THE EMBO JOURNAL

Manuscript EMBO-2009-72231

Protein disulfide isomerase is required for signal peptide peptidase-mediated protein degradation

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Review timeline:

Submission date:	24 January 2009
Editorial Decision:	18 February 2009
Resubmission Received :	14 August 2009
Editorial Decision:	21 September 2009
Revision received:	01 October 2009
Editorial Decision:	29 October 2009
Revision received:	01 November 2009
Accepted:	04 November 2009

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.)

1st Editorial Decision 18 February 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees, whose comments are enclosed below. As you will see, all three referees recognise the potential interest in your study, but express significant concerns with the conclusiveness and the interpretation of your results. I hope you understand if I do not go into the details of the reviewers' reports, but a major criticism regards the SPP-PDI interaction - you do not demonstrate this at the endogenous level, and it is not clear whether this interaction is important for ERAD as opposed to other ER functions of the two proteins. Since the major interest in the paper comes from the potential identification of an SPP-PDI complex involved in ERAD, clearly it is of critical importance to demonstrate this conclusively.

In the light of these recommendations from three experts in the field, I am afraid we see little choice but to reject your manuscript at this stage - we feel that the amount of work required to resolve the issues highlighted is significantly beyond the scope of a normal revision. However, we do recognise the potential interest of your findings. Therefore, if you are able to fully address the criticisms raised by the referees, we would certainly be willing to look at a new version of your manuscript. This would, however, have to be considered as a new submission, rather than a revision, and would be re-evaluated at that stage (perhaps also involving new reviewers), also in the context of any literature published in the interim period.

I am sorry we can not be more positive at this stage, but we hope that you find the referees' comments helpful when revising the manuscript for future submission here or elsewhere.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The manuscript by K.Ahn's group shows that protein disulfide isomerase (PDI) is involved in SPP-mediated dislocation of misfolded proteins and that a substrate binding-deficient mutant, but not a catalytic mutant of PDI, impairs the degradation of both class I MHC and CD3delta. While their data are consistent with this interpretation, there are a number of improvements that are required before this ms can be considered further.

1. The interaction of SPP with wild-type PDI is a key concept used to explain the involvement of PDI in SPP-mediated degradation of misfolded proteins (Figure 3). However, the authors only used over-expressed PDI-myc to demonstrate PDI association with SPP. Given that PDI is a fairly abundant ER-resident protein, the authors should show the interaction of SPP with endogenous PDI. More importantly, the authors must observe and quantify the interaction of SPP with wild-type PDI and PDI mutants (PDI C36, 39, 380, 383S and PDI F258W/I272W) as well as that of SPP with US2 in the presence of either wild-type PDI-myc or the PDI mutants. For example, if the interaction of endogenous SPP with either wild type PDI-myc or PDI C36, 39, 380, 383S-myc is stronger than that of endogenous SPP with PDI F258W/I272W-myc, then the wild-type PDI-myc and the catalytic site mutant of PDI could be binding competitors of SPP. This would lead to a reduced pool of endogenous SPP in the cells, which is important for US2-mediated dislocation of misfolded proteins. I conclude that the importance of the peptide binding site of PDI for SPP-mediated degradation of class I MHC in the US2 pathway is not convincingly demonstrated, as the figure lacks data that allow a comparison of the interaction of endogenous SPP with wild type PDI-myc and the PDI mutants. This is clearly a key issue; without addressing this by experiment, the conclusions are too speculative, and therefore additional data are required, especially where it concerns the intreracting partners of wild type PDI. It will not be sufficient to do this simply by immunoprecipitation/immunoblot, and ideally some less biased method (mass spec?) should be used.

2. In Figure 5B, the authors used W6/32 to measure degradation of class I MHC in US2-expressing cells. In the figure, the amount of b2m increases after 30 minutes of chase when a decrease would be expected. Why is b2m-associated class I MHC so stable in these cells? In most relevant pubications, folded MHC class I is barely detectable by W6/32 in US2 and US11 expressing cells. Since Figure 5B is critical for explaining a specific function of PDI in SPP-mediated degradation of misfolded proteins, the authors need to repeat this experiment with increased expression levels of US2. I am not convinced by the authors' conclusion that the two bands for MHC class I HC observed after a 30 minute chase in cells expressing PDI F258W/I272A-myc represent differentially glycosylated forms (Figure 5B), since they do not show any data that perain to the deglycosylated form of HC in the presence of proteasome inhibitor. To draw such conclusions, the authors must use cells that express wild-type or mutant PDI, expose them to proteasome inhibitor, then immunoprecipitate with an antibody that recognizes free heavy chains, instead of using W6/32, to demonstrate the presence of the deglycosylated forms of HC.

3. The authors suggest that PDI is involved only in SPP-dependent ERAD without addressing whether it may not be able to play a role in SPP-independent ERAD. To support of this conclusion that PDI would not be involved in SPP-independent ERAD, they should examine whether perturbing PDI function affects other ERAD substrates. This point is essential to palce the observations reported here on a more genral footing, rather than be centered so heavily on the US2 model system.

4. I'm still puzzled by the lack of a proposal for a specific mechanism of involvement of PDI in SPP mediated ERAD. The discussion is rather deficient on this point.

Referee #2 (Remarks to the Author):

In this paper (EMBOJ-2009-70469) Lee and colleagues report on a requirement for PDI in dislocation of proteins from the endoplasmic reticulum via the signal peptide peptidase-mediated pathway. The pathway disposes of MHC class I heavy chain in the presence of the viral protein US2, but also of CD3delta independently of viral proteins, suggesting that this is a general mechanism for protein disposal. The authors demonstrate PDI associates with SPP, and suggest that PDI is an integral component of the SPP-mediated ERAD pathway.

Although this paper is potentially interesting, the experimental design has considerable weaknesses, some of the data are of insufficient quality, and some controls are missing as specified below.

Introduction: Htm1p in yeast has recently been shown to have mannosidase activity and the pathway of disposal of glycoproteins by ERAD has been well defined by Aebi and colleagues (JCB 184, 159-72; 2009). The 2nd and 3rd paragraph of the introduction are confusing and imprecise; the role of US2 and US11 in viral immune evasion needs to be explained more clearly here.
Fig 1:
- depletion of PDI by si did not restore classI surface levels to a level 'similar' to that of wt HeLa cells; the scale is a log scale, and there is still a significant difference between the si curve and the wildtype.
- K.o. efficiencies for each target should be specified.

Fig. 2:
- what do the authors mean with 'disrupting N-linked glycosylation at position 52'? There is no reference to this anywhere else in the description of the figure or discussion.
- why do the bands in C look so different from A (no visible reduced and cytosolic forms)? the quality of this figure is not sufficient to state whether or not there is more reduced US2 in siPDI cells or not.

Fig. 3:
- PDI-myc needs to be characterized as PDI is normally retained in the ER via its KDEL sequence at the extreme C-terminus. The myc-tag will allow PDI to be secreted which is potentially problematic in the interpretation of the subsequent experiments.
- the specific precipitated (US2, SPP, etc) for each panel should be shown as IP controls
- given the tag at the C-terminus how can the authors be sure that PDI interacts with SPP to promote ERAD of other substrates rather than being a substrate itself?

