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Hydrophobicity as a driver of MHC class I antigen processing

Lan Huang, Matthew Kuhls, Laurence Eisenlohr

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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 01 October 2010

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

While referee #1 is not persuaded that the advance and insight provided is sufficient to consider publication in the EMBO Journal, referee #2 and 3 are supportive and find the topic of the analysis of great interest. However all three referees also raise significant concerns and find that further analysis would be needed in order to strengthen the findings reported. Given the support provided by referees #2 and 3, I would like to ask you to submit a revised manuscript should you be able to address the concerns raised in full (including the specific points raised by referee #1). I should remind you that it is EMBO Journal policy to allow a single major round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. 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

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Huang cs studied the role of hydrophobic TM segments in the efficiency of peptide generation for MHC class I. They convincingly show that incorporation of transmembrane sequences in cytosolic exposed proteins results in somewhat faster presentation when the antigen is introduced by vaccinia virus.

However, the differences between transmembrane containing versus control proteins are usually small and such differences are difficult to consider in the absence of any statistical information

I have major problems with the data. No SD is shown for any of the experiments and how is an MFI from a Gaussian curve really defined; does this really allow accurate determination of differences of -say- 3 and 4? If the wideness of the flow signal is considered as an SD, are the differences then still significant? These are important though not discussed/incorporated points.

The authors also assume that the various constructs are comparable since they are still recognized by specific antibodies and similarly sensitive to proteases. Fluoresecence microscopy for detection could be important to exclude that a different fraction of the various molecules escapes detection by forming aggregates.

The authors argue that these finding may be helpful in vaccine design (last sentence Abstract). However, one could argue that the rate of production of peptides is not too relevant but merely the steady state levels (those exposed around two times the MHC class I Kb half-life). Such data are not provided. Still these are relevant as it may show differences as well which would indicate that one of the various protein constructs made yield more/less efficient peptides.

Figure 3. Why is the expressed peptide somewhat sensitive to epoxomycin and still presented by RMA-S cells?

In conclusion: this manuscript adds another signal to the process of antigen processing which may be of interest (when all problems mentioned above are solved) for immunologists but not for a broader scientific audience.

Referee #2 (Remarks to the Author):

It is becoming increasingly clear that most class I peptide ligands derive from defective forms of proteins. What are these defective forms, and how do different defective forms differ in accessing the class I pathway? Little is know about these questions, which are important for both antigen presentation and also the general issue of protein degradation. Huang et al. provide the first report that the presence of unshielded hydrophobic domains favor the rapid degradation of proteins and also give the substrate good access to the processing pathway.
I have a number of comments for the authors to consider.
General comments.
Philosophical comment: There is no way to unambiguously know whether a degraded protein was or wasn't truly defective. This may be uncomfortably intellectually, but it is nonetheless true. By what criteria can perfection be assured (defectiveness, of course is routinely achieved with antibodies or proteases, as so nicely documented in this paper).? The authors might consider this, and not just dismiss it as semantics (not that they do in the paper, and I may be unfairly anticipating their response).
Technical: The study hinges on the use of 25D1-1.16 to measure cell surface expression of Kb-SIINFEKL complexes. It would be much better to use Alexa 647 conjugated 25D1-1.16 this would assure a linear relationship between the flow signal and the number of complexes. Further, why are cells fixed after staining....doesn't this reduce the S/N, which is already low? Also makes gating dead cells difficult, which can contribute to background staining. Further, the data for the most part

