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Hop/Sti1 phosphorylation inhibits its co-chaperone function

Alina Roehl, Franziska Toppel, Evelyn Bender, Andreas Schmid, Klaus Richter, Tobias Madl and Johannes Buchner

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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: Nonia Priente

1st Editorial Decision

15 July 2014

Thank you for your submission to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email. As you will see, the referees find the topic of interest and that the study could become suitable for publication here, but they also find the study is not sufficiently conclusive as it stands.

As the reports are included below, I will not detail them here. However, all referees request that non-phosphorylatable alanine mutants are also analyzed. Referee 3 brings up two additional important points by requesting a demonstration of the effect of phospho-mimicking mutations on Sti1-Hsp70 association, and on the composition of Hop complexes, which need to be addressed. In addition, please also experimentally address the other points brought up by referees 1 and 2 and discuss the issues mentioned. If the referee concerns are adequately answered, we would be happy to accept your manuscript for publication. However, please note that it is EMBO reports policy to undergo one round of revision only and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review.

I look forward to seeing a revised form of your manuscript when it is ready. In the meantime, please contact me if I can be of any assistance.

REFeree REPORTS:

Referee #1:

Rohl and colleagues describe species specific negative regulation of the Hsp90 co-chaperone Hop/Sti1 mediated by distinct phosphorylations of Hop/Sti1. The experiments are clearly described and for the most part the data support the authors' conclusions. My major concern is the lack of evaluation of relevant non-phosphorylatable mutants in the assays described. Although the authors do present some data that the E mutants grossly retain structural integrity, it would greatly strengthen their argument as to the dynamic importance of phosphorylation *in vivo* (since no phospho-specific antibodies are used in the study) if they examined the behavior of comparable non-phosphorylatable mutants in the *in vivo* GR activity assay. I am not suggesting that they test all of their mutants, only those two in which E mutation most strongly affects Hsp70 interaction - e.g., S410A (Sti1) and Y354F (Hop). In this context, it would also be interesting

Other comments:

1. Along the lines of what is mentioned above, it would be useful to assess GR expression/protein stability in the yeast harboring the Sti1/Hop phospho-mutants (at least Sti1-S410E and Hop-Y354F).
2. In the discussion, on page 11, the authors state that "tyrosine phosphorylation is absent in yeast". While tyrosine phosphorylation of Hop may not occur in yeast, Swel-dependent tryosine phosphorylation of Hsp90 has been reported previously (Mollapour et al. Mol Cell 2010; 37, 333). The authors should clarify the sentence in question either to make clear that they are referring only to tyrosine phosphorylation of Hop in yeast or to correct the state that tyrosine phosphorylation does not occur in yeast.

Referee #2:

In this study the authors compared the homologous Hsp90 cochaperones yeast Sti1 and human Hop and characterize effects of phosphorylation at sites previously found in global mass spectrometry (MS) screens. They show convincing evidence that Sti1 and Hop have similar affinities to Hsp90 and Hsp70, they both inhibit Hsp90's ATPase activity and both support client activation in a yeast model system. In particular, the finding that human Hop is able to inhibit the ATPase activity of human Hsp90 is important, because it had been claimed previously that Hop deviates from Sti1 in this respect despite the high sequence conservation (McLaughlin 2002). This discrepancy could be due to the very weak ATPase activity of human Hsp90. The authors also analyzed uncharacterized phosphorylation sites of Sti1 and Hop by constructing phosphor-mimetic variants. Most of them exhibited diminished activation for glucocorticoid receptor (GR) in yeast and some showed slightly reduced affinity to Hsp70. They also highlighted a tyrosine phosphorylation site in Hop that resulted in a distinct change of conformation. Consistently, this variant exhibited the strongest defects in respect to affinity to Hsp70 and GR activation *in vivo*.

This study is an important contribution to the field of chaperone research and posttranslational modifications. The quality of the data is very high throughout the study and most conclusions are justified. The results are discussed in sufficient detail in the context of previous publications. There are only a few comments that authors should address.

