceptable in my view. Furthermore, the new structural features should be worked out more thoroughly.

We appreciate that Referee #1 considered that "*The structure of PI4K IIalpha is interesting, noteworthy and of general interest*. "In response to his/her suggestion, we now have generated mutants and performed kinase assays both in isolated enzymes either form bacterial expression, mammalian expression or performed *in situ* in permeabilized cells. The results of these experiments are now included in the revised manuscript (Fig 2C, Fig 3B, Fig 4). They nicely complement our structural analysis and strengthen our manuscript. We have also put significant efforts to improve our structural figures.

Introduction, p3 (starting with the title page): the new structure, which revealed both a novel fold …' Is this really a novel fold (for example, according to the definition in the SCOP database)? Or a non-typical kinase fold? Please make a qualified statement, e.g. by proper comparisons.

We have changed the text accordingly and now state in the introduction that "the structure, which revealed non-typical kinase fold and an unexpected hydrophobic pocket". We have also included comparison with the more typical lipid kinase domain (Fig 1D) of another PI4K, that of PI4K IIIβ that was published while this manuscript was under review (Burke et al., Science 2014). We also show a comparison (EV Fig1) with the closest crystallized homolog, a Ser/Thr kinase ctkA (cell translocating kinase A) from *Helicobacter pylori*, that shows possible evolutionary connection between the type II PI4Ks and Ser/Thr protein kinases.

Figure 1, Structure: It is very difficult from Figure 1A to recognize the fold of the structure and the relation to other published kinase structures. Please add a topology plot showing schematically the domain architectures of both domains and their relation to each other. Are the ATP-binding pockets the only features of this structure worth highlighting (I guess not)? For example, what about the linker regions between the domains? Some comparisons (schematically or superpositions) to other PI kinase or protein kinase structures would greatly help to appreciate the value of the new structure. Please improve the figures and put more work into this part!

We have included a plot showing the primary structure of the wt kinase, the construct used for biochemical analysis (pseudo-wt) and that of the crystallized construct (Fig 1A) and the topology

plot (Fig 1C) to make the figure clearer. We now also highlight the putative PI binding site with docked inositol (Fig 3C). As mentioned above we have also added figures comparing (superpositions) PI4K IIα with the typical lipid kinase domain of PI4K IIIβ (Fig 1D) and with the closest protein kinase domain homolog ctkA (EV Fig 1).

Figure 2a: Please label all residues and atoms shown in the figure (it is not very clear). The color scheme of the residues is also not very helpful (all in green), maybe color carbons of amino acid residues according to domain assignment? Is the organization of the first ATP-binding site similar to other kinases?

All residues are now labeled. We have changed the color scheme (and state it explicitly in the figure legend) to color carbon atoms according to domain assignment, carbon atoms in the ligand in silver, oxygen in red, nitrogen in blue, phosphor in orange and water as gray ball. We now show the unbiased Fo-Fc map in green contoured at two sigma.
We now state that the catalytic site is similar to catalytic sites of other kinases in the respect that the ATP is locked between the N-lobe and C-lobe and to other lipid kinases in the respect that it brings the ATP in close proximity to the membrane. Specifically we have added these sentences in the Results and Discussion section: "Its similarity to other kinases is confined to the position of ATP being locked between the N-lobe and C-lobe and that similar to other lipid kinases, the ATP is in close proximity to the membrane.".

Figure 3: Monte-Carlo simulations. The simulations are neither introduced nor discussed in detail. What one can take out of it, maybe the membrane interaction which is not surprising given the location of the lipid anchor. The second suggested conformation looks interesting, but without some experimental data confirming the physiological significance, this is too speculative. That the second conformation is related to nucleotide exchange, is not evidenced by any data in the manuscript.

We agree with the reviewer that the lipid anchor is key determinant for the membrane binding mode of the PI4K IIalpha enzyme. We now highlight it in the Results and Discussion section, specifically we state: "Based on these data we propose that the enzyme is kept at the membrane by multiple interactions: First, its palmitoylation plays a very important role. Second, the amphipathic segment 165-KWTKWLQK-172 adjacent to the palmitoylation sites also makes a significant contribution…**".**

We have included a detailed introduction of our simulation method. Due to space limitation it is in the Expanded View in Materials and Methods section. We have also included two figures (EV Fig3A, EV Fig3B) explaining how the simulation data were analyzed.

