Structural features within the nascent chain regulate alternative targeting of secretory proteins to mitochondria

Natalie V Pfeiffer, Daniela Dirndorfer, Sven Lang, Ulrike K Resenberger, Lisa M Restelli, Charles Hémion, Margit Miesbauer, Stephan Frank, Albert Neutzner, Richard Zimmermann, Konstanze F Winklhofer and Jörg Tatzelt

Corresponding author: Joerg Tatzelt, Ludwig-Maximilians-University Munich

Review timeline:

- Submission date: 31 August 2012
- Editorial Decision: 08 October 2012
- Revision received: 21 December 2012
- Editorial Decision: 21 January 2013
- Revision received: 31 January 2013
- Accepted: 01 February 2013

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.)

Editors: Isabel Arnold

1st Editorial Decision 08 October 2012

Thank you for submitting your manuscript for consideration by The EMBO Journal. Three referees have now evaluated it, and their comments are shown below. Given the heterogeneous picture with respect to requirements for the revision resulting from these reports, I have now also had a chance to discuss the case further internally and to consult with an external editorial advisor. We would now like to invite you to submit a revised version of the manuscript that addresses the referees' concerns along the lines detailed below.

We all agree with referees 1 and 2 that deeper analysis of the structural determinants as well as the mitochondrial sublocalisation along the lines suggested will be key to strengthen the study sufficiently and will be required. I should point out that referee 2 refers to 'uncleavable' ER signal peptides, which, we think, is probably a misunderstanding, but does not affect the other points he/she raises. While we do not think that deeper mechanistic analysis with respect to the events occurring at the ER would necessarily increase the significance and impact of the study, we would still agree with referee 2 that looking into the physiological or (patho-)physiological significance of alternative targeting of certain endogenous proteins within the cell would clearly increase the importance of this study. We would like to encourage you to strengthen the study in this direction, however, we will not insist on such additional data.

I should add that it is EMBO Journal policy to allow only a single round of revision and that acceptance of your manuscript will therefore depend on the completeness of your responses in this
revised version. When preparing your letter of response to the referees’ comments, please bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

REFEREE REPORTS

Referee #1

While previously thought to be exceptional, protein targeting to more than one compartment is now recognized to be a quite common occurrence. The manuscript by Pfeiffer et al. adds a new twist to the problem of how newly synthesized proteins choose between the ER and mitochondria: they show that the some ER signal peptides have the unanticipated ability to target proteins to mitochondria, and that the mature part of the nascent polypeptide chain, by influencing translocation efficiency across the ER membrane, may determine whether a polypeptide is diverted to mitochondria by an ambivalent signal peptide. This is an interesting finding, and well suited to the interests of the EMBO J readers. However, there are two gaps in the story, which must be filled before the manuscript is suitable for publication.

1. In previous work (Miesbauer et al., 2009), the group reported that translocation into the ER is adversely affected by the intrinsically disordered portion of the mature sequence of Prion Protein (PrP), and positively affected by the alpha-helical domains, including the C-terminal GPI addition signal sequence, which is predicted to be alpha helical. Here, they extend this observation, by testing the effect of alpha helical domains on the translocation of two other intrinsically disordered proteins: shadoo and somatostatin. While they interpret their results to lend further support to the concept that alpha helical domains in mature portions of the polypeptide facilitate translocation, other interpretations are possible. In the case of shadoo, they clearly demonstrate that the C-terminal GPI addition sequence favours ER translocation, but can we be sure that this is because of its propensity to form an alpha-helix? The results of this study suggest that the GPI signal sequence is MORE efficient than other investigated predicted alpha-helical domains. So, what are the features of this sequence that make it the most efficient translocation inducer? In the case of prosomatostatin, a comparison is made between two constructs (both containing the protein's N-terminal targeting sequence), one containing only the pro domain (predicted to be alpha helical) with the C-terminal, intrinsically disordered, hormone domain deleted, and the other one composed only of the hormone domain. The first of these is translocated, while the second one is not. However, these two constructs are not really comparable, as the first one has 108 residues vs 17 residues the second one (not including the N-terminal signal sequence). Because of the short length of the second construct (SomDeltaPro), the signal sequence will be poorly available to co-translational targeting, and this could strongly affect the subsequent translocation event.