show a singe time point post-infection: why not show 20 or 30 min intervals? This would increase confidence in the conclusions, which are based on perilously small differences (but fine if they are reproducible).
Which brings up another important concern: there is considerable assay to assay variation in gene expression from different VV stocks. For this reason, it is best to ratio 25D1-1.16 staining to VV gene expression, which can even be done on a per cell level by flow cytometry software. In a perfect world, the authors could express the various proteins with GFP (or other color), but this has its own problems, particularly for this study, where differences in GFP folding negate its use. There is no reason, however, for the authors not to ratio 25D1-1.16 staining to staining with another VV-protein (e.g. B5R). This is critical for experiments in which the differences in 25D1-1.16 staining are small.
Paper is wonderfully written for the most part, but it would be easier on the reader to put the essential details of the experiments in the Results section itself: e.g.. time of infection, time of drug addition, what detergents were used.
Specific comments:
Figure 1B. (and elsewhere). Using the term half-life, implies first order degradation kinetics, which is tested by linear regression of a semi-log plot between amount remaining (log) vs. time. This should be done for all of the degradation curves.
Figure 2B. No reason not to make a small plot the degradation data.
Figure 3: Serious problem. How are RMA or RMA/S cells expressing VV proteins? This is really surprising, since these cells, typical for mouse B and T cell lines, are perversely resistant to VV infection (EL4 cells, the true identity of RMA cells aren't infected either). Do the authors have a subclone that behaves differently?.
Figure 5B: the difference in degradation kinetics between delta 6 and delta 13 seems negligible...is it really reproducible?
Degradation rate of cytoTac (N-TM)?
Figure 7: Panel A Con A experiment is cool-good thinking.
Panel C. what's going on with TAC chase...loss of protein? For the immunoblots, how do we know what fraction of proteins are extracted with NP40? Why not extract with boiling sample buffer to maximize recovery and minimize proteolysis. Speaking of which, where are the ubiquitylated species rescued by the inclusion of NEM in the extraction buffer?
Why is so much HA endo H sensitive? Most should be endo H resistant in an immunoblot. Was endo F used? Otherwise, something is not right. Important point: The experiment with HA to confirm the TAC hydrophobic domain results has not been performed: the valid comparison is between cytoHA and cytoHA TM-, not HA and HA TM:
Disucssion:
How could rapid degradation increase the supply of amino acids: it's just wasting energy to make proteins to recover the amino acids. An organism cannot grow by eating itself!
It might be useful for the authors to point out that in mammalian cell (unlike yeast, where Varshavky established the rule) , the N-end rule is not generally functional, i.e. in many contexts proteins are not rapidly degraded, even with Arg at the N-terminus. Townsend was very lucky in using NP to extend Varshavsky results, the N-terminus of NP is one of a few proteins in mammalian cells known to work (see papers from Eric Long and Bob Siliciano on this exact issue).
The authors might consider comparing their interpretation of DRiPs with the immunoribosome update to the DRiP hypothesis. Also, it would be fair to point out that the DRiP hypothesis does not stipulate that peptides must be generated with super rapid kinetics. For membrane proteins (like TAC) with no known pathway back to the cytosol once departed from the ER, peptides almost have to derive from DRiPs that come from TAC that never made it out of the ER to the Golgi complex, or into the ER in the first place (indeed, they must refer to Schlosser et al's work on ER mistargeting and antigen presentaiton: EMBO Rep 8, 945.)

Referee #3 (Remarks to the Author):

Huang et al
In this manuscript, the authors explore propose that an exposed hydrophobic transmembrane domain may be a driver for rapid protein degradation and thus a mechanism for rapid supply of peptides for presentation by MHC class I molecules. They utilise mutants of the Il-2 receptor alpha subunit (tac), which are targeted to the cytosol and rendered unstable due to ablation of the signal sequence, as model substrates to determine the influence of the transmembrane domain on rate of degradation and peptide presentation.

The principal finding is that the transmembrane domain of cytosolic Tac appears to be a potent degradation motif. CytoTac is stabilised by deletion of the transmembrane domain, the rate of degradation is proportional to the length of the transmembrane domain and transfer of the cytoTac transmembrane domain to influenza nucleoprotein NP13-498 induces rapid degradation.

This work is of interest and exploration of the effect of an exposed transmembrane domain on protein degradation and supply of peptides to MHC I is novel. However, the data presented for the effect of the transmembrane domain on the degradation of ER localised proteins, the more physiologically relevant scenario, are not quite as convincing:-