We agree with the reviewer that the second, loose membrane binding, configuration is too speculative. We mention it now as a second group of solutions of our simulations (EV Fig3C) in which the physiological significance is unclear. Please, see the Expanded View Results and Discussion section where the loose membrane binding configuration is now described.

Functional data:
At a minimum, the authors need to probe the functional significance of the two ATP-binding pockets, using biochemical and cell-based assays. What is the suggested mechanism of PI phosphorylation? For example, is D308 part of a pocket accommodating the PI headgroup? Can this be supported by mutagenesis and functional data? Is the function of the second ATP binding pocket indeed related to cholesterol / inositol head group binding? Test by ITC, biacore or other methods! Are other kinetic factors influenced by mutations in this pocket?

We have performed mutagenesis analysis of selected residues. These targeted the ATP binding site, the hydrophobic pocket and the unique solvent-exposed hydrophobic loop (Fig 2C, Fig 3B, Fig. 4). We show that residues defining the ATP site (e.g. S134, V150, I345) are important for catalytic activity whereas mutation of residue further away (F139A) is not significant. We also show that a residue making direct hydrogen bond (K152) with the α-phosphate of the ATP molecule is indispensable for catalytic activity. Since the D308 is too far away from the ATP, the known D308A kinase dead mutation can be only explained by D308 being part of the inositol binding pocket. Unfortunately, despite our substantial efforts we were unable to get crystal structure with inositol or inositol-1-P bound. However, modeling suggests that inositol can be fitted nicely in a pocket in

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close vicinity to the ATP's γ-phosphate (Fig3C).

Characterization of the interaction of cholesterol and the kinase *in vitro* has proven to be difficult as the enzyme precipitated in any buffer where cholesterol was at least partially soluble. To address the functional relevance of the hydrophobic pocket we have performed experiments in the permeabilized cells (Fig. 4C) and *in vitro* assay (Fig 2C, Fig. 4D).experiments (Fig 4) showing that mutations within the hydrophobic pocket significantly reduces but does not eliminate the kinase activity. We also tested the localization of the mutant enzyme and found no obvious difference compared to wild type (EV Fig. 4). The same mutations also inhibited the enzyme in *in vitro* kinase assays suggesting that the hydrophobic pocket contributes to the lipid binding and proper alignment of the enzyme with the membrane. This membrane interaction might be quite non-specific as our data show reduced activity even by the *in vitro* assays against lipid micelles suggesting that this site may be promiscuous in its membrane interaction.

Methods: What about the first 76 first amino acids? Anything known about the function of these?

The function of the proline rich N-terminus is known, at least to some extent. We now state at the beginning of the Results and Discussion section: “The proline rich N-termini of the enzyme contains physiologically important binding sites for ubiquitin ligase Itch (Mossinger et al., 2012) and clathrin adaptor complex 3 (Craige et al., 2008) (Fig. 1A) but is predicted disordered and its deletion does not affect the kinase activity (Barylko et al., 2002) .”.
We have also indicated these binding sites on the linear cartoon shown in Fig1A.

Data collection statistics
Space group 18 should be conventionally described as P21 21 2 with a, b, c = 104.8, 79.5, 78.7 (this needs a molecular replacement in the correct space group and one more refinement run).

The space group 18 is now described as P21 21 2. We have also performed few additional rounds of refinement, which slightly improved the model.

Referee #2:

BAUMLOVA ET AL
EMBOR-2014-38841V1

This interesting manuscript describes a series of new findings regarding the functional architecture of the PtdIns 4-OH kinase IIa enzyme responsible for synthesis of a significant pool of PI4P in yeast and mammalian systems. Because the authors describe a new structure for an enzyme of large interest to the lipid signaling and membrane trafficking communities, this MS promises to be of interest to a broad readership. It is generally written in a clean and scholarly manner. However, as it stands, the MS is limited to the report of a crystal structure of a non-canonical kinase fold and some coarse-grain simulations that address how the enzyme may associate with membranes. Some functional data need to be further developed to solidify what promise to be important conclusions.

We appreciate the Referee’s statement that our manuscript “*promises to be of interest to a broad readership. It is generally written in a clean and scholarly manner.* “

As detailed to our reply to Reviewer 1, we have performed extensive studies and now included functional data to complement our structural analysis (Fig. 2C, Fig. 3B, Fig. 4).