2. The authors find that under conditions of reduced translocation (either because of features of the mature part of the nascent chain or because of reduced translocation capacity of the ER) the ER signal peptides of shadoo, prosomatostatin, and APP are all three capable of redirecting a polypeptide to mitochondria. This is interesting, but a deficiency of the paper is the lack of characterization of the submitochondrial localization of the construct. The authors present one blot concerning only one of the constructs as evidence that they are translocated at least across the outer mitochondrial membrane. This conclusion is based on the resistance of the construct APP.115alpha2alpha3 to proteolysis in isolated intact mitochondria and on its sensitivity in the presence of detergent. This experiment does not rule out the possibility that the construct is
aggregated and therefore protease-resistant and that detergent, by disrupting protein-protein interactions, allows better access to the protease. My suspicion is that the investigated constructs are on the outer mitochondrial membrane exposed to the cytosol, with the ER signal peptide functioning as a signal anchor. This would be interesting as well, but a far shot from concluding that an ER signal peptide is functioning as a mitochondrial import signal. It is known that the features that distinguish N-terminal ER targeting sequences from outer mitochondrial membrane signal anchors are quite subtle (e.g., Kanaji S et al., 2000, JCB 151:277). Therefore, an interpretation of the mechanism of the observed mitochondrial targeting by the investigated signal peptides depends critically on a rigorous definition of the submitochondrial localization and membrane topology of the constructs.

In conclusion, it is my opinion that before publication the study must be improved, to: (i) present more evidence, possibly with synthetic sequences, on the role of alpha-helical sequences in translocation, or, at the least, recognize that the remarkable activity of the GPI attachment signal may depend on features other than its alpha-helicity; for the somatostatin work, constructs of similar length must be compared; and (ii) investigate more in depth the submitochondrial localization of representative mitochondria-targeted constructs (more than one), for instance by antibody accessibility in semi-permeabilized cells.

Referee #2

It is generally believed that secretory proteins are targeted to and imported into the ER in a cotranslational manner. Interestingly, Pleifffer et al demonstrated in this paper that uncleavable ER signal peptides (ER-SP) of Shadoo (Sho), Somatostatin (Som) and App have the property to mediate alternative targeting to mitochondria; they target nascent proteins with α-helical domains or with the C-terminal GPI-anchor signal correctly to the ER, but target unstructured nascent proteins to mitochondria. Mitochondria-mistargeted proteins induce dissipation of mitochondrial membrane potential. In contrast, ER-SP of Prion Protein (PrP) misdirects nascent chains with random structures to the cytoplasm but not to mitochondria. These findings are novel and provide new insights into the recognition of the ribosome-nascent chain complex (RNC) by the ER translocation machinery, although the precise mechanism and physiological significance remain unknown.

Comments:
- Structural characteristics of ER-SP of Sho, Som, and APP should be compared with ER-SP of PrP. How about their propensity of forming basic bipartite α-helix? It will be important to determine amino acid residues in ER-SP essential for mitochondria targeting.
- It is surprising that alpha-helical structures or C-terminal placed GPI-anchor signal directs cotranslational ER translocation of proteins that are otherwise post-translationally targeted to mitochondria. Since basic amino acid cluster placed downstream of ER-Signal-Anchor is known to inhibit recognition by SRP and directs nascent chains to mitochondria, it would be informative to analyze by SRP-induced translation arrest whether affinity of SRP to ER-SP in RNC is affected by random or α-helical structures in nascent chains.
- The authors should perform cell fractionation for appropriate constructs directed to the ER and directed to mitochondria.
- Fig.5B: submitochondrial fractionation should be performed for APP115α2α3, APP115α2α3AA, and UCR115α2α3 to know if they are correctly imported into the matrix or missorted to the other mitochondrial compartments.
- Fig. 6A or C: Dissipation of membrane potential across the inner membrane does not represent mitochondrial transport. The import should be shown by cell fractionation.