Fig. 5:
- do the authors have data to support their claim that the 2 bands visible in 5B are differentially glycosylated forms of MHCclassI HC?

Fig. 6: the graphs are peculiar in that absence of SPP or PDI only causes a delay in degradation of CD3 delta, but no change in the rate; this could be an indirect effect. Are other secretory pathway functions normal? Is the rate of protein secretion affected? what about the rate of degradation of a substrate that is PDI and SPP-independent?

Discussion:
- does overexpression of dominant negative PDI have an effect on classI HC degradation in US11 cells?

- there is no SPP in yeast, but PDI has been shown to play a decisive role in ERAD in yeast as well; this should be discussed.

- p12: oxidoreductase function in the ER is partially redundant; since the authors knocked down only one reductase at a time, they cannot say that the reductase activities in the ER are not essential for HC dislocation.

Referee #3 (Remarks to the Author):

PDI is required for signal peptide peptidase-mediated dislocation of misfolded proteins from endoplasmic reticulum

Lee et al

This manuscript details studies on the mechanisms of US2-induced degradation of MHC class I heavy chains. The authors reach the conclusions that PDI is involved in this process for US2, but not US11 mediated-degradation and that signal peptide peptidase is involved in the same process via an interaction with PDI.

The study is interesting and the conclusions are potentially significant. However, there are a number of concerns regarding the methodology and interpretation of the results.

1) There is the use throughout the manuscript of the term "dominant negative", both with respect to PDI mutants and reported derlin-1 mutants. None of the PDI mutants have been shown to be "dominant negative" i.e. when expressed to not only be inactive but also to inhibit the activity of the endogenous protein, and when reporting other constructs the authors should be very precise in which activity/activities are inhibited.

2) The interpretation of the results shown in figure 1 are open to question.

a) There should be a Normal HeLa control in the western blots in figure 1A and 1B
b) There appears to be a down-regulation of MHCI levels in many of the siRNA experiments compared with the siGFP control in Figure 1A. This is not commented on nor is it reflected in the data on cell surface expression shown in the other panel of figure 1A. It is notable that in figure 4, where a similar reduction in intracellular MHCI levels are observed, a decrease in cell surface exposed MHCI is observed in parallel.
c) The authors discuss the data in terms of a role for PDI in US2-mediated degradation. However, the authors show no evidence that the effects is linked to degradation per se. Similar results could be obtained from many other effects e.g. transcription levels, translation efficiency, SRP-based transport into the ER of MHCI etc. If the authors want to conclude the effect is on degradation direct supporting evidence is required. The same is true for the results shown in figure 4 and figure 5. The authors should also show clearly whether the siPDI effect arises due to it being a direct effect on US2 levels or on altering the distribution of the different forms of US2 (this is partially covered in Figure 2).
d) Connected with the above, in figure 1B the authors should consider showing the effects of the siRNA on US11 levels.
e) The authors state in the results section that "depletion of PDI by siPDI restored the surface MHC class I level to a level similar to that of wild type HeLa cells". From figure 1A this is clearly not the case, the levels increases, but only to a level that is intermediate between the two controls.

3) With the sequence of US2 and a good knowledge of N-glycosylation it is possible to work out why a C52A rather than C52S mutation was made in US2, but this needs a much better explanation for the non-specialized reader.

4) The interpretation of the results shown in figure 2 are open to question.

a) The authors should discuss more why such a small proportion of the US2 (+CHO) is in the oxidised state in Figure 2A lane 3 - is it usual for half of the ER resident protein to be fully reduced? Furthermore the authors should discuss why there is such a large difference in the ratios of the US2 states in Figure 2A compared with figure 2C and the implications of this difference for their results. The authors should also explain what they mean by "partially oxidized US11" (figure 2A) and how they determined this.
b) The effects of US2 expression on MHCI surface expression in figure 2B appear to be much smaller than the effects in figure 1A. Since transient transfection often results in very high expression levels, this is unlikely to be due to the differences in US2 levels (this could be tested, with appropriate controls). This implies that there are additional effects on MHCI surface expression levels, beyond US2 expression per se in the stable cell lines. The authors should consider fully the

implications of this.

5) The interpretation of the results shown in Figure 3 are open to question

a) The authors should provide appropriate controls that the apparent associated between PDI and SPP is not due to cross reactivity of the SPP antibody with PDI.
b) It is unclear why the Ab HC chain band in Figure 3A is so variable between lanes and why it is so variable between figure 3A and figure 3B.
c) The authors state that the results imply that "PDI did not interact with Derlin-1". There are multiple possible reasons why a negative result was obtained here, it is more accurate to say that the authors did not see an interaction between PDI and Derlin-1 by co-IP.
d) SPP is involved in cleaving the signal sequences of ER resident proteins. Since PDI is a highly abundant ER resident protein the authors should do appropriate controls to show that the SPP-PDI complex is linked to ERAD and not a simply consequence of SPP action on import of PDI to the ER i.e. PDI being a substrate for SPP.

6) The role of F258 and I272 in PDI function is confused. These residues are required not only for the "chaperone function", but also play a role in many of the other functions of this protein folding catalyst.

7) The results in figure 5B imply that in the PDI F258A/I272A over-expressing strain there is a greater proportion of the ER resident high mannose form and yet there is also (from figure 4D) a higher level of surface exposed MHCI. How can greater ER retention and simultaneously greater ER export be reconciled?

8) The data in Figure 6 are open to question.

a) The data in Figure 6 is of lower quality than in the rest o the paper with the bands being extremely weak against the background.
b) It is unclear why the amount of protein in the chase is significantly greater after 1 hour than 0hr in figure 6A and 6C. This suggests that all of the protein that is synthesized during the pulse is not in the 0 hr time point (for whatever reason) and hence it is unclear what the half-time for disappearance of the ERAD substrate is in the siSPP cell line. It is noteworthy that the % change for the 1-3 hour time-points for the siSPP is approximately the same as for the 0-2 control time-points implying no differences in the rate of degradation (though there is a possible difference in the lag phase before degradation starts).
c) Since SPP is required for the efficient import of a large number of proteins into the ER, it is very unclear whether the effects of siSPP are due to changes in ER-import efficiency, changes in ER protein composition or, as the authors claim, directly to changes in the rate of ER associated degradation.

9) Since PDI is an essential protein linked to the folding of a multitude of proteins the authors should carefully consider whether any effects they observe based on siPDI are direct or indirect. The authors should also consider the effects of the starvation of the cells prior to pulse-chase on the redox state of the ER and how with siPDI this may take significantly longer to recover from.

Resubmission Received 14 August 2009

Thank you very much for your letter of February 18, 2009 regarding our manuscript (EMBOJ-2009-70469) and comments from the three reviewers. Enclosed is a revised manuscript by Lee *et al.* entitled “Protein disulfide isomerase is required for signal peptide peptidase-mediated protein degradation”.