Major Comments:

This MS totally rests on solution of the PtdIns 4-OH kinase IIa crystal structure and some crude molecular dynamics simulations to model how the enzyme docks onto a membrane surface. The progress is important because the work describes the first structure for a PtdIns 4-OH kinase, and the structure describes in detail what was expected to be (and is) an unusual kinase fold. The resolution of the structure is OK, and the statistics report a quality model. Two other interesting pieces of nformation are reported by the new structure. First, there is an important clarification

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regarding the defect in the previously assumed kinase dead mutant. Second, the authors find a second ATP binding site that they propose is actually a cholesterol (or other lipid) binding site filled by ATP in the crystallization process. Some important functional experiments flow from these new insights but none of these are developed in this MS. This is a significant weakness. Comments follow:

1. Can the second ATP binding site accommodate the headgroup of PtdIns? This is the obvious possibility for a lipid ligand and could be addressed by modeling. Does soaking crystals in inositol or inositol-1-P fill the site?

The hydrophobic pocket could indeed accommodate an inositol headgroup (SI Fig2B) but our docking studies indicate that it is energetically unlikely. Despite our significant efforts in obtaining inositol or inositol-1-P containing crystals, found the hydrophobic pocket still occupied by ATP and no crystals were obtained with inositol. We attempted a similar approach with cholesterol but the recombinant PI4K IIα protein precipitates in any buffer we tried where cholesterol was at least partially soluble and the crystals have cracked when moved to drops with dissolved cholesterol probably due to rather high ethanol or DMSO concentration needed to solubilize cholesterol.

Now we state in the Results and Discussion section:
"Sterically speaking the hydrophobic pocket could accommodate the head group of several phospholipids, including the inositol ring of PI, (EV Fig. 2A). However, the proximity of the hydroxyl groups of an inositol ring to the hydrophobic side chains of F364 and W359 would make such interaction energetically unfavorable."

2. In a related vein, there are no mutagenesis data to show the second 'ATP-binding' site is of any relevance to in vitro or in vivo activity. If the site is important for activity, it is interesting. If not, then a potentially important new insight fades away.

As indicated above in our response to Reviewer 1, we have now included mutagenesis data showing relevance of the hydrophobic pocket both *in vitro* (Fig 2C) and *in situ* in cells (Fig 4). Mutation of any of the conserved residues of the hydrophobic pocket (W359, F364, W368, Q445) significantly impairs the kinase activity *in vitro* probably due to lower affinity for membranes. We have also performed *in situ* cellular analysis (Fig 4) which confirmed that the combined mutation of W359A, W369A significantly reduced but not eliminated kinase activity and did not change the localization of the enzyme (EV Fig. 4).

3. Regarding the coarse-grain MDS runs, it is not clear to this reviewer exactly what the two configurations of the enzyme on the membrane surface mean. It seems these are two families of solutions, but the actual parameters for the MDS are inadequately described. If one initiates the MDS with either solution as a starting structure in an all-atom MDS, does one observe interconversion between the two? These points have to be made more clear in the MS. Interconversion during an MDS run would be interesting.

The two groups of configurations of the membrane-bound enzyme emerge from the analysis that is now described in detail in Expanded View section. Briefly, we first identified which residues are immersed in the membrane (EV Fig. 3A) in the course of the simulations. Segments 165-KWTKWLQK-172 and 173-LCCPCCF-179 as well as the hydrophobic pocket are embedded in the membrane most often. Also helices H5 and H7 frequently interact with the membrane. We then selected several residues in these membrane-interacting regions of the enzyme, and analyzed how their distance from the membrane changes during a simulation run (EV Fig. 3B). The residues forming the hydrophobic pocket always remain in the membrane region. In contrast, the residues in helices H5 and H7 exhibit much larger variations in the distance to the membrane; we observe altering periods when they are in close contact with the membrane, and periods when they are well separated from the membrane. This observation indicates that the kinase fluctuates between different membrane-bound states during the simulation run. Indeed, direct visual inspection of the simulation configurations revealed the two groups of configurations.

In our study we used the coarse-grained model to simulate large-scale motions of the enzyme. With our computer resources, we could afford all-atom simulations of the membrane-bound kinase on the time scales shorter than 100 ns, which probably would be enough for docking the enzyme to the

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membrane but definitely not enough to observe unbinding events. Although the coarse-grained model is less precise than all-atom models, it permits thorough sampling of possible physical interactions of the kinase with the membrane. In fact, during the simulations we observe frequent interconversion between the open and tight configurations of the enzyme (top panel in EV Fig. 3B).