Referee #3

In this study, the authors illustrate that GPI anchor signal sequences and alpha-helical domains can promote ER translocation of unstructured signal peptide-containing proteins that would otherwise fail translocation. In addition, the authors show that some ER signal sequences that fail translocation target to mitochondria and cause mitochondrial functional defects. This supports the idea that an ER signal sequence is not always sufficient to ensure proper translocation and suggests that there is something about intrinsically unstructured domains that make them difficult to translocate. A second
implication is that under conditions of reduced ER translocation, aberrant targeting to mitochondria may contribute to their dysfunction, perhaps due to the ER signal peptide in mitochondria. While these observations are interesting and highlight the complexity of cytosolic targeting pathways, the study would benefit from some greater mechanistic insight into why unstructured domains cannot be translocated. In the absence of this, the findings are relatively descriptive; while this will certainly be useful for stimulating future work in this area, its appropriateness for EMBO seems diminished.

Some specific suggestions for improving the study:

1) There is an extensive amount of mechanistic information about how the early steps in ER protein translocation occur (or at least thought to occur). I was therefore surprised to see so little of this information taken into account in trying to explain a central part of the study: failure of an otherwise functional signal peptide to translocate an unstructured domain. Is the failure at the step of SRP recognition, signal-Sec61 interaction, insertion of the nascent chain into the translocation channel, binding to luminal proteins to drive translocation, or something else? Obviously, the study would be improved considerably if this issue were addressed experimentally, but it is at least worth discussing. In particular, all the above steps are thought to occur by around 70-90 residues of synthesis (see Jungnickel and Rapoport, 1995, Cell) raising the puzzling question of how a GPI signal at the C-terminus of a rather long protein affects translocation. A scholarly discussion of these issues is warranted.

2) It is worth determining the length constraints of this effect: how far downstream of the signal will a GPI anchor or helical domain still work?

3) It seems important to establish whether unstructured segments are able to ever be translocated. In particular, will a type II transmembrane domain at the N-terminus allow translocation of a downstream unstructured domain?

4) The authors' data suggest that the signal peptide is a substantial contributor to mitochondrial toxicity. It is therefore worth determining rigorously whether the signals are cleaved or not when they target to mitochondria. A much better experiment would be to determine whether the signal is 'clogging' the mitochondrial translocon.

5) Fig. 4B would benefit from showing the matched constructs lacking the structured domains for comparison.

6) Recent work suggests that signal peptides can mediate post-translational translocation into the mammalian ER, and it seems likely that many of these substrates are unstructured. The authors may wish to comment on whether any of their substrates are likely to use this pathway (since many of them seem rather small).

Reply to reviewers: Manuscript EMBOJ-2012-83163

Referee #1

While previously thought to be exceptional, protein targeting to more than one compartment is now recognized to be a quite common occurrence. The manuscript by Pfeiffer et al. adds a new twist to the problem of how newly synthesized proteins choose between the ER and mitochondria: they show that the same ER signal peptides have the unanticipated ability to target proteins to mitochondria, and that the mature part of the nascent polypeptide chain, by influencing translocation efficiency across the ER membrane, may determine whether a polypeptide is diverted to mitochondria by an ambivalent signal peptide. This is an interesting finding, and well suited to the interests of the EMBO J readers. However, there are two gaps in the story, which must be filled before the manuscript is suitable for publication.

1. In previous work (Miesbauer et al., 2009), the group reported that translocation into the ER is adversely affected by the intrinsically disordered portion of the mature
sequence of Prion Protein (PrP), and positively affected by the alpha-helical domains, including the C-terminal GPI addition signal sequence, which is predicted to be alpha helical. Here, they extend this observation, by testing the effect of alpha helical domains on the translocation of two other intrinsically disordered proteins: shadoo and somatostatin. While they interpret their results to lend further support to the concept that alpha helical domains in mature portions of the polypeptide facilitate translocation, other interpretations are possible. In the case of shadoo, they clearly demonstrate that the C-terminal GPI addition sequence favours ER translocation, but can we be sure that this is because of its propensity to form an alpha-helix? The results of this study suggest that the GPI signal sequence is MORE efficient than other investigated predicted alphahelical domains. So, what are the features of this sequence that make it the most efficient translocation inducer?

