EMBO reports

Manuscript EMBOR-2013-38142

A novel import route for an N-anchor mitochondrial outer membrane protein aided by the TIM23 complex

Jiyao Song, Yasushi Tamura, Tohru Yoshihisa and Toshiya Endo

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Review timeline:

Submission date:	23 October 2013
Editorial Decision:	27 November 2013
Revision received:	26 February 2014
Accepted:	11 March 2014

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.)

Editor: Barbara Pauly

1st Editorial Decision 27 November 2013

Thank you very much for the submission of your research manuscript to our editorial office. Please accept my apologies for the delay in getting back to you, which was due to the fact that for back-to-back submissions like yours and Dr. Pfanner's, we allow extra time for the reviewers to submit both sets of reports to our editorial office. We have now received the full set of reviews on your manuscript.

As the detailed reports are pasted below I will only repeat the main points here. You will see that the referees are in principle positive about the publication of your study in our journal and I would therefore like to invite you to revise your manuscript according to their suggestions. While referee 1 feels that the study should be developed further with regard to the mechanism by which the TOM and TIM complexes mediate Om45 translocation, we feel that this would go beyond the scope of the current manuscript and we would therefore not insist on these additional insights. If you, however, already have data at hand that address some of the concerns of this reviewer we do encourage you to include them or at least discuss the possible mechanism by which TOM/TIM mediate the insertion of Om45 into the outer membrane. The other issues of the referees should be addressed as far as possible, in particular the additional experiments suggested by reviewers 2 and 3. With regard to the suggestion of referee 3 to test whether Mim1 and Mim2 are involved in Om45 insertion, I would recommend referring to the data in the related manuscript by Pfanner and colleagues.

Acceptance of the manuscript will depend on a positive outcome of a second round of review and I

should also remind you that it is EMBO reports policy to allow a single 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.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. If you feel that this period is insufficient for a successful submission of your revised manuscript I can potentially extend this period slightly. Also, the length of the revised manuscript should not exceed roughly 28,000 characters (including spaces and references). This might be achieved by combining the results and discussion section, as this avoids unnecessary redundancies.

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We also welcome the submission of cover suggestions or motifs that might be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised form of your manuscript when it is ready. Should you in the meantime have any questions, please do not hesitate to contact me.

REFEREE REPORTS:

Referee #1:

The manuscript by Song et al reports work defining the import pathway of the protein Om45 of the mitochondrial outer membrane that contains a single membrane spanning helix. Several results are reported. First, experimental data support the idea that the soluble domain of Om45 is in the intermembrane space (IMS); previously published work were contradictory, with the majority indicating that this domain faced the cytosol. Secondly, through the use of a variety of mutants, a strong case is made for a role for the Tom20/22 outer membrane receptors and the Tim23/50 proteins of the inner membrane translocon for the import of Om45. While the requirement for the translocon components (and a membrane potential) is required for import of full-length protein into the IMS, the N-terminal 50 residues, which contains both the transmembrane segment and sequences important for targeting to mitochondria does not. The experimental results are clearly and thoughtfully presented.

The authors correctly state that a role of inner membrane translocase components proteins in translocation of an outer membrane protein is novel. However, this manuscript leaves many questions regarding this mode of translocation. The results are intriguing, but unfortunately leave the reader wanting for some mechanistic insight.

1. The authors nicely demonstrate that the N-terminal 50 residues of Om45 are sufficient for entry into the IMS via a Tom20/22 dependency? What sequences are required for this translocation and what distinguishes those from the more common targeting sequences that drive translocation (in a Tom20/22 dependent manner) into the matrix through the Tim23 translocon?

2. The data is clear that it is the C-terminal region that requires Tim23, Tim50 and membrane potential for translocation into the IMS. This is a surprising and interesting result. But no mechanistic connection is made. For example: What is the relationship between sequences of Tim23/Tim50 that bind presequence of "typical matrix targeted proteins" and those required for Om45? Is there something unique about the C-terminus of Om45, or would any carrier protein fused

to the N-terminal region of Om54 require these components for import?