Major comments:

1- Fig. 3C: all phospho-mimetic variant of Sti1 showed diminished levels of GR activation in yeast as compared to the wild-type protein, demonstrating that Sti1 is not phosphorylated to a high degree at these positions in yeast under the conditions tested or phosphorylation of these sites is only very transient. The authors may want to use non-phosphorylatable alanine variants *in vivo* to see whether they are now more active than wild-type Sti1.

Similarly, in Fig. 3D, phospho-mimetic variants of Hop show a decreased potential to activate GR in yeast as compared to the wild-type protein. However, the responsible kinase(s) may be absent in yeast.

The authors should discuss this.

2- Fig. 5B and 5C: to further validate the dissociation constants by phospho-mimetic variants to Hsp70, the authors could use data from analytical ultracentrifugation (integration of peaks for free and bound Sti1/Hop) to calculate the K_d and compare them with values obtained by SPR.

It is worth noting that the differences in K_d for Hsp70 of the phospho-mimetic variants as compared with wild-type are relatively small. Would that be sufficient to account for the near-complete

abrogation of GR activation of some variants in yeast?

Minor comments:

1- Fig. 1C: the absolute values of ATPase activity should be given in the legend. Fig. 1D and 1E: original SPR data including the fits should be provided as supplementary figure.

2- Fig. 4C: The different conformations of Hop in the phosphorylated state are not very well visualized. TPR2A and TPR2B should be colored differently in the unphosphorylated and the phosphorylated conformation and the author should overlay one of the two domains (e.g. TPR2B) while changing the color of the other domain according to each different conformation. They should also indicate what is represented by the spheres.

3- On page 8 the authors compare the reduced levels of GR activation of phospho-mimetic and wild-type Sti1 proteins with the results for phospho-mimetic and wild-type Hop proteins. They write "While for Sti1, different degrees of inactivation were observed, the effect of phosphorylation on Hop seems stronger and more like a complete inactivation" This conclusion does not seem to be justified the differences are more gradual. Furthermore, GR activation is determined in yeast and human Hop might be slightly stronger affected because the interaction with yeast Hsp90 and yeast Hsp70 already make it more sensitive to small changes in activity. "Regulation" may be used instead of "inactivation" in this context.

4- On page 11, the authors write " ... as tyrosine phosphorylation is absent in yeast." This is not quite true. There was a report by Mollapour et al in 2010 on a phosphorylation site by the tyrosine kinase Swe1 in yeast. Moreover, Ser/Thr kinases in yeast can have dual functionality (Stern et al 1991 Mol Cell Biol; Mendelhall et al 1998 Microbiol Mol Bio Reviews; Malathi et al 1999 Genetics; Zhu et al 2000 Nature Genetics; Magherini et al 2006 Int J Biochem Cell Biol; etc)

5- Fig. 2 A and B: the authors might want to include also the color label in the figure as well as separate the descriptions for A and B in the legend to make it unambiguous.

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8- The authors should mention in the Method part which yeast Hsp90 protein they have used in their assays.

9- It would be helpful to indicate the phosphorylation sites in the alignment shown in supplemental figure 1

Referee #3:

Although the yeast Sti1 protein and human Hop are conserved in primary sequence, the amino-acids used to regulate this cochaperone differ. The presented manuscript attempts to demonstrate that the p60-cochaperones are regulated through different phosphorylation sites. While the work is potentially interesting, the current data does not fully support the conclusions.

A) The authors use glutamate substitutions as phospho-mimicks and tested the mutants in vivo (Figure 3). Yet, they do not perform the equally important experiment of testing alanine substitutions. In the absence of the alanine mutants it is not possible to conclude whether the glutamate substitutions are actually mimicking phosphorylation events or whether the substitutions are acting as mutations causing pleiotropic effects on the Sti1 or Hop proteins.

B) The authors state, "the two Hsp70 binding sites of Sti1 implicating a regulatory role for the interaction of Hsp70". Rather than just suggesting this mechanism, the authors should directly show that the T37E and S410E mutants alter the association of Sti1 and Hsp70 in vivo by performing pull-down experiments to assess the interaction level between these proteins for the wild type and mutant proteins.

