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A PH domain within OCRL bridges clathrin mediated membrane trafficking to phosphoinositide metabolism

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(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

27 February 2009

Thank you for submitting your manuscript for consideration by the EMBO Journal. We initially sent your manuscript to four referees, and have now received the reports of three of them, which are enclosed below. The fourth report is still outstanding, but we feel we can make a decision - to invite you to submit a revised version of your manuscript - based on the three reports we have in hand. Should the fourth report reach us, we will of course transmit the comments on to you, and may ask that these should also be addressed.

As you will see, referees 1 and 2 are broadly supportive of publication, although while referee 1 raises only minor issues, referee 2 has concerns as to the functional implications of your work. Referee 3 is more negative, again based primarily on issues as to functional significance. However, given the positive comments of two reviewers, we are prepared to invite you to revise the manuscript if you are able to address these concerns. In particular, both referees 2 and 3 feel that the contribution of the N-terminal clathrin binding motif, to clathrin binding in particular and OCRL function more generally, is not sufficiently well characterised. I appreciate that the recent publication from the Lowe lab, to which you drew my attention, has conducted some analysis in this direction, but as you also noted, it focusses more on the C-terminal motif, which differs between the two isoforms. I would like to stress again that the publication of this work does not impact upon our assessment of yours, and we certainly would not want you to repeat experiments that have been done in this manuscript. However, we feel that a more detailed analysis of the functional relevance of the N-terminal clathrin binding motif, along the lines suggested by reviewer 3, would be important. In addition, it will of course be necessary to refer to the Lowe lab manuscript in your discussion. Finally, I have to admit I agree with referee 3's comment that, to a non-structural biologist, the similarity of the OCRL N terminus to a PH domain is not obvious. It would be very useful if this could be better illustrated.

In the light of the referees' positive recommendations, I would therefore like to invite you to submit a revised version of the manuscript. I should add that it is EMBO Journal policy to allow only a single round of revision. Therefore, acceptance of your paper will depend on the content of the next, final version, which will have to be assessed again by at least some of the referees.

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

Best wishes,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This is a thorough structural and functional analysis of the N-terminal domain of OCRL, the last remaining uncharacterized region of this protein. The domain is shown to be a PH domain and to bind clathrin, but not lipids. The clathrin binding is functionally important for OCRL targeting. As a bonus, the structure of the N-terminal domain of a related lipid phosphatase, INPP5B, was also determined. The INPP5B N-terminal domain is also shown to have a PH domain fold, despite a lack of sequence similarity. The study constitutes a significant advance in understanding the structural organization and regulation of OCRL. The paper is well written and illustrated, and the technical quality is excellent. The paper is suitable for publication following a few minor improvements.

1. To make the characterization complete, it would be helpful to measure the K_d of the clathrin heavy chain terminal domain for the unusual sequence in the OCRL PH domain (or a peptide model thereof) and to compare it to conventional clathrin binding signals.
2. Pg 12, para 4, line 2, "figure 5" should be "Figure 7".
3. Incorporate a structure-based sequence alignment of OCRL and INPP5B into the alignment of INPP5B orthologs in Figure 3D.
4. Replace kD with kDa on pages 6 and 12.

Referee #2 (Remarks to the Author):

This manuscript describes the identification of a PH domain structure in the N-terminal regions of the PI(4,5)P₂ and PI(3,4,5)P₃ 5-phosphatases OCRL and INPP5B. This structure was not predicted from the amino acid sequences of these proteins. Also, the N-terminal regions of the two proteins are distinct, yet have a similar structure, albeit with some small differences. This implies a common or similar function, but this remains to be identified. The PH domain do not appear to bind lipids, and may therefore interact with proteins. A novel clathrin binding site is identified in the N-terminal region of OCRL, which is on a flexible loop protruding out of the folded PH domain structure. This site, which is absent in INPP5B, is unusual in that it differs from the typical clathrin box sequence by having an alanine at position 5. It is required for efficient recruitment of OCRL to clathrin coated pits at the plasma membrane. It is speculated that OCRL may have an important function in regulating phosphoinositide levels during clathrin vesicle endocytosis, possibly to regulate uncoating or signaling from early endocytic membranes.