We currently have no direct experimental evidence that the open configuration is physiological. Therefore, we emphasize that the coarse-grained model correctly predicted the tight configuration, which we were able to confirmed by mutagenesis experiments (Fig 3B) and we now show the open configuration only as a second group of solutions obtained (EV Fig 3C).

4. The relevance of the simulated open conformation is easily testable by mutating the 165-172 substructure. This should be done. The authors should also decide how to test docking predictions from the tight structure and do those experiments.

We now show that when the segment 165-KWTKWLQK-172 (predicted most important for membrane binding) was mutated to alanines, the kinase became inactive (Fig. 3B). We have also tested two additional residues at the predicted membrane interface (N163, R275) and two control residues (N249, V339) predicted not to interact with the membrane. As predicted, the mutations N163A or R275A significantly lowered the kinase activity whereas the mutations N249A or V339A had no effect (Fig 3B).

Referee #3:

This study reports for the first time a structure for a family member of the novel family of PI4K. The study provides some insight into how this specific PI4K might function. However the manuscript lacks any functional data that might be gleaned from the novel structure that the authors describe. I suggest that the authors follow up on the observation of a novel hydrophobic pocket using mutational studies coupled with localization and enzymatic function assays to assess the role of this hydrophobic pocket in the regulation/maintenance of activity. This would strengthen the manuscript in terms of relating the structural data observed with functional enzymatic output.

We appreciate the constructive feedback of Referee #3.
As detailed in our replies to Reviewers 1 and 2, we performed a series of functional studies addressing the roles of the hydrophobic pocked and some other possibly important features indicated by the structure. These experiments included both *in vitro* and *in vivo* studies and are now shown in the revised ms. (Fig 2C, Fig 3B, Fig 4) These results and their ramification are now included in the revised manuscript in the Results and Discussion sections.

2nd Editorial Decision 22 July 2014

Thank you for your patience while we have reviewed your revised manuscript. As you will see from the reports below, the referees appreciate the efforts in the improvement of the study. Although referee #2 still raises concerns about the -in his/her opinion- limited analysis of the functional role played by the newly described hydrophobic pocket, we have decided to nevertheless accept your manuscript without requesting further experimental work, under the condition that the minor changes still requested by the reviewers are addressed in the text for clarification. Specifically, referee 1 feels that in some instances additional clarifications and re-structuring of the manuscript is needed. With regard to referee 2 I would suggest that you briefly discuss the issue that differences in protein folding could account for the observed differences in the catalytic activity of the wild type and mutant enzyme(s).

If possible, could you maybe also try to shorten the text slightly as with over 36,000 characters is currently exceeds our length restrictions.

If all remaining corrections have been attended to, you will then receive an official decision letter

from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports.

REFEREE REPORTS:

Referee #1:

Along the reviewers' comments, additional experiments were performed which greatly increase the impact of the study. Now, the manuscript contributes important structural and mechanistic insights into PI4II-kinases, with implications for cell signaling, membrane trafficking and drug development. I therefore believe that the manuscript is a strong candidate for EMBO Reports. Some more formal issues, such as the presentation and explanation of the results, could still be improved before publication, as outlined below.

The text is not always logically structured and some figures need reordering, according to their appearance in the text (for example, Figure 4 is described before Figure 3). Three examples:
p2 (p1 starts with the abstract) bottom: the ATP molecule is in close proximity to the membrane
At this point, the orientation of the kinase to the membrane is not clear.

p4, middle: a surface-exposed hydrophobic loop not predicted to be important for membrane binding... AND ... no effect was observed when the solvent exposed hydrophobic loop was mutated. On which model is this prediction based?

Molecular simulations show that when the kinase is tightly bound to the membrane, its ATP molecule (which one?) directly points directly towards the lipids. ???
What do you mean? What are the assumptions, what is the rationale to do these experiments, what is the approach? (see also below).