Specific Points

Fig 4 might be easier to follow if the y axis was on the same scale - so that the difference between cytotac and cytotac(DeltaTM) were visually more obvious. Again from this figure, they don't really comment on the fact that the degree of presentation they get from the Tac and Tac(delta TM) (C and D) is much less than what they see in A and B.
Fig 7 - this figure is less convincing than some of the others, in particular Fig 7C would benefit from some clarifications. They show that on withdrawal of MG-132 the cytosolic deglycosylated Tac-TM appears to be more stable than the deglycosylated wild-type Tac but they don't show any other data e.g. pulse-chase on the overall t1/2 of Tac TM vs wild-type Tac. In a recent JCB paper (J Cell Biol. 2010 January 25; 188(2): 223-235), the TM versions of CD3 and -secretase were both significantly less stable than wild-type. If the Tac TM was more unstable and continued to be dislocated more quickly than the wild-type Tac then this might make it appear more stable in the cytosol in their experiment? - this would also depend on how much faster the rate of proteasomal degradation is versus the rate of dislocation?
It would be helpful if this could be clarified and perhaps shown in a more convincing way ?metabolic labelling and pulse chase analysis
Figure 7D/E: The difference between the amount of deglycosylated influenza haemaglutinin (HA) versus deglycosylated HA TM in the presence of MG-132 is difficult to clearly visualise on the immunoblot shown. A time-course demonstrating increased stability of deglycosylated HA TM would considerably strengthen the evidence presented. The inclusion of molecular weight markers in figure 7 would also be helpful. Again, this figure might be clarified by pulse chase analysis?

1st Revision - authors' response 07 January 2011

We thank the reviewers for their thorough reading of our manuscript and valuable suggestions. Changes to the text are indicated with yellow highlight. Exceptions to this are the supplementary figures which were so altered as to make highlighting of minimal value. Following are responses to each of the concerns.

Referee #1 (Remarks to the Author):

Huang cs studied the role of hydrophobic TM segments in the efficiency of peptide generation for MHC class I. They convincingly show that incorporation of transmembrane sequences in cytosolic exposed proteins results in somewhat faster presentation when the antigen is introduced by vaccinia virus.

However, the differences between transmembrane containing versus control proteins are usually small and such differences are difficult to consider in the absence of any statistical information

Re: We appreciate the need for statistical information on the various comparisons made throughout the manuscript. In the original submission, statistical information was provided for several key findings (Figs. 1B, 4C-D, and 5C, see Methods and Figure legends). In all other cases, we provided representative experiments that were reproducibly repeated. In the revised manuscript, we have now

provided statistical analysis for representative data (Figs. 1C-D, 5B-C, new 5E-F, 6B-D, 7C, new 7E) according to the recommendations of a statistician. As for the magnitude of the effect, we would argue that it is actually substantial in many cases. For example, in Figure 1C, we show that the potency of the TM domain substantially enhances peptide supply above that for the control construct. The perception is that differences are small because all of the constructs are subjected to some degree of basal processing (discussed at the outset of the kinetics section within the results) that clearly contributes an appreciable amount of peptide. Thus, we are never comparing to a value of zero and, consequently, the range is limited.

I have major problems with the data. No SD is shown for any of the experiments and how is an MFI from a Gaussian curve really defined; does this really allow accurate determination of differences of -say- 3 and 4? If the wideness of the flow signal is considered as an SD, are the differences then still significant? These are important though not discussed/incorporated points.

Re: Vaccinia virus efficiently infects the target cells (L-Kb, MC57G). Thus, the MFI is derived from essentially the entire sample (live gate > 95%) in a 5-hr infection/presentation assay. For the sake of transparency, we now include the raw flow data in one main figure (Fig. 3) and several supplemental figures (new supplemental Figs. 1-3) to demonstrate how MFI is derived. Although the differences of raw flow signals (or MFI) are highly reproducible from multiple repeats, we agree with the reviewer's concern that SD and statistical analysis are important for accurate assessment of differences. Therefore, we determined SD from single experiments with triplicate samples or from multiple repeats that were normalized to the control and expressed as "fold change". Differences between constructs have been determined by statistical analyses in the revised manuscript (Figs. 1, 4-7). Our conclusions still hold under these stringent tests.

The authors also assume that the various constructs are comparable since they are still recognized by specific antibodies and similarly sensitive to proteases. Fluoresecence microscopy for detection could be important to exclude that a different fraction of the various molecules escapes detection by forming aggregates.