This is a well written and interesting manuscript, with clear and compelling data. They add to our knowledge of the OCRL and INPP5B proteins with the identification of an unsuspected common PH domain fold in their N-termini. Unfortunately, the functional significance of this structural

similarity is unclear, and not investigated in this study. The identification of an unsuspected novel clathrin binding site in OCRL is also of interest, and as shown in the manuscript is important for association of OCRL with clathrin coated pits at the plasma membrane. The data all point to a function for OCRL in endocytosis, but again this does not appear to have been investigated. Therefore the functional significance of the two major findings reported here (PH domain fold in OCRL and INPP5B and a second clathrin binding site in OCRL) remains unclear. The manuscript would be significantly strengthened if this could be addressed.

Additional points:

- 1.) Although the core fold of the PH domains from OCRL and INPP5B are very similar, the surface density map looks quite different to me, suggesting they may have distinct interaction partners or functions. This possibility if valid could be described in more detail in the text.
- 2.) The relative contribution of the C-terminal clathrin binding site and the alpha-adaptin binding sites to OCRL localization are not studied. It would be interesting to compare mutations of each of these domains to that of the LIDI motif.

Referee #3 (Remarks to the Author):

In this manuscript, Mao et al report the 3D structures of the N-terminal regions of two related inositol 5-phosphatases, OCRL (aa 1-119) and INPP5B (aa 1-156). Despite weak sequence homology between the two regions, the overall fold is conserved. One peculiar exception is the existence of a loop within OCRL that, as Mao et al demonstrate with biochemical assays, engages the terminal domain of clathrin heavy chain. Mutagenesis of residues in the loop suggest a partial match with a previously described "clathrin-binding box" with the exception of the residue at position 5. Although both N-terminal regions are described as PH domains, none of them bound phospholipids. Overexpression of the N-terminal domain of OCRL resulted in HeLa cells that failed to internalize transferrin, and mutation in the N-terminal clathrin-binding box decreased association to clathrin-coated pits by more than 50%.

As stated at the beginning of the Discussion, "this study completes the characterization of the modular structure of OCRL and INPP5B and corroborates evidence for a role of OCRL in ... clathrin-dependent endocytic membrane trafficking." I basically agree: it completes a molecular characterization and corroborates previously published evidence. The main novel findings are the two 3D structures, which are vaguely described as PH domains, and the definition of a clathrin-box variant with evidence of its biological significance.

Besides a general concern about the extent to which this study advances our understanding of these inositol 5-phosphatases, specific issues are:

- 1) What are the bases for calling these two structures "PH domains"? I could only find a vague statement about the folds being "similar" with no figure showing superimposition of structures or information about RMSD's with canonical PH domains. The surface representations in Fig 4 do convince the reader of the differences, but not of any significant similarity.
- 2) The description of the N-terminal clathrin box is consistent with published data on the ability of OCRL to bind clathrin even after removal of the C-terminal box (Choudhury et al, MBC 2005), and the cell biological data argues for its importance for OCRL function, yet its characterization as "unusual clathrin box" is shallow. How does its affinity towards clathrin terminal domain compare against "usual" clathrin boxes? The data shown in Fig 1C is not quantitative and, thus, it is hard to interpret. Does it engage the same binding site in the terminal domain? If so, how could one reconcile this with the published structural data suggesting that a negative charge is required at position 5 (ter Haar et al. PNAS 2000)?
- 3) The assay used in Fig. 5 to argue for a dominant-negative effect of the N-terminal region of OCRL is too rudimentary (even when in the past it has been published in high-profile journals). Besides being qualitative, basically the same result would be obtained if the overexpressed fragment just made the cells sick. Robust quantitative assays for clathrin-dependent internalization have been used for over two decades.

