Sequestration of the PKC ortholog Pck2 in stress granules as a feedback mechanism of MAPK signaling in fission yeast

Yuki Kanda, Ryosuke Satoh, Teruaki Takasaki, Naofumi Tomimoto, Kiko Tsuchiya, Chun An Tsai, Taemi Tanaka, Shu Kyomoto, Kozo Hamada, Toshinobu Fujiwara and Reiko Sugiura
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MS TITLE: Stress Granules as a Feedback Mechanism of MAPK Signaling by Sequestering PKC/Pck2

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ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript. Unfortunately we only have one review but i have also looked at the paper in the light of this report.

To see the reviewer's report and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to

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all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this paper, Kanda et al. investigate the effects in fission yeast cells on stress granule (SG) formation and translocation of Pck2 (protein kinase C), of the kinase activity of Pck2 itself during heat stress. Their findings provide another example of modulating effects on the stress response at the post-transcriptional level. The work is a valuable contribution to the field, and also nicely illustrates how SGs can function as signaling hubs, not mere repositories of inactive RNAs, integrating intracellular signaling with subcellular localization.

Pck2 kinase activity affects Pmk1 signaling, putting Pmk1 downstream of Pck2 with respect to heat stress response. Previous screens for suppression of toxicity of Pmk1 hyperactivation turned up components implicated in SG formation, leading the authors to probe for a link between Pck2 and SGs. They find that Pck2 is localized to SGs under severe (45oC) but not moderate (43oC) heat stress (Fig 6). To study the Pck2 kinase activity-dependence of movement of Pck2 into SGs, a kd allele of Pck2 was used. This reduces co-localization of Pck2 with SGs but does not eliminate it. A hyperactive allele of Pck2 (pck2-R389A) gives more SGs per cell under heat stress (Fig. 3E) and more long-lasting dispersal from cell tips, whereas a pck2Δ null allele does the opposite, arguing for a role for Pck2 in SG formation. Heat shock caused Pck2 to disperse from cell tips within 5 min, so precedes formation of SGs. This is consistent with Pck2 going to the insoluble fraction at 45oC (Fig. 6F,G). By dampening the Pck2 activity, SGs gives a delay in relocalization of Pck2 to cell tips that allows cells to recover after heat shock. Pck2 kinase activity does not affect SG formation per se, only the localization of Pck2 to SGs.

Comments for the author

In my opinion, two major issues in the paper require attention however:

1. It is intrinsically difficult to show that any process is dependent on SG formation, as all gene products required to form SGs either as structural SG components or by regulating SG assembly, are also involved in other cellular processes. The authors use the dynamics of SG formation and dissolution to demonstrate that reactivation of Pmk1 signaling after a first heat shock goes faster when there is less SGs (Fig. 5). These experiments could have been more convincing if instead performed in other mutant backgrounds known to be defective in SG formation.

2. The authors argue repeatedly in the paper that active Pck2 preferentially locates to SGs. Yet, what is shown in most instances is evidence that constitutive or stress-induced activation of Pck2 does promote its dispersal from cell tips and translocation to SGs, while deactivation does the opposite. So far so good. But to say that it is the activated fraction of the Pck2 molecules in the population is a different matter. These would presumably be the ones phosphorylated in its activation loop, and to distinguish those from the rest would require e.g. a reagent like a Pck2-specific anti-phospho-antibody. The authors use this to quantitate active Pmk1, and something analogous would be required to support this statement for Pck2. The closest they come to demonstrating such a preferential localization of active Pck2 to SGs is where they show Pck2 activity relocating from the soluble to the insoluble fraction upon heat shock in Fig. 6. However, this does not show localization to SGs.

The authors should clarify if they mean that the activated fraction of Pck2 preferentially localizes to SGs, and if yes, provide clear evidence for this.

Moreover, the legend to Fig. 6 E is not clear. The graph shows Pck2 kinase activity during moderate or high heat stress, and is taken to mean that active Pck2 is preferentially located to the insoluble SG fraction at high heat stress. What we see in the graph is decreasing Pck2 activity at 45oC, and unchanged Pck2 activity at 43oC. If this panel is to show active Pck2 localizing to SGs, then what

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the graph must show is the remaining activity in the soluble fraction, which decreases. Is this interpretation correct? This has to be clarified.

