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Recycling of Peroxiredoxin IV Provides a Novel Pathway for Disulphide Formation in the Endoplasmic Reticulum

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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 08 October 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments are shown below. You will see that the referees are generally positive about your work and that they would support its ultimate publication in The EMBO Journal after minor revision. I would thus like to invite you to prepare a revised manuscript in which you need to address the points raised by the referees in an adequate manner.

There is also one editorial issue that needs further attention. We routinely check for figures that are containing lanes of gels that are assembled from cropped lanes. While cropping and pasting may be considered acceptable practices in some cases (please see Rossner and Yamada, JCB 166, 11-15, 2004) there needs to be a proper indication in all cases where such processing has been performed according to our editorial policies. Please note that it is our standard procedure when images appear like they have been pasted together without proper indication (like a white space or a black line between) to ask for the original scans (for our records). In the case of the present submission there is one panel that does not appear to fully meet these requirements: figure 5B, panel "Anti-ERp57". I therefore like to kindly ask you to suitably amend the panel in question and to explain in the figure legend that all lanes come from the same gel. Please be reminded that according to our editorial policies we also need to see the original scan for the figure in question.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

This is a thorough study on the role of peroxiredoxin IV in protein disulfide bond formation in the ER. It uses purified proteins, in vitro translation, and silencing in intact cells to probe whether interactions and effects are direct and whether they have in vivo relevance and reproducibility. Redox balance is not a trivial issue to address, as oxygen is everywhere and many molecules can contribute to the reactions. This study was very carefully done, well-designed and well-executed. The writing is crystal clear and so are the data. The discussion is insightful and covers all one would wish. It implicitly suggests that our focus should move from Ero1alpha as main oxidase in the mammalian ER to a more intricate system where both Ero1alpha and peroxiredoxin IV, in combination with glutathione, form a network that ensures disulfide formation in folding proteins. I have one minor issue the authors should address.

Page 12 bottom, figure 6
I am not convinced that ERp46 and P5 do not form mixed disulfides with DM. The amount of peroxiredoxin IV trapped with ERp46 appears lower in lane 2 than in lane 1, and lane 4 is lower than lane 3 (and lane 2). The quantitation of lanes 1 and 2 does not seem to fit with these 2 observations.

It is the same for P5, except that here the quantitation matches the gel and shows a lower signal in lane 6 than in lane 5. Removal of C51 halves the interaction of P5. Can really more than 50% of the interaction be attributed to the peroxidatic cysteines?

Referee #2 (Remarks to the Author):

"Recycling of peroxiredoxin IV provides a novel pathway for disulphide bond formation in the endoplasmic reticulum" by Tavender, Springate, and Bulleid

PrxIV is an endoplasmic reticulum (ER) peroxiredoxin that catalyzes the reduction of hydrogen peroxide to water and gains a disulfide bond in the process. This disulfide must be reduced to recycle the enzyme to its active state. The authors of this manuscript tested a set of PDI-family oxidoreductases for their ability to reduce PrxIV (and become oxidized in the process). They also tested the effects of increasing and decreasing the amounts of these ER oxidoreductases on the rate of PrxIV reduction in semi-permeabilized cells. The authors observed that ERp46, PDI, P5, and ERp57 reduced PrxIV in vitro. Of the oxidoreductases tested, only ERp18 was unable to reduce PrxIV. In semi-permealized cells, ERp46, PDI, and P5 (but not ERp57) over-expression enhanced PrxIV reduction, whereas RNA interference resulted in a more dramatic decrease in PrxIV reduction rate for the ERp46 and P5 knockdowns compared to the PDI knockdown.

It would seem that the conclusions from this study should be that PDI-family proteins can reduce and recycle PrxIV and that they differ in their abilities to do so. However, the authors make a small leap from their findings to the last sentence of the abstract:

"Oxidation of PrxIV therefore provides a novel pathway to introduce disulphides into enzymes involved in oxidative protein folding within the endoplasmic reticulum."

The authors have clearly shown that electron transfer from reduced PDI-family oxidoreductases to PrxIV occurs. However, they have not shown that formation of disulfides in these oxidoreductases contributes significantly to oxidative protein folding in the ER. It is quite possible that those proteins that best reduce PrxIV are least likely to oxidize other substrates. In fact, it seems that there is no correlation between the ability of a PDI-family oxidoreductase to reduce PrxIV and its general importance in oxidative folding (see for example Rutkevich et al., Mol. Biol. Cell 21, 3093-3105 (2010)). Although it was shown in Jessop et al., 2009 that ERp46 and P5, for example, form mixed disulfides with various proteins folding in the ER (e.g., beta-1 integrin and LDLR), these interactions are on a reductive pathway, since they were trapped using single-cysteine mutants of the oxidoreductases.