Re: In the revised manuscript, we have provided confocal immunofluorescent images as a supplemental figure to exclude aggregate formation by various constructs (new supplemental Fig. 4). While cytoTac (TM) is diffusely distributed throughout the cell, cytoTac appears to localize to the ER membrane. This is not unexpected since it has been reported that access to the cytosolic face of the ER is required for the degradation stimulated by hydrophobicity (Metzger, MB, et al. JBC, 2008, 283: 32302-32316).

The authors argue that these finding may be helpful in vaccine design (last sentence Abstract). However, one could argue that the rate of production of peptides is not too relevant but merely the steady state levels (those exposed around two times the MHC class I Kb half-life). Such data are not provided. Still these are relevant as it may show differences as well which would indicate that one of the various protein constructs made yield more/less efficient peptides.

Re: The reviewer has raised an important question with respect to vaccine relevance. To address this, we examined class I peptide presentation after extended vaccinia virus infection (24-h vs. 5-h). The pattern of class I peptide supply holds at this time point as well (new supplemental Fig. 1C). This is consistent with a report that incorporation of an N-degron improves performance of a tumor vaccine through enhancement of class I peptide supply (Tobery T, et al. JI, 1999, 162: 639-642). Therefore, the novel degradation signal we identified in this manuscript is expected to have a meaningful impact upon CD8 T cell responses in vivo. This argument is clarified in the discussion section of the revised manuscript.

Figure 3. Why is the expressed peptide somewhat sensitive to epoxomycin and still presented by RMA-S cells?

Re: The legend for Fig. 3 has been clarified. The synthetic SIINFEKL peptide (pOVA257-264, not

SIINFEKL-expressing virus mOVA257-264) was used as a positive control in RMA-S cells. This peptide binds directly to surface MHC class I molecules and does not require intracellular processing. It is therefore presentable by TAP-deficient RMA-S cells.

With respect to epoxomicin treatment, a vaccinia virus expressing the mini-gene SIINFEKL, mOVA257, (not synthetic peptide, pOVA257-264) was used as a positive control. Proteasomal processing of this gene product is not required for presentation. The minor sensitivity to epoxomycin was likely due to the toxicity of epoxomicin used at a high dose (10 uM) and extremely high expression of minigene SIINFEKL. In the revised manuscript we employed epoxomicin at a lower dose (3 uM) and utilized a vaccinia virus that expresses the SIINFEKL-encoding mini-gene at a much lower level via a 5' UTR hairpin technique (m/S-HP18) (new Fig. 3A). The results showed that presentation of three Tac-based constructs is completely proteasome-dependent. To avoid this kind of confusion, in the revised manuscript we utilized biosynthesized constructs for both the positive and negative controls. Thus, in the revised figure, the positive control is ss-m/S, a biosynthesized peptide that is targeted to the ER independent of TAP function (via signal sequence-mediated translocation). In response to a concern by Reviewer 2, we added TAP-deficient B6 fibroblast cells as target cells to the analysis (new Fig. 3B). These cells also consistently demonstrated that presentation of three Tac-based constructs is TAP-dependent.

In conclusion: this manuscript adds another signal to the process of antigen processing which may be of interest (when all problems mentioned above are solved) for immunologists but not for a broader scientific audience.

Re: We submitted our paper to EMBO specifically because we thought many of the conclusions would be of interested to a broader audience. Our report is the first to identify a property other than an N-degron that can drive rapid peptide supply. It also points to the substantial delay in the processing of ER-targeted substrates, an observation that is at odds with the conclusion that all peptides are generated from very short-lived proteins. Furthermore, it reveals important themes in fundamental cell biology, e.g. intracellular proteolysis for both cytosolic and ER-targeted proteins via exposure of a transmembrane domain, and brings into focus the basic force that exposed hydrophobicity provides for intracellular proteolysis.

Referee #2 (Remarks to the Author):

It is becoming increasingly clear that most class I peptide ligands derive from defective forms of proteins. What are these defective forms, and how do different defective forms differ in accessing the class I pathway? Little is know about these questions, which are important for both antigen presentation and also the general issue of protein degradation. Huang et al. provide the first report that the presence of unshielded hydrophobic domains favor the rapid degradation of proteins and also give the substrate good access to the processing pathway.