Referee #2:

This is an impressive study revealing a novel import pathway of mitochondrial outer membrane proteins. It shows that protein translocation machinery of the inner membrane is required for import of a signal anchored mitochondrial outer membrane protein.

There have been controversial claims of regarding the topology of the N-terminally anchored mitochondrial Om45. Using protease protection assays in intact mitochondria and in mitoplast as well as subcellular fractionation Song et al. convincingly show that the N-terminus of Om45 is exposed to the cytosol, whereas the large soluble domain resides in the intermembrane space. Next a collection of deletion mutants was used to show that the N-terminal approx. 50 amino acids are sufficient to direct the Om45 variants to the intermembrane space. In elegant assay based on cell lines expressing variants of Tom20 and Tom22 whose cytosolic domains can be removed by the TEV protease the authors demonstrate that both proteins are required for Om45 import. Moreover, competition experiments with chemical amounts of a precursor protein establish that Om45 and matrix protein precursors compete for the same outer membrane pore. Further experiments show that import of Om45 is dramatically reduced in Tim23 and Tim50 mutants. Finally, studies using valinomycin and CCCP demonstrate that import of the full length Om45 but not the C-terminal truncation thereof is abolished in the absence of the membrane potential. In line with these finding import of Om45 depended on Pam17 but surprisingly not on mitochondrial Hsp70.

This is an excellent manuscript. The topic is clearly of general interest as it presents the first case of a non-beta barrel mitochondrial outer membrane protein that is inserted into the outer membrane from the intermembrane space side. The results are of high quality and I agree with the authors conclusions. A particular strength of the mansucript ist that essentially most experiments have carefully been quantified. In summary this results in a consistent and convincing story of how Om45 is imported into yeast mitochondria.

I have very few specific comments all of which I consider minor:
Have all experiment for which a graph is shown been performed in triplicate? If yes, is it correct that in the cases were no error bar is visible in the graphs, it is simply to small to be seen? Should this bet he case the experiments show very little variation indeed. - Please clarify.

It is claimed in the manuscript (page 7, bottom) that import of Om45 did not strongly depend on Tim22 and Tim40. I think this needs more explanations. In both cases a clear and apparently reproducible effect is seen. For the Tim40 (Fig. S1D) the reduction of import appears to be as strong as for the Tim23 mutant (Fig. 4A).

Referee #3:

Om45 is the most abundant but function unknown yeast mitochondrial outer membrane (OM) protein. It has a single hydrophobic segment at the N-terminus, although its membrane topology as well as its topogenesis remains controversial. Song et al in this manuscript demonstrated that Om45 is integrated to the OM via a unique pathway; Its precursor is imported first into the intermembrane space (IMS) dependent on the TOM complex and the TIM23 complex very much like the import of matrix-targeted presequence-containing precursors. It is then released from the TIM23 complex and integrated into the OM from the IMS.

This report revealed an unexpected new pathway of topogenesis of an OM protein through sequential collaboration of protein tranlocation machineries. This will have a significant impact on the organelle biogenesis field.

COMMENTS:

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- It would be informative to know the requirement of Tom70, because hydrophobicity of the N-terminal segment of Om45 is relatively high.

- Fig. 3D: This experiment does not exclude the possibility that the import inhibition was caused at the step of Tom20 and Tom22 but not at the Tom40 channel. The authors should demonstrate that recombinant pSu9-DHFR indeed stuffed up the Tom40 channel. Were these experiments performed in the presence of methotrexate?

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- Fig. 4E: The authors' finding that CCCP does not inhibit the import of Om45-100 into the IMS is quite interesting. It would be very informative to analyze intra-mitochondrial localization of Om45-100. The authors should also discuss what would be the driving force of this import.

- It will be very important to know whether Mim1 and Mim2 are involved in the topogenesis of Om45.

- The authors should discuss the mechanism by which the Tim23 complex (plus membrane potential) triggers retrograde insertion of Om45 into the OM.