Therefore, the title is not the most accurate description of what the results show. The focus of the paper is on recycling of PrxIV and not how recycling relates to downstream disulfide formation in ER client proteins.

The authors do show in the current manuscript that GSH can reduce the oxidized forms of the various oxidoreductases after they have transferred electrons to PrxIV. So, in principle, peroxiredoxin-to-oxidoreductase-to-glutathione-to-folding protein does represent a pathway for introducing disulfides into the ER, even if the effect on oxidative protein folding is not shown directly. Furthermore, the above criticisms do not really detract from the results presented. At worst they build false expectations in the reader or slightly obscure the likelihood that not all ER oxidoreductases function physiologically on pathways that result in net oxidation of protein substrates.

Additional questions/corrections relating to the text and figures are listed below.

Page 8, bottom: should be "... where one third of total PrxIV is typically fully reduced."

Page 11, bottom: the explanation that excess ERp57 does not increase PrxIV reduction in semi-permeabilized cells does not appear to take into account that ERp57 is over-expressed in this experiment. Is there enough excess calnexin and calreticulin around to complex the excess ERp57 and prevent it from reducing PrxIV as ERp57 was observed to do alone in vitro? In any case, this should be explained.

Page 12: It was previously shown by the same group that PrxIV translated into semi-permeabilized cells containing single-cysteine trapping mutants of oxidoreductases forms mixed disulfides preferentially with P5 and ERp46 (among the six mutant oxidoreductases tested). The authors should state more clearly how the current study adds to the previously reported observations. Is it merely confirmatory? The statement that "It was impossible to conclude whether the mixed disulphides were formed during nucleophilic attack by the PDI family members of peroxidatic disulphides or non-catalytic disulphides within PrxIV" is confusing, since the authors then report doing what was claimed to be "impossible." Rather than impossible, it seems that this experiment was just something left over from the previous report, in other words a control that should have been done then.

Page 15: The authors state that they cannot prepare reduced PrxIV in aerobic buffers because the peroxidatic cysteine residues oxidize. However, it seems from the Materials and Methods that assays monitoring reduction of PrxIV by reduced oxidoreductases were done in aerobic buffers. The timescales of these experiments (i.e., Fig. 2C) are very short. However, to what extent is PrxIV being re-oxidized and re-reduced during these reactions? It seems odd that a peroxiredoxin would be so easily oxidized by oxygen rather than "saving itself" for peroxide. Is this reviewer misunderstanding something here?

Page 16: The authors refer to "The apparent preferential oxidation of ERp46 by PrxIV and the greater effects of over-expression or knockdown of this enzyme rather than P5 or PDI on the reduction of PrxIV in the ER..." However from the results section and figures, it is not clear that over-expression of ERp46 has a greater effect than over-expression of P5 (Fig. 5C). Same goes for the knockdown in Fig. 7C. Therefore, to which results does this statement refer?

Page 17, bottom: The double negative construction is confusing. Perhaps change "The lack of broad effects of PrxIV knockout upon mouse physiology does not indicate a substantially compromised antioxidant defence" to something like, "The lack of broad effects of PrxIV knockout upon mouse physiology suggests that a functional antioxidant defence remains even in the absence of PrxIV." Or "... suggests that additional antioxidant defences exist." Also, in the next sentence, do the authors really want to use "one part of a multi-component system" to suggest why PrxIV is not more essential physiologically? Removing one part of a multi-component system could easily cause the whole system to shut down. The authors presumably meant that PrxIV may be one of a number of co-existing systems that facilitate disulfide formation.

Page 33, top: The figure legend reads that MALDI-TOF of PDI was done "as detailed in methods," but the methods don't provide ANY details, just a reference to another paper. So perhaps the figure legend should read, "...as described (Chambers et al, 2010)." Also, the full reference for this paper can now be provided in the reference list.

Page 33: a comma is missing between "NEM" and "harvested."

Referee #3 (Remarks to the Author):

This is a nice piece of work. It is no doubt that the occurrence of multiple protein disulfide formation pathways in mammalian cells is currently a hot topic of study. This paper demonstrates that PrxIV, which was known to be a H2O2 scavenger in the ER, has a significant catalytic activity to oxidize several PDI family members, leading to the establishment of a novel pathway to introduce protein disulfide bonds. Importantly, the PrxIV-mediated disulfide formation is coupled to the reduction of H2O2, which may contribute to attenuating the ER stress. Taking into account the highly competitive circumstance in this field of research, I believe that this paper should be published in EMBO Journal very urgently through consideration for the following minor issue.