Other issues:

3) Co-localization of Pck2 with SGs under heat stress was done without quantitation (Fig. 1 A) – this could easily be added.
4) Pck2 is required for Pmk1 activation at early, but not late stages of heat shock (Fig. 3A). Could this be due to a redundant function of Pck1?
5) Discussion, line 385-386: If the model that Pmk1 activity underlies SG formation is supported by deleting an inactivating phosphatase for Pmk1leading to enhanced translocation of Pck2 to SGs, then this data should be shown e.g.in a supplementary figure. What is the JCS standpoint of "data not shown", is it not required to show this data?

Minor remarks:

Why show the kinase activity of mutant Pck2 after showing its effects in the cell (Supplementary Fig. 4A) rather than before?
Line 277: is the decrease in the pmk1 deletion background compared to the actual wild-type, or compared to the pck2-R389A allele without the pmk1Δ deletion?
Fig. 1C: Change the label "Control" to "-CHX"

First revision

Author response to reviewers' comments

Referee: 1
We are grateful to Reviewer 1 for providing useful suggestions and insightful comments that helped us to considerably improve our manuscript. As indicated in the following responses, we have incorporated these comments and suggestions in our revised manuscript.

The reviewer made the following observations: In this paper, Kanda et al. investigate the effects in fission yeast cells on stress granule (SG) formation and translocation of Pck2 (protein kinase C), of the kinase activity of Pck2 itself during heat stress. Their findings provide another example of modulating effects on the stress response at the post-transcriptional level. The work is a valuable contribution to the field, and also nicely illustrates how SGs can function as signaling hubs, not mere repositories of inactive RNAs, integrating intracellular signaling with subcellular localization.

Pck2 kinase activity affects Pmk1 signaling, putting Pmk1 downstream of Pck2 with respect to heat stress response. Previous screens for suppression of toxicity of Pmk1 hyperactivation turned up components implicated in SG formation, leading the authors to probe for a link between Pck2 and SGs. They find that Pck2 is localized to SGs under severe (45°C) but not moderate (43°C) heat stress (Fig 6). To study the Pck2 kinase activity-dependence of movement of Pck2 into SGs, a kd allele of Pck2 was used. This reduces co-localization of Pck2 with SGs but does not eliminate it. A hyperactive allele of Pck2 (pck2-R389A) gives more SGs per cell under heat stress (Fig. 3E) and more long-lasting dispersal from cell tips, whereas a pck2Δ null allele does the opposite, arguing for a role for Pck2 in SG formation. Heat shock caused Pck2 to disperse from cell tips within 5 min, so precedes formation of SGs. This is consistent with Pck2 going to the insoluble fraction at 45°C (Fig. 6F,G). By dampening the Pck2 activity, SGs gives a delay in relocalization of Pck2 to cell tips that allows cells to recover after heat shock. Pck2 kinase activity does not affect SG formation per se, only the localization of Pck2 to SGs.

The reviewer also stated, "In my opinion, two major issues in the paper require attention however."

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Reviewer 1 Comments for the author
1. The reviewer mentioned, “It is intrinsically difficult to show that any process is dependent on SG formation, as all gene products required to form SGs either as structural SG components or by regulating SG assembly, are also involved in other cellular processes. The authors use the dynamics of SG formation and dissolution to demonstrate that reactivation of Pmk1 signaling after a first heat shock goes faster when there is less SGs (Fig. 5). These experiments could have been more convincing if instead performed in other mutant backgrounds known to be defective in SG formation.”

Re: We thank the Reviewer for these pertinent comments. We submit that this work uncovers a novel and important new finding that Pck2, an evolutionarily conserved mediator of MAPK signaling, is spatiotemporally regulated via its SG recruitment in a MAPK activity-dependent manner. We also believe that it goes into substantial depth by showing a requirement of Pck2’s kinase activity for its potent SG localization as well as the uniqueness of Pck2 recruitment into SGs upon severe HS, but not moderate HS.

We have followed this reviewer’s excellent suggestion and expressed Pck2-GFP in several mutant backgrounds known to be defective in SG formation in S. pombe and/or in mammals. These include 1) nrd1 deletion cells that are reported to display impaired SG formation upon HS (Satoh et al., PLoS ONE, 2012), and 2) the phosphorylation mutant of the translation initiation factor eIF2α (eIF2α S52A) (Nilsson and Sunnerhagen, RNA, 2011), as in mammalian cells, the key event leading to the formation of SGs is the stress-induced phosphorylation of the translation initiation factor eIF2α.