The reviewers’ comments were constructive and very helpful for improving the manuscript. On the basis of the reviewers’ criticisms, we have worked extensively in the last 6 months and have added a significant amount of new experimental data. We have made all necessary changes to respond to the reviewers’ comments. We have made modifications to the original figures (Figure 1A, 1B, Figure 2C, Figure 3A, and 3B), and included completely new data in Figure 1 (1C), Figure

3 (3C), Figure 4 (4C and 4D), Figure 5 (5B and 5E), and Figure 6 (6A-6D). Importantly, we now demonstrate the association between PDI and SPP at the endogenous level. We also show that PDI is vital for degradation of CD3δ but not for degradation of CFTR DeltaF508, a Derlin-1 dependent ERAD substrate, suggesting that involvement of PDI in ERAD is not limited to the US2 pathway, but rather that PDI plays a general role in SPP-mediated ERAD of certain misfolded substrates. These revisions made it necessary for us to rewrite a significant portion of the manuscript.

A detailed point-by-point response to the comments of the reviewers is provided below. I believe the manuscript has been improved satisfactorily and hope it will be accepted for publication in EMBO J.

Thank you very much for your consideration.

Point-by-point responses to the comments of each reviewer

We greatly appreciate the reviewers' valuable and constructive comments on our paper,

Reviewer #1

Point 1) The interaction of SPP with wild-type PDI is a key concept used to explain the involvement of PDI in SPP-mediated degradation of misfolded proteins (Figure 3). However, the authors only used over-expressed PDI-myc to demonstrate PDI association with SPP. Given that PDI is a fairly abundant ER-resident protein, the authors should show the interaction of SPP with endogenous PDI.

-We performed coimmunoprecipitation of SPP with endogenous PDI and now show the interaction of SPP with endogenous PDI (Figure 3C).

Point 2) More importantly, the authors must observe and quantify the interaction of SPP with wild-type PDI and PDI mutants (PDI C36, 39, 380, 383S and PDI F258W/I272W) as well as that of SPP with US2 in the presence of either wild-type PDI-myc or the PDI mutants.

-According to the reviewer's suggestion, we quantified the interaction of SPP with PDI-myc, PDI C36,39,380,383S-myc, and PDI F258W/I272A-myc (Figure 5B). We observed stronger association of SPP with PDI F258W/I272A-myc than with PDI-myc. This observation suggests that PDI F258W/I272A-myc competes with endogenous PDI for SPP.

Point 3) In Figure 5B, the authors used W6/32 to measure degradation of class I MHC in US2-expressing cells. In the figure, the amount of b2m increases after 30 minutes of chase when a decrease would be expected. Why is b2m-associated class I MHC so stable in these cells? In most relevant publications, folded MHC class I is barely detectable by W6/32 in US2 and US11 expressing cells.

-Figure 5B was relocated to Figure 5D.

-The b2m molecules dissociate from MHC class I HCs before dislocation and remain stably in the ER or associate with newly synthesized MHC class I HCs. Thus, the W6/32 immunoprecipitates after chase include b2m molecules associated with MHC class I HCs labeled during pulse and with newly synthesized ones. Therefore, the amount of b2m detected by W6/32 antibody is barely changed before and after chase.

-The degradation of MHC class I molecules by US2 or US11 has been shown by pulse-chase experiments using antibody against b2m-free MHC class I heavy chains or the W6/32 antibody

(Nature. 1996 Dec 5;384(6608):432-8.). The amount of MHC class I molecules detected by both antibodies was dramatically decreased after chase regardless of their folding status.

Point 4) I am not convinced by the authors' conclusion that the two bands for MHC class I HC observed after a 30 minute chase in cells expressing PDI F258W/I272A-myc represent differentially glycosylated forms (Figure 5B), since they do not show any data that perain to the deglycosylated form of HC in the presence of proteasome inhibitor. To draw such conclusions, the authors must use cells that express wild-type or mutant PDI, expose them to proteasome inhibitor, then immunoprecipitate with an antibody that recognizes free heavy chains, instead of using W6/32, to demonstrate the presence of the deglycosylated forms of HC.

-I believe that the reviewer misinterpreted the data. The lower of the two bands for MHC class I HC does not represent a dislocated deglycosylated form. The dislocated deglycosylated form is expected to be lower than this band by about 2 kDa. Instead, the lower band represents an ER resident high mannose form and the upper band represents an ER exported form, as evidenced by EndoH assay of aliquots (Figure 5D and E).

Point 5) The authors suggest that PDI is involved only in SPP-dependent ERAD without addressing whether it may not be able to play a role in SPP-independent ERAD. To support of this conclusion that PDI would not be involved in SPP-independent ERAD, they should examine whether perturbing PDI function affects other ERAD substrates. This point is essential to place the observations reported here on a more general footing, rather than be centered so heavily on the US2 model system.

-According to the reviewer's suggestion, we now provide new data. Because it is not known whether SPP is involved with ERAD substrates other than MHC class I in the US2 pathway, we initially determined whether SPP plays a role in ERAD using two representative ERAD substrates. We showed that knockdown of SPP significantly inhibited the degradation of CD3δ but not CFTR DeltaF508. Knockdown of PDI affected the degradation of SPP-dependent CD3δ but not CFTR DeltaF508 (Figure 6B and C).

-Since Derlin-1 has been reported to associate with CFTR DeltaF508 to facilitate its degradation, similar to US11-mediated degradation of MHC class I, we concluded that PDI is involved specifically in SPP-dependent ERAD (Figure 6B and C).

Point 6) I'm still puzzled by the lack of a proposal for a specific mechanism of involvement of PDI in SPP mediated ERAD. The discussion is rather deficient on this point.

-We now propose a mechanism of PDI function in SPP-mediated ERAD in the Discussion.

"Based on a previous study (Loureiro et al, 2006) and on our results obtained using US2-modified MHC class I HC and CD3δ as model substrates, we present the following model for the role of PDI in the SPP-mediated ERAD pathway: misfolded proteins are recruited to SPP where PDI bound to SPP unfolds the proteins into a dislocation-competent structure through its chaperone function, allowing the misfolded substrates to be escorted to the dislocation channel." (page 14, lines 27-32).

Reviewer #2

Point 1) Introduction: Htm1p in yeast has recently been shown to have mannosidase activity and the pathway of disposal of glycoproteins by ERAD has been well defined by Aebi and colleagues (JCB 184, 159-72; 2009). The 2nd and 3rd paragraph of the introduction are confusing and imprecise; the role of US2 and US11 in viral immune evasion needs to be explained more clearly here.

-In response to the reviewer's comments, we rewrote the Introduction section.

"Although EDEM (Htm1p in yeast) and OS-9/XTP3-B (Yos9p in yeast) have been shown to recognize some ERAD substrates and deliver them to the dislocation machinery (Christianson et al, 2008; Clerc et al, 2009; Cormier et al, 2009; Eriksson et al, 2004; Oda et al, 2003), how cells distinguish misfolded proteins from folding intermediates is not fully understood." (page 2, lines 31-page 3, lines 2).