To simplify the argumentation I would consider reordering the manuscript: After the description of the structure and the mutagenesis of the ATP-binding site, the membrane binding mode was of interest. Thus, molecular dynamics simulations were performed resulting in two models which were then tested by mutagenesis. In the confirmed model, the 'hydrophobic' pocket was pointing towards the membrane; mutations in this pocket show reduced membrane binding indicating that the pocket may bind some lipid headgroups. Molecular modelling studies indicate that it could accommodate a cholesterol molecule. And finally, the deduced membrane binding mode is compared to that of other PI kinases. And please also label the SE loop and the hydrophobic pocket in the modeled membrane-bound structure.

Structural similarity
The topology plot is very helpful in this regard, but the superposition with PI4K III is too crowded and needs to be done more carefully. It appears from the figure that mainly the C-lobes, but not the N-lobes, of the two enzymes bear structural similarity? Please superimpose them separately and analyze the similarity in more detail. You could, for example, provide a comparison of the topology plots of related proteins in the EV. Do both lobes bear structural similarity to ctkA and Ser/Thr kinases? Also search for structural similarities with the separate lobes.

Figure legend 1C: In vitro soluble kinase assays
What is an ADP-Glo kinase assay? Please shortly summarize in the figure legend the experimental setup, e.g. what has been measured at which temperature. Was substrate present in these reactions?

Figure 2A, residue D308
From Figure 2A, the distance of D308 to the gamma phosphate is not clear, please mention the

accurate distance. Is it too far away to position a water molecule for catalysis? Why should the alanine mutation of an acidic residue have such a profound effect for binding the negatively-charged inositol head group? Are there other examples of acidic residues crucially involved in binding an inositol headgroup?

Hydrophobic pocket
I was astonished that a hydrophobic pocket can harbor a polar ATP molecule. How hydrophobic is the pocket? You could provide a hydrophobic surface representation in the EV, for example with VASCO.

The molecular modelling is still not well explained and integrated, and I found it hard to understand from reading the results how these studies were performed (see also above). This accounts especially for the expanded view where enough space is provided to explain the detailed rationale of these calculations. I would suggest to move some of the EV method part (which, in fact, gives a nice overview of what has been done) to the EV results to explain the approaches in an intelligible way for a general molecular biology readership.

Refinement table:
You could add some more details to the refinement statistics, such as number of reflections, number of refined atoms (protein, ligand, water), resolution range for refinement, highest resolution shell, Rwork / Rfree in the highest resolution shell.

Figures (general):
Increase font sizes, especially in Figure 2.

Figure 1B
Please separately label the two ATP molecules in all figures for better orientation (for example, ATP1, ATP2).

p4 top: sterically speaking
Better: The hydrophobic pocket could sterically accommodate ...
The following paragraph (possible ligands) could be shortened.

Referee #2:

BAUMLOVA ET AL
EMBOR-2014-38841V2

The major criticisms of this referee regarding the original submission were that the authors failed to adequately support their interesting work on the functional architecture of the PtdIns 4-OH kinase IIa enzyme with functional data to further develop several potentially important, but highly speculative, conclusions they had made. While the authors have included some such functional data in the revised MS, this reviewer is not persuaded that they have actually done the key experiments to merit publication of this MS in EMBO R. It is the opinion of this reviewer that, while the MS should be published, it belongs in a kore specialized journal. It is important to understand that the publication of a PtdIns 4-OH kinase IIa structure by another group does not contribute to this judgment. Comments follow:

Much is made of the authors find a second ATP binding site that they propose is actually a cholesterol (or other lipid) binding site filled by ATP in the crystallization process. They now include some data to indicate this is a functionally important site on the basis of site-directed mutagenesis experiments. However, the effects are partial, and there are no controls for protein folding or membrane binding with the mutant proteins. So, the data as these stand are suggestive only. It is surprising the authors did not test whether the remaining activity of these second binding site mutants are regulated by cholesterol or not. This would at least give some indication of whether the idea has any merit. Because the mutants are more defective in kinase activity in the context of the construct purified from bacteria vs the palmitoylated version produced by mammalian cells does not necessarily mean that palmitoylation and the independent membrane binding signal this provides

compensates for the membrane association function of the site mutants in a context which lacks the palmitoylation site. He mutant proteins may simply fold better when expressed in the mammalian system. The lack of a clear conclusion regarding this second site remains a significant weakness in this work.

The authors mutate the 165-172 substructure as was requested in the original review. However, they never look at membrane binding ability of the mutants but only the kinase activity. Why? The hypothesis points to membrane binding and this property is what needs to be tested. Again, maybe the mutants just fold more poorly.