1st Revision - authors' response 26 February 2014

Referee 1

The manuscript by Song et al reports work defining the import pathway of the protein Om45 of the mitochondrial outer membrane that contains a single membrane spanning helix. Several results are reported. First, experimental data support the idea that the soluble domain of Om45 is in the intermembrane space (IMS); previously published work were contradictory, with the majority indicating that this domain faced the cytosol. Secondly, through the use of a variety of mutants, a strong case is made for a role for the Tom20/22 outer membrane receptors and the Tim23/50 proteins of the inner membrane translocon for the import of Om45. While the requirement for the translocon components (and a membrane potential) is required for import of full-length protein into the IMS, the N-terminal 50 residues, which contains both the transmembrane segment and sequences important for targeting to mitochondria does not. The experimental results are clearly and thoughtfully presented.

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1. The authors nicely demonstrate that the N-terminal 50 residues of Om45 are sufficient for entry into the IMS via a Tom20/22 dependency? What sequences are required for this translocation and what distinguishes those from the more common targeting sequences that drive translocation (in a Tom20/22 dependent manner) into the matrix through the Tim23 translocon?

As for translocation across the OM, we have now found that Tom70N-Om45, a fusion protein between the N-terminal 30 residues of OM-targeted Tom70 and Om45 lacking its N-terminal 30 residues, also follows the Om45 sorting pathway in a $\Delta\Psi$ and TIM23 complex-dependent manner (Fig. 2D and E, Fig. 3D, and Fig. 4B and F in the revised manuscript). This shows that a short positively charged segment followed by the hydrophobic TM segments of Tom70 and Om45 (for the sequences, see Fig. 2A in the revised manuscript) contain signals to direct protein translocation across the OM. However, probably folding of the C-terminal domain may prevent translocation across the OM since Tom70 is more resistant against protease digestion than Om45 (Fig. S2 in the revised manuscript).

As for the translocation across the IM, we tested the import of typical matrix-targeting presequences, pSu9 and pb_2(80)Δ19, followed by full-length Om45, and found that they could not cross the IM (see the attached figure). Probably, the N-terminal TM segment of Om45 may prevent translocation across the IM. Since we feel that the detailed mechanism behind this observation is beyond the scope of this work, we did not add these results to the revised manuscript.

2. The data is clear that it is the C-terminal region that requires Tim23, Tim50 and membrane potential for translocation into the IMS. This is a surprising and interesting result. But no mechanistic connection is made. For example: What is the relationship between sequences of Tim23/Tim50 that bind presequence of "typical matrix targeted proteins" and those required for Om45? Is there something unique about the C-terminus of Om45, or would any carrier protein fused to the N-terminal region of Om54 require these components for import?

The TIM23 complex and $\Delta\Psi$ may play a role in pulling Om45, perhaps by binding to the N-terminal segment of Om45, across the OM to IMS. Then, requirement of the TIM23 complex and $\Delta\Psi$ for the truncated Om45 proteins may not be simply related to the presence of a signal for the TIM23 complex recognition, but may be related to the folding states or polypeptide lengths of the truncated Om45 domains. We now added this discussion to the revised manuscript.

Referee 2

This is an impressive study revealing a novel import pathway of mitochondrial outer membrane proteins. It shows that protein translocation machinery of the inner membrane is required for import of a signal anchored mitochondrial outer membrane protein.

There have been controversial claims of regarding the topology of the N-terminally anchored mitochondrial Om45. Using protease protection assays in intact mitochondria and in mitoplast as well as subcellular fractionation Song et al. convincingly show that the N-terminus of Om45 is exposed to the cytosol, whereas the large soluble domain resides in the intermembrane space. Next a collection of deletion mutants was used to show that the N-terminal approx. 50 amino acids are sufficient to direct the Om45 variants to the intermembrane space. In elegant assay based on cell lines expressing variants of Tom20 and Tom22 whose cytosolic domains can be removed by the TEV protease the authors demonstrate that both proteins are required for Om45 import. Moreover, competition experiments with chemical amounts of a precursor protein establish that Om45 and matrix protein precursors compete for the same outer membrane pore. Further experiments show that import of Om45 is dramatically reduced in Tim23 and Tim50 mutants. Finally, studies using valinomycin and CCCP demonstrate that import of the full length Om45 but not the C-terminal truncation thereof is abolished in the absence of the membrane potential. In line with these finding import of Om45 depended on Pam17 but surprisingly not on mitochondrial Hsp70.