In these mutant backgrounds, the endogenous Pabp was visualized, and the effects of moderate (43˚C) and severe (45˚C) heat shock on the localization of Pabp were examined. In nrd1 deletion cells, the number of Pabp-GFP dots was significantly decreased upon moderate heat shock (43˚C) as compared with that of the WT cells. However, the number of Pabp dots upon severe heat shock (45˚C) was not significantly affected by Nrd1 deletion. Moreover, Pck2-GFP dots were similarly observed both in Nrd1 deletion and WT cells upon HS (45˚C).

The number of the Pabp-tdTomato dots was significantly decreased in the eIF2α S52A mutant cells upon moderate heat stress (43˚C), but not upon severe heat stress conditions (45˚C). In addition, dynamic translocation of Pck2-GFP from the cell tips to the SGs upon severe HS (45˚C) was similarly observed in the eIF2α S52A mutant cells and the WT cells.

These results indicate that Pck2 translocation to SGs upon severe HS was independent of Nrd1 or the phosphorylation of eIF2α and suggest the distinct regulatory mechanism and/or the structural components of SGs achieved by severe HS. These results further implicate the uniqueness of Pck2-positive SGs upon severe HS.

We also performed experiments utilizing the dynamics of SG formation and dissolution upon first and second HS (45˚C) and investigated the dynamics of Pck2-GFP and Pmk1 MAPK reactivation in Nrd1 deletion in comparison with the WT cells. The results showed that Nrd1 deletion does not significantly affect the Pck2-GFP localization and Pmk1 MAPK reactivation.

Collectively, these results reveal the heterogeneity of SGs upon distinct HS conditions and highlight the uniqueness of Pck2-positive SGs induced by severe HS. Thus, although we appreciate the importance of this issue raised by the reviewer, we would prefer the identification of components/regulators that would specifically affect Pck2 recruitment into SGs and/or the cis-elements/sequences required for Pck2 SG localization as well as the physiological significance of the unique feature of the severe heat stress-mediated SG formation the focus of a future study.

These Figures and descriptions have been incorporated in the revised manuscript (Figure 6A, 6B, 7C, 7D, S3A, S3B, and S3C).

2. The reviewer mentioned “The authors argue repeatedly in the paper that active Pck2 preferentially locates to SGs. Yet, what is shown in most instances is evidence that constitutive or stress-induced activation of Pck2 does promote its dispersal from cell tips and translocation to SGs,

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while deactivation does the opposite. So far so good. But to say that it is the activated fraction of the Pck2 molecules in the population is a different matter. These would presumably be the ones phosphorylated in its activation loop, and to distinguish those from the rest would require e.g. a reagent like a Pck2-specific anti-phospho-antibody. The authors use this to quantitate active Pmk1, and something analogous would be required to support this statement for Pck2. The closest they come to demonstrating such a preferential localization of active Pck2 to SGs is where they show Pck2 activity relocating from the soluble to the insoluble fraction upon heat shock in Fig. 6. However, this does not show localization to SGs. The authors should clarify if they mean that the activated fraction of Pck2 preferentially localizes to SGs, and if yes, provide clear evidence for this."

Re: We thank this reviewer for the constructive comments. We are sorry for the misunderstanding/misusage of the phrase "active Pck2 preferentially locates to SGs". Accordingly, we editorially modified the text to minimize the phrase "active Pck2 preferentially locates to SGs". We also removed the word "active" from the model to show our hypothesis (Figure 8). We also performed experiments as suggested by the reviewer. For this, we investigated if we could use the mammalian anti-phospho-PKC antibodies (anti-PKC (phospho T497): ab59411) to distinguish phosphorylated Pck2 from the rest in S. pombe. It has been reported that Ksg1 (fission yeast PDK1)-mediated phosphorylation of the activation loop of Pck2 at T842 was essential to regulate its biological activity (Madrid et al., JCS, 2015). We, therefore, created strains expressing the phosphorylation mutant Pck2 T842A-GFP wherein Thr842 in the Pck2 activation loop was replaced with Ala. Immunoblotting showed that anti-phospho-PKC T497 antibodies detected Pck2 phosphorylation in cells harboring the WT Pck2-GFP, but not in cells harboring the unphosphorylated Pck2 T842A-GFP or the kinase-dead version of Pck2 K712W-GFP (A), consistent with a previous report (Madrid et al., 2015, JCS).