Reply: The reviewer raises the interesting possibility that a C-terminal GPI signal sequence might be a more efficient translocation inducer than α-helical domains. To analyze this aspect in detail we tested the activity of different C-terminal domains to promote ER import of Sho:

1. a GPI signal sequence in which the cleavage/attachment site (ω site) for the GPI anchor is mutated (Sho-ω mtGPI). In this way, the original length of the nascent Sho polypeptide chain is preserved without allowing the GPI anchor attachment to occur.
2. a heterologous CD4 transmembrane domain (Sho-CD4). The transmembrane domain is predicted to form an α-helix and the mature protein is membrane-anchored.
3. a GPI signal sequence in which the cleavage/attachment site (ω site) for the GPI anchor is mutated (Sho-ω mtGPI). In this way, the original length of the nascent Sho polypeptide chain is preserved without allowing the GPI anchor attachment to occur.
4. helices 2 and 3 of PrP (Sho-α2α3). In this context it is important to note that in vitro experiments indicated that isolated PrP fragments comprising helix 2 and helix 3 show complete structural autonomy, i.e. independently adopt an alphahelical conformation (Eberl & Glockshuber, 2002; Gallo et al, 2005).

This analysis revealed that all of these domains can efficiently promote ER import of Sho (new Figure 1F, G).

In the case of somatostatin, a comparison is made between two constructs (both containing the protein's N-terminal targeting sequence), one containing only the pro domain (predicted to be alpha helical) with the C-terminal, intrinsically disordered, hormone domain deleted, and the other one composed only of the hormone domain. The first of these is translocated, while the second one is not. However, these two constructs are not really comparable, as the first one has 108 residues vs 17 residues the second one (not including the N-terminal signal sequence). Because of the short length of the second construct (SomΔPro), the signal sequence will be poorly available to co-translational targeting, and this could strongly affect the subsequent translocation event.

REPLY: Indeed, it appears plausible to assume that impaired ER import of SomΔpro is due to the reduced length of the construct. However, data from different experimental approaches speak against such a concept.

1. PrP•115-115 is 207 amino acids in length, yet not imported. This example illustrates that increasing the length of the polypeptide chain does not necessarily restore ER import of an unstructured protein (new Figure 2A, B).
2. Employing in vitro assays with stalled ribosome—nascent chain complexes (RNCs) and pancreatic microsomal membranes, we show that a short unstructured protein (PrP•115) is cotranslationally targeted to the ER membrane with a targeting efficiency comparable to that of a structured protein of similar length (PrP•α2α3) (new Figure 4F).

3. As shown in the 'Figure 1 for Reviewers', increasing the length of SomΔpro by fusing the unstructured domain of Sho to its C-terminus does not restore ER import (SomΔpro-Sho, 167 amino acids). Similarly to SomΔpro, SomΔpro-Sho is targeted to mitochondria.

2. The authors find that under conditions of reduced translocation (either because of features of the mature part of the nascent chain or because of reduced translocation capacity of the ER) the ER signal peptides of shadoo, somatostatin, and APP are all three capable of redirecting a polypeptide to mitochondria. This is interesting, but a deficiency of the paper is the lack of characterization of the submitochondrial localization of the construct. The authors present one blot concerning only one of the constructs as evidence that they are translocated at least across the outer mitochondrial...
membrane. This conclusion is based on the resistance of the construct APP 115alpha2alpha3 to proteolysis in isolated intact mitochondria and on its sensitivity in the presence of detergent. This experiment does not rule out the possibility that the construct is aggregated and therefore protease-resistant and that detergent, by disrupting protein-protein interactions, allows better access to the protease. My suspicion is that the investigated constructs are on the outer mitochondrial membrane exposed to the cytosol, with the ER signal peptide functioning as a signal anchor. This would be interesting as well, but a far shot from concluding that an ER signal peptide is functioning as a mitochondrial import signal. It is known that the features that distinguish N-terminal ER targeting sequences from outer mitochondrial membrane signal anchors are quite subtle (e.g., Kanaji S et al., 2000, JCB 151:277). Therefore, an interpretation of the mechanism of the observed mitochondrial targeting by the investigated signal peptides depends critically on a rigorous definition of the submitochondrial localization and membrane topology of the constructs.

REPLY: The reviewer raised a very important point. We employed three different approaches, including antibody accessibility in semi-permeabilized cells as suggested, to show that the ER signal peptides mediate targeting to the mitochondrial matrix (new Figure 7B, C, D, new Suppl. Figure 3).

