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Mammalian proapoptotic factor ChaC1 and its homologues function as γ-glutamyl cyclotransferases acting specifically on glutathione

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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 15 May 2012

Thank you very much for the submission of your research manuscript to our editorial office. We have now received the full set of reports from the referees that were asked to assess it.

As the detailed reports are pasted below I will only repeat the main points here. You will see that while the reviewers acknowledge the potential interest of the findings, they all agree that more work is needed to support the idea that ChaC functions as a gamma-glutamyl cyclotransferase for glutathione in the ER stress pathway. Both referees 1 and 3 feel that the physiological relevance of the reported activity of ChaC on glutathione in ER stress and the UPR response needs to be strengthened, either in a mammalian or a yeast system. Reviewer 3 also states that the cell death and cell viability assays would need to be strengthened. Referees 2 and 3 point out some instances in which further clarifications, additional controls and a more substantial statistical analysis are needed.

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 main concerns of the referees (as outlined above and in their reports) must be addressed. 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.

I look forward to seeing a revised form of your manuscript when it is ready.

Yours sincerely

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1

The submitted manuscript details an interesting observation that a previously uncharacterised UPR target called ChaC1 can deplete glutathione when overexpressed in yeast and could, therefore, potentially be involved in the process of glutathione depletion that may result in apoptosis following a UPR. The data, as far as it goes, is solid though the text is difficult to follow at times due to bad grammar. The main issue with the paper is that while it may show that this enzyme can carry out the first step of degradation of glutathione in yeast when overexpressed this does not mean that it carries out this reaction under normal physiological conditions in either yeast or mammalian cells. There are a couple of crucial experiments that are ommitted from the paper which would strengthen the case. For publication in this journal you would need to show more conclusively that this enzyme carries out the proposed function, rather than just provide suggestive evidence. Specifically one would need to look at the phenotype of the yeast knock out and to show that the glutathione depletion seen during later stages of the UPR is due to the activity of this enzyme by carrying out a knockdown in mammalian cells.

Referee #2

This report concerns a cytosolic protein Chac1 that is conserved from E. coli to mammals.
A paper published in 2009 described certain intriguing features of Chac1 (PMID: 19109178): The mRNA encoding the mammalian protein is expressed at higher levels in cells exposed to endoplasmic reticulum stress. This affect is apparently transcriptional, as it can be traced to the CHOP-ATF4 branch of the unfolded protein response, a branch whose activity is implicated in cell death. Knockdown to Chac1 by siRNA is protective of HEK cells exposed to $CdCl_2$ and attenuates the development of cell-death markers also in response to tunicamycin - an agent that causes unfolded protein stress in the ER. Finally, Chac1 over-expression is sufficient to trigger certain aspects of the cell-death program, such as activation of PARP and enhanced TUNEL staining. The biochemical basis of these observations has been obscure.
Enter, Kumar and colleagues. Firstly they discover that the amino acid sequence of Chac1 and that of its yeast homologue YER163c can be threaded through the structure of human gamma-glutamyl cyclotransferase (γ-GCT, PDB:2pn7 and 2rbh). Furthermore, key residues implicated in the active site of γ-GCT are conserved in Chac1. This suggested that Chac1/YER163c may also have gamma glutamyl cyclo-transferease activity.

To test this idea, they exploited the fact that budding yeast deleted in enzymes that metabolize cysteine-containing amino acids (met15Δ ecm38Δ dug3Δ) are auxotrophic for methionine. However, they can be coaxed to live on other sources of sulfur, when provided the necessary catabolic enzymes. Thus expression of γ-GCT allowed the yeast to live on γ-glutamyl-methionine as well as γ-glutamyl-cysteine. Interestingly, expression of mouse Chac1, or YER163c or their E. coli homologue, ChaC, also allowed survival on γ-glutamyl-cysteine but not on γ-glutamyl-methionine. This suggested that Chac1 is a selective gamma-glutamyl cyclotransferase. Interestingly, these three Chac1 homologues were aslo able to support yeast growth on glutathione (γ-glutamyl-cysteine-glycine) but γ-glutamyl-cyclotransferease is unable to do so. Together these observations support the idea that Chac1 is able to catalyze the cyclyzation of the gamma-glutamyl to oxoproline and the liberation of cysteine-glycine dipeptide, which is further cleaved to its constituents by Dug1p providing the yeast with metabolizable sulfur in the form of cysteine.

Purified Chac1, cleaved glutathione in vitro with a km within the physiological range of glutathione's concentration in cells.