"MHC class I molecules consist of a heavy chain (HC), β_2 microglobulin, and an 8-10mer peptide, and function on the cell surface to present antigenic peptides to cytotoxic T lymphocytes. The human cytomegalovirus (HCMV) evades cytotoxic T lymphocytes through binding of HCMV glycoproteins US2 and US11 to newly synthesized MHC class I HC, inducing their dislocation into the cytosol for subsequent degradation (Barel et al, 2006a; Wiertz et al, 1996a; Wiertz et al, 1996b)." (page 3, lines 7-12).

Point 2) Fig 1: Depletion of PDI by siRNA did not restore class I surface levels to a level 'similar' to that of wt HeLa cells; the scale is a log scale, and there is still a significant difference between the siRNA curve and the wild type.

-In response to the reviewer's comment, we rewrote.

"whereas depletion of PDI by siPDI restored the surface MHC class I level to close to that of wild type HeLa cells" (page 5, lines 1,2).

Point 3) K.o. efficiencies for each target should be specified.

-We now indicate the knockdown efficiencies (Figure 1).

Point 4) What do the authors mean with 'disrupting N-linked glycosylation at position 52'? There is no reference to this anywhere else in the description of the figure or discussion.

- We clarified this point in the manuscript

"Replacement of the cysteine residue at position 52 by alanine was specifically designed to avoid creation of a new N-linked glycosylation motif (NX(S/T)X, where X represents any amino acid other than proline" (page 6, lines 6-8).

Point 5) why do the bands in C look so different from A (no visible reduced and cytosolic forms)? the quality of this figure is not sufficient to state whether or not there is more reduced US2 in siPDI cells or not.

–The difference between A and C originated from discrepancy of pulse time. We have replaced this figure with a new one (Figure 2C).

Point 6) PDI-myc needs to be characterized as PDI is normally retained in the ER via its KDEL sequence at the extreme C-terminus. The myc-tag will allow PDI to be secreted which is potentially problematic in the interpretation of the subsequent experiments.

-We now provide immunofluorescence data showing that the myc-tag does not affect the intracellular distribution of PDI (Figure 4D).

Point 7) the specific precipitated (US2, SPP, etc) for each panel should be shown as IP controls.

-We now provide the IP controls (Figure 3A and B).

Point 8) given the tag at the C-terminus how can the authors be sure that PDI interacts with SPP to promote ERAD of other substrates rather than being a substrate itself?

-We have reinforced the explanation of this point.

"Depletion of PDI did not influence the steady-state levels of SPP dimer or monomer (Figure 3C, compare lane 4 and 5). Our data indicate that SPP is not a substrate for PDI but rather a specific binding partner. Conversely, PDI is probably not a substrate for SPP because SPP is a member of the presenilin (PS)/SPP-Like (SPPL) superfamily of intramembrane-cleaving aspartic proteases and PDI is a soluble ER resident protein." (page 8, lines 7-12).

Point 9) Do the authors have data to support their claim that the 2 bands visible in 5B are differentially glycosylated forms of MHC class I HC?

-A similar question was raised by reviewer # 1 (point 4). We now provide supporting data, which show that the two bands are differentially glycosylated forms of MHC class I HC (Figure 5E).

Point 10) Fig. 6: the graphs are peculiar in that absence of SPP or PDI only causes a delay in degradation of CD3 delta, but no change in the rate; this could be an indirect effect.

-We now provide new experimental data. To improve knockdown efficiency we used a retroviral infection system. After enrichment of transfectants by selection in puromycin for 2 weeks, cells expressing GFP-, SPP-, PDI-specific siRNA were used for experiments. The absence of SPP or PDI reduced the degradation rate of CD3δ, but not CFTR DeltaF508 (Figure 6).

Point 11) Are other secretory pathway functions normal? Is the rate of protein secretion affected?

- Previously, we demonstrated that PDI knockdown did not affect the surface expression of several glycoproteins (Cell 127:369-82, 2006). We also confirmed that SPP depletion did not affect the surface expression of MHC class I molecules in HeLa cells, suggesting that the secretory pathway functions normally in those cells (data not shown).

Point 12) what about the rate of degradation of a substrate that is PDI and SPP-independent?

- This issue was also raised by reviewer #1 (point 5).

Point 13) Discussion: does overexpression of dominant negative PDI have an effect on class I HC degradation in US11 cells?

-Overexpression of dominant negative PDI or other PDI constructs did not affect the degradation of MHC class I by US11 (data not shown). This result emphasizes that PDI is specific to US2-mediated ERAD.

Point 14) there is no SPP in yeast, but PDI has been shown to play a decisive role in ERAD in yeast as well; this should be discussed.

-In response to the reviewer's comment, we have now added a discussion of this point.

"There is no SPP in yeast although a PDI homologue has been shown to play a decisive role in ERAD in yeast as well (Gillece et al, 1999). It is therefore possible that the evolutionary expansion of the number and complexity of secretory and membrane proteins necessitated the simultaneous expansion of ERAD pathways in higher eukaryotes. The multiple functional homologues and ERAD E3 ubiquitin ligase membrane complexes found in the mammal seem to support that view (Christianson et al, 2008; Wang & Ng, 2008; Yoshida, 2007)." (page 13, lines 21-27).

Point 15) oxidoreductase function in the ER is partially redundant; since the authors knocked down only one reductase at a time, they cannot say that the reductase activities in the ER are not essential for HC dislocation.

-We revised the manuscript in response to the reviewer's comment. We have limited our claim to say that the reductase activity of PDI is not essential for HC dislocation because knockdown of each protein did not affect the dislocation of HC.

"the above results suggested that US2-mediated degradation of MHC class I HC does not require the catalytic activity of PDI" (page 9, lines 24-25).

Reviewer #3

Point 1) There is the use throughout the manuscript of the term "dominant negative", both with respect to PDI mutants and reported derlin-1 mutants. None of the PDI mutants have been shown to be "dominant negative" i.e. when expressed to not only be inactive but also to inhibit the activity of the endogenous protein, and when reporting other constructs the authors should be very precise in which activity/activities are inhibited.

- We have corrected the manuscript as the reviewer suggested and only use the term "dominant negative'' for PDI F258W/I272A-myc and Derlin-1 GFP. These mutants are inactive and inhibit the activity of the endogenous proteins (Figure 4B and C, Nature. 2004 Jun 24;429(6994):834-40.).

"Collectively, we concluded that PDI F258W/I272A-myc operates dominant negatively and PDI plays a role as a molecular chaperone in US2-mediated degradation of MHC class I HC." (page 9, lines 18-20).

Point 2) There should be a Normal HeLa control in the western blots in figure 1A and 1B.

-We provided new data including a normal HeLa control (Figure 1A and 1B).

Point 3) There appears to be a down-regulation of MHCI levels in many of the siRNA experiments compared with the siGFP control in Figure 1A. This is not commented on nor is it reflected in the data on cell surface expression shown in the other panel of figure 1A.

-We now provide new data from several independent experiments. We confirmed that the amount of MHC class I HC was not affected by siRNAs other than siPDI (Figure 1A).