In conclusion, it is my opinion that before publication the study must be improved, to:
(i) present more evidence, possibly with synthetic sequences, on the role of alpha-helical sequences in translocation, or, at the least, recognize that the remarkable activity of the GPI attachment signal may depend on features other than its alpha-helicity; for the somatostatin work, constructs of similar length must be compared; and
(ii) investigate more in depth the submitochondrial localization of representative mitochondria-targeted constructs (more than one), for instance by antibody accessibility in semi-permeabilized cells.

REPLY: As presented in detail above, we think that we have been able to experimentally address all points raised by the reviewer.

Referee #2

It is generally believed that secretory proteins are targeted to and imported into the ER in a co-translational manner. Interestingly, Pfeiffer et al demonstrated in this paper that uncleavable ER signal peptides (ER-SP) of Shadoo (Sho), Somatostatin (Som) and App have the property to mediate alternative targeting to mitochondria; they target nascent proteins with α-helical domains or with the C-terminal GPI-anchor signal correctly to the ER, but target unstructured nascent proteins to mitochondria. Mitochondriamistargeted proteins induce dissipation of mitochondrial membrane potential. In contrast, ER-SP of Prion Protein (PrP) misdirects nascent chains with random structures to the cytoplasm but not to mitochondria. These findings are novel and provide new insights into the recognition of the ribosome-nascent chain complex (RNC) by the ER translocation machinery, although the precise mechanism and physiological significance remain unknown.

Comments:
- Structural characteristics of ER-SP of Sho, Som, and APP should be compared with ER-SP of PrP. How about their propensity of forming basic bipartite α-helix? It will be important to determine amino acid residues in ER-SP essential for mitochondria targeting.

REPLY: Based on the reviewer's suggestion we examined the biochemical and biophysical properties of the ER signal peptides, but did not find any features that could be specifically linked to their activity to mediate mitochondrial targeting. For example, these signal peptides did not have a propensity of forming a basic bipartite α-helix. Since this analysis did not provide a clue to structural characteristics specific for the ER-SP of Sho, Som, or APP, we decided to not include the data in the manuscript. However, the data are available for the reviewers (Figure 2 for Reviewers).
In addition, we generated chimeric PrP/APP and PrP/Som signal peptides and evaluated their targeting activity in transiently transfected cells. This approach revealed that the ability to mediate mitochondrial targeting was linked to the N-terminal half of the Som signal peptide, whereas it was located in the C-terminal half of the APP signal peptide (new Figure 5E, F).

- It is surprising that alpha-helical structures or C-terminal placed GPI-anchor signal directs co-translational ER translocation of proteins that are otherwise posttranslationally targeted to mitochondria. Since basic amino acid cluster placed downstream of ER-Signal-Anchor is known to inhibit recognition by SRP and directs nascent chains to mitochondria, it would be informative to analyze by SRP-induced translation arrest whether affinity of SRP to ER-SP in RNC is affected by random or α-helical structures in nascent chains.

REPLY: Our experimental data do not support the notion that a C-terminal GPI anchor or α-helical domains re-direct proteins destined for mitochondria to the ER. Instead, it appears that mitochondrial targeting is a consequence of an aborted ER import. Such a scenario is supported by the following experimental approaches:
1. Co-expression of p58IPK decreases ER import and increases mitochondrial targeting (Figure 8C).
2. Interfering with Sec61 function by treating cells with eeyarestatin I directed wildtype Sho containing a C-terminal GPI signal sequence to mitochondria (Figure 9A).
3. In cells functionally depleted of Sec61alpha by using siRNA-mediated silencing of the SEC61A1 gene, wildtype Sho, wildtype Som, and wildtype APP are targeted to mitochondria (Figure 9B, C, D, E).
4. New data included in the revised manuscript show that a short unstructured protein (PrP115), which is not productively imported into the ER, is co-translationally targeted to the ER membrane with a targeting efficiency comparable to that of a structured protein of similar length (PrPα2α3) (new Figure 4F). This indicates that structural features of the nascent chain do not affect co-translational targeting of ribosome–nascent chain complexes to the ER.

- The authors should perform cell fractionation for appropriate constructs directed to the ER and directed to mitochondria.

REPLY: This is a valid concern. While ER import was already shown in the initial manuscript by analyzing N-linked glycosylation as a marker for productive translocation into the ER lumen, we only showed that APP115α2α3 co-fractionated with mitochondria and was protected after proteolytic digestion of intact mitochondria. In the revised manuscript we included a comprehensive analysis to determine the mitochondrial sublocalization of the constructs. We employed three different approaches to show that the ER signal peptides can mediate targeting to the mitochondrial matrix (new Figure 7B, C, D, new Suppl. Figure 3).