Immunofluorescence microscopy was performed using anti-GFP and anti-phospho-PKC antibodies to detect the subcellular localization of total or phosphorylated Pck2 with or without severe heat stress as shown below. Anti-GFP antibodies successfully detected the immunofluorescence of Pck2 protein (B: anti-GFP: red), as the fluorescence of GFP-Pck2 (B: Pck2-GFP: green) merged with that detected by anti-GFP antibodies enriched in the cell tips in the absence of HS (B: Merge). In contrast, anti-phospho-PKC antibodies failed to detect the Pck2 cell-tip-localization both in cells harboring the WT-Pck2-GFP and Pck2 T842A-GFP (C: Anti-p(T842)). Instead, anti-phospho-PKC antibodies stained the cytoplasmic region of both strains. Importantly, upon HS, Pck2 dots as detected by the fluorescence of GFP were barely detectable by anti-GFP-antibodies (D), indicating that the immunostaining process, including fixation (formaldehyde) and Zymolyase treatment for cell wall digestion and cell permeabilization, might intervene the cellular architecture implicated in the integrity of SG assembly. Although we appreciate that the reviewer's suggestion to visualize active Pck2 in SGs by immunofluorescent staining using an anti-phospho-Pck2 antibody would be ideal to unequivocally demonstrate that the activated fraction of Pck2 preferentially localizes to SGs, it is technically difficult at present.

As we consider this aspect to be too preliminary for inclusion in the result section, I would like to show the data for the Reviewer's and the Editor's reference, only as below.

We can provide the data from the URL below:
https://drive.google.com/file/d/1kvkR-j_NJ_D0SO2bThFMG7BU_0f-RSD_/view?usp=sharing

To address this issue by alternative methods, we further undertook experiments aiming to test if the activated Pck2 preferentially localizes to SGs by visualizing both active and inactive Pck2 fused to a distinct fluorescent tag, respectively as shown below.

For this, we visualized the endogenous kinase-dead (inactive) Pck2 K712W-GFP and investigated the effect of exogenous expression of the constitutively active version of Pck2 RA-fused to mCherry (A). In addition, we visualized the endogenously expressed active RA-Pck2-GFP and investigated the effect of exogenous expression of inactive Pck2 K712W-mCherry (B).

The results showed that the inactive Pck2 K712W-GFP was observed to persist in the plasma membrane and less translocated to the SGs as compared with the exogenously expressed active Pck2-mCherry (A). In contrast, the endogenously expressed active Pck2RA potently translocated to

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SGs whereas exogenously expressed inactive Pck2 barely translocated to SGs (B). We believe that these data corroborate the possibility that active Pck2 preferentially localized to SGs. However, as these experiments are an indirect demonstration of the issues raised by the reviewer, we carefully revised the manuscript by taking special caution regarding the limitations of this alternative experiment. These Figures and descriptions have been incorporated into the revised manuscript (Supplementary Figure S4).

We can provide the data from the URL below:
https://drive.google.com/file/d/1WhDxObf2E9l7TQetuvzMJ0EJfDC_vPo-/view?usp=sharing

The reviewer also mentioned, “Moreover, the legend to Fig. 6 E is not clear. The graph shows Pck2 kinase activity during moderate or high heat stress, and is taken to mean that active Pck2 is preferentially located to the insoluble SG fraction at high heat stress. What we see in the graph is decreasing Pck2 activity at 45°C, and unchanged Pck2 activity at 43°C. If this panel is to show active Pck2 localizing to SGs, then what the graph must show is the remaining activity in the soluble fraction, which decreases. Is this interpretation correct? This has to be clarified.”

Re: Our apologies for the inappropriate labeling of the graph (Figure 6E), as well as insufficient description regarding the legend to Figure 6E. We labeled the graph as “Remaining kinase activity in the soluble fraction” and described the legend to Figure 6E appropriately to indicate that Pck2 activity decreases at 45°C and unchanged Pck2 activity at 43°C in the soluble fraction.
These Figures and descriptions have been incorporated into the revised manuscript (Figure 5E).

Other issues:
3. Co-localization of Pck2 with SGs under heat stress was done without quantitation (Fig. 1 A) – this could easily be added?

Re: Accordingly, we quantified the co-localization of Pck2 with SGs and incorporated this Figure in the revised manuscript (New Figure 1B, lower panel).

4. Pck2 is required for Pmk1 activation at early, but not late stages of heat shock (Fig. 3A). Could this be due to a redundant function of Pck1?

