Interaction of calmodulin with Sec61α limits Ca\(^{2+}\) leakage from the endoplasmic reticulum

Frank Erdmann, Nico Schäuble, Sven Lang, Martin Jung, Alf Honigmann, Mazen Ahmad, Johanna Dudek, Julia Benedix, Anke Harsman, Annika Kopp, Volkhard Helms, Adolfo Cavalié, Richard Wagner and Richard Zimmermann

Corresponding author: Richard Zimmermann, Saarland University

Review timeline:

- Submission date: 25 May 2010
- Editorial Decision: 28 June 2010
- Revision received: 26 September 2010
- Editorial Decision: 11 October 2010
- Revision received: 21 October 2010
- Accepted: 22 October 2010

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 28 June 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments to the authors are shown below. As you will see while referees 1 and 3 are more positive and would support publication of the study here after appropriate, but significant revision referee 2 raises major concerns regarding the conclusiveness of the electrophysiological data. On balance I have come to the conclusion that there is sufficient support from the referees that we will be able to consider a revised version of this manuscript. However, it will be indispensable to address referee 2’s concerns regarding the channel recordings in full and in a fully convincing manner. Also, stronger data on the direct interaction of CaM with endogenous Sec61α along the lines suggested by the referees is needed and the Sec61α siRNA data need to be controlled as put forward by referee 1 (and 2). The other issues raised by the referees need to be addressed or responded to as well in an adequate manner an to their satisfaction.

I should remind you that it is EMBO Journal 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 as well as on the final assessment by the referees.

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 initiative, please visit our website:
http://www.nature.com/emboj/about/process.html
Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFeree comments

Referee #1 (Remarks to the Author):

This manuscript by Erdmann and colleagues reports on a novel interaction between calmodulin (CaM) and a cytosolically facing IQ-motif in Sec61a. This interaction is proposed to be physiologically relevant based on electrophysiology data showing that CaM can block currents through the Sec61 channel, and in vivo Calcium imaging in cultured cells treated with siRNAs against Sec61. The results are used to argue that CaM is an important regulator of Calcium leak from the ER through the Sec61 channel.

The findings are clearly novel and could be of substantial interest to both the Calcium field and protein translocation field. Some aspects of the data are especially strong and convincing, including the identification of the IQ-motif, binding of the IQ-motif peptide to CaM in a Ca+2-dependent manner, and the electrophysiology analysis. If two less compelling (but very important) aspects of the manuscript are addressed as outlined below, this paper would make a nice contribution to the EMBO Journal. Some other minor issues are also noted.

1) While the interaction between CaM and the IQ-motif peptide from Sec61a is clear, the data showing a direct interaction between CaM and native Sec61a is not especially convincing. The Nycodenz gradient experiment in Fig. 2 with purified Sec61 proteoliposomes has several problems. It is unclear why Sec61/SecY migrates differently in the three panels, but this raises the issue of whether the fractionation is reliable and reproducible. Lane 2 in panel D looks overloaded, which is problematic because this is really the only lane that suggests a Ca+2-dependent interaction. Furthermore, the total amount of CaM seems different in panels D and E, again suggesting a potential loading artifact. And finally, the lack of clear Ca+2-dependence is puzzling given the data in Fig. 1 and Fig. 3. For all these reasons, I feel unambiguous data showing interaction between CaM and Sec61a is essential. I strongly suggest demonstrating this directly by crosslinking. There are multiple lysines in and near the IQ-motif, as well as on CaM, and it should be straightforward to crosslink the two using amine-reactive reagents. Showing this direct interaction on native ER microsomes would substantially strengthen the claim of an interaction.

2) The analysis in cultured cells is lacking a key control in which the siRNA knockdown cells are rescued with a Sec61a plasmid refractory to the siRNA. I strongly suggest this rescue experiment be performed with both wild type Sec61a and one with the IQ-motif mutated. The latter seems critical because otherwise, the observation could easily be an indirect effect: for example, Sec61 depletion might lead to defective insertion of some other protein involved in the observed effects.

Minor issues:

1) Are proteoliposomes reconstituted from total RM proteins immunodepleted of Sec61 defective in CaM binding? In other words, is this the only (or major) binding site for CaM?

2) IQ-motifs are typically Ca+2-independent in their binding to CaM. The authors may wish to discuss this given their proposed interaction is highly Ca+2-dependent.

3) Some of the Ca+2 and EGTA concentrations seem very high while others are quite low. The authors may wish to explain their choices and discuss in the text how this relates to normal Ca+2 concentrations in the cytosol.
Referee #2 (Remarks to the Author):

The authors address an interesting and important question, namely whether calmodulin regulates gating of the translocon. The quality of the work, particularly that relating to single-channel analyses, and is, however, insufficient to justify the conclusions.