- Fig.5B: submitochondrial fractionation should be performed for APP115α2α3, APP115α2α3AA, and UCR115α2α3 to know if they are correctly imported into the matrix or missorted to the other mitochondrial compartments.

REPLY: See above.

- Fig. 6A or C: Dissipation of membrane potential across the inner membrane does not represent mitochondrial transport. The import should be shown by cell fractionation.

REPLY: In the revised version we show that the activity of Sho•115α2α3, Som•115α2α3, and APP•115α2α3 to disrupt the mitochondrial membrane potential correlates directly with their mitochondrial targeting (new Figure 8A).

Referee #3

In this study, the authors illustrate that GPI anchor signal sequences and alpha-helical domains can promote ER translocation of unstructured signal peptide-containing
proteins that would otherwise fail translocation. In addition, the authors show that some ER signal sequences that fail translocation target to mitochondria and cause mitochondrial functional defects. This supports the idea that an ER signal sequence is not always sufficient to ensure proper translocation and suggests that there is something about intrinsically unstructured domains that make them difficult to translocate. A second implication is that under conditions of reduced ER translocation, aberrant targeting to mitochondria may contribute to their dysfunction, perhaps due to the ER signal peptide in mitochondria. While these observations are interesting and highlight the complexity of cytosolic targeting pathways, the study would benefit from some greater mechanistic insight into why unstructured domains cannot be translocated. In the absence of this, the findings are relatively descriptive; while this will certainly be useful for stimulating future work in this area, its appropriateness for EMBO seems diminished.

Some specific suggestions for improving the study:
1) There is an extensive amount of mechanistic information about how the early steps in ER protein translocation occur (or at least thought to occur). I was therefore surprised to see so little of this information taken into account in trying to explain a central part of the study: failure of an otherwise functional signal peptide to translocate an unstructured domain. Is the failure at the step of SRP recognition, signal-Sec61 interaction, insertion of the nascent chain into the translocation channel, binding to luminal proteins to drive translocation, or something else? Obviously, the study would be improved considerably if this issue were addressed experimentally, but it is at least worth discussing. In particular, all the above steps are thought to occur by around 70-90 residues of synthesis (see Jungnickel and Rapoport, 1995, Cell) raising the puzzling question of how a GPI signal at the C-terminus of a rather long protein affects translocation. A scholarly discussion of these issues is warranted.

REPLY: The reviewer raised the interesting and important question of why ER import of intrinsically disordered protein is impaired. The main focus of the current manuscript is on alternative targeting of secretory proteins to mitochondria. Therefore, we feel that experiments to study underlying mechanisms of an aborted ER import of intrinsically disordered proteins is beyond the scope of this study.

Nonetheless, we included in vitro data to show that a short unstructured nascent chain (PrP •115), which is not productively imported into the ER, is co-translationally targeted to the ER membrane with an efficiency comparable to that of a structured nascent chain of similar length (PrP •α2α3) (new Figure 4F).

2) It is worth determining the length constraints of this effect: how far downstream of the signal will a GPI anchor or helical domain still work?

REPLY: In the revised manuscript we show that ER import of an intrinsically disordered protein of 186 amino acids (PrP•115-115) can be restored by both a C-terminal GPI anchor and a C-terminal α-helical domain (new Figure 2A, B).

3) It seems important to establish whether unstructured segments are able to ever be translocated. In particular, will a type II transmembrane domain at the N-terminus allow translocation of a downstream unstructured domain?

REPLY: As suggested by the reviewer, we fused the N-terminal transmembrane domain of the MHC class II invariant chain (aa 1-66) to ΔSP•ShoΔGPI to generate IVC•ShoΔGPI. The Western blot analysis of transiently transfected cells indicated that this construct is not glycosylated, indicating that it is not imported into the ER (Figure 3 for Reviewers).

