Coordination of Grp1 recruitment mechanisms by its phosphorylation

Jian Li, David Lambright, and Victor Hsu

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Review Timeline:

Submission Date:	2020-03-04
Editorial Decision:	2020-04-13
Revision Received:	2020-09-16
Accepted:	2020-09-23

Editor-in-Chief: Matthew Welch

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.)

RE: Manuscript #E20-03-0173
TITLE: Coordination of Grp1 recruitment mechanisms by its phosphorylation

Dear Victor, I have just received the reviews of your study "Coordination of Grp1 recruitment mechanisms by its phosphorylation". Overall, they are positive but there are issues raised, especially by reviewer 1, that need to be addressed. I hope these will mostly be possible without further experimentation. Please send the revised manuscript along with a cover letter that explains how you addressed each issue. Thanks for submitting this excellent study, I hope you and your team are healthy and doing well.
Sincerely,
Adam Linstedt

Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Hsu,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

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Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

The cytohesins are a family of ARF-specific guanine nucleotide exchange factors (GEFs) that catalyze the loading of ARFs with GTP and are important regulators of carrier vesicle formation. The four cytohesins contain PH domains that mediate their recruitment and activation to specific cellular locations. One of these, Grp1, has been reported to bind PI(3,4,5)P3, and is recruited to the plasma membrane at least in part through this mechanism. Here the authors report the surprising finding that Akt-dependent phosphorylation of Grp1 at Thr280, which resides within its PH domain, alters its phosphoinositide-binding specificity, promoting interaction with PI4P rather than PIP3. T280 phosphorylation also enhances binding of an N-terminal coiled-coil domain to two accessory proteins, GRASP and IPCEF, which together with PI4P binding by the PH domain, drives recruitment of Grp1 to recycling endosomes in adipocytes, where it promotes recycling of the insulin-sensitive glucose transporter Glut4. Others (Hiester and Santy, 2013) have shown that phosphorylation of a corresponding threonine in another cytohesin (ARNO) relieves an autoinhibitory conformation, and that the free coiled-coil domain binds both GRASP and IPCEF, so this aspect of the current study is not entirely novel. However, here the authors show that a combinatorial interaction between Grp1, PI4P, GRASP and IPCEF is necessary for recruitment to recycling endosomes. Knockdown of either PI4KIIIa, GRASP or IPCEF substantially impairs recruitment and attenuates insulin-stimulated Glut4 recycling to the plasma membrane.

In general the study is well-designed, the data are convincing and support the conclusions drawn by the authors. There are, however, several issues that need to be addressed:
1. A major issue is that all of the immunoblots need to be quantified across at least 3 experiments. Currently only single blots are shown with no quantification. This is essential to demonstrate reproducibility.
2. How were the phosphoinositide-specific antibodies validated?
3. It is clear that both GRASP and IPCEF bind the coiled coil domain of Grp1, but do they bind each other?
4. While the authors show that a phosphomimetic mutant, T280D, localizes to recycling endosomes, does the corresponding alanine substitution inhibit localization? This is never shown. Does it inhibit Glut4 translocation?
5. Fig. 5 shows the interaction between recombinant coiled-coil domain and recombinant

GRASP/IPCEF/Grp1 PH domain to demonstrate direct interaction. First, are these blots or stained gels? If they are blots, how were the non-GST-tagged proteins indentified? Are they tagged or were antibodies used?
6. The composition of the liposomes used for binding studies should be described in more detail in the text. What percentage of each lipid is incorporated? This information is in the Methods section but should also be stated in main text.
7. It would be useful to show a cartoon of Grp1 as part of Fig. 1, showing the location of T280.
8. The identity of the PI4P biosensor (P4M) should be mentioned in the text.
9. What are the specificities of the PI4K inhibitors PAO, PIK93 and adenosine? This is not described anywhere.
10. The authors mention in several places that the Hiester and Santy study showed only that phosphorylation promoted membrane interaction. That study also clearly demonstrated that the coiled-coil domain of ARNO/cytohesin-2 bound directly to GRASP and IPCEF. This should be stated more clearly in the text, both in the introduction and the discussion.

Reviewer #2 (Remarks to the Author):

Hsu and colleagues analyze the mechanism by which an ARF-GEF, Grp1, is recruited to the recycling endosomes (RE). They have previously shown that insulin activates the AKT kinase which phosphorylates Grp1 in position 280 and that this induces the recruitment of Grp1 to the RE, a step that in turn promotes the transport of GLUT-4 from the RE to the plasma membrane.

Others have previously shown that ARNO, an ARF-GEF similar to Grp1, which binds to cell membranes through the interaction between its PH domain and phosphoinositides, requires, for membrane binding, the release, mediated by a phosphorylation in a site analogue to T280 in Grp1, of an inhibitory mechanism due to intramolecular bonds between the ARNO PH domain and a coil coil (CC) region.

The authors show that a partially similar mechanism occurs in Grp1. Phosphorylation by AKT in position 280 causes the release of the intramolecular bond between the PH domain and the CC region, but in addition activates a complex mechanism that leads a switch of the selectivity of the PH domain from PI3P to PI4P; that this lipid localizes in recycling endosomes (in addition to other membranes); and that this switch mentioned involves a second mechanism, by which the CC domain in Grp1, once freed form the interaction with the PH domain, binds to two protein residents of the recycling endosomes. Each of these effects is modest, but the sum of the effects on the PH domain and on the CC region results in a strong recruitment of Grp1 on the endosomes and in the activation of transport of GLUT-4 to the plasma membrane.