Major

1. No data is presented to justify the claim (page 6) that calmodulin binding to the IQ peptide is via a single binding site. The authors need to present the Hill slopes of the curve fits.

2. The results shown in figure 2A provide no controls to indicate where GST calmodulin would migrate in the absence of microsomes in a sucrose gradient. This lack of control, together with the lack of effect of calcium on migration in sucrose gradients, challenge the conclusion that calmodulin associates with the Sec61 beta complex in these gradients. Why in, figure 2B, is the migration of GST calmodulin so erratic in the upper western blot? How many times were the experiment shown in figure 2 replicated?

3. The bilayer recordings are neither clear nor convincing. I can make little sense of figure 3A. Firstly the y axis appears to be incorrectly labelled. Are the authors suggesting that in the absence of calmodulin, the first open state has a current amplitude of about 25pA, the second about 30pA, and the third about 36pA? What do they suggest that these three open states mean? The shapes of the i-v curve in figure 3C is rather strange with very abrupt changes at about +/-10mV; how do the authors explain these observations? It looks as if there are 2 components to the i-V curves are looks like a large leak current with superimposed single channel openings. The most dramatic effect of calmodulin appears to be on the leak current. These observations would need to be much carefully interpreted to justify the present conclusion that calmodulin regulates ion transport through Sec61. Why was calmodulin added to both sides of the bilayer, surely it is expected to act only from the cytosolic surface?

4. Figures 3E and F are suggested to report the behaviour of single channels, but it seems far from clear what the single channel current amplitude is (point 3). I see no justification in fitting the lines shown in either figure. The data appear to provide no evidence for any voltage-dependent control of open probability.

5. Do the authors have reason to suppose that the purified Sec61 consistently orients into the bilayer in a single orientation? If not it seems surprising that calmodulin applied only in the trans-chamber is as effective as calmodulin applied to both chambers (figure 3H). Please comment. The authors appear not to have assessed the effects of calmodulin applied from the cis side and are therefore not justified in concluding that there is a side-specificity in the actions of calmodulin.

6. Calcium-calmodulin-dependent closure of the sec61 complex after dissociation of ribosomes appears not to provide a plausible mechanism to limit calcium influx from the ER. Although local leakage of calcium may provide a local calcium signal, as soon as the sec 61 complex closes, the calcium signal would dissipate, the calmodulin would dissociate, and the channel would open again. The authors should discuss their scheme more fully.

Minor

1. The introduction (page 4, bottom) includes examples of calcium channels regulated by calmodulin, but omits reference to voltage gated calcium channels w understanding is properly achieved the highest level of sophistication. Addition of appropriate reference (e.g. from David Yue, one such reference is cited elsewhere) seems advisable.

2. A wide readership of EMBO Journal may find the description of FCS measurements too technical. The methods, and particularly the abbreviations, need to be more carefully described.
3. The methods include too many undefined abbreviations. These make it difficult for the specialised reader, and probably almost impossible for the non-specialist reader, to understand the methods.

4. The authors report (page 15) using dog pancreas to prepare rough microsomes. Does the journal require a fuller description of these methods and a specific statement to indicate compliance with local animal use legislation?

5. Figures 1D and 1E would show the data more clearly if the X axes were either logarithmic or a split scale.

6. The authors should state how many times the experiment shown in figure 1F was replicated.

Referee #3 (Remarks to the Author):

The manuscript by Erdmann and co-workers identifies a novel role for calmodulin in preventing calcium leakage from the ER via the Sec61 translocon. The authors used bioinformatics to identify an "IQ" motif at the N-terminus of Sec61alpha and provide convincing evidence that this site can bind calmodulin in vitro. The binding appears to be dependent on calcium binding, although when Sec61 or ER membranes are used CaM is associated with the membrane fraction to some degree even in the absence of calcium. There is no association with the bacterial equivalent SecY, which lacks the IQ motif. The key experiment for me is that knocking down Sec61 can reverse a CaM agonist dependent calcium leakage identified by the authors. This provides strong correlative support for their hypothesis. Calmodulin binding does not appear to affect Sec61 dependent protein translocation, at least in vitro, and the authors provide a working model that is a very useful summary of their data in context.

The gating of the ER translocon has been a controversial area with segments of the Sec61alpha subunit, the ribosome and BiP all previously suggested to contribute to this process. This study provides convincing evidence that calmodulin may also contribute to the gating or regulation of the Sec61 complex, specifically with respect to the movement of small molecules across it.