I have a number of comments for the authors to consider.
General comments.

Philosophical comment: There is no way to unambiguously know whether a degraded protein was or wasn't truly defective. This may be uncomfortably intellectually, but it is nonetheless true. By what criteria can perfection be assured (defectiveness, of course is routinely achieved with antibodies or proteases, as so nicely documented in this paper).? The authors might consider this, and not just dismiss it as semantics (not that they do in the paper, and I may be unfairly anticipating their response).

Re: We couldn't agree more with the reviewer's assessment of this knotty problem. Our contention is that, while defectiveness cannot be discounted as a driving force for rapid peptide supply, one can discount the conventional kinds of defectiveness that entail quality control decision making and relatively slow degradation. We have modified the discussion to clarify this position.

Technical: The study hinges on the use of 25D1-1.16 to measure cell surface expression of Kb-SIINFEKL complexes. It would be much better to use Alexa 647 conjugated 25D1-1.16 this would assure a linear relationship between the flow signal and the number of complexes.

Re: We appreciate this excellent suggestion. In the revised manuscript, we used Alexa 647 conjugated 25D1-1.16 to confirm our key observations and provide representative data in new Supplemental Fig. 2.

Further, why are cells fixed after staining....doesn't this reduce the S/N, which is already low? Also makes gating dead cells difficult, which can contribute to background staining.

Re: VV-infected cells are fixed after staining, as mandated by our Institutional Biosafety Committee, in order to prevent direct contact with the virus or infected cells. Fixation does reduce the fluorescent signal to some extent but not enough to confound statistical analyses of the results. Furthermore, dead cells are a minimal concern in the 5-h infection assays that were performed.

Further, the data for the most part show a singe time point post-infection: why not show 20 or 30 min intervals? This would increase confidence in the conclusions, which are based on perilously small differences (but fine if they are reproducible).

Re: We thank the reviewer for this helpful suggestion. In the revised manuscript, we have added a time-course presentation assay (30-min intervals) in new Supplementary Fig. 1B which confirms our key finding.

Which brings up another important concern: there is considerable assay to assay variation in gene expression from different VV stocks. For this reason, it is best to ratio 25D1-1.16 staining to VV gene expression, which can even be done on a per cell level by flow cytometry software. In a perfect world, the authors could express the various proteins with GFP (or other color), but this has its own problems, particularly for this study, where differences in GFP folding negate its use. There is no reason, however, for the authors not to ratio 25D1-1.16 staining to staining with another VV-protein (e.g. B5R). This is critical for experiments in which the differences in 25D1-1.16 staining are small.

Re: The reviewer raises a critical concern that we were certainly aware of. In the initial submission, we briefly addressed this issue in Supplementary Fig. 1 A-B, in which virus titration was further confirmed by anti-VV (B5R) staining. In the revised manuscript, we addressed this concern more thoroughly. First, we stained the same virus-infected samples with either 25D1.16 Ab or anti-B5R Ab (new Supplemental Fig. 1A). The results showed that the three Tac-based constructs showed very similar levels of B5R staining but significantly different levels of Kb/SIINFEKL on cell surface. Second, we followed the reviewer's suggestion that we carry out the analysis by flow cytometry (new Supplemental Fig. 2). We gated co-stained cells for the same level of B5R expression and then analyzed levels of Kb/SIINFEKL complexes. This approach also confirmed that differences in 25D1.16 staining are not attributable to differences in virus loading (as indicated by anti-B5R staining).

Paper is wonderfully written for the most part, but it would be easier on the reader to put the essential details of the experiments in the Results section itself: e.g.. time of infection, time of drug addition, what detergents were used.

Re: We have added these details to the Results section in the revised manuscript.

Specific comments:
Figure 1B. (and elsewhere). Using the term half-life, implies first order degradation kinetics, which is tested by linear regression of a semi-log plot between amount remaining (log) vs. time. This should be done for all of the degradation curves.

Re: We have made this suggested change to the manuscript (Figs. 1B, 2B, 5B, 5E, 6B, 6D, 7C, 7E).

Figure 2B. No reason not to make a small plot the degradation data.