Over-expression of wildtype, but not catalytically dead mutants of Chac1 and its homologues was able to deplete yeast of their content of glutathione and compromised their viability - in a manner that was reversed by glutathione supplementation.

In their discussion, the authors note that CHOP over-expression has been reported to lead to glutathione depletion (McCullough 2001), the notion that this might occur via induction of an enzyme that degrades glutathione is most beguiling and gives the important discoveries reported here a greater context.

Specific points of critique:

Figure 2 is key.
A clearer explanation for the requirement for a combined met15Δ, ecm38Δ dug3Δ genotype in construction of the assay that provided clues to ChaC's substrate should be provided. This can be explained in simple terms and with no colloquialism:
It is my understanding that the various complemented strains were tested for their ability to survive on various sources of organic sulfur. All could grow on methionine (this is presumably expected) but complementation with certain genes imparted the ability to grow of certain sources of sulfur. This experiment needs to be explained such that a non-expert who has never heard of the mutations in question would be convinced by the significance of the observation.
Why are the levels of endogenous YER163c insufficient to endow the yeast the ability to exploit glutathione as a source of sulfur?

Figure 3:
It is unclear why oxoprolinase would lead to AUR oxidation? Presumably H2O2 is generated in a coupled reaction (coupled to the production of phosphate?), a few words of explanation in the results section would help.
In explaining the apparent discrepancy between the apparent inability of Chac1 to cleave gamma-Glu-Cys in vitro (Figure 3D) and the ability of the mutant yeast to grow on gamma-Glu-Cys when over-expressing Chac1 (Figure 2A), is it possible that gamma-Glu-Cys is converted to glutathione (by Gsh2p) in vivo?

Referee #3

In the current study, Akhilech Kumar and co-workers described a novel finding indicating that members of the ChaC family of γ-glutamyl cyclotransferases from mouse and S Cerevisiae, cleave the γ-glutamyl bond of glutathione to yield 5-oxoproline and cys-gly. Moreover, overexpression of mouse WT ChaC1 but not its catalytically inactive mutant (E>Q) led to glutathione depletion and enhanced apoptosis in yeasts. The author's findings using in vitro models described a novel pathway for glutathione modulation levels through its degradation. Most of experiments are well performed but still some additional experiments and controls are required to improve the quality of the work to be published on EMBO Reports.

General comments:

Overall this is a well-done study that provides new and useful information about the regulation of the Glutathione levels by degradation and the cell decision to apoptotic cell death; however, there are some additional required experiments that will be useful in this study to better explain the observed phenomena. One key question remains opne, What are the implication of WT and mutant ChaC proteins to the UPR in yeast? (Methods Enzymol. 2011, 491:261-92). is there more or less ROS induction in the model?. In the case of ChaC-induced

apoptotic cell death, additional experiments need to confirm these findings (i.e. Tunel assay, Nucleus morphological changes etc.).

Specific comments:

In general, all data presented here needs at least three independent experiments and to have proper statistical analysis.

In Figure 1, to show a diagram indicating functional and similarities between ChaC family members in terms of structural similarity will be helpful.

In Figure2 it is important to confirm that the overexpression of the different ChaC genes cloned in the vector p426GPD is working, for example by PCR or Western blot. Do these WT and/or mutants have a different response against to ER stress? and/or ROS? How is the susceptibility of these clones against apoptotic inducers?

Figure 3 needs further attention in terms of resolution and to uniform size of all graphics, as well as to maintain thesame scales for Y-axis. It is also important to include positive and negative controls for mouse and yeast ChaC1 proteins.

In the case of Figure 4, why is there a 40-50%reduction of glutathione levels and 30% of apoptotic cells is due leak expression of ChaC1? Also for viability assay it is important to complement these findings with additional cell proliferation assays (i.e. yeast cell cycle assay by FACS).

Minor points:

Several plots and fonts are not with the same size and some images have decreased resolution (i.e. supplementary figure S5 scales and axis names are illegible) .

1st Revision - authors' response 27 August 2012

(Please see next page.)

Referee No. 1:

1. The referee has commented that the text needs improvement in writing/grammar.

-We have made an effort to improve this to the best of our ability.

2. The referee comments that "this enzyme can carry out the first step of degradation of glutathione in yeast when overexpressed does not mean that it carries out this reaction under normal physiological conditions in either yeast or mammalian cells."

- We agree that the enzyme would only carry out the reaction when it is induced. In mammals it is known that one of the conditions under which it is induced is during UPR, but other conditions may exist. The exact conditions of the induction of the protein in yeast are not yet known.