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We performed most experiments more than three times except for those in Fig. S1 in the original manuscript (Fig. S4 in the revised manuscript). We thus performed additional experiments to achieve triplicate assays and put error bars in all the panels, except for the panel for BN-PAGE of AAC in C, in Fig. S4 in the revised manuscript. Now all the quantified panels (except for AAC in Fig. S4C) in the figures in the main body of the manuscript and supplemental materials have error bars based on triplicated experiments. Graphs with no visible error bar simply mean that the error bars are too small to be seen in the revised manuscript.

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We now performed import assays for Om45 into Tim22 depleted mitochondria three times, and put error bars. Now it looks clear that import of AAC (a typical substrate for the TIM22 pathway) is more affected by depletion of Tim22 than Om45 (Fig. S4 in the revised manuscript). We also performed import assays for Om45, pSu9-DHFR and Tim9 three times, and put error bars. We think that Tim9 (a typical substrate for the Tim40 pathway) is more affected by depletion of Tim40 than Om45 (Fig. S4 in the revised manuscript). The reason for the slight impairment of import of Om45 and pSu9-DHFR could be the decreased $\Delta\Psi$ in Tim40 depleted mitochondria.

Referee 3
Om45 is the most abundant but function unknown yeast mitochondrial outer membrane (OM) protein. It has a single hydrophobic segment at the N-terminus, although its membrane topology as well as its topogenesis remains controversial. Song et al in this manuscript demonstrated that Om45 is integrated to the OM via a unique pathway; Its precursor is imported first into the intermembrane space (IMS) dependent on the TOM complex and the TIM23 complex very much like the import of matrix-targeted presequence-containing precursors. It is then released from the TIM23 complex and integrated into the OM from the IMS.

This report revealed an unexpected new pathway of topogenesis of an OM protein through sequential collaboration of protein tranlocation machineries. This will have a significant impact on the organelle biogenesis field.

COMMENTS:

1. Relative hydrophobicity of the N-terminal segment of Om45 is higher than the signal anchor (SA) of Tom20 and Tom70. It is reported that the N-terminal segment of Om45 and Tom70 SA can functionally replace the SA of Tom20. Nevertheless, in this report, the N-terminal hydrophobic segment of Om45 does not function as SA but functions like matrix-targeted presequence. The reason for this difference should be discussed.

Now we found that the N-terminal segment of Tom70 can also function as the signal for directing the Om45 C-terminal domain to the IMS (Fig. 2D and E in the revised manuscript). Therefore the matching between the N-terminal segment of OM proteins and the C-terminal domain appears important for sorting between the SA pathway for Tom70 and Tom20 and the new Om45 pathway. This is now discussed in the revised manuscript.

2. It would be informative to know the requirement of Tom70, because hydrophobicity of the N-terminal segment of Om45 is relatively high.

Now we tested import of Om45 into mitochondrial lacking Tom70 and Tom71, and found that Om45 import is not affected by depletion of Tom70 and Tom71 (Fig. S3 in the revised manuscript).

3. Fig. 3D: This experiment does not exclude the possibility that the import inhibition was caused at the step of Tom20 and Tom22 but not at the Tom40 channel. The authors should demonstrate that recombinant pSu9-DHFR indeed stuffed up the Tom40 channel. Were these experiments performed in the presence of methotrexate?

We did not use methotrexate (Mtx), since even in the absence of Mtx, the tightly folded DHFR domain appeared to resist crossing the OM (see below). Indeed, it is practically difficult to convincingly block the channel alone, not the receptors of the TOM40 complex. Therefore we mentioned the possibility of competition taking place at the receptor and/or channel level in the revised manuscript. However, together with the antibody-shift in BN-PAGE and crosslinking experiment (Fig. 3F and G), it is highly likely that competition takes place mainly at the level of the Tom40 channel.