Re: This plot has been added to the revised manuscript (Fig. 2B).

Figure 3: Serious problem. How are RMA or RMA/S cells expressing VV proteins? This is really surprising, since these cells, typical for mouse B and T cell lines, are perversely resistant to VV

infection (EL4 cells, the true identity of RMA cells aren't infected either). Do the authors have a subclone that behaves differently?.

Re: We neglected to address this important detail in the initial submission. We did observe that RMA cells are highly resistant to VV infection, but not perversely so. A small fraction (5-10%) of RMA cells is infected, as indicated by SIINFEKL presentation following infection with recombinant viruses. Thus, we gated on this population for flow analysis. In the revised manuscript, we have provided the raw flow data and the calculated MFI (Supplemental Fig. 3 A-B). To avoid any concerns about analyses of minor populations, we repeated the experiments with TAP-/- B6 fibroblast cells, which are efficiently infected by VV. The results of these experiments, which are quite similar to those obtained with RMA and RMA-S cells, appear in the main section of the revised manuscript (Fig. 3B). The RMA/RMA-S data results have been moved to Supplemental Fig. 3.

Figure 5B: the difference in degradation kinetics between delta 6 and delta 13 seems negligible...is it really reproducible? Degradation rate of cytoTac (N-TM)?

Re: Although the differences among the truncated TM constructs are reproducible in three independent assays, we did not perform the assay a sufficient number of times to achieve statistical significance between delta 6 and delta 13, and the same applies to the difference between delta 13 and delta TM. Neither is critical to our point. We have added the statistical information to the revised manuscript (Fig. 5B).

We did not show the degradation data of cytoTac (N-TM) in the initial submission due to inclusion of these data in another manuscript. In the revised manuscript, we add the degradation data of cytoTac (N-TM) from an unreported experiment in Fig. 5D-E.

Figure 7: Panel A Con A experiment is cool-good thinking.

Re: Thank you, Reviewer 2.

Panel C. what's going on with TAC chase...loss of protein? For the immunoblots, how do we know what fraction of proteins are extracted with NP40? Why not extract with boiling sample buffer to maximize recovery and minimize proteolysis. Speaking of which, where are the ubiquitylated species rescued by the inclusion of NEM in the extraction buffer?

Re: We were not sufficiently clear about this part of the work. Panel C specifically tracks the fate of the retrotranslocated/deglycosylated Tac species. The cohort of Tac protein that passes quality control (subsequently trafficking to the cell surface) was not depicted in the original manuscript. We have altered the wording to clarify this point and have also included an experiment that tracks the stability of mature Tac (Supplemental Figure 9). This cohort is very stable under the experimental conditions that were employed.

We appreciate the reviewer's concern, that the disappearance of the ERAD species is due to partitioning into a detergent-insoluble fraction rather than proteolysis (a concern also expressed by Reviewer 1). However, the stabilization of the Tac ERAD substrate by proteasome inhibitor (Figure 7, panel A) argues against this as do the confocal images that we have provided in the revision (Supplementary Fig. 4) We have modified the text to emphasize this point.

The ubiquitinated species are not visible in the panels that comprise Figure 7. To alleviate the reviewer's concern, we immunoprecipitated the model antigens with anti-HA Ab, and then blotting with anti-Ub Ab (FK1 clone, specific for polyubiquitinated proteins). Supplemental Fig. 10 confirms that polyubiquitinated species become apparent with MG132 treatment.

Why is so much HA endo H sensitive? Most should be endo H resistant in an immunoblot. Was endo F used? Otherwise, something is not right. Important point: The experiment with HA to confirm the TAC hydrophobic domain results has not been performed: the valid comparison is between cytoHA and cytoHA TM-, not HA and HA TM:

Re: We were also surprised by the endo H sensitivity of HA (moved to new Supplemental Fig. 11B).

Endo H (not endo F) was used in the experiments. To further validate our results, we first performed the assay with flu-expressed HA, which demonstrated partial endo H resistance (data not shown). Next, we confirmed surface HA expression after 6-h virus infection by flow cytometry using anti-HA (H18E23) Ab (Supplemental Fig. 11A). A fully glycosylated HA band resistant to Endo H digestion was barely detected at 6-h post infection but became a major species at 18-h post infection (Supplemental Fig. 11C). Our speculation is that maturation of HA is different at different phases of VV infection.