Additional correspondence

03 March 2009

I have now received the comments of the fourth reviewer, which are attached below. As you will see, his/her report is in good agreement with those of the other reviewers: namely that further characterisation of the N-terminal clathrin binding motif would be very valuable. As he/she recognises, structural analysis of the interaction is probably asking too much, but the alternative experiments suggested would potentially strengthen the paper considerably, and go along the same lines as the comments of the other referees. Therefore, I would ask that you also address the criticisms raised in this report in the revised version of your manuscript.

Apologies for the delay in getting this report back to you, and I look forward to receiving your revision.

Reviewer 4 comments:

This paper by Mao et al is a mainly structural study on the related amino terminal domains of OCRL and INPP5B, which both turn out to have unexpected PH domain folds. The interesting part of the paper come from the identification of a clathrin binding motif in an OCRL PH domain loop, which the authors go on to show functions in vivo. The data as it stands is of a suitable quality for publication in EMBO but, in my opinion, the work does not go far enough to justify publication as a whole in EMBO at present. However by carrying out a few more studies I would feel able to recommend publication. The major shortfall in this paper is that it does not characterize and identify where and how this 'novel' calthrin binding motif binds to the clathrin beta propeller. Is it in fact a different version of the standard LLDLD clathrin box or does it bind somewhere else, for instance between another pair of blades or on the top face. What is the relative strength of the interaction of this motif as compared to the known standard clathrin box and the so-called WxxW box.

The ideal experiments would be to study by NMR or crystallography the binding of this peptide with help from the published clathrin propeller structures from the Kirchhausen lab. However this will be extremely difficult I suspect and so I would recommend the following :

Charaterise the strength of binding between the clathrin beta propeller and the new peptide and compare it with the binding of the standard clathrin box where the kd is around 20 micromolar. This could be done by isothermal titration calorimetry or by using a fluorescence based binding assay.

Compare the effect of mutations known to abolish binding of standard LLDLD box peptides as demonstrated by the Kirchhausen laboratory on the binding of an LLDLD box peptide and the 'novel motif' identified here.

Finally it would also be very nice to see that transplanting the 'novel motif' here into another context conferred clathrin binding. An obvious candidate would be the PH domain of INPP5B. Another possibility would be to replace the LLDLD box in a construct of say AP2 beta ear+hinge and show that this construct can still drive the polymerisation of clathrin as shown by the McMahon laboratory

=====Referee #1 (Remarks to the Author)=====

The study constitutes a significant advance in understanding the structural organization and regulation of OCRL. The paper is well written and illustrated, and the technical quality is excellent. The paper is suitable for publication following a few minor improvements.

We thank the reviewer for his/her very positive comments.

1. To make the characterization complete, it would be helpful to measure the Kd of the clathrin heavy chain terminal domain for the unusual sequence in the OCRL PH domain (or a peptide model thereof) and to compare it to conventional clathrin binding signals.

We performed surface plasma resonance (SPR) experiments and measured the Kd of the interaction between clathrin's γ -propeller domain and the PH domain of OCRL. The value obtained (2.5 μ M) is in fact higher than the Kd calculated for a conventional clathrin box (see Miele et al. 2004; PMID: 14981508). The new SPR experiments confirmed the specificity of the interaction and its dependence from the unconventional clathrin box (binding was abolished by the I74N mutation). They also revealed competition with binding to clathrin mediated by a conventional clathrin box, the clathrin box of the clathrin adaptor AP-2. These results are reported in figures 1D and 1E)

2. Pg 12, para 4, line 2, "figure 5" should be "Figure 7".

Thank you for pointing out these mistakes that we corrected.

3. Incorporate a structure-based sequence alignment of OCRL and INPP5B into the alignment of INPP5B orthologs in Figure 3D.

We have revised figure 3D and added the OCRL PH sequence to the alignment based on structural elements.

4. Replace kD with kDa on pages 6 and 12.

We have changed the two "kD"s to kDa.

=====Referee #2 (Remarks to the Author)=====

This reviewer concurs with reviewer #2 in the opinion that the manuscript is "well written and interesting" and that it reports "clear and compelling data".

