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ATPase-driven oligomerization of RIG-I on RNA allows optimal activation of type-I interferon

Jenish R. Patel, Ankur Jain, Yi-ying Chou, Alina Baum, Taekjip Ha and Adolfo Garcia-Sastre

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(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 03 June 2013

Thank you for your patience during the peer-review of your study for EMBO reports. We have now received reports from the two referees that were asked to evaluate your study, which can be found at the end of this email. As you will see, both referees find the topic of interest and would consider a suitably revised version appropriate for publication in EMBO reports.

As you will see, referee 2 finds the claim of RIG-I oligomerization on dsRNA insufficiently supported by the data, but also suggests how to address this issue, whereas referee 1 highlights two experiments and points out some minor inaccuracies/overstatements. In view of these comments, I would like to give you the opportunity to revise your manuscript. If the referee concerns can be adequately addressed, we would be happy to accept your manuscript for publication. Please note that it is EMBO reports policy to undergo one round of revision only and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review.

Revised manuscripts must be submitted within three months of a request for revision unless previously discussed with the editor and be a maximum of 28,500 characters (including spaces). When submitting your revised manuscript, please also include editable TIFF or EPS-formatted figure files, a separate PDF file of any Supplementary information (in its final format) and a letter detailing your responses to the referees.

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I look forward to seeing a revised form of your manuscript when it is ready. In the meantime, do not hesitate to get in touch with me if I can be of any assistance or there are any questions/concerns regarding the revision.

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REFEREE REPORTS:

Referee #1:

In their manuscript, Patel et al. confirm and expand earlier findings on the activation of the innate immune receptor RIG-I. Through a series of biochemical analyses, the authors further investigate RIG-I oligomerization upon ligand binding. Their data demonstrate that the length of the 5' base-paired region in IVT-RNA defines the status of RIG-I oligomerization, which correlates with interferon induction. Furthermore, in vitro oligomerization of RIG-I was also dependent on the presence of hydrolyzable ATP. Authors propose a model of RIG-I activation in which, ATPase-activity drives RIG-I oligomerization on stimulatory RNA. Altogether, these data are interesting, well presented and relevant to the field.

Specific comments
1. The term Sendai virus "DI RNA" is confusing as it suggests that it was isolated from DI particles when in fact it is in vitro transcribed RNA using a DI sequence as the template. Any RNA sequence with a 5' triphosphate and a panhandle structure should in theory be capable of inducing the same response.
2. Although their data are compatible with previous reports on RIG-I tramslocation, the authors should refrain from referring to the RIG-I translocase activity when this has not been explicitly investigated.
3. The color scheme (such as the different colors on the DI "loop") should be explained.
4. In Figure 1B, the synthetic 5'ppp RNA is not stimulatory. It is unclear why that is. What is the source of this RNA and it is base-paired or not?
5. Authors demonstrate in Figure 4 that ATP is important in driving RIG-I oligomerization. Although the data are very clear and convincing, it could be strengthened by investigating the role of the RIG-I ATPase domain (although this may be complicated by the fact that RIG-I ATPase mutants such as the K858A-K861A do not appear to bind to stimulatory RNA).
6. Authors could also strengthen their data by determining whether the digestion of agonistic RNA following RIG-I oligomerization affect multimerization status.
7. Page 3: there is a typo in "requirements".
8. Page 3: "10 b" should be corrected.

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Referee #2:

Patel et al. demonstrate that RIG-I-mediated induction of type I IFN is dependent on the length of dsRNA in addition to the presence of 5' triphosphate by using sendai virus DI RNA as the model. RIG-I oligomerizes on the dsRNA in an ATPase activity dependent manner, and the oligomerization of RIG-I correlates with the strength of type I IFN response.

Overall, this study is novel and potentially interesting. The data are mostly convincing and the manuscript is concisely written. However, the scheme shown in Fig. 5C is still too speculative unless the authors visualize RIG-I multimer formation on the dsRNA. The reported translocase activity of RIG-I ATPase domain is bidirectional and might move back and forth, which is not clear if this fits with the story. Furthermore, RIG-I is also reported to harbor helicase activity in a manner dependent on ATP.
Further specific comments are required to be addressed.

Specific comments
1. The authors claim that oligomerization of RIG-I on a dsRNA is essential for strong type I IFN responses. Although the authors adjusted numbers of dsRNA for transfection, it is not appropriate to compare the length of dsRNA required for RIG-I activation. Thus, the authors should use same weight of dsRNAs with different lengths for inducing cellular activation. Since many reports have shown that relatively short (about 25 mer) dsRNAs with 5' triphosphate end are strong type I IFN inducer via RIG-I activation, it is not clear why this study is conflicted with the previous studies.

2. Based on EMSA data shown in Figures 3 and 4, the authors claim that RIG-I binding with 94 bp triphosphate dsRNA are origomeric. However, the blot requires dsRNA staining, since it is not clear if the band is truly a dsRNA-RIG-I complex.

1st Revision - authors' response 06 June 2013

Responses to referee comments are in blue.