The purpose of the HA experiment was to confirm the proteasome-dependent degradation of ERAD substrates (HA vs. HA TM-) in order to strengthen our observation with the ER-targeted Tac-based proteins. We do not have cytoHA TM- virus on the hand for this comparison in any event. We confirmed the TM effect on cytosolic proteins with the NP-based experiments in Figure 6.

Disucssion:
How could rapid degradation increase the supply of amino acids: it's just wasting energy to make proteins to recover the amino acids. An organism cannot grow by eating itself!

Re: This discussion point was not made with sufficient clarity. We were simply reiterating Wheatley's speculation that degrading a fraction of nascent protein ensures a ready supply of amino acids so that proteins can be rapidly produced in response to environmental changes (see references in the text). We did not mean to imply that this increases the supply of amino acids. Wording has been changed to clarify this point.

It might be useful for the authors to point out that in mammalian cell (unlike yeast, where Varshavky established the rule) , the N-end rule is not generally functional, i.e. in many contexts proteins are not rapidly degraded, even with Arg at the N-terminus. Townsend was very lucky in using NP to extend Varshavsky results, the N-terminus of NP is one of a few proteins in mammalian cells known to work (see papers from Eric Long and Bob Siliciano on this exact issue).

The authors might consider comparing their interpretation of DRiPs with the immunoribosome update to the DRiP hypothesis. Also, it would be fair to point out that the DRiP hypothesis does not stipulate that peptides must be generated with super rapid kinetics. For membrane proteins (like TAC) with no known pathway back to the cytosol once departed from the ER, peptides almost have to derive from DRiPs that come from TAC that never made it out of the ER to the Golgi complex, or into the ER in the first place (indeed, they must refer to Schlosser et al's work on ER mistargeting and antigen presentaiton: EMBO Rep 8, 945.)

Re: Responses to these valuable suggestions have been incorporated into the revised manuscript.

Referee #3 (Remarks to the Author):

Huang et al
In this manuscript, the authors explore propose that an exposed hydrophobic transmembrane domain may be a driver for rapid protein degradation and thus a mechanism for rapid supply of peptides for presentation by MHC class I molecules. They utilise mutants of the Il-2 receptor alpha subunit (tac), which are targeted to the cytosol and rendered unstable due to ablation of the signal sequence, as model substrates to determine the influence of the transmembrane domain on rate of degradation and peptide presentation.

The principal finding is that the transmembrane domain of cytosolic Tac appears to be a potent degradation motif. CytoTac is stabilised by deletion of the transmembrane domain, the rate of degradation is proportional to the length of the transmembrane domain and transfer of the cytoTac transmembrane domain to influenza nucleoprotein NP13-498 induces rapid degradation.

This work is of interest and exploration of the effect of an exposed transmembrane domain on protein degradation and supply of peptides to MHC I is novel. However, the data presented for the effect of the transmembrane domain on the degradation of ER localised proteins, the more physiologically relevant scenario, are not quite as convincing:-

Specific Points

Fig 4 might be easier to follow if the y axis was on the same scale ñ so that the difference between cytotac and cytotac(DeltaTM) were visually more obvious. Again from this figure, they don't really comment on the fact that the degree of presentation they get from the Tac and Tac(delta TM) (C and D) is much less than what they see in A and B.

Re: To show the kinetic trends more obviously, we converted the y axis to the scale of fold change compared to the MFI at PSI treatment (3 h post infection) in the revised manuscript

Although the major purpose of Fig 4 is to compare the difference of presentation kinetics (not presentation levels), we do agree that there is value in discussing the degree of presentation from different constructs. We have now added text to the Results section that addressed this issue and provides a potential explanation (the comparatively flattened and extended kinetics of peptide production of ER-targeted proteins).