Re: Accordingly, we evaluated the functional involvement of Pck1 in Pmk1 activation at a later stage of heat shock, by performing immunoblotting using Pck1 deletion strains harboring Pmk1-GST from its endogenous promoter. The results showed that Pck1 deletion rather increased Pmk1 phosphorylation at early, and no significant change of Pmk1 activation was observed at a later stage of HS, thus indicating that Pck1 is not involved in Pmk1 activation at the late stage of HS. Because the simultaneous deletion of Pck1 and Pck2 is lethal, Pck1 and Pck2 share an essential function for cell viability, but regarding Pmk1 activation upon HS, the functional redundancy was not detected (Toda et al., 1993).

These Figures and descriptions have been incorporated in the revised manuscript (Figure 3B).

5. Discussion, line 385-386: If the model that Pmk1 activity underlies SG formation is supported by deleting an inactivating phosphatase for Pmk1 leading to enhanced translocation of Pck2 to SGs, then this data should be shown e.g.in a supplementary figure. What is the JCS standpoint of “data not shown”, is it not required to show this data?

Re: Accordingly, we incorporated the data showing enhanced translocation of Pck2 to SG in cells deleted for Pmp1, an inactivating phosphatase for Pmk1. This further supports our model that Pmk1 MAPK activity underlies Pck2 recruitment to SGs.

These Figures and descriptions have been incorporated in the revised manuscript (Figure S7A and S7B).

Minor remarks:
1. Why show the kinase activity of mutant Pck2 after showing its effects in the cell (Supplementary Fig. 4A) rather than before?

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Re: Accordingly, we reversed the order of the Figure panels so that the kinase activity assay of mutant Pck2 appeared first, then examined its effects in the cell.
These changes have been incorporated in the revised manuscript (Figure 2B, 2C, 2D, 2E, 2F, and 2G).

2. Line 277: is the decrease in the pmk1 deletion background compared to the actual wild-type, or compared to the pck2-R389A allele without the pmk1Δ deletion?

Re: we clarified the text by describing that "the constitutively active Pck2 R389A-GFP, which exhibited a higher kinase activity and an enhanced SG localization ability in WT cells, markedly decreased the localization at the SGs in the Pmk1 deletion background as compared with that of the Pck2R389A-GFP in WT cells (Figure 4D, E). In the Pmk1 deletion background, the number of the WT-Pck2 dots and that of the constitutively active Pck2 R389A-GFP dots are almost the same (Figure 4B, lower panel, 4E, lower panel, and Supplementary Table S2).

3. Fig. 1C: Change the label "Control" to "-CHX"

Re: Accordingly, we amended the error in the label.
In addition, following the JCS guideline for the manuscript preparation that "The number of supplemental figures must be equal to or less than the total number of main-text figures", we incorporated two panels of the Supplementary Figures in the previous manuscript in the main-text Figures in the revised manuscript (Figure 1C and 3E).

We hope that the aforementioned new findings would satisfy your requirements. If you would still find issues to be addressed and suggest better descriptions or phrases appropriate to further strengthen our conclusion, we would respectfully take your advice.

Your generous consideration is highly appreciated.

Sincerely yours,
Reiko Sugiura, M.D. & Ph.D.
Professor

Second decision letter

MS ID#: JOCES/2020/250191

MS TITLE: Stress Granules as a Feedback Mechanism of MAPK Signaling by Sequestering PKC/Pck2

AUTHORS: Yuki Kanda, Ryosuke Satoh, Teruaki Takasaki, Naofumi Tomimoto, Kiko Tsuchiya, Chun An Tsai, Taemi Tanaka, Shu Kyomoto, Kozo Hamada, Toshinobu Fujiwara, and Reiko Sugiura
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

In their revised version, the authors have addressed both the major concerns that I had in an ambitious way.

The question whether reactivation of Pmk1 is altered in an SG-deficient background was investigated in nrd1 and tif211 mutants. For both of them, there was an effect on SG number under

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moderate heat stress, but not under high heat (45oC). The translocation of Pck2 granules under high heat was also unaffected. This is indeed an indication that a separate control is active under the high heat conditions studied here.

The question whether only activated (phosphorylated) Pck2 localizes to SGs. The authors clarify that this is not what they meant, nevertheless they have now made a laudible effort with additional research tools (anti-mammalian phospho-PKC) to ascertain if this is the case. They acknowledge that it has not been quite possible with these methods to settle this issue, on the other hand they apparently never originally made that claim.

In both cases, the authors have made considerable efforts to bring light on these questions, and have come up with some new information.

I also brought up several smaller issues, which have all been corrected or addressed in a satisfactory way by the authors.

In summary, I am content with the revisions made by the authors, and now recommend publication.

Comments for the author

No remaining suggestions

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