C) Similar to point 2, the authors state, "implying that either the affinity for Hop is reduced or the conformation of the ternary complex is altered". The authors should directly demonstrate if the phospho-mimicks alters the composition of the Hop complexes in vitro.

D) Minor-Last sentence of abstract seems to missing "human" in the line "yeast and especially their...".

Referee#1:

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In response to the request to analyze mutants which cannot be phosphorylated *in vivo*, we created nine Ala-mutants and carried out the *in vivo* experiments regarding their effect on maturation of the glucocorticoid receptor in $\Delta sti1$ yeast cells. In the case of Sti1 the alanine substitutions did not lead to a decrease of GR activation except for S95A. The Hop alanine/phenylalanine variants affected GR maturation similar to the wild-type protein (see Figure 3 manuscript).

Other comments:

1. Along the lines of what is mentioned above, it would be useful to assess GR expression/protein stability in the yeast harboring the Sti1/Hop phospho-mutants (at least Sti1-S410E and Hop-Y354E).

To address this issue we used yeast cell lysates of a $\Delta sti1$ yeast strain harboring the respective plasmids for phospho-mimicking variants as well as alanine substitutes and detected the protein level of GR as well as Sti1 and Hop. We detected GR expression in all immunoblot experiments in the presence of Sti1/Hop wild-type, phospho-mutants as well as alanine/phenylalanine variants. As a negative control, we used Sti1 Δ yeast cells harboring an empty vector and as positive $\Delta sti1$ yeast cells expressing Sti1/Hop wild-type (see below). Comparable levels of the variants were obtained. The slight variations obtained in this experiment cannot be correlated to differences in activity. The experience obtained in these experiments suggests that a more detailed analysis using additional techniques would be required which goes beyond the scope of this study. Therefore we add the information on this experiment only in this reply.

Data not shown.

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and Hsp70, they both inhibit Hsp90's ATPase activity and both support client activation in a yeast model system. In particular, the finding that human Hop is able to inhibit the ATPase activity of human Hsp90 is important, because it had been claimed previously that Hop deviates from Sti1 in this respect despite the high sequence conservation (McLaughlin 2002). This discrepancy could be due to the very weak ATPase activity of human Hsp90. The authors also analyzed uncharacterized phosphorylation sites of Sti1 and Hop by constructing phosphor-mimetic variants. Most of them exhibited diminished activation for glucocorticoid receptor (GR) in yeast and some showed slightly reduced affinity to Hsp70.

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Similarly, in Fig. 3D, phospho-mimetic variants of Hop show a decreased potential to activate GR in yeast as compared to the wild-type protein. However, the responsible kinase(s) may be absent in yeast. The authors should discuss this.

In response to this query we mention in the revised manuscript that the natural phosphorylation of Hop will not occur in yeast. In agreement with this notion the alanine mutants and the wild-type behave similarly. The use of phospho-mimetic mutants allows circumventing this issue.

2- Fig. 5B and 5C: to further validate the dissociation constants by phospho-mimetic variants to Hsp70, the authors could use data from analytical ultracentrifugation (integration of peaks for free and bound Sti1/Hop) to calculate the K_d and compare them with values obtained by SPR. It is worth noting that the differences in K_d for Hsp70 of the phospho-mimetic variants as compared with wild-type are relatively small. Would that be sufficient to account for the near-complete abrogation of GR activation of some variants in yeast?

This is an interesting point. Actually, we performed the SPR experiments as it seemed impossible to extract quantitative data from the auc experiments. The reason is that upon complex formation there is not a defined shift from one peak to another (with a baseline in between). Rather we observed a gradual shift of the s-value. This complicates the application of mathematical models to extract a K_D . In our analysis, the change in affinity for Hsp70 is the major difference observed between wild-type and variants. In the revised version we added the dc/dt profiles showing the gradual peak shift in response to increasing concentrations of the respective variant as figure S3. It seems therefore reasonable to assume that this difference is the basis for the observed *in vivo* effects. Once further

assays are available such as single molecule FRET assays for Sti/Hop conformation it will be certainly worth revisiting this question.