3. The referee comments we "would need to show more conclusively that this enzyme carries out the proposed function, rather than just provide suggestive evidence."

-We would like to respond by stating that in providing evidence that ChaC1 is an enzyme degrading glutathione, we have demonstrated quite convincingly that the ChaC1 protein (which hitherto had no assigned molecular function) is carrying out this glutathione-degrading activity through the following:

a. Homology modeling on known g-glutamylcyclotransferases, and confirming this loss of function through catalytic site mutations (E>Q)

b. Demonstration of glutathione-degradation activity through in vivo growth assays

c. Identification of the products of the reaction of ChaC1 on glutathione as 5-oxoproline and Cys-Gly through HPLC - using the in vitro purified enzymes.

d. .Demonstration of glutathione-degradation activity through 2 *in vitro* assays (coupled enzyme assays that we have developed- one based on 5-oxoproline detection, and the other on Cys-Gly detection) . Both ChaC1 and Yeast YER163c display this activity as seen by both these assays
e. Determining of the kinetic parameters of the enzyme – a key aspect to show its physiological relevance to the substrate (glutathione)

We hope that the referee will now agree that the enzymatic function conclusive in nature and not only suggestive.

4. The referee comments "would need to look at the phenotype of the yeast knock out and to show that the glutathione depletion seen during later stages of the UPR is due to the activity of this enzyme by carrying out a knockdown in mammalian cells.

Our evidence indicates that the ORF YER163c does not appear to be induced by UPR response (our unpublished preliminary observations) and this confirms earlier microarray experiments of Travers et.al, Cell. 101:249-58, 2000 whose UPR induced microarray data set also do not reveal induction of YER163c. The induction in mammals (shown by others) by UPR is however conclusive. In the present manuscript, we addressed the question raised by the referee –though differently- by looking at the phenotypic effects of ChaC1 and the catalytically dead ChaC1(that does not degrade glutathione), and demonstrated that only wildtype ChaC1 but not the mutant causes an enhanced apoptosis.

Referee No. 2

6. The referee has asked for a clearer explanation for used the combined met15D ecm38D dug3D and for a for a clearer explanation of the growth experiment with different analogues

We have now provided a clear description of the genetic background. We hope this explanation of the use of met15D ecm38D dug3D background to evaluate the functions of the ChaC1 family would now be clear. The changed version (page 6, line 13 onwards):

"This strain is an organic sulphur auxotroph owing to a *met15*Δ. The strain also carries a deletion in *ECM38* (which encodes for γ-glutamyl transpeptidase, γ-GT) and also carries a deletion in *DUG3* (which degrades glutathione by the alternative DUG pathway [22]). As a consequence of the absence of the two enzymes capable of cleaving the γ-glutamyl bond the strain is deficient in glutathione and γ-glutamyl amino acids utilization."

7. The referee has asked why have the endogenous levels of YER163c insufficient to endow the yeast the ability to exploit GSH as a source of reply

The reason for this is that the endogenous levels of YER63c appear to be extremely low. We are unable to detect any YER163c transcript by RT PCR under a variety of conditions. Only when YER163c is overexpressed can we actually see the transcript. The reason for this is not clear, but clearly the basal expression is too low to allow GSH to be used as a source of sulphur. And yet, the continued presence of this gene in yeast (with no development of stop codons or pseudogenes) suggests that it has some important role to play .

8. The referee asks in relation to Fig3 why 5-oxoprolinase leads to Cys-Gly oxidation..

- It is not the 5-oxoprolinase, but spontaneous auto-oxidation that is occurring. Even pure cys-gly gets auto-oxidized with time.

9. The referee asks for an explanation of the discrepancy with Fig 2 A and Fig3D and whether this could be due to conversion to glutathione by Gsh2 followed by degradation (*in vivo*).

We have checked the possibility by creating a *gsh2Δ* in the same genetic background (*S.cerevisiae met15*Δ *ecm38*Δ *dug3*Δ *gsh2Δ*) but it did not change the in vivo growth phenotype (data not shown). Our explanation for the apparent discrepancy is that there is a residual activity towards g-glu-cys that is not seen in a kinetic assay done over a few minutes, but becomes apparent in a growth assay done over a few days. This is the explanation already given in the text, and we think that the explanation still stands and we have retained it as such.