Referee #1:

In their manuscript, Patel et al. confirm and expand earlier findings on the activation of the innate immune receptor RIG-I. Through a series of biochemical analyses, the authors further investigate RIG-I oligomerization upon ligand binding. Their data demonstrate that the length of the 5' base-paired region in IVT-RNA defines the status of RIG-I oligomerization, which correlates with interferon induction. Furthermore, in vitro oligomerization of RIG-I was also dependent on the presence of hydrolyzable ATP. Authors propose a model of RIG-I activation in which, ATPase-activity drives RIG-I oligomerization on stimulatory RNA. Altogether, these data are interesting, well presented and relevant to the field.

We thank the referee for appreciating the relevance of our findings.

Specific comments

1. The term Sendai virus "DI RNA" is confusing as it suggests that it was isolated from DI particles when in fact it is in vitro transcribed RNA using a DI sequence as the template. Any RNA sequence with a 5' triphosphate and a panhandle structure should in theory be capable of inducing the same response.

We agree that the term “DI RNA” can be confusing since the RNA was *in vitro* transcribed (IVT). Therefore, we now simply refer to the ligand as RNA or as IVT SeV DI RNA but also explain the nature of this RNA in the first paragraph of “results and discussion” section (page

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2). We wanted to use the sequence (and therefore the structure) of an RNA species that has been shown to be a genuine ligand for RIG-I during viral infection of cells, such as the Sendai DI RNA. However, we agree with the referee that any RNA sequence with the 5'-triphosphate and panhandle structures should induce the same response. Importantly, our data indicate that all panhandle 5'-triphosphate RNAs induce IFN-I but the level of induction depends on the length of the dsRNA stem of the panhandle.

2. Although their data are compatible with previous reports on RIG-I translocation, the authors should refrain from referring to the RIG-I translocase activity when this has not been explicitly investigated.

We have now checked the instances referring to the translocase activity and rather emphasize on the ATPase activity of RIG-I where necessary to be more consistent with the findings of this study.

3. The color scheme (such as the different colors on the DI "loop") should be explained.

We thank the referee for pointing this out as we missed to include an explanation for using the different colors. The colors on the RNA cartoons are based on the RNAfold structure prediction base-pairing probability gradient, where the red and purple colors form the extremes of low and high probabilities of base-pairing respectively, as indicated in Fig. 1A. This is now noted in the figure legend of Fig. 1.

4. In Figure 1B, the synthetic 5'ppp RNA is not stimulatory. It is unclear why that is. What is the source of this RNA and it is base-paired or not?

We apologize for not including pertinent information on the synthetic 5'-ppp RNA in Figure 1B. This 19-mer 5'-ppp dsRNA (Invivogen) induces IFN-I but requires very high amounts up to 2 μg to be transfected into the reporter cells for a detectable response. We find that this synthetic 5'ppp RNA is not stimulatory at the concentration tested (Fig. 1B). We have now clarified in the text that we have used a low amount of RNA (50 ng) to compare the different RIG-I substrates.

5. Authors demonstrate in Figure 4 that ATP is important in driving RIG-I oligomerization. Although the data are very clear and convincing, it could be strengthened by investigating the role of the RIG-I ATPase domain (although this may be complicated by the fact that RIG-I ATPase mutants such as the K858A-K861A do not appear to bind to stimulatory RNA).

We thank the referee for suggesting this important experiment. We have now included data showing that a motif II (DECH/ATPase domain) mutant of RIG-I, D372A is not able to form high molecular weight oligomers on RNA like the WT RIG-I in presence of ATP (Fig. 4H), confirming that ATP hydrolysis is indeed required for oligomerization. Furthermore, we show that like WT RIG-I, the D372N RIG-I is also capable of binding to RNA in an indirect ELISA based binding assay (Fig. S3A), in accordance with the minimal binding unit observed in native PAGE experiment (Fig. 4H). We also confirm that this mutant does not show ATP hydrolysis activity in presence of RNA using a colorimetric ATPase assay (Fig. S3C). In addition, in the revised Fig. 5B, we show that substituting D372N for WT RIG-I prevents binding of additional units of RIG-I on existing RIG-I-RNA complexes in presence of ATP, confirming that ATP hydrolysis by existing RIG-I allows recruitment of new RIG-I on RNA.

6. Authors could also strengthen their data by determining whether the digestion of agonistic RNA following RIG-I oligomerization affect multimerization status.

We thank the referee for suggesting this interesting experiment. We find that post-treatment with RNase V1, which digests dsRNA, disrupted ATP-driven multimers of RIG-I on RNA, suggesting that the oligomers are transient and dynamic *in vitro*, which allows enough exposure of the dsRNA stem for endonuclease digestion (Fig. 4I).

7. Page 3: there is a typo in "requirements".

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We thank the referee for pointing out this overlooked error. We have corrected the typo on page 3.

8. Page 3: "10 b" should be corrected.

We have corrected this to make it clear.

Referee #2:

Patel et al. demonstrate that RIG-I-mediated induction of type I IFN is dependent on the length of dsRNA in addition to the presence of 5' triphosphate by using sendai virus DI RNA as the model. RIG-I oligomerizes on the dsRNA in an ATPase activity dependent manner, and the oligomerization of RIG-I correlates with the strength of type I IFN response.

Overall, this study is novel and potentially interesting. The data are mostly convincing and the manuscript is concisely written. However, the scheme shown in Fig. 5C is still too speculative unless the authors visualize RIG-I multimer formation on the dsRNA. The reported translocase activity of RIG-I