The authors should address the observation that the fraction of PrxIV C51A forming a mixed disulfide with P5 was significantly smaller than that of PrxIV WT (Fig. 6, lanes 1 and 2). Is this observation true or reproducible? If so, does it mean that P5 have a lower specificity against the peroxidatic disulfide of PrxIV than PDI and ERp46? Indeed, the reductase activity of P5 against the peroxidatic disulfide seems significantly lower compared to that of ERp46 and PDI at least in the in vitro assays (Fig. 2C and 2D).

1st Revision - authors' response 12 October 2010

Reviewer #1

Page 12 bottom, figure 6
We do not claim in the text that mixed disulphides between ERp46/P5 and PrxIV DM are absent. Our stated observation is simply that mixed disulphides accumulate more readily with C51A than with DM. It is clear from the data that mixed disulphides are trapped with both C51A and DM.

For figure 6 it is true that the amount of PrxIV trapped with ERp46 is lower in lane 2 than lane 1. However, the quantified values express the trapped material as a proportion of the total PrxIV translated and translocated to the ER (lower band in upper panels). Total PrxIV is visibly less for lane 2 than for lane 1, hence the similarity of the final values. In contrast, the amount of PrxIV trapped with P5 declines across lanes 5-7 while the total translated and translocated material remains fairly constant. It is this difference between the ‘Total’ controls which accounts for the disparity in the quantified values.

It is clear that PrxIV C51A accumulates proportionally less mixed disulphides with P5 than does PrxIV WT, however we deliberately avoid further conjecture regarding the substrate trapping experiment. Ability of PDI family members to trap mixed disulphides is not only affected by the

affinity of the oxidoreductase for its substrate but is also due to the propensity for third party thiols to resolve the resulting complex. Consequently over-interpretation of data generated from such experiments has been criticised in the past. Moreover, we do not know how P5 selects its substrates (directly or via BiP?). Thus we can speculate that the effect of C51A mutation upon mixed disulphide accumulation could be a result of altered reactivity of PrxIV with P5, altered affinity of BiP for PrxIV or simply a consequence of easier access to outside thiols for resolution of the P5-PrxIV C51A disulphide. The experiment in Figure 6 was designed to verify whether PDI family members could interact directly with PrxIV containing only one species of disulphide. The experiment was therefore deemed successful and further speculation considered errant.

Reviewer #2

General points

We would agree that the ability of an oxidoreductase to reduce PrxIV is not necessarily an indication that the oxidoreductase will in turn oxidise client proteins. It is also true that ERp46 may be on a reductive pathway as we have previously published. For this reason we avoid suggesting that ERp46 has a prevalent role in oxidation rather than reduction. It is important to remember however that ERp46 has three redox-active domains and we have little idea of their individual activities.

In contrast we feel it is safe to consider oxidation of PDI by PrxIV to be a clear route by which hydrogen peroxide can drive oxidation of nascent ER proteins. We agree however that there appears to be a jump at the end of the abstract and so would like to replace the last sentence with; "Oxidation of protein disulphide isomerase by peroxiredoxin IV may therefore increase efficiency of disulphide formation by Ero1 and also allows disulphide formation via alternative sources of hydrogen peroxide".

As indicated by the reviewer, our data also suggest oxidised glutathione may be a major product of PrxIV activity irrespective of the PDI family member employed. Characterisation of Ero1 around ten years ago led to GSSG being largely discounted as an oxidant *in vivo*. This view is beginning to change once again with the increasing acceptance of an ER redox buffer/multiple oxidant hypothesis.

Specific points

Page 8, bottom: 'reduce' changed to 'reduced'.

Page 11, bottom: We can not say for sure whether the Cnx/Crt machinery is saturated with ERp57 during ERp57 over-expression. However, our results suggest that most ERp57 is unable to react with PrxIV as ERp57 over-expression does not enhance PrxIV recycling. We have added a sentence in acknowledgement:

"The fact that ERp57 does not enhance PrxIV reduction in the ER therefore suggests that ERp57 may be adequately sequestered by calnexin, calreticulin even when ERp57 is over-expressed".

Page 12: The context of the statement has been altered. It now reads:

"Likewise, when translated *in vitro* in the presence of SP cells produced from these cell lines, radioactively labelled PrxIV formed prevalent mixed disulphides with ERp46 and P5. In each case the native PrxIV sequence was translated, potentially containing both peroxidatic disulphides and non-catalytic disulphides. Consequently it was not possible to conclude whether the mixed disulphides were formed during the nucleophilic attack by the PDI family members on peroxidatic disulphides or on non-catalytic disulphides within PrxIV. To address this issue a similar experiment was undertaken using PrxIV cysteine to alanine mutants which could only form one of the two species of disulphide."