Understanding the physiological significance and contribution of each of these components remains the long-term challenge.

Major comments.

I. The authors do not describe the effects of the Sec61alpha knock-down on the HeLa cells used for the study. One might imagine that a significant loss of Sec61 would be a problem - do they see cell death or any evidence of toxicity? There is also no indication of the concentration of siRNA that was used for the knock-downs.

II. I found the last experiment using preprolactin to look at the effect of CaM on ER translocation a bit convoluted, mainly because calcium has a direct effect on Sec61 translocation. The authors indicate they have seen such an effect before with lanthanum, and looking at this paper I find they used several different proteins to study ER translocation. A similar analysis of the effect of CaM binding on another Sec61 dependent precursor would strengthen the case.

Minor

P3 line2: should read as well as acting as..?

P3 line 6: almost a third of proteins ... Clarify this is based on an estimate of genes encoding this type of protein, and is not a measure of number or mass of actual polypeptides?

P3 line 19: how can the translocon be ribosome bound yet also ribosome depleted - I am not sure what this is supposed to mean?
P4 line 4: when the authors state the Sec61 complex comprises an "active" ion channel do they mean it uses energy to transport ions or that it simply functions as a (passive) channel. Clarify or rephrase.

P4 para 2: ER calcium is largely bound to high and low affinity sites on proteins - when then authors talk about calcium concentration do they mean total calcium or freely available calcium?

P13-14: I found the discussion was a little on the short side.

Figure 7: This could be supplementary. Worth including, but not in the main body of the text.

Answers to the comments of the referees:

Referee #1
1) We have replaced the Nycodenz gradients in Figure 2 with additional sucrose gradients and show the negative control that was requested by referee #2.

In addition, we carried out a flotation analysis after incubation of RM with GST-CaM in the presence of a CaM-antagonist. The observation was that the antagonist prevents the interaction of CaM with RM, demonstrating that a substrate interaction of CaM is involved. This is shown in new Figure 2D. We feel that this result nicely links our biochemical, biophysical and cell biological analyses.

As suggested, we also carried out an approach that directly demonstrates the interaction between CaM and native Sec61α. We employed the artificial protease strategy that is described in the revised manuscript. Here we observed direct interaction as shown in new Figure 2H. However, the interaction did not show the Ca²⁺-dependence that was observed in the CaM/IQ-peptide interaction and the sucrose gradients.

We suggest that binding of CaM to the IQ motif is Ca²⁺-dependent and that there is a second Ca²⁺-independent CaM binding site present in the Sec61 complex. Indeed, bioinformatic analysis detected a potential CaM binding site in Sec61γ that would reside in the cytosol (KPDRKEFQKIAMAT). According to our gradient data, CaM has lower affinity for this second site as compared to the IQ motif and, therefore, was not detected when free CaM was removed during flotation. This suggestion is consistent with the fact that there are Ca²⁺-dependent (myosin light chain kinase, constitutive nitric oxide synthase, connexin 43, TRPC4) and independent interactions of CaM (neuromodulin, neurogranin, unconventional myosins).

2) When we submitted the original manuscript we were convinced that seeing the effects with two different and independent siRNAs is sufficient to exclude indirect effects. However, we carried out the requested experiments and observed that expression of the SEC61A1 cDNA that lacks the UTR of the SEC61 gene in the presence of the SEC61A1–UTR siRNA indeed rescues the phenotype of SEC61 silencing (new Figure 6, A and B). Furthermore, we attempted the requested rescue experiment with an IQ mutant version of SEC61A1 cDNA and observed that it cannot rescue the phenotype of SEC61 silencing (new Figure 6, C and D). We feel that this result nicely wraps up the whole story.

Minor issues:
1) This is a very good but difficult experiment. For lack of time, we did not try.
2) We discussed this issue above and in the new Discussion.
3) Originally, the experiments were carried out at millimolar concentrations of CaCl₂. In order to get to physiologically more relevant concentrations, the crucial experiments were repeated at lower concentrations.

In case of the protein transport experiments, high concentration had to be employed in order to exceed the EGTA that is present in the lysate in order to inhibit the micrococcal nuclease that had been used in the pre-treatment of the lysate for the digestion of endogenous mRNAs.