The experimental demonstration of this complex series of events is carried out by the authors at two levels: one is in vitro, in suitably prepared liposomes, for the mechanistic analysis of the phenomena; and the second is in live cells, for of the conclusions in live cells. The experiments are complete and in my opinion professionally performed. In my opinion, the conclusions represent an important advance in the specialized but important field of ARF and other GTPases that control membrane trafficking and many other cellular phenomena.

A gap that could and should be filled (not with new experiments but using molecular modeling approaches) concerns the question of how the phosphorylation in T280 can modify the specificity of binding of Grp1 from PI3P to PI4P. Molecular models of the interaction of Grp1 with PI3P are

available, which should make this analysis quite possible. Explaining the switch at this level would greatly support the conclusions and increase the overall strength and elegance of the study.

RESPONSES (MBC Manuscript #E20-03-0173)

We thank the reviewers for their thoughtful comments on our manuscript. Below, we provide a point by point response to all critical comments that were provided.

Reviewer #1

1. A major issue is that all of the immunoblots need to be quantified across at least 3 experiments. Currently only single blots are shown with no quantification. This is essential to demonstrate reproducibility.

- As requested, we have now added quantitation in addition to showing representative immunoblots. Quantitation involves at least 3 experiments as requested.

2. How were the phosphoinositide-specific antibodies validated?

- They have been validated by the vendor, Echelon Biosciences. Their website also list publications that have used these antibodies.

3. It is clear that both GRASP and IPCEF bind the coiled coil domain of Grp1, but do they bind each other?

- As requested, we have now performed this additional experiment and find that GRASP and IPCEF1 also bind directly to each other. See new Figure S7A and S7B.

4. While the authors show that a phosphomimetic mutant, T280D, localizes to recycling endosomes, does the corresponding alanine substitution inhibit localization? This is never shown. Does it inhibit Glut4 translocation?

- We have shown in our previous publication (Li et al 2012, Dev Cell, 22: 1286-96) that the alanine mutant (T280A) prevents Grp localization to the recycling endosome and also inhibits glut4 recycling.

5. Fig. 5 shows the interaction between recombinant coiled-coil domain and recombinant GRASP/IPCEF/Grp1 PH domain to demonstrate direct interaction. First, are these blots or stained gels? If they are blots, how were the non-GST-tagged proteins identified? Are they tagged or were antibodies used?

- Gels showing Grp1 forms and 6xHis-tagged IPCEF1/GRASP are immunoblots, with the former using an anti-Grp1 antibody and the latter using an anti-6xHis tag antibody. Gels showing GST-tagged proteins are Coomassie stained. We have now added this clarification to the Methods section.

6. The composition of the liposomes used for binding studies should be described in more detail in the text. What percentage of each lipid is incorporated? This information is in the Methods section but should also be stated in main text.

- As requested, we have now stated the liposome composition also in the main text.

7. It would be useful to show a cartoon of Grp1 as part of Fig. 1, showing the location of T280.

- We wish to point out that the requested cartoon, indicating the location T280 in Grp1, has been shown previously in Figs S1A and S6A. Moreover, because Fig 1 has become quite crowded, due to the quantitation of all gels requested by the reviewer, we hope the reviewer agrees that the illustrations in Figs S1A and S6A are sufficient.

8. The identity of the PI4P biosensor (P4M) should be mentioned in the text.

- As requested, we have now added this description to the text.

9. What are the specificities of the PI4K inhibitors PAO, PIK93 and adenosine? This is not described anywhere.

- The specificities of the inhibitors are detailed in the review that we had cited in the text (Balla and Balla 2006 Trends Cell Biol. 16:351-361).

10. The authors mention in several places that the Hiester and Santy study showed only that phosphorylation promoted membrane interaction. That study also clearly demonstrated that the coiled-coil domain of ARNO/cytohesin-2 bound directly to GRASP and IPCEF. This should be stated more clearly in the text, both in the introduction and the discussion.

- We wish to clarify that the Hiester study incubated the CC domain of ARNO with cell lysate followed by immunoblotting for GRASP and IPCEF to show protein interactions. This approach cannot distinguish between direct versus indirect binding. In contrast, we have used purified proteins to show direct interactions.

Reviewer #2

A gap that could and should be filled (not with new experiments but using molecular modeling approaches) concerns the question of how the phosphorylation in T280 can modify the specificity of binding of Grp1 from PI3P to PI4P. Molecular models of the interaction of Grp1 with PI3P are available, which should make this analysis quite possible. Explaining the switch at this level would greatly support the conclusions and increase the overall strength and elegance of the study.

- As requested, we have now added molecular modeling to explain how the phosphorylation of T280 can modify the specificity of phosphoinositide binding by Grp1. See new Figure 6 and its accompanying text in the Discussion section.

RE: Manuscript #E20-03-0173R
TITLE: "Coordination of Grp1 recruitment mechanisms by its phosphorylation"

Dear Dr. Hsu:

I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Sincerely,
Adam Linstedt
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Hsu:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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