Page 15 (now page 16): This does require clarification. Based on our results, the ratios of proteins we use, our reaction construction and the timescales involved, we do not believe that we are witnessing extensive re-oxidation in our time courses. PrxIV can be successfully reduced in aerobic

buffers but this state is not stably maintained as introduction of fresh buffer/reagents leads to significant re-oxidation. It is currently unclear whether this is caused solely by contaminating peroxides or also by other oxygen species that may be present. As such, under aerobic conditions PrxIV can not be reliably and adequately reduced for further experiments beyond analysis of the reduction pathway itself.

To clarify this we have altered the sentence based around "...the inability to prepare reduced PrxIV in aerobic buffers" so that is covers the above points more comprehensively.

Page 16 (now page 17): This sentence was misleading. It should have indicated that ERp46 performed best overall in terms of *in vitro* reduction and mixed disulphides in SP cells, while also scoring solidly in terms of modulated cellular expression. The first few sentences have been altered to accurately reflect the data presented.

Page 17 (now page 18/19): Agreed. Final sentences altered in keeping with both suggestions.

Page 33: Amended on all counts.

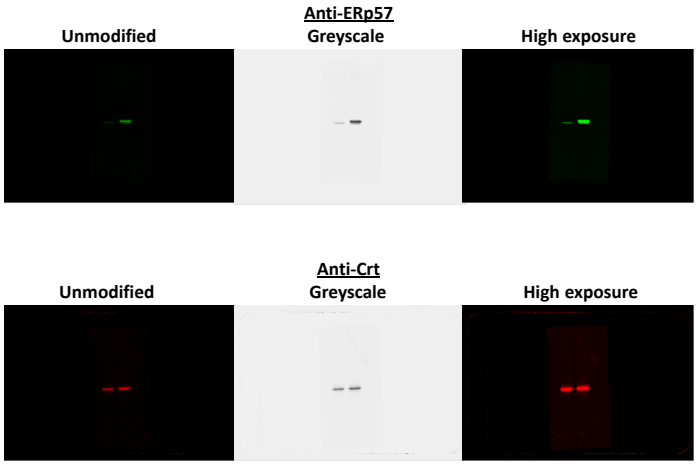
Reviewer #3

As touched upon previously, it is always wise to employ discretion when interpreting data involving mixed disulphides between PDI family CXXA mutants and substrate proteins. In particular, the diminished fraction of PrxIV C51A which forms a mixed disulphide with P5 (relative to PrxIV WT) is difficult to discuss without immediately straying into pure speculation.

We do not know how the specificity of redox exchange between PrxIV and the PDI family is conferred. One hypothesis is that P5 relies upon substrate selection by BiP in the ER, perhaps explaining why the effects of modulating P5 expression in cells are more dramatic than the *in vitro* data would suggest. If true, binding of PrxIV by BiP may be affected by topological changes in PrxIV caused by the absence of Cys51. Alternatively P5 may directly recognise PrxIV yet somehow rely upon interaction with Cys51 in order to most effectively reduce peroxidatic disulphides. Native PrxIV may therefore provide a better substrate for P5 than PrxIV C51A. A third possibility is that specificity is actually conferred by PrxIV towards the PDI family and this may change between PrxIV WT and C51A for different proteins. Finally, differences in proportions of mixed disulphides may be due to altered accessibility of third party thiols for resolution of the mixed disulphide complex.

Presently we have no favoured hypothesis as to the mechanism controlling specificity between P5 and PrxIV. Consequently any speculation we offer to explain detailed aspects of figure 6 is based upon further speculation. We find this situation unsatisfactory so decided to err on the side of caution by offering no conjecture beyond acknowledging that P5 does undergo direct redox-exchange with the peroxidatic disulphides. However, to acknowledge the fact that the specificity issue has yet to be addressed we have added a line to the discussion which reads; "Further dissection of the mechanisms of specificity for P5 and ERp46 are essential for full understanding of their individual roles in recycling PrxIV".

Regarding the issue of the panel 'Anti-ERp57' in figure 5B; these samples are from a single gel and are not cropped and pasted from separate sources. As confirmation we include a figure in the attached PowerPoint file containing the original scans (left panels) from which the greyscale images (centre) are derived, as well as higher exposure images which illuminate the background (right panels). This should clarify that the panel in question is indeed from a single gel image. Please inform us if there remains a problem so that we can be sure it is not associated with file conversion/transfer. The legend for figure 5B has also been amended to include "Samples presented within each individual panel are from the same gel. Anti-Crt panels presented are from the same gel as the corresponding anti-PDI family blot".



Acceptance letter 15 October 2010

Thank you for sending us your revised manuscript. You will be pleased to learn that you have now addressed the criticisms in a satisfactory manner and that the paper will now be publishable in The EMBO Journal. You will receive a formal acceptance letter shortly.

Thank you very much again for considering our journal for publication of your work.

Yours sincerely,
Editor
The EMBO Journal