In addition, we generated additional constructs to analyze the activity of different C-terminal domains to promote ER import of Sho:
1. A GPI signal sequence in which the cleavage/attachment site (ω site) for the GPI anchor is mutated (Sho-mtGPI). In this way, the original length of the nascent Sho polypeptide chain is preserved without allowing the GPI anchor attachment to occur.
2. A heterologous CD4 transmembrane domain (Sho-CD4). The transmembrane domain is predicted to form an α-helix and the mature protein is membrane-anchored.
3. Helix 2 of PrP (Sho-α2) and
4. Helices 2 and 3 of PrP (Sho-α2α3). The data presented in new Figure 1F and G revealed that all of these constructs are imported into the ER.

4) The authors' data suggest that the signal peptide is a substantial contributor to mitochondrial toxicity. It is therefore worth determining rigorously whether the signals are cleaved or not when they target to mitochondria. A much better experiment would be to determine whether the signal is 'clogging' the mitochondrial translocon.

REPLY: In the revised manuscript we present a comprehensive analysis on the submitochondrial localization of the constructs (new Figure 7B, C, D, new Suppl. Figure 3). This analysis indicated that the ER signal peptides can mediate targeting to the mitochondrial matrix.

The reviewer raised the interesting idea that the toxic constructs, or a fraction thereof, cause clogging of the mitochondrial translocon. Unfortunately, we have not been able to acquire experimental evidence for such a scenario. A possible reason for this might be the localization of the 3F4 epitope for antibody detection. This epitope is located in front of the α2α3 domain, which is likely to cause 'clogging'. Thus, after a limited PK digestion of mitoplasts this epitope would be degraded together with the non-translocated part of APP•115α2α3.

5) Fig. 4B would benefit from showing the matched constructs lacking the structured domains for comparison.

REPLY: We included the respective Western blots in the revised manuscript. In addition, we show that the unstructured constructs are not secreted and contain an uncleaved ER signal peptide (new Suppl. Figure 1C, D).

6) Recent work suggests that signal peptides can mediate post-translational translocation into the mammalian ER, and it seems likely that many of these substrates are unstructured. The authors may wish to comment on whether any of their substrates are likely to use this pathway (since many of them seem rather small).

REPLY: Indeed, recent work indicates that post-translational targeting/translocation in mammalian cells is more abundant than previously appreciated. As mentioned above, we started to address this interesting issue by analyzing co-translational targeting of stalled ribosome–nascent chain complexes (RNCs) in vitro (new Figure 4F).

2nd Editorial Decision 21 January 2013

Thank you for submitting your revised manuscript to the EMBO Journal. Your study has now been re-reviewed by the referees and their comments are provided below. As you can see the referees appreciate the introduced changes and support publication here. There are a few minor issues raised that I would appreciate your response to before acceptance here. Most of these can be resolved with appropriate text changes.

Once we receive the revised version, we will proceed with its acceptance. Thank you for submitting your interesting study to the EMBO Journal!

REFEREE REPORTS

Referee #1

The authors have responded in a satisfactory manner to all the concerns raised in my review of the previous version of the manuscript. The revised manuscript is much improved and is now suitable for publication in EMBO J. I only have three suggestions: 1) Reviewer Figure 1 could be included as supplementary figure, as it supports the hypothesis of the role of the folding status of the pro-domain in prosomatostain translocation (although this reviewer would have preferred the unfolded Sho domain to be placed in front of and not after the hormone domain!); 2) It would be nice to show
a better positive control for Figure 4F, as the DeltaSP.115 translation products appears to be completely lost during the fractionation procedure; 3) Some of the gels have irrelevant bands that might be mentioned in the Figure legends (e.g., Fig. 2B, first gel of second row, lowest band).

Referee #2

The paper is well revised and answers satisfactorily the points that reviewers have raised. I believe this is an important report providing new insights into the recognition mechanism of nascent precursor proteins by the ER and mitochondrial protein translocation machinery.

Referee #3

This revised manuscript contains several additions that more thoroughly examine the authors' initial observation. Most notable among these are: (1) display of matched controls for various constructs and experiments, (2) characterization of the sub-mitochondrial localization of the proteins with ER signal peptides that are targeted to mitochondria, (3) some more in-depth investigation into possible properties that may mediate the ability of ER signal peptides to target to mitochondria. Given these improvements, I support publication in EMBO J.