Referee #2
1) The requested data were added.
2) The requested control was added as new Figure 2A. We also included a statement in the figure legend that gives the number of repeats (3 to 5).
3 and 4) Figure 3 was re-configured in order to meet the demands. We apologize for mislabeling the y axis of former Figure 3A. In addition, we have included a new panel to Supplementary Figure S2 that addresses the question of potential leak currents. Basically, we recorded single channel currents immediately after switching to the desired voltage and these currents reflected the multiple open states of the Sec61 channel, as previously described (Wirth et al., 2003). We controlled for leak after run down of the channels at the end of the experiment and the resistance of the bilayers was > 1 G Ohm in the presence and absence of CaM, indicating that CaM has no effect on the resistance of the bilayers.
5) We have reason to believe that Sec61 complexes are present in both orientations in proteoliposomes. Even so, the topology of the CaM effect was addressed in a series of experiments, where CaM was added to a single channel on one side. As expected, the inhibitory effect of CaM was not seen in all trials. When there was no effect CaM was added to the same channel on the other side and the inhibitory effect was observed. Therefore, we concluded that CaM acts only from one side. For lack of space we showed only one result from this series of experiments in the original manuscript. Since this point is not essential for the present manuscript and would take up too much space we omitted it from the revised manuscript.
6) The idea is that binding of Ca²⁺-CaM triggers a conformational change in Sec61α that is reversed in the absence of Ca²⁺-CaM. This results in another cycle of Ca²⁺-leakage and subsequent inhibition by Ca²⁺-CaM. This is explained in the new Discussion.
7) In addition, we carried out the electrophysiological analysis in the presence of a CaM-antagonist. The observation was that the antagonist prevents the inhibitory effect of CaM, demonstrating that a substrate interaction of CaM is involved. This is shown in new Figure 4. We feel that this result nicely links our biochemical, biophysical and cell biological analyses.

Minor issues.
1) The references were added.
2 and 3) The description was improved, but moved to the Supplement for lack of space.
4) This is the standard source in the protein transport field. In any case, we buy canine pancreas from a company in the US.
5) The figure was changed as requested.
6) We have added the requested information to the figure legend and give mean values in the corresponding text.

Referee #3
1) We have added a new Supplemental Figure S3 and the requested information to the Results section, documenting cell number and cell viability as measured by automated cell counting and Nuclear-ID cell viability assay, respectively.
2) The requested data were added as new Figure 7E and are discussed in the corresponding section of the Results.

Minor issues:
1) Line 2 was corrected
2) Line 3 was corrected.
3) We did not mean to suggest that this occurs simultaneously but alternatively and tried to rephrase accordingly.
4) No we did not mean an active mechanism in terms of energetics and tried to rephrase accordingly.
5) We made it clear that we are talking about freely available calcium.
6) We have added some beef to the Discussion.
7) We would prefer to keep Figure 7 (new Figure 9) but are prepared to take it out if you insist.
Thank you for sending us your revised manuscript. Our original referees 1 and 3 have now seen it again. Referee 2 was not available to look at the revised manuscript at this time. We have therefore involved a new referee, referee 4, to look at referee 2's report and the revisions made in response to it. Also, we asked the other referees to comment.

In general, the referees are now positive about publication of your paper. However, referee 1 (and referee 3) still express(es) concerns regarding the evidence provided for an interaction between CaM and native Sec61, and feels that further amendments will be required. Referee 4 has issues with figure 4 and recommends removing it from the manuscript. I would therefore like to ask you to deal with the issues raised in an amended version of the manuscript.

In addition, there is one remaining editorial issue that needs further attention. Prior to acceptance of every paper we perform a final check for figures containing lanes of gels that are assembled from cropped lanes. While cropping and pasting may be considered acceptable practices in some cases (please see Rossner and Yamada, JCB 166, 11-15, 2004) there needs to be a proper indication in all cases where such processing has been performed according to our editorial policies. Please note that it is our standard procedure when images appear like they have been pasted together without proper indication (like a white space or a black line between) to ask for the original scans. In the case of the present submission there is a panel that does not fully meet these requirements: Figure 2 H. I therefore like to kindly ask you to correct this figure along these lines within the amended manuscript. It would also be important to explain the in the figure legend that all lanes come from the same gel. Please be reminded that according to our editorial policies we also need to see the original scan for the panel in question. I am sorry to have to be insistent on this at this late stage. However, we feel that it is in your as well as in the interest of our readers to present high quality figures in the final version of the paper.

Thank you very much for your cooperation.

We are looking forward to receiving a suitably amended manuscript as soon as possible.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE COMMENTS

Referee #1 (Remarks to the Author):

In revising the manuscript, the authors have provided additional experiments regarding my two criticisms. The Sec61-knockdown was rescued with a wild type versus mutant Sec61, and a direct interaction between Sec61 and CaM was evaluated more thoroughly.