2nd Revision - authors' response 06 August 2014

Referee #1:

Along the reviewers' comments, additional experiments were performed which greatly increase the impact of the study. Now, the manuscript contributes important structural and mechanistic insights into PI4II-kinases, with implications for cell signaling, membrane trafficking and drug development. I therefore believe that the manuscript is a strong candidate for EMBO Reports. Some more formal issues, such as the presentation and explanation of the results, could still be improved before publication, as outlined below.

The text is not always logically structured and some figures need reordering, according to their appearance in the text (for example, Figure 4 is described before Figure 3). Three examples:
p2 (p1 starts with the abstract) bottom: the ATP molecule is in close proximity to the membrane
At this point, the orientation of the kinase to the membrane is not clear.

Now we discuss the position of the ATP in respect to the membrane in the ***Membrane binding mode of PI4K IIα*** *paragraph. Specifically we say: " Molecular simulations show that when the kinase is tightly bound to the membrane, the ATP molecule, located in the catalytic site between the N- and C-lobes, points directly towards the lipid bilayer."*

p4, middle: a surface-exposed hydrophobic loop not predicted to be important for membrane binding... AND ... no effect was observed when the solvent exposed hydrophobic loop was mutated. On which model is this prediction based?

Now we clarify that the prediction is based on our simulations. Specifically we say: "...surface exposed (SE) hydrophobic loop not predicted to be important for membrane binding in our simulations was mutated (332-WVVV-335 mutated to 332-SAAA-335) (Fig. 4)."

Molecular simulations show that when the kinase is tightly bound to the membrane, its ATP molecule (which one?) directly points directly towards the lipids. ???
What do you mean? What are the assumptions, what is the rationale to do these experiments, what is the approach? (see also below).

We are now more specific we say: " The ATP molecule, located in the catalytic site between the N- and C-lobes, points directly towards the lipid bilayer."

To simplify the argumentation I would consider reordering the manuscript: After the description of the structure and the mutagenesis of the ATP-binding site, the membrane binding mode was of interest. Thus, molecular dynamics simulations were performed resulting in two models which were then tested by mutagenesis. In the confirmed model, the 'hydrophobic' pocket was pointing towards the membrane; mutations in this pocket show reduced membrane binding indicating that the pocket may bind some lipid headgroups. Molecular modelling studies indicate that it could accommodate a cholesterol molecule. And finally, the deduced membrane binding mode is compared to that of other PI kinases. And please also label the SE loop and the hydrophobic pocket in the modeled membrane-bound structure.

We agree with the referee. Now the Membrane binding mode is discussed before the 'hydrophobic' pocket.

The SE loop and the hydrophobic pocket in the modeled membrane-bound structure are now labeled.

Structural similarity
The topology plot is very helpful in this regard, but the superposition with PI4K III is too crowded and needs to be done more carefully. It appears from the figure that mainly the C-lobes, but not the N-lobes, of the two enzymes bear structural similarity? Please superimpose them separately and analyze the similarity in more detail. You could, for example, provide a comparison of the topology plots of related proteins in the EV. Do both lobes bear structural similarity to ctkA and Ser/Thr kinases? Also search for structural similarities with the separate lobes.

Following the Reviewer's suggestion, we have added an EV Figure (EV Figure 1, panels C and D) showing superpositions of the N- C-lobes of PI4K IIα and the more typical PI4K IIIβ separately and comparing the topology plots of PI4K IIα and PI4K IIIβ.

Figure legend 1C: In vitro soluble kinase assays
What is an ADP-Glo kinase assay? Please shortly summarize in the figure legend the experimental setup, e.g. what has been measured at which temperature. Was substrate present in these reactions?

We are now more specific and we say: "...kinase activity was measured in vitro using recombinant proteins and the luminescent ADP-Glo kinase assay (Tai el al., 2011).". However, due to space limitations, we feel that details such as concentrations, temperature, volume, plate reader used, type of substrate, manufacturer of the substrate etc. are better presented in the EV Materials and Methods section for a reader interested in details.

Figure 2A, residue D308
From Figure 2A, the distance of D308 to the gamma phosphate is not clear, please mention the accurate distance. Is it too far away to position a water molecule for catalysis? Why should the alanine mutation of an acidic residue have such a profound effect for binding the negatively-charged inositol head group? Are there other examples of acidic residues crucially involved in binding an inositol headgroup?