Minor comments:
(1) The authors use stalled RNCs in an in vitro assay to show that short proteins can still undergo co-translational targeting to the ER (Fig. 4F). However, the primary impediment to efficient co-translational targeting of short secretory proteins is time, where translation termination occurs before targeting. The stalled RNCs are not an accurate representation of this situation since they are at steady state where translation termination is prevented, which may artificially suggest more efficient targeting than might occur if translation termination were possible. A suitable caveat seems appropriate.
(2) Pg. 10, 2nd paragraph, last sentence: spelling mistake (impaitment should be impairment).

2nd Revision - authors' response 31 January 2013

Referee #1

The authors have responded in a satisfactory manner to all the concerns raised in my review of the previous version of the manuscript. The revised manuscript is much improved and is now suitable for publication in EMBO J. I only have three suggestions: 1) Reviewer Figure 1 could be included as supplementary figure, as it supports the hypothesis of the role of the folding status of the pro-domain in prosomatostain translocation (although this reviewer would have preferred the unfolded Sho domain to be placed in front of and not after the hormone domain!)

Reply: As suggested by this reviewer, Figure 1 has been added to the supplementary information.

2) It would be nice to show a better positive control for Figure 4F, as the DeltaSP.115 translation products appears to be completely lost during the fractionation procedure

Reply: We are sorry for this misunderstanding, possibly we did not describe the experimental approach in sufficient detail. The fractionation procedure is designed to separate ribosome-nascent chain complexes (RNCs) bound to ER membranes from untargeted nascent chains by sedimentation. Targeting to the ER membrane is mediated by the signal recognition particle (SRP) that binds to both the ER signal peptide and the SRP receptor in the ER membrane. Since deltaSP.115 does not contain an ER signal peptide, RNCs are not targeted to the ER membrane and consequently are not found in the pellet fraction after centrifugation. Importantly, deltaSP.115 is efficiently translated as shown in the lane labeled "t".

3) Some of the gels have irrelevant bands that might be mentioned in the Figure legends (e.g., Fig. 2B, first gel of second row, lowest band).
Reply: Under conditions of partial ER import several bands can occur corresponding to molecule +/- ER signal peptides and/or +/- N-linked glycans. In addition, inhibition of N-linked glycosylation by tunicamycin and deglycosylation by PNGaseF treatment is not always complete, resulting in additional bands. We think that labelling of each of these fractions in the figures would be rather reader unfriendly and confusing. Instead, we specifically labelled bands that are relevant in the context of the respective experiment.

Referee #2

The paper is well revised and answers satisfactorily the points that reviewers have raised. I believe this is an important report providing new insights into the recognition mechanism of nascent precursor proteins by the ER and mitochondrial protein translocation machinery.

Reply: Thank you.

Referee #3

This revised manuscript contains several additions that more thoroughly examine the authors’ initial observation. Most notable among these are: (1) display of matched controls for various constructs and experiments, (2) characterization of the sub-mitochondrial localization of the proteins with ER signal peptides that are targeted to mitochondria, (3) some more in-depth investigation into possible properties that may mediate the ability of ER signal peptides to target to mitochondria. Given these improvements, I support publication in EMBO J.

Minor comments:
(1) The authors use stalled RNCs in an in vitro assay to show that short proteins can still undergo co-translational targeting to the ER (Fig. 4F). However, the primary impediment to efficient co-translational targeting of short secretory proteins is time, where translation termination occurs before targeting. The stalled RNCs are not an accurate representation of this situation since they are at steady state where translation termination is prevented, which may artificially suggest more efficient targeting than might occur if translation termination were possible. A suitable caveat seems appropriate.

Reply: We fully agree with the reviewer 3. Therefore, we have already addressed this point in our manuscript by stating in the Results section that "the structure of the nascent chain does not seem to modulate ER targeting, at least under the in vitro conditions tested (Results, Fig. 4F)". Moreover, we mentioned in the Discussion section that "We cannot rule out that structural features within the nascent chain modulate targeting of the ribosome-nascent chain complex to the ER".

In addition, we have now added the following sentence in the Discussion to indicate that the data were obtained with stalled RNCs: "We showed that a short unstructured protein, which is not productively imported into the ER, can be cotranslationally targeted to ER membranes under in vitro conditions employing stalled RNCs".

(2) Pg. 10, 2nd paragraph, last sentence: spelling mistake (impairment should be impairment).

Reply: Thank you, corrected.