Of these two, the knockdown rescue experiment was clear and convincing. However, the binding experiments continue to be less than clear, and this is quite puzzling to me given the very high affinity between CaM and the IQ peptide. In the flotation experiments with RM and PKRM, the amount of floated CaM is very small and similar (albeit fainter) bands are visible in even the negative control samples. This is worrisome because subtle differences in blotting efficiency, exposure times, and so forth could easily explain such small differences. Yet, the authors provide no quantification or explanation. In the experiment with purified Sec61, flotation is much more efficient, but the profile of CaM doesn't match with that of Sec61 (are they not from the same samples or blot?) and a key control with SecY is not provided (the liposome control is less convincing since they are claiming a sequence-specific interaction). Similarly, the induced proteolysis experiment is not especially clear given the weak signal, the lack of Ca+2 dependence, and the lack again of a non-binding control with SecY.

I realize the authors have taken the criticisms seriously and done quite a bit of work to address them,
but clear evidence for an interaction between CaM and native Sec61 is lacking. While interaction with purified proteins or peptides is clearer, the very high promiscuity of CaM really requires better evidence of an interaction with Sec61 in the native context (e.g., in RM or in cells) to make a compelling case. Given the peptide binding and the biological effects, a direct physical interaction seems plausible, but not directly demonstrated (at least very convincingly).

Nonetheless, I do still feel this is an interesting study and will be of interest. As a compromise, perhaps they could at the least do two small things: (1) quantify the flotation experiments and provide some numbers with standard deviations to make their case, and (2) insert a caveat in the text that evidence for a direct interaction between CaM and Sec61 in its native context remains to be fully explored.

Referee #3 (Remarks to the Author):

The revised manuscript by Erdmann and co-workers provides convincing evidence that calmodulin plays a novel role in preventing calcium leakage from the ER via the Sec61 translocon. The authors have convincingly addressed all of the issues raised in my original review, and the rescue of the Sec61 siRNA depletions by a wild-type but not an IQ mutant version of Sec61 is a very strong addition to the work. The in vitro nature of the field as a whole has led to rather simplistic views of the ER translocon and the combination of biochemistry and cell biology used by the Authors provides good evidence for a physiologically relevant regulatory process that was previously unsuspected.

Minor comment.

Page 8: the authors state that GST-CaM floats with RMS in the presence and to a lesser extent in the absence of Calcium (Figs. 2B and 2C). From the images I have I agree there is association in the presence of Calcium but the levels seen in the absence of calcium do not appear to be above the background seen in the control (Fig2A) given the relative exposures. I would suggest the use of "perhaps" or "may" would be sensible here - this is a very minor detail.

Response to Referee 2.

I have been asked to comment on the Authors response to Referee 2. The authors provide convincing responses and additional data for points 1, 2, 5 and 6, and all of the minor issues raised. Points 3 and 4 relate to detailed technical aspects of the electrophysiology experiments - an area where I simply have no expertise and am unable to comment.

Referee #4 (Remarks to the Author):

This is a nice paper with significant message that address an unresolved problem in Ca2+. The authors adequately addressed most of the points raised in the review. The results in Fig. 5 are quite compelling, in particular with the added rescue experiments. However, the new results in Fig. 4 actually detract rather than add to the manuscript. The leak current is very large and OphA clearly increases the leak or worse affect the function of Sec61. I strongly recommend deleting this Fig. since it does not add much to the message. If the authors insist in including the Fig. they have to show the effect of OphA on bilayer resistance and control for the leak.

The discussion includes many speculations resulting in some interpretation of the results, especially the paragraph in page 17. Suggest revising/removing this speculation.

I would like to thank you and the referees very much for considering our manuscript for publication in The EMBO Journal and for giving us the opportunity to make some final amendments.
We have added statements to the legends to Figures 2 and 7E that point out that only the areas of interests are shown from single blots/gels, as requested. In addition, a white space was inserted into Figure 2H, as requested. Enclosed you will find three different exposures for the complete Western blot that is partially shown in Figure 2H.

Answers to the comments of the referees:

Referee #1
We have followed your advice and added the quantification of the gradients as well as the requested statement on pages 8 and 9, respectively. In addition, we have replaced Figure 2G (we note that we always showed the same blot).

Referee #3
We have followed your advice and added the requested “perhaps” to the Results section that is concerning Figure 2C.

Referee #4
We have removed Figure 4 (plus the corresponding statements from Introduction and Results) and the paragraph on page 17, as suggested.

Original gels

![Image of original gels]