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The mycobacterial Mpa-proteasome unfolds and degrades pupylated substrates by engaging Pup's N-terminus

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1st Editorial Decision 08 November 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. As you will see, all three reviewers express significant interest in your study, and are supportive of publication, pending suitable revision. However, while referees 1 and 2 suggest only minor text changes, referee 3 does raise a number of concerns that would need to be addressed, some experimentally, in a revised version of your manuscript. One concern brought up by all three referees is the fact that your in vitro system works only with the truncated proteasome; while we do of course appreciate that there are various possible explanations for this, and do not think it essential that your resolve this issue fully, any data you can provide to demonstrate that the conclusions reached using this truncated version are likely to hold true for the in vivo situation would clearly be very valuable.

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. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the 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 REPORTS:

Referee #1 (Remarks to the Author):

In this manuscript, Striebel et al. develop an in vitro system to study the unfolding (by Mpa) and the degradation (by an open gate proteasome variant) of a pupylated model (Pup-GFP) and native (PanB) substrate. This manuscript is extremely well put together and the fluorescence-based assay elegant. Moreover, the results are of high impact and of general significance to the current understanding of proteasomal degradation, as they are likely to be applicable to the eukaryotic 26S proteasome.

The authors demonstrate that as anticipated the N-terminal unstructured region of Pup is important for its unfolding by Mpa and its degradation by the proteasome. Surprisingly however, even deletion of the eight N-terminal amino acids stabilizes Pup-GFP and pupylated PanB from degradation and unfolding (for Pup-GFP). The effects are not due to a specific sequence requirement; however, some sequences are unable to substitute for the lost amino acids, as demonstrated in Fig 5E. Other important observations are also described including the degradation of Pup and the required Mpa-proteasome binding, as demonstrated by the effect of truncating the two C-terminal residues of Mpa.

The experiments are well designed and implemented. Although the final model is highly compelling, the only suggestion this reviewer has is to offer the possibility that the length requirement for Pup's unstructured region may be influenced by the in vitro assay, as an open gate variant of the proteasome is used. Presumably this requirement stems from a missing attribute in the in vitro system. This same attribute could change the length requirement for the unstructured region of Pup. These considerations should be addressed in the Discussion section.

Referee #2 (Remarks to the Author):

The manuscript by Striebel and colleagues describes the mechanism by which the actinobacterial proteasome recognizes and processes pupylated substrates and in doing so provides some exciting new insights into the molecular mechanism of the degradation system. The manuscript reconstitutes mycobacterial proteasome degradation in vitro and then demonstrates that this proteasome unfolds proteins in an ATP-dependent manner. It shows that substrates are recognized when the central domain of Pup binds to the coiled coil domain of the ATPase ring of the proteasome. Degradation then initiates when the N-terminal region of Pup is engaged by specific loops in the ATPase ring. Thus, strikingly, the proteasome initiates degradation not on the substrate but on the degradation tag. As a result, the tag itself is degraded.
These findings are novel, interesting, and important. The manuscript puts them into context with what is known about other ATP-dependent protease systems and the similarities and differences between the systems are fascinating.
The experiments are elegant and convincing. The conclusions are fully supported by the experiments. The paper is well written and was a pleasure to read and I recommend it for publication.
I have two suggestions:
1) In the middle of page 6, it is explained that the protease system only functioned when the first seven residues of the protease subunits were truncated. Left by itself, this detail is somewhat disconcerting to the reader. I recommend that this facet of the experimental design is discussed

briefly.
2) At the end of page 9, the manuscript mentions that small amounts of a protein fragment transiently accumulate during the in vitro degradations. The manuscript tentatively assigns this fragment to correspond to the C-terminal half of Pup. It is somehow confusing to contemplate how a sequential degradation mechanism that starts at the N-terminus of Pup and degrades the rest of the substrate produces a C-terminal Pup fragment. I recommend that this is somewhat better explained. However, this is a minor issue and not a serious distraction from the paper.

Referee #3 (Remarks to the Author):

This is a sound study on an important topic (in vitro degradation of pupylated proteins) although with fairly expected findings. The authors did propose a partially novel model regarding how pup binds to the CC domain of MPA, but some control experiments have to be performed to really support the major conclusions of their model. For the most, part of the appropriate experiments were done to show how the MPA (ARC) functions (just like all the other AAA ATPases) to unfold substrates (i.e. mutation of the poor loops abrogates unfolding activity). However, it is important that they have reconstituted degradation of a Pup-GFP fusion protein in a convincing way, which had not been accomplished for the Mtb proteasome and this AAA ATPase). My enthusiasm is reduced because the WT Myco-20S does not function with MPA to degrade the GFP fusion substrate, but instead only works with the gateless Myco-20S! This is quite surprising, and thus the system is rather artificial. Presumably there is some missing factor.

Major issues:
1) As noted above, it is a serious concern that the MPA doesn't bind to or function with the WT Myco20S.

2) Deletion of the CC domain from MPA prevents binding of pup, and thus blocks unfolding and degradation of pub-GFP. However, the authors never demonstrated or mentioned that the deltaCC-MPA was functional or even hexamerized. In order to conclude that this mutation proves that pup binds to MPA via the CC domain, the authors should verify that the deltaCC-MPA properly hexamerizes and can hydrolyze ATP with the same efficiency as WT MPA. If deltaCC-MPA hydrolyzes ATP like WT, and still cannot bind/unfold pup-GFP, then the model presented is acceptable.

3) The authors demonstrate that pup (a natively unfolded protein), by itself, in unconjugated form, is rapidly degraded by the Myco-20S proteasome. While this is perhaps expected for natively unstructured proteins, this observation raises questions about the biological function of pup. Pup is thought to co-function like ubiquitin and should be conjugated to proteins that need to be selected for proteasomal degradation. However if pup by itself is degraded rapidly, and has the same affinity for MPA in conjugated and unconjugated form, then how could it be used as a degradation tag, if it is always being degraded. In contrast, ubiquitin is not degraded by the proteasome, and its affinity for the proteasome is low, until it is conjugated to a protein as a chain. Then and only then, it becomes a tag for degradation. If pup constantly targets itself for degradation, how can it be found in amounts in the cell sufficient to conjugate to proteins for targeting?

4) The degradation curve for figure 3 does not appear to follow expected decay kinetics, especially for a substrate that has a uM affinity (i.e. the loss of fluorescence is completely linaer for ~95% of the curve, and then sharply levels off). Typically, as substrate concentration decreases, the rate of catalysis gradually decreases. Did the authors try and fit the expected decay curve to this data? The other degradation curves in this MS look normal.

Minor points
1) The authors are hypothesizing that pup has a specific domain for recognizing MPA-CC domain. It would be nice to replace pup with a unstructured arbitrary sequence of equal size to see if the unstructured region was sufficient to catalyze degradation. Alternatively it would be nice to see a replacement of the MPA recognition domain with an arbitrary sequence of similar length. According to the authors' model, such a construct should still be unfolded by MPA, though its affinity might be less (e.g. Does Casein-GFP function?).

2) The authors indicated that during degradation, a fragment of pup with a C-terminal truncation was generated from a pup conjugated substrate, it's not clear why the authors think that the band observed in figure 6 is such. In addition, it is not clear how a C-terminal truncated pup could be generated, since the C-terminus of pup is conjugated to the substrate, and thus is presumably blocked.

1st Revision - authors' response 29 January 2010

We thank the reviewers for their insightful comments on our manuscript.
These are the point-by-point answers:

Referee 1:

- *The length requirement for Pup's unstructured region may be influenced by the in vitro assay, as an open gate variant of the proteasome is used. Presumably this requirement stems from a missing attribute in the in vitro system. This same attribute could change the length requirement for the unstructured region of Pup. These considerations should be addressed in the Discussion section.*

The open-gate proteasome variant is used here, because the recombinantly produced wt proteasome particle exhibits only very low degradation activity, most likely due to poor interaction with the ATPase Mpa. We have added a comment on this in the result and discussion section. However, in agreement with our findings using the open-gate proteasome variant, PanB that is conjugated to the truncated Pup 8N can also not be degraded by the wild type proteasome, even if incubated for extended amounts of time, while in the same time frame PanB-Pup is degraded (see supplementary Figure S9). This indicates that the role of the Pup N-terminus is likely the same for both the open-gate proteasome as well as the wild type proteasome. We agree with the reviewer that an additional factor might be required, perhaps transiently, to allow gate opening. We have added a paragraph to the discussion addressing this issue.

Referee 2:

- *In the middle of page 6, it is explained that the protease system only functioned when the first seven residues of the protease subunits were truncated. Left by itself, this detail is somewhat disconcerting to the reader. I recommend that this facet of the experimental design is discussed briefly.*

We are now commenting on the reasons for the use of the open-gate variant, both in the result section as well as in the discussion. In addition we have added a figure to the supplement (Figure S9), showing the very low degradation activity of the wt proteasome towards the natural substrate PanB-Pup (see also answer to referee 1).

- *At the end of page 9, the manuscript mentions that small amounts of a protein fragment transiently accumulate during the in vitro degradations. The manuscript tentatively assigns this fragment to correspond to the C-terminal half of Pup. It is somehow confusing to contemplate how a sequential degradation mechanism that starts at the N-terminus of Pup and degrades the rest of the substrate produces a C-terminal Pup fragment. I recommend that this is somewhat better explained. However, this is a minor issue and not a serious distraction from the paper.*

We did not mean to imply that Pup is degraded from the C-terminus. We agree with the referee that calling it a C-terminal Pup fragment would suggest that and that it thus is a poor choice of terminology. The cleavage occurs after Tyr58, which appears to be a preferred site. However,

eventually, this fragment is cleaved further, since it disappears later. We have now included ESI-MS data of the degradation of Pup by Mpa and the open-gate proteasome as Supplementary Figure S8.

Referee 3:

- *As noted above, it is a serious concern that the MPA doesn't bind to or function with the WT Myco20S.*

It was shown by negative-stain EM that Mpa does not interact substantially with the wild-type proteasome (Wang et al, 2009). The very N-termini are shorter in the bacterial subunits than those from their archaeal and eukaryotic homologs, leading to a clustering of aromatic and hydrophobic residues (MSFPYFI for Mtb, see Supplementary Figure 10), which might contribute to the difficulties with gate opening. An additional factor might be required, perhaps transiently, to promote the correct conformation of the N-termini. Please see also answers to referees 1 and 2.

- *Deletion of the CC domain from Mpa prevents binding of pup, and thus blocks unfolding and degradation of pub-GFP. However, the authors never demonstrated or mentioned that the deltaCC-Mpa was functional or even hexamerized. In order to conclude that this mutation proves that pup binds to MPA via the CC domain, the authors should verify that the deltaCC-MPA properly hexamerizes and can hydrolyze ATP with the same efficiency as WT Mpa. If deltaCC-Mpa hydrolyzes ATP like WT, and still cannot bind/unfold pup-GFP, then the model presented is acceptable.*

It has been shown previously by size-exclusion chromatography and native-gel electrophoresis, that deletion of the coiled-coil domain does not influence the oligomeric state of Mpa (Zhang et al, 2004; Wang et al, 2009). We have added a statement about this to the result section. We have also added a supplementary figure (Figure S1) showing the ATPase activities of all used Mpa variants. These data demonstrate that deletion of the CC domain does not influence the ATPase activity.

- *The authors demonstrate that pup (a natively unfolded protein), by itself, in unconjugated form, is rapidly degraded by the Myco-20S proteasome. While this is perhaps expected for natively unstructured proteins, this observation raises questions about the biological function of pup. Pup is thought to co-function like ubiquitin and should be conjugated to proteins that need to be selected for proteasomal degradation. However if pup by itself is degraded rapidly, and has the same affinity for MPA in conjugated and unconjugated form, then how could it be used as a degradation tag, if it is always being degraded. In contrast, ubiquitin is not degraded by the proteasome, and its affinity for the proteasome is low, until it is conjugated to a protein as a chain. Then and only then, it becomes a tag for degradation. If pup constantly targets itself for degradation, how can it be found in amounts in the cell sufficient to conjugate to proteins for targeting?*

To address this question, we have carried out a competition experiment, in which Mpa and 7CP are simultaneously incubated with free Pup and PanB-Pup. The results shows that degradation of free Pup starts only after PanB-Pup has been degraded nearly completely (Supplementary Figure S6). This suggests that the affinity of Mpa towards pupylated substrates is likely higher than to free Pup. Furthermore, the relative cellular levels of free Pup versus pupylated substrates will clearly play a role. This is, amongst other factors, also influenced by the affinities of the conjugation machinery towards Pup.

- *The degradation curve for figure 3 does not appear to follow expected decay kinetics, especially for a substrate that has a uM affinity (i.e. the loss of fluorescence is completely linaer for ~95% of the curve, and then sharply levels off). Typically, as substrate concentration decreases, the rate of catalysis gradually decreases. Did the authors try and fit the expected decay curve to this data? The other degradation curves in this MS look normal.*

As the competition experiment between PanB-Pup and free Pup degradation shows, the affinity for pupylated substrates is likely higher. In addition, the curves in Figure 3 represent degradation curves, while the other curves in the manuscript are unfolding curves and are thus carried out in absence of the core particle. In other chaperone-protease systems, like for example ClpAP, the

ATPase alone has a lower affinity for tagged substrates than when in complex with the protease. Consistent with this notion, we show that binding of the proteasome to Mpa stimulates the ATPase activity of Mpa.

- *The authors are hypothesizing that pup has a specific domain for recognizing Mpa-CC domain. It would be nice to replace pup with a unstructured arbitrary sequence of equal size to see if the unstructured region was sufficient to catalyze degradation. Alternatively it would be nice to see a replacement of the Mpa recognition domain with an arbitrary sequence of similar length. According to the authors' model, such a construct should still be unfolded by Mpa, though its affinity might be less (e.g. Does Casein-GFP function?).*

Pup proteins display the highest level of conservation in the C-terminal half of the sequence. We have previously shown by pull-down experiments using Pup-decorated beads, that the CC domain is the site of recognition for Pup on Mpa (Sutter et al, 2009). Using NMR analysis we also recently showed that the central region of Pup, spanning residues 21ñ58 is recognized by the proteasomal chaperone Mpa (Sutter et al, 2009). Our model does not predict that an arbitrary, unfolded region can target proteins for degradation. For the ubiquitin proteasome system it has been shown that two requirements have to be met for degradation to occur: one, polyubiquitin must be recognized at the regulatory particle and second, an unstructured initiation site must be available on the substrate. However, without polyubiquitin, the unstructured initiation site is not sufficient to target the substrate for degradation.

- *The authors indicated that during degradation, a fragment of pup with a C-terminal truncation was generated from a pup conjugated substrate, it's not clear why the authors think that the band observed in figure 6 is such. In addition, it is not clear how a C-terminal truncated pup could be generated, since the C-terminus of pup is conjugated to the substrate, and thus is presumably blocked.*

In supplementary Figure S7, we show that in an anti-Pup western blot, the fragment is visible, demonstrating that it is a Pup-fragment. Calling the fragment a C-terminal fragment was indeed an unfortunate choice of words. We did not mean to suggest that Pup is degraded from the C-terminus. We have now changed the wording and have also added additional mass spectrometric data characterizing the fragment. Please refer also to our answer to referee 2.

2nd Editorial Decision 01 February 2010

Many thanks for submitting the revised version of your manuscript EMBOJ-2009-73115R. I have now had the chance to look through your revision and your point-by-point response carefully. I can see that you have responded well to most of the reviewers' comments, and I do not think it necessary to have your manuscript re-reviewed at this stage. In principle, therefore, I am pleased to be able to tell you that we will be able to accept your manuscript for publication in the EMBO Journal.

However, there is one issue that I would like to ask you to modify first. All three referees raised concerns as to your use of the open-gate proteasome for all your assays. You now show, in Figure S9, that the full-length proteasome does also degrade your model substrate in vitro, albeit very inefficiently. This strikes me as an important result, and I think it deserves a place in the main figures, rather than being relegated to the final supplementary figure and only being mentioned in the discussion. I would therefore suggest that you incorporate the data presented in figure S9 into the main text and figures, perhaps as additional panels in Figure 3. If you feel strongly against this, please let me know and I'm sure we can reach an agreement about how to proceed, but I do think it essential that the data minimally be mentioned in the Results section.

If you are happy to modify the manuscript as I suggest, please could you do so and submit your new files as a formal revision. If you would like to discuss this further, please just get in touch.

2nd Revision - authors' response 02 February 2010

Thank you for your response to our revised manuscript 'The mycobacterial Mpa-proteasome unfolds and degrades pupylated substrates by engaging Pup's N-terminus'.
We have now made the additional changes you suggested. We have included the degradation experiments carried out with the full-length proteasome in Figure 3 of the main text as an additional panel (Fig. 3C), presenting them in the same format as the degradation data with the open-gate proteasome. We decided to also maintain the data with the full-length proteasome in the supplement in their current state to provide the full degradation gels and also the degradation reaction using the truncated Pup variant. The role of the Pup N-terminus is mentioned only in the paragraph following the degradation and it would be out of context to show the degradation with Pup Δ8N already in Figure 3. As both full-length and truncated proteasome are now mentioned in the main text, we labeled the respective alpha subunits as 'prcA' and 'prcAΔ7N'.
We have now also included former Figure 4 (the ATPase bar diagram) in Figure 3 as a fourth panel (Fig. 3D). This reduces the number of figures in the main text by one, but at the same time increases the size of Figure 3. We find that the layout of the figures is more balanced this way.
We have adjusted the figure referencing in the text accordingly and have also changed the method section to include the expression and purification procedure for the full-length proteasome. Furthermore, we adjusted the order of the supplementary figures to match their order of appearance in the main text. We have not made changes to the main text, since the results with full-length proteasome were mentioned in the result section and discussed extensively in the discussion section already in the previous version.