4. Fig. 3E: The CL/IP signal seems to be too poor to conclude that the stacked Om45 precursor interacted with Tom40. The experimental rational for using the precursor with such a complicated structure should be provided. Again, is methotrexate included in the reaction mixture here?

In the import assay, Om45-DHFR was gone after PK treatment while Om45-Om45-DHFR partially resisted PK digestion. This may suggest that Om45-Om45-DHFR is more tightly associated with the TOM40 complex than Om45-DHFR, so that we decided to use Om45-Om45-DHFR for BN-PAGE and crosslinking experiments. We did not added methotrexate in this experiment since addition of methotrexate did not change the results (we thus did not mention methotrexate in the legend). Now we analyzed the stuck fusion protein by antibody-shift assays in BN-PAGE, which successfully showed that the fusion protein got stuck at the TOM40 complex level. According the referee's comments, we also made efforts to enhance the signal from the crosslinking products by addition of methotrexate etc, but failed probably because the crosslinking efficiency is very low. Nevertheless in combination with the competition experiments and antibody-shifts in BN-PAGE, we believe that our conclusion that Om45 use the Tom40 channel to cross the OM can be validated.

5. Fig. 4E: The authors' finding that CCCP does not inhibit the import of Om45-100 into the IMS is quite interesting. It would be very informative to analyze intra-mitochondrial localization of Om45-100. The authors should also discuss what would be the driving force of this import.

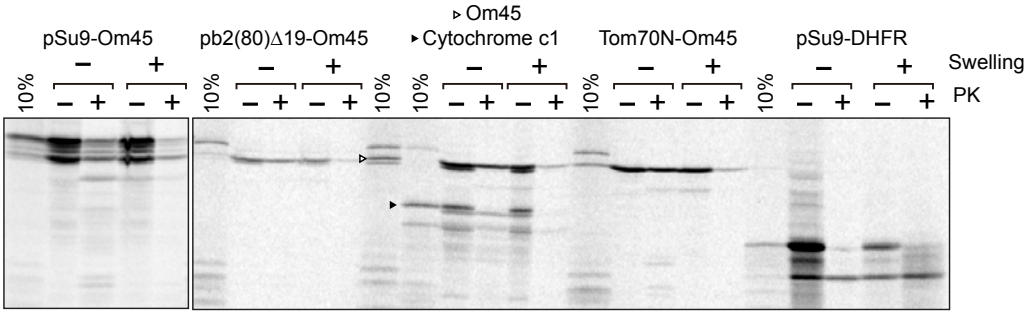
Om45-100 is localized in the IMS (Fig. 4H in the revised manuscript). At the moment, we do not have any hint for the driving force for $\Delta\Psi$-independent import of Om45-100. Perhaps interaction of Om45-100 with a component in the IMS (such as Om14, a partner protein for Om45) could trap the imported Om45. However, we are afraid that it is too early to discuss such mechanisms.

6. It will be very important to know whether Mim1 and Mim2 are involved in the topogenesis of Om45.

Since we were informed that Pfanner's group found that the complex formation of Om45 in the OM requires Mim1, we have now mentioned this by referring to their results in the manuscript submitted in parallel with ours instead of repeating the experiments they already did.

7. The authors should discuss the mechanism by which the Tim23 complex (plus membrane potential) triggers retrograde insertion of Om45 into the OM.

We think that the interaction with the TIM23 complex in a $\Delta\Psi$-dependent manner plays the role of pulling of Om45 across the OM. However, the TIM23 complex powered by $\Delta\Psi$ may not contribute to the insertion of Om45 into the OM from the IMS side. Insertion of Om45 into the OM may be facilitated by Mim1 as described above.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2nd Editorial Decision 11 March 2014

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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REFEREE REPORTS:

Referee #2:

After reading the rebuttal letter and the revised manuscript I came to the conclusion that the authors addressed all the concerns of the three reviewers. - The manuscript is now ready to be published.

Referee #3:

The paper is well revised and answers satisfactorily the points that reviewers have raised. I believe this is an important report providing new insight into the mechanism of mitochondrial outer membrane biogenesis.