The distance is 4.55A from the D308 carboxyl oxygen to the closest oxygen of the ATP's gama phosphate which would allow to position a water for catalysis. On the other hand, there are examples where the D308 is part of the Inositol binding pocket. For instance the crystal structure of the yeast PX-domain protein grd19p (sorting nexin 3) complexed to phosphatidylinosytol-3-phospahte (pdb accession number 1OCU). Now we mention these possibilities.
We now say in the Figure legend: "The catalytically important residue D308 surprisingly does not form a direct contact with ATP as it is spaced 4.55Å away from the ATP's closest γ-phosphate oxygen."
And in the discussion: "...the known D308A kinase dead mutation can be only explained by D308 being part of the inositol binding pocket and/or being important for positioning a water molecule for catalysis. "

Hydrophobic pocket
I was astonished that a hydrophobic pocket can harbor a polar ATP molecule. How hydrophobic is the pocket? You could provide a hydrophobic surface representation in the EV, for example with VASCO.
The molecular modelling is still not well explained and integrated, and I found it hard to understand from reading the results how these studies were performed (see also above). This accounts especially for the expanded view where enough space is provided to explain the detailed rationale of these calculations. I would suggest to move some of the EV method part (which, in fact, gives a nice overview of what has been done) to the EV results to explain the approaches in an intelligible way for a general molecular biology readership.

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We have moved some of the EV method part to the EV results as suggested.

Refinement table:
You could add some more details to the refinement statistics, such as number of reflections, number of refined atoms (protein, ligand, water), resolution range for refinement, highest resolution shell, Rwork / Rfree in the highest resolution shell.

We have added more details as suggested.

Figures (general):
Increase font sizes, especially in Figure 2.

Font sizes were increased.

Figure 1B
Please separately label the two ATP molecules in all figures for better orientation (for example, ATP1, ATP2).

The ATP molecules are now labeled ATP1 and ATP2 as suggested.

p4 top: sterically speaking
Better: The hydrophobic pocket could sterically accommodate ...
The following paragraph (possible ligands) could be shortened.

Now we say: "The hydrophobic pocket could sterically accommodate ..."
The following paragraph was moved to the EV section.

Referee #2:

BAUMLOVA ET AL
EMBOR-2014-38841V2

The major criticisms of this referee regarding the original submission were that the authors failed to adequately support their interesting work on the functional architecture of the PtdIns 4-OH kinase IIa enzyme with functional data to further develop several potentially important, but highly speculative, conclusions they had made. While the authors have included some such functional data in the revised MS, this reviewer is not persuaded that they have actually done the key experiments to merit publication of this MS in EMBO R. It is the opinion of this reviewer that, while the MS should be published, it belongs in a kore specialized journal. It is important to understand that the publication of a PtdIns 4-OH kinase IIa structure by another group does not contribute to this judgment. Comments follow:

Much is made of the authors find a second ATP binding site that they propose is actually a cholesterol (or other lipid) binding site filled by ATP in the crystallization process. They now include some data to indicate this is a functionally important site on the basis of site-directed mutagenesis experiments. However, the effects are partial, and there are no controls for protein folding or membrane binding with the mutant proteins. So, the data as these stand are suggestive only. It is surprising the authors did not test whether the remaining activity of these second binding site mutants are regulated by cholesterol or not. This would at least give some indication of whether the idea has any merit. Because the mutants are more defective in kinase activity in the context of the construct purified from bacteria vs the palmitoylated version produced by mammalian cells does not necessarily mean that palmitoylation and the independent membrane binding signal this provides compensates for the membrane association function of the site mutants in a context which lacks the palmitoylation site. He mutant proteins may simply fold better when expressed in the mammalian system. The lack of a clear conclusion regarding this second site remains a significant weakness in this work.

The authors mutate the 165-172 substructure as was requested in the original review. However, they never look at membrane binding ability of the mutants but only the kinase activity. Why? The hypothesis points to membrane binding and this property is what needs to be tested. Again, maybe the mutants just fold more poorly.

To clarify we discuss the poor folding hypothesis now. Specifically, we now say: "...even thought that the mutants expressed at the same level as wild type enzyme both in mammalian and bacterial cells and that the recombinant mutant proteins behaved as wild type during size exclusion chromatography we can not completely rule out that the mutants had somewhat lower kinase activity due to folding problems.".

3rd Editorial Decision 07 August 2014

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

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