Minor comments:

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The data requested were added.

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We agree and "regulation" is now used instead of "inactivation".

4- On page 11, the authors write " ... as tyrosine phosphorylation is absent in yeast." This is not quite true. There was a report by Mollapour et al in 2010 on a phosphorylation site by the tyrosine kinase Swe1 in yeast. Moreover, Ser/Thr kinases in yeast can have dual functionality (Stern et al 1991 Mol Cell Biol; Mendelhall et al 1998 MicrobiolMol Bio Reviews; Malathi et al 1999 Genetics; Zhu et al 2000 Nature Genetics; Magherini et al 2006 Int J Biochem Cell Biol; etc)

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Done

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The typos were corrected.

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Done

8- The authors should mention in the Method part which yeast Hsp90 protein they have used in their assays.

We added this information at the beginning of the methods section. Hsp82 was used.

9- It would be helpful to indicate the phosphorylation sites in the alignment shown in supplemental figure 1

Phospho-sites in *S. cerevisiae* Sti1 and human Hop are highlighted with red squares in the alignment shown in Figure S1.

Referee #3:

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As suggested we performed pull-down experiments with Sti1 wild-type and the phospho-mutants T37E, S410E coupled covalently to CNBr activated sepharose beads (see detailed method description below). The coupling reaction of the Sti1 variants to the sepharose beads were verified by SDS-PAGE (Figure 2B). Sti1 wild-type as well as the phospho-mutants were able to bind Hsp70 and Hsp90 (Figure 2A). We could not detect consistent differences in the interaction by the pull-down/western blotting approach. To this end we would need a more sensitive and more direct assay involving less processing steps.

Data not shown.

C) Similar to point 2, the authors state, "implying that either the affinity for Hop is reduced or the conformation of the ternary complex is altered". The authors should directly demonstrate if the phospho-mimicks alters the composition of the Hop complexes *in vitro*.

This is indeed an important point. However, it cannot be resolved experimentally at the moment. This would require to extract quantitative data from the aUC experiments. This is impossible as upon complex formation there is not a defined shift from one peak to another (with a baseline in between). Rather we observed a gradual shift of the s-value. This complicates the application of mathematical models to extract a K_D . In the revised version we added the dc/dt profiles showing the

gradual peak shift in response to increasing concentrations of the respective variant as figure S3.

D) Minor-Last sentence of abstract seems to missing "human" in the line "yeast and especially their...".

Corrected

2nd Editorial Decision

06 November 2014

Thank you for your patience while we have reviewed your revised manuscript. As you will see from the reports below, the referees are now all positive about its publication in EMBO reports. I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor issues/corrections have been addressed, as follows.

The description of the statistical analyses performed in the legends to several of the figure panels is incomplete. Please go through your manuscript carefully once more and ensure that all relevant figures and supplementary figures have been generated according to proper statistical analysis procedures, and all figure legends include information on the number of independent experiments measured (which should be at least three if errors are indicated), the type of error bars used and what the bar represents (mean, median, etc).

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. Please contact me if you have any questions or I can help getting the study ready for publication.

REFeree REPORTS:

Referee #1:

The authors have adequately addressed all of the comments raised by the reviewers. Further revisions are not necessary.

Referee #2:

The authors answered all our concerns satisfactorily. They added new data that strengthened their conclusions. I would like to add that pull-down experiments are only semi-quantitative and would not be able to resolve the observed K_d differences. Overall the study is very interesting to the chaperone field in particular because we are just about to grasp the extent of the regulation of chaperones by posttranslational modifications.

Referee #3:

The authors have met or exceed in their responses to the criticisms raised during the initial review. The work represents an important contribution to the molecular chaperone and protein folding fields.

2nd Revision - authors' response

18 November 2014

We have prepared our manuscript for final acceptance according to your suggestions. Specifically we

- added descriptions of the statistical analyses in the figure legends
- added missing error bars

We hope the paper is now ready to be accepted and we are looking forward to hearing from you.

3rd Editorial Decision

20 November 2014

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

Thanks for your contribution to EMBO reports and congratulations on a successful publication.