Point 4) The authors discuss the data in terms of a role for PDI in US2-mediated degradation. However, the authors show no evidence that the effects is linked to degradation per se. Similar results could be obtained from many other effects e.g. transcription levels, translation efficiency, SRP-based transport into the ER of MHCI etc. If the authors want to conclude the effect is on degradation direct supporting evidence is required. The same is true for the results shown in figure 4 and figure 5. The authors should also show clearly whether the siPDI effect arises due to it being a direct effect on US2 levels or on altering the distribution of the different forms of US2 (this is partially covered in Figure 2). Connected with the above, in figure 1B the authors should consider showing the effects of the siRNA on US11 levels.

-We have provided additional data. HeLa cells expressing US2 or US11 were depleted for PDI expression by siRNA and labeled, chased, and then immunoprecipitated with antibodies against MHC class I, US2, and US11. While MHC class I HC was efficiently degraded after 30-min chase in US2 control cells, PDI knockdown significantly inhibited degradation of class I HC at the same chase time point (Figure 1C). PDI knockdown did not affect US2 synthesis and stabililty (Figure 1C) or MHC class I synthesis.

-Knockdown of PDI did not inhibit the degradation of MHC class I in US11 cells or affect US11 synthesis and stability (Figure 1C).

Point 5) The authors state in the results section that "depletion of PDI by siPDI restored the surface MHC class I level to a level similar to that of wild type HeLa cells". From figure 1A this is clearly not the case, the levels increases, but only to a level that is intermediate between the two controls.

- This issue was raised by Reviewer #2 (point 2).

Point 6) With the sequence of US2 and a good knowledge of N-glycosylation it is possible to work out why a C52A rather than C52S mutation was made in US2, but this needs a much better explanation for the non-specialized reader.

- This issue was raised by Reviewer #2 (point 4). We emphasized the explanation of this in the manuscript.

Point 7) The authors should discuss more why such a small proportion of the US2 (+CHO) is in the oxidised state in Figure 2A lane 3 - is it usual for half of the ER resident protein to be fully reduced? Furthermore the authors should discuss why there is such a large difference in the ratios of the US2 states in Figure 2A compared with figure 2C and the implications of this difference for their results. The authors should also explain what they mean by "partially oxidized US11" (figure 2A) and how they determined this.

-This was raised by Reviewer #2 (point 5). We replaced the Figure with a new one to clarify the point (Figure 2C).

-The reduced forms of US2 in figure 2A lane 3d arose from short labeling time (5 min). The majority of US2 was oxidized after a 10-min chase.

-We are afraid we have badly misjudged the partially oxidized US11 in figure 2A. Subtle differences in migration mobility between US11 and US11 mutants under the non-reducing condition originated from amino acid substitutions since US11 and US11 mutants also migrated differentially under reducing condition (data not shown).

Point 8) The effects of US2 expression on MHCI surface expression in figure 2B appear to be much smaller than the effects in figure 1A. Since transient transfection often results in very high

expression levels, this is unlikely to be due to the differences in US2 levels (this could be tested, with appropriate controls). This implies that there are additional effects on MHCI surface expression levels, beyond US2 expression per se in the stable cell lines. The authors should consider fully the implications of this.

-The differences in downregulation of MHC class I level by US2 between stable cells and transient transfectants might be a matter of which clone is selected for further use during establishment of the stable cell line. We chose a clone that highly expressed US2 to ensure a clear-cut phenotype regarding MHC downregulation.

- We performed identical experiments to Figure 1A using cells cotransfected with US2 and siPDI or siGFP and obtained similar results to those shown in Figure 1A (data not shown). Thus we believe that the effect of PDI depletion in US2 pathway did not result from using stable cell lines.

Point 9) The authors should provide appropriate controls that the apparent associated between PDI and SPP is not due to cross reactivity of the SPP antibody with PDI.

-We provide new data. We now demonstrate that there was no cross reactivity of the SPP antibody with PDI and vice versa (Figure 3C).

Point 10) It is unclear why the Ab HC chain band in Figure 3A is so variable between lanes and why it is so variable between figure 3A and figure 3B.

- The variation in antibody heavy chain- or light chain bands between lanes was due to the different animals in which antibodies were raised (Antibodies for US2, SPP or Derlin-1 are rabbit polyclonal and antibody for p97 is monoclonal and BiP antibody is goat polyclonal).

Point 11) The authors state that the results imply that "PDI did not interact with Derlin-1". There are multiple possible reasons why a negative result was obtained here, it is more accurate to say that the authors did not see an interaction between PDI and Derlin-1 by co-IP.

- We have corrected the manuscript according to the reviewer's comment.

"We did not observe an association of PDI-myc with Derlin-1, a key component in the US11 pathway (Figure 3A, lane 6), or with p97 or BiP, which are known to be involved in the US2 pathway (Chevalier & Johnson, 2003; Hegde et al, 2006) (Figure 3A, lanes 7 and 8)." (page 7, lines 14-17).

Point 12) SPP is involved in cleaving the signal sequences of ER resident proteins. Since PDI is a highly abundant ER resident protein the authors should do appropriate controls to show that the SPP-PDI complex is linked to ERAD and not a simply consequence of SPP action on import of PDI to the ER i.e. PDI being a substrate for SPP.

- This issue was raised by reviewer #2 (point 8).

Point 13) The role of F258 and I272 in PDI function is confused. These residues are required not only for the "chaperone function", but also play a role in many of the other functions of this protein folding catalyst.

- Although it is controversial, we believe that the b' domain of PDI, especially the F258 and I272 resides, is essential for the chaperoning function because mutation of PDI within this site greatly reduces the binding affinity for small peptide substrates, with the greatest effect being a I272W substitution, which appears to have no structural effect (J Biol Chem. 279:10374-81, 2004).

Point 14) The results in figure 5B imply that in the PDI F258A/I272A over-expressing strain there is a greater proportion of the ER resident high mannose form and yet there is also (from figure 4D) a higher level of surface exposed MHC I. How can greater ER retention and simultaneously greater ER export be reconciled?

-We now provide the new data (Figure 5E). US2 cells expressing PDI constructs were pulse-chased, lysed, and immunoprecipitated with W6/32 antibody. The immunoprecipitates were digested with Endo H before SDS-PAGE analysis. After 30-min chase, a significant proportion of the MHC class I molecules were degraded in cells expressing PDI-myc or PDI C36,39,380,383S-myc and half of the remaining class I molecules escaped the ER as evidenced by EndoH assay (Figure 5E, lanes 2 and 4). In contrast, overexpression of PDI F258W/I272A-myc in US2 cells inhibited degradation of MHC class I molecules and thus the steady-state levels of both the ER resident form and the ER-escaped form (likely the surface exposed form) increased relatively (Figure 5E, compare lane 6 with 2 and 4). Thus we assume that a higher level of surface exposed MHC I (Figure 4B) simply represents the higher steady-state level of MHC escapees on the surface in US2 cells expressing PDI F258W/I272A-myc.

Point 15) It is unclear why the amount of protein in the chase is significantly greater after 1 hour than 0 hr in figure 6A and 6C. This suggests that all of the protein that is synthesized during the pulse is not in the 0 hr time point (for whatever reason) and hence it is unclear what the half-time for disappearance of the ERAD substrate is in the siSPP cell line. It is noteworthy that the % change for the 1-3 hour time-points for the siSPP is approximately the same as for the 0-2 control time-points implying no differences in the rate of degradation (though there is a possible difference in the lag phase before degradation starts).