We thank the reviewer for his/her very positive comments.

Although the core fold of the PH domains from OCRL and INPP5B are very similar, the surface density map looks quite different to me, suggesting they may have distinct interaction partners or functions. This possibility if valid could be described in more detail in the text.

We agree with the opinion of the reviewer that the surface density maps of the PH domains of OCRL and INPP5B look quite different in spite of the similarity of the core fold. We suspect that binding to clathrin in the case of OCRL may not be the only functional difference. Yet, so far, this is the only difference that we have found. We have searched for the occurrence of potential other binding partners, but with negative results.

The relative contribution of the C-terminal clathrin binding site and the alpha-adaptin binding sites to OCRL localization are not studied. It would be interesting to compare mutations of each of these domains to that of the LIDI motif.

We have followed this suggestion and we have complemented the previous TIRF microscopy

experiments with an analysis of the effect of the disruption of the COOH-terminal clathrin box of OCRL. This new result reveals a similar contribution of both clathrin boxes to the localization of OCRL at clathrin coated pits. (see revised figure 6).

====Referee #3 (Remarks to the Author)=====

What are the bases for calling these two structures "PH domains"? I could only find a vague statement about the folds being "similar" with no figure showing superimposition of structures or information about RMSD's with canonical PH domains.

We revised Figure 2 and included the structure of the pH domains of PLC for comparison. We have also calculated the RMSD between these two PH domains, which is about $2.2 \approx$ over 65 overlapping residues.

How does its affinity towards clathrin terminal domain compare against "usual" clathrin boxes? The data shown in Fig 1C is not quantitative and, thus, it is hard to interpret. Does it engage the same binding site in the terminal domain?

We have now performed quantitative SPR studies and found an affinity of 2.5 M which is in the range, in fact higher, than that of "usual" clathrin boxes. We have also found that a conventional clathrin box (from the clathrin adaptor AP-2) competes with the PH domain of OCRL in the binding to clathrin, thus suggesting a similar mode of binding (New figures 1D and E).

If so, how could one reconcile this with the published structural data suggesting that a negative charge is required at position 5 (ter Haar et al. PNAS 2000)?

We are surprised too by our results in view of the structural results of ter Haar et al. However, our results have now been supported 1) by a new method, SPR, and 2) by competition experiments (Figure 1D and E). As shown in Figure 1E, a classical clathrin box peptide competes with OCRL PH domain for binding to clathrin. We note that in ter Haar the importance of the acidic residue at the 5 position had not been confirmed by mutagenesis.

The assay used in Fig. 5 to argue for a dominant-negative effect of the N-terminal region of OCRL is too rudimentary (even when in the past it has been published in high-profile journals). Besides being qualitative, basically the same result would be obtained if the overexpressed fragment just made the cells sick. Robust quantitative assays for clathrin-dependent internalization have been used for over two decades.

We have performed new transferrin uptake experiments. We show new microscopy images (new Figure 5) and we have quantified the reduced transferrin uptake by an Elisa assay.

====Reviewer 4 comments=====

Is it in fact a different version of the standard LLDLD clathrin box or does it bind somewhere else, for instance between another pair of blades or on the top face. What is the relative strength of the interaction of this motif as compared to the known standard clathrin box and the so-called WxxW box.

The competition shown by the SPR results indicates that the clathrin binding peptide of OCRL binds at a site in clathrin that overlaps with the binding site for the standard LLDLD box. We found a K_d of $2.5 \mu\text{M}$, which is higher than the calculated affinity for an LLDLD peptide of about $20 \mu\text{M}$, as the reviewer states. Such higher affinity may be explained by a contribution of flanking amino acids in the peptide (ETLLIDIASNS) used in the SPR experiments.

Comments referee #4

The authors have addressed most of the points raised by carrying out additional experiments rather than 'arguing the points away' which is a welcome change these days. In my opinion this article is now suitable for publication in EMBO J