Referee No 3

10. The referee asks what are the implications of ChaC and WT to the UPR response in yeast.

As indicated earlier, although the mammalian homologue is induced under UPR, so far evidences suggest that YER163c does not play a role in UPR response in yeast. Thus YER163c is not induced under UPR in yeast (in genome wide microarray experiments by Walter and coworkers), and also by initial preliminary experiments done by us under a variety conditions known to induce UPR in yeast (Tunicamycin, DTT).

11. The referee asks for more experiments to confirm these findings, Tunel Assay, Nuclear fragmentation.

We have now provided additional data using the TUNEL assay (Fig 4E)

12. The referee has indicated that the data need at least 3 independant experiments and to have proper statistical analysis

We would like to mention that the glutathione estimation experiments have been done 4 times, each in triplicate (and has now been mentioned in the legends). The growth assays on plates have been done several times (more than 3). The TUNEL and Annexin V assays for apoptosis have also been done in replicates. Standard error bars have been provided on all these figures.

13. The referee has indicated that In figure 1 it would help to show a diagram indicating functional similarities between chac famly members in terms of structural similarity

- We have now included a Table (Table 1) that includes the required information.

14. The referee has raised the concern that in figure 2 there is a need to confirm that the overexpression of the different Chac genes in the vector G426GPD is working.

The proteins are functional as can be seen from Fig 2. Only the g-GACT clone does not have a positive indication of being expressed. We have now HA-tagged the g-GACT protein and shown expression in yeast. This is indicated in the text as data not shown (Page 6,2nd last line from the bottom). The E>Q mutant has also been HA-tagged and expression demonstrated (along with its wildtype). This is also indicated as data not shown (page 7, line 13).

15. The referee has asked if the WT and/or mutants have a different response against to ER stress? and/or ROS? How is the susceptibility of these clones against apoptotic inducers?

We have demonstrated that the WT but not the mutant ChaC1 leads to enhanced apoptosis. In mammalian cells ChaC1 is already known to be induced under ER stress, and knockdown experiments have shown to have a consequent effect on apoptosis (using apoptotic inducers). As the main goal was to establish the molecular function of ChaC1 in glutathione degradation and relate it to apoptosis in vivo, we have restricted our analysis to measuring the glutathione levels and the final readout, apoptosis.

16. Referee has indicated that Figure 3 needs attention in terms of resolution and to uniform size of all graphics, as well as to maintain the same scales for Y-axis. It is also important to include positive and negative controls for mouse and yeast ChaC1 proteins.

- The absorption was done at 210nm. Since the different peptides have different absorptions, we have used different scales, to accentuate the formation or lack of formation of product in each case. This has been retained. We would like to point out that this was not a quantitative assay. The quantitation has been demonstrated by the two in vitro assays in figures (Fig 3D, Supplementary Figure S7).

17. The referee mentions that in the case of Figure 4, why is there a 40-50% reduction of glutathione levels and 30% of apoptotic cells is due leak expression of ChaC1? Also for viability assay it is important to complement these findings with additional cell proliferation assays (i.e. yeast cell cycle assay by FACS).

-The precise reason for this is not clear at this stage. The viability is clear from the plate growth assays, that we have followed up by assays for apoptosis using both AnnexinV and TUNEL assays. We do not think cell cycle assays would add much at this point since it is the cellular glutathione concentrations in the populations that would be important. In *Saccharomyces cerevesiae* we have also observed a delayed growth stasis upon glutathione depletion that results from an overlapping role played by Thioredoxin. It is possible that this might be a factor, but it needs much more investigation (Sharma et.al.,

"Glutathione depletion leads to delayed growth stasis in Saccharomyces cerevisiae: evidence of a partially overlapping role for thioredoxin." Curr Genet. 38:71-7. 2000)

Minor points:

18. Several plots and fonts are not with the same size and some images have decreased resolution (i.e. supplementary figure S5 scales and axis names are illegible)

- Figure S5 has now been changed to Fig S5A and S5B and the figure sizes have been increased, and resolution has been increased, and axis names more legible.

Other changes made in the text:

1. Ref 22 has been changed to provide a more recent reference to the Dug3 protein : Kaur H, Ganguli D, Bachhawat AK. (2012). Glutathione degradation by the alternative pathway (DUG pathway) in Saccharomyces cerevisiae is initiated by (Dug2p-Dug3p)2 complex, a novel glutamine amidotransferase (GATase) enzyme acting on glutathione. J Biol Chem. 287:8920-31
2. The oligos list in the supplementary table has been expanded to include the new oligos used.

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

Yours sincerely,

Editor
EMBO Reports