-A similar criticism was raised by reviewer #2 (point 10).

Point 16) Since SPP is required for the efficient import of a large number of proteins into the ER, it is very unclear whether the effects of siSPP are due to changes in ER-import efficiency, changes in ER protein composition or, as the authors claim, directly to changes in the rate of ER associated degradation.

- This criticism was also raised by reviewer #2 (point 12).

Point 17) Since PDI is an essential protein linked to the folding of a multitude of proteins the authors should carefully consider whether any effects they observe based on siPDI are direct or indirect. The authors should also consider the effects of the starvation of the cells prior to pulse-chase on the redox state of the ER and how with siPDI this may take significantly longer to recover from.

- The reviewer's concern is quite reasonable but difficult to clarify. Depletion of PDI by siPDI does not affect the surface expression of several disulfide-bonded glycoproteins (Park et al, cell, 2006). Based on this observation, we imagine that siPDI is unlikely to affect the redox state of the ER and that the observed effect of siPDI is probably direct.

2nd Editorial Decision 21 September 2009

Thank you for resubmitting your manuscript for consideration by the EMBO Journal. Firstly, please let me apologise for the delay in getting back to you with a decision: I sent the manuscript to the original three referees, and have now received the reports from two of them, but the original referee 1, despite numerous reminders, has still not returned his/her report. It is unclear at this point whether he/she will do so, and therefore in order to save you from a further loss of time, I am making a decision based on the two reports we have. As you will see, referee 2 (originally #3) finds the manuscript to be substantially improved and now supports publication, pending a number of text changes. Referee 1 (originally #2), however, still raises a significant concern regarding the myc-tagged constructs used in the study. Having looked at the data myself, I am in agreement with this referee that the immunofluorescence data are not sufficient to make the point that the myc-PDI constructs are appropriately localised and are functional. It would therefore be critical to address this issue using biochemical means.

In the light of the referees' positive recommendations, I would therefore like to invite you to submit a revised version of the manuscript, addressing all the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses included in the next, final version of the manuscript.

Should we receive the missing report, we will forward it on to you, and may also ask you to respond to any concerns raised by this reviewer.

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

Yours sincerely,
Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

In the revised version of their manuscript on the role of PDI in SPP-mediated ERAD the authors responded to a number of my criticisms of the previous version satisfactorily.

I remain, however, unconvinced by their characterization of myc-tagged PDI: Immunofluorescence is not an adequate method to show whether or not PDI-myc is functional and remains in the ER. The authors need to show biochemically that the tagged protein is not secreted and that redox functions in the ER are comparable to cells expressing untagged PDI.

Referee #2 (Remarks to the Author):

Protein disulfide isomerase is required for signal peptide peptidase-mediated protein degradation.

This manuscript details studies on the mechanisms of US2-induced degradation of MHC class I heavy chains. The authors reach the conclusions that PDI is involved in this process for US2, but not US11 mediated-degradation and that signal peptide peptidase is involved in the same process via an interaction with PDI.

The study is interesting, the conclusions are potentially significant and the manuscript is very significantly improved over the original.

I still have some qualms about the degree to which this is a direct vs indirect effect, especially

relating to potential changes in the redox state of the ER and on the exact role of PDI in the process if it is a direct effect. However, I have similar qualms about a large proportion of the in vivo literature in this field.

Since SPP has only 2 significant luminal regions, both of which are short and could be easily added to a marker protein and since the literature seems to be full of PDI domain constructs, I would suggest that the authors investigate further the nature of the interaction between SPP and PDI. However, this would be for another manuscript and should not be required for publication of this manuscript.

On due consideration I feel that the manuscript exceeds the current standard in the field for publication at this level.

There are a few corrections to the text that are suggested, the most important of which relates to Page 8 line 20.

Page 4 line 16: Replace "chaperone" with "protein folding catalyst" (the primary function of PDI is as a protein folding catalyst)

Page 4 line 23: Since there are no prior references to PDI in the manuscript this line reads as though PDI was first identified by Loureiro et al in 2006. A sentence with one or more appropriate references about PDI should be added prior to this, preferably in the introduction.

Page 5 line 2: Replace "close to that" with "closer to that"

Page 5 line 14: Delete "specifically" (there is the possibility that it is an indirect effect. Also PDI has other functions and other proteins are involved in US2-mediated degradation)

Page 5 line 28: Delete "specifically"

Page 6 lines 1-2: Replace "is to oxidize or reduce substrates" with "is to oxidize or isomerize protein substrates"

Page 6 lines 20-21: The implication from the line is not quite the same as the implication from the corresponding figure. The authors should modify the statement to include that fact that only a fraction of the total are shown to contain intra-molecular disulfide bonds.

Page 6 lines 27-28: Examination of Figure 2B suggests that the Cys mutants have an effect. While this is greatly reduced compared with the wild type protein the results do not imply that "the formation of intra-disulfide bonds in US2 and US11 is essential"

Page 7 line 2: The time relevant to the statement "relatively long pulse duration" needs stating in the legend to figure 2.

Page 7 line 21: Delete "specifically"

Page 7 line 25: Delete "specific"

Page 8 line 8: Delete "specific"

Page 8 lines 20 and 26-27 and associated text through the rest of the results and discussion: Reading Pirneskoski et al 2004, I can find no evidence to support the statement that the b' domain of PDI "is responsible for chaperone activity" nor that the "F258W/I272A has decreased chaperone activity". The substrate binding domain appears to be responsible for just that - binding substrates - which would imply that it would be defective in many of the associated activities of PDI, but without singling out the "chaperone activity".

Page 9 lines 14 and 17: Add after "replace endogenous PDI" the words "in this process".

Page 9 line 31: Delete "even"

Page 10 line 31 to page 11 line 2: Is there a statistically significant difference between "half" and "59%". If not then these lines should be deleted or highly modified.

Page 11 line 30: Replace "indicating" with "suggesting" since the authors do not directly show that PDI is involved in the SPP-dependent pathway.

Page 12 line 33: N-ethylmaleimide is not an oxidant.

Page 13 line 13: Delete "specifically"

Finally, I must apologize to the authors and editors for an erroneous comment I made in my original review, fortunately this was corrected by the authors.

1st Revision – Authors' Response 01 October 2009

Reply to the reviewers' comments

We deeply appreciate the reviewers' constructive comments on our study.

Referee #1

Point) I remain, however, unconvinced by their characterization of myc-tagged PDI: Immunofluorescence is not an adequate method to show whether or not PDI-myc is functional and remains in the ER. The authors need to show biochemically that the tagged protein is not secreted and that redox functions in the ER are comparable to cells expressing untagged PDI.