Fig 7 - this figure is less convincing than some of the others, in particular Fig 7C would benefit from some clarifications. They show that on withdrawal of MG-132 the cytosolic deglycosylated Tac- TM appears to be more stable than the deglycosylated wild-type Tac but they don't show any other data e.g. pulse-chase on the overall t1/2 of Tac TM vs wild-type Tac. In a recent JCB paper (J Cell Biol. 2010 January 25; 188(2): 223-235), the TM versions of CD3 and -secretase were both significantly less stable than wild-type. If the Tac TM was more unstable and continued to be dislocated more quickly than the wild-type Tac then this might make it appear more stable in the cytosol in their experiment? - this would also depend on how much faster the rate of proteasomal degradation is versus the rate of dislocation? It would be helpful if this could be clarified and perhaps shown in a more convincing way ?metabolic labelling and pulse chase analysis

Re: Both Reviewers 2 and 3 were concerned about the fates of the mature, non-ERAD species of the Tac constructs. In the revised manuscript, we report on the stabilities of mature wild-type Tac and Tac TM in a protein synthesis inhibitor (PSI)-chase assay following MG132 withdrawal (new Supplemental Fig. 9). As expected, the glycosylated proteins are very stable during the 60-min chase.

We have gone to great lengths to strengthen the ERAD/TM part of the work. "Pulse/chase" analyses of Tac and HA, and their TM counterparts have now been carried out for several time points and error analyses have been applied. Because the ERAD species in both cases are in trace amounts, we have been unable to utilize metabolic labeling methods, which are less sensitive for these experiments than the western blot approach that we used. This is now substantiated in Supplemental Figure 7. Our conclusions about the influence of the TM domain on degradation rate post-retrotranslocation remain the same.

We agree with Reviewer 3 that commentary on the recent JCB paper by Bernasconi et al. is important. In that paper, the TM versions of BACE476 and CD3 were both less stable than their wild-type counterparts. We have added a section to the Discussion that cites the Bernasconi paper and provides several potential explanations for the apparent disparity. In brief, we do not believe that this is due to the fact that we looked only at the cytosolic phase of ERAD because the kinetics of peptide production from Tac and Tac (TM) suggest that the overall degradation rate of Tac (as governed by all phases of ERAD) is faster. Rather, we point to the fact that the BACE476 and CD3 are homogeneously defective and may not represent most of the defective variants of Tac. We also speculate that the high expression of a 100% ERAD substrate may put different stresses on the ERAD system than our system, thereby shifting the rate limiting step.

Figure 7D/E: The difference between the amount of deglycosylated influenza haemaglutinin (HA) versus deglycosylated HA TM in the presence of MG-132 is difficult to clearly visualise on the immunoblot shown. A time-course demonstrating increased stability of deglycosylated HA TM would considerably strengthen the evidence presented. The inclusion of molecular weight markers in figure 7 would also be helpful. Again, this figure might be clarified by pulse chase analysis?

Re: We concur with this concern. Due to detection issues already discussed, we did not compare the

amount of deglycosylated HA versus deglycosylated HA TM directly. Rather, we compared the deglycosylated fraction in the absence or presence of proteasome inhibitor (Fig. 7D). This method demonstrated a clear increase of deglycosylated species in the HA sample but not in HA TM sample, suggesting the ERAD substrates from HA sample are subjected to more rapid proteasomal degradation.

In response to the reviewer's suggestion, we carried out a time-course of HA ERAD. This required systematic optimization of MG132 withdrawal and anti-S1 Ab immunoblotting. The eventual experiments consistently demonstrated, with statistical significance, that the deglycosylated HA fraction is more unstable than the deglycosylated HA TM fraction. This can be seen in updated Fig. 7E in the revised manuscript.

We have added molecular weight markers in the revised manuscript (Fig. 7D and other figures).

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Thank you for submitting your revised manuscript to the EMBO Journal. I asked the original referee #1 and 3 to review the revised manuscript and I have now received their comments back. As you can see below, both referees appreciate the introduced changes.

I am therefore pleased to proceed with the acceptance of the paper for publication here. You will receive the formal acceptance letter shortly

Best wishes

Editor
The EMBO Journal

Referee #1

The authors have done an exemplary job of positively responding to the reviewers' suggestions for strengthening the manuscript.

Referee #3

I am satisfied with the efforts the authors have made to respond to all the criticisms of the 3 reviewers and would now recommend publication.