- As stated in 'Materials and Methods' section of the original manuscript, PDI-myc represents the construct in which myc-tag is inserted at a position before the C-terminal KDEL sequence (an ER retention motif) and thus is expected to be localized in the ER. Nevertheless, in response to the reviewer's comment, we proceeded to test whether PDI-myc is secreted or not. We now provide strong evidence for ER retention of PDI-myc ('Referee-only' Supplementary Figure 1A). The cDNAs encoding wild-type PDI (WT PDI) and PDI-myc were transfected into HeLa cells and labeled for 6 h. Cell lysates and 'medium' fractions were subjected to immunoprecipitation with anti-PDI antibodies. Endogenous PDI was exclusively immunoprecipitated from the cell lysates but not from 'medium' ('Referee-only' Supplementary Figure 1A, compare lanes 1 and 4) as reported previously by Bulleid group (Curr. Biol. 11:1114-8, 2001). Almost all of the ectopically overexpressed WT PDI was immunoprecipitated from cell lysates ('Referee-only' Supplementary Figure 1A, lane 5) and trace amount of WT PDI was detected in medium ('Referee-only' Supplementary Figure 1A, compare lanes 2 and 5), suggesting that the overexpression of PDI resulted in a leakage of ER retention. Similar to WT PDI, most PDI-myc was immunoprecipitated from lysates and only negligible amount of PDI-myc was immunoprecipitated from medium ('Referee-only' Supplementary Figure 1A, lanes 3 and 6). These data support that myc-tagged PDI is not secreted.

-To examine whether overexpression of PDI-myc constructs bring about potential changes in the redox state of the ER, we examined the surface expression level of several disulfide-bonded glycoproteins (CD47, CD59 and C71) in cells overexpressing PDI constructs ('Referee-only' Supplementary Figure 1B). Compared with the mock control, overexpression of WT PDI or myc-tagged PDI constructs did not affect the surface expression of glycoproteins (CD47, CD59 and CD71). Furthermore, the expression level of BiP, a molecular chaperone of which expression is increased upon ER stress such as redox purtabation and accumulation of misfolded proteins, was not changed despite 2-5-fold overexpression of ectopic WT PDI or PDI-myc ('Referee-only'

Supplementary Figure 1C). Thus, these data provide, albeit still indirect, evidence that ER redox functions in cells overexpressing ectopic PDI are comparable to those of mock.

Referee #2

Point 1) I still have some qualms about the degree to which this is a direct vs indirect effect, especially relating to potential changes in the redox state of the ER and on the exact role of PDI in the process if it is a direct effect. However, I have similar qualms about a large proportion of the in vivo literature in this field.

-Similar criticism was raised by reviewer #1. Because it is difficult to determine effect of myc-tagged PDI overexpression on redox state of the ER, we could not help doing with indirect experiments.

Point 2) Page 4 line 16: Replace "chaperone" with "protein folding catalyst" (the primary function of PDI is as a protein folding catalyst)

-According to reviewers' suggestion, we replaced "chaperone" with "protein folding catalyst".

Point 3) Page 4 line 23: Since there are no prior references to PDI in the manuscript this line reads as though PDI was first identified by Loureiro et al in 2006. A sentence with one or more appropriate references about PDI should be added prior to this, preferably in the introduction.

-We added a reference to PDI and rewrote the manuscript.

"These findings indicate that the protein folding catalyst PDI (Goldberger et al, 1964) is a component of the SPP-mediated ERAD machinery." (page 4, lines 15-16)

"PDI has been implicated in the degradation of misfolded proteins (Gillece et al, 1999; Molinari et al, 2002) and was identified as an US2-associated protein through large-scale affinity purification (Loureiro et al, 2006)."

Point 4) Page 5 line 2: Replace "close to that" with "closer to that"

-We replaced "close to that" with "closer to that".

Point 5) Page 5 line 14: Delete "specifically" (there is the possibility that it is an indirect effect. Also PDI has other functions and other proteins are involved in US2-mediated degradation)

-We deleted "specifically".

Point 6) Page 5 line 28: Delete "specifically"

-We deleted "specifically".

Point 7) Page 6 lines 1-2: Replace "is to oxidize or reduce substrates" with "is to oxidize or isomerize protein substrates"

-We replaced "is to oxidize or reduce substrates" with "is to oxidize or isomerize protein substrates".

.

Point 8) Page 6 lines 20-21: The implication from the line is not quite the same as the implication from the corresponding figure. The authors should modify the statement to include that fact that only a fraction of the total are shown to contain intra-molecular disulfide bonds.

-We modified the manuscript.

“Wild-type US11 displayed both the oxidized and the reduced forms under the non-reducing condition (Figure 2A, lane 8), whereas US11 cysteine mutants showed only the reduced form under the non-reducing condition (Figure 2A, lanes 9 and 10).” (page 6, lines 16-19).

Point 9) Page 6 lines 27-28: Examination of Figure 2B suggests that the Cys mutants have an effect. While this is greatly reduced compared with the wild type protein the results do not imply that "the formation of intra-disulfide bonds in US2 and US11 is essential"

-We completely agree with the reviewer. In previous study, we did not show the expression level of proteins because anti-US2 antibodies for immunoblotting were not available. Now we provide new experimental data (‘Referee-only’ Supplementary Figure 2). Cells transiently expressing US2, US11, or their cysteine mutants were labeled and chased for 40 min. The cell lysates were immunoprecipitated with W6/32 antibody, anti-US2 or anti-US11 serum. MHC class I molecules were efficiently degraded after 40-min chase in cells expressing US2 or US11 (‘Referee-only’ Supplementary Figure 2, lanes 1-2 and 7-8), whereas overexpression of Cys mutants did not induce the degradation of MHC class I after chase (‘Referee-only’ Supplementary Figure 2, lanes 3-6 and 9-12). The expression level was comparable for all constructs (bottom panels).

Point 10) Page 7 line 2: The time relevant to the statement "relatively long pulse duration" needs stating in the legend to figure 2.

-We specified the pulse duration in the legend to Figure 2C.

“After puromycin selection, cells were labeled for 10 min” (page 23, line 29).

Point 11) Page 7 line 21: Delete "specifically"

-We deleted “specifically”.

Point 12) Page 7 line 25: Delete "specific"

-We deleted “specific”.

Point 13) Page 8 line 8: Delete "specific"

-We deleted “specific”.

Point 14) Page 8 lines 20 and 26-27 and associated text through the rest of the results and discussion: Reading Pirneskoski et al 2004, I can find no evidence to support the statement that the b' domain of PDI "is responsible for chaperone activity" nor that the "F258W/I272A has decreased chaperone activity". The substrate binding domain appears to be responsible for just that - binding substrates - which would imply that it would be defective in many of the associated activities of PDI, but without singling out the "chaperone activity".

-According to the reviewers’ comment, we have corrected the manuscript through the results and discussion. We replaced “chaperone activity” for b’ domain of PDI and F258W/I272 mutants with “substrate binding activity”.

Point 15) Page 9 lines 14 and 17: Add after "replace endogenous PDI" the words "in this process".

-We added after "replace endogenous PDI" the words "in this process".

Point 16) Page 9 line 31: Delete "even"

-We deleted “even”.

Point 17) Page 10 line 31 to page 11 line 2: Is there a statistically significant difference between "half" and "59%". If not then these lines should be deleted or highly modified.

-According to reviewers' suggestion, we deleted the sentence.

Point 18) Page 11 line 30: Replace "indicating" with "suggesting" since the authors do not directly show that PDI is involved in the SPP-dependent pathway.

-We replaced "indicating" with "suggesting".

Point 19) Page 12 line 33: N-ethylmaleimide is not an oxidant.

-In response to the reviewers' comment, we rewrote the page 12 line 33.

"Agents that affect intracellular redox potential and/or free thiol status, such as diamide and N-ethylmaleimide inhibit the dislocation of MHC class I HC by US2 and US11" (page 13, lines 5-7).

Point 20) Page 13 line 13: Delete "specifically"

-We deleted "specifically".

3rd Editorial Decision 29 October 2009

Many thanks for submitting the revised version of your manuscript EMBOJ-2009- 72231R. I am sorry for the slight delay in getting back to you with a decision on this. As I told you, neither of the referees from the previous round of review were able to look at your revision, and therefore I involved an external advisor, specifically to comment on the adequacy of your responses to the previous reviews. His/her comments are appended below. He/she is satisfied that the data provided in your point-by-point response does address the concerns of the reviewers, and therefore finds that the paper is suitable for publication. From our side, however, it would be essential that you incorporate these data into the manuscript, as supplementary figures, so that they are available to the reader. I am therefore pleased to say that we can now commit to accepting your manuscript for publication in the EMBO Journal, pending minor changes as detailed here.

However, our advisor does also make a number of additional comments on the manuscript. At this stage, I do not think it fair to bring up new concerns and insist that you address them, particularly since this advisor had not reviewed the previous versions of the manuscript. All the same, I would like to give you the opportunity to respond to the criticisms raised. Obviously, should you have data to answer these concerns, it would be beneficial that you incorporate them into the manuscript, but I do not think it necessary at this stage. Minimally, please could you submit a point-by-point response to these comments when you upload the revised manuscript? The advisor also remarks on typographical and grammatical errors in the manuscript. Before you submit your final version, I would therefore ask that you (or, if possible, a native English-speaking colleague) go through the manuscript once more and correct any errors in the text.

I look forward to receiving your final version of the manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFFEREE REPORTS

Advisor comments:

The paper can be published, provided however that the many typos are corrected and the wording

made more fluent and concise.

Although the data are overall convincing, taking into account the consensus about MHC Class I folding and maturation, especially regarding TAP complex, I still feel a little bit unease for the following reasons:

1. Figure 1 A shows that only PDI siRNA rescues from the US2-associated MHC class I degradation. However, it was previously shown that both ERp57 and PDI have an important role in MHC class I peptide loading, regulating the oxidation state or selecting optimal peptides, etc. (the same authors showed the PDI involvement in a Cell paper, 2006).
Using FACS analyses, they couldn't detect these effects in the experiments shown. Yet, knocking down ERp57 or PDI should inhibit somehow MHC class I productive folding. This result might be derived from the involvement of US2 in an unknown mechanistic manner, or FACS analysis is not sensitive enough.
The most efficient way to detect the productive folding or degradation of some substrates is pulse-chase analyses. FACS shows only the accumulation of these substrates and could not be so effective to know the accurate involvement of these chaperones or enzymes.

Relating to the reviewers' comments, PDI knockdown no doubt affects Ero1alpha activation and alters redox in the ER. However, more important than the redox change itself would be to know whether this really affects the process of productive folding and degradation or not, an issue that should be addressed by pulse-chase assays. This question is quite important and both reviewers pointed at it.
However, it could be beyond the scope of their paper, so I guess it is not essential for them to clarify this point, even if it would strengthen the conclusions.

Anyway, the authors should at least comment about the fact that ERp57 siRNA knockdown did not affect MHC class I maturation. If mAb H10 (which recognize free class I heavy chains) yield similar results to the conformational antibodies, the conclusions would be reinforced.

2. Is UPR is induced by PDI siRNA?
Addressing issues related to ERAD, the authors should show that the UPR level under PDI siRNA is not altered with respect to other experimental conditions.

2nd Revision - Authors' Response 01 November 2009

Thank you very much for your favorable comments on our manuscript by Lee et al., entitled "Protein disulfide isomerase is required for signal peptide peptidase-mediated protein degradation."

As you suggested, we incorporated "Figures for referees only" into "the Supplementary Figures": “Figure for Referees # 1 is now “Supplementary Figure 2”, and “Figure for Referees # 2“ is now “Supplementary Figure 1”. This manuscript has been edited by professional editing company (BioScience Writers, LLC lweber@biosciencewriters.com). We think that the advisor’s comments are valuable but minor ones that can be handled minimally in the level of text. A point-by-point response to the advisor’s comments is provided below. I hope it will be accepted for publication in The EMBO Journal.

Thank you very much for your consideration.

Reply to the external advisor’s comments

Point 1) The paper can be published, provided however that the many typos are corrected and the wording made more fluent and concise.

-This manuscript had been edited by professional editing company.

Point 2) Figure 1A shows that only PDI siRNA rescues from the US2-associated MHC class I

degradation. However, it was previously shown that both ERp57 and PDI have an important role in MHC class I peptide loading, regulating the oxidation state or selecting optimal peptides, etc. (the same authors did showed the PDI involvement in a Cell paper, 2006). Using FACS analyses, they couldn't detect these effects in the experiments shown. Yet, knocking down ERp57 or PDI should inhibit somehow MHC class I productive folding. This result might be derived from the involvement of US2 in an unknown mechanistic manner, or FACS analysis is not sensitive enough. The most efficient way to detect the productive folding or degradation of some substrates is pulse-chase analyses. FACS shows only the accumulation of these substrates and could not be so effective to know the accurate involvement of these chaperones or enzymes.

Relating to the reviewers' comments, PDI knockdown no doubt affects Ero1alpha activation and alters redox in the ER. However, more important than the redox change itself would be to know whether this really affects the process of productive folding and degradation or not, an issue that should be addressed by pulse-chase assays. This question is quite important and both reviewers pointed at it. However, it could be beyond the scope of their paper, so I guess it is not essential for them to clarify this point, even if it would strengthen the conclusions.

Anyway, the authors should at least comment about the fact that ERp57 siRNA knockdown did not affect MHC class I maturation. If mAb H10 (which recognize free class I heavy chains) yield similar results to the conformational antibodies, the conclusions would be reinforced.

-In normal HeLa cells, cell surface expression of MHC class I should be decreased by knockdown of ERp57 or PDI. In US2 stable cells, however, unfolded MHC I molecules derived from knockdown of ERp57 or PDI are expected to be degraded by US2 pathway because, like wild-type HLA-A2.1, HLA-A2.1 C101S mutant (unfolded) was efficiently degraded in US2 stable cells (Figure 5A). Thus, in the US2 stable cells in which MHC class I molecules are degraded in the ER and already downregulated at the cell surface, knockdown of ERp57 or PDI would not further affect MHC class I maturation.

Point 3) Is UPR is induced by PDI siRNA? Addressing issues related to ERAD, the authors should show that the UPR level under PDI siRNA is not altered with respect to other experimental conditions.

- Based on our observation that PDI siRNA slightly induces BiP, we think that UPR might be induced by prolonged PDI knockdown. Note that attenuation of ERAD activates the UPR (Mol. Biol. Cell, Vol. 14, pp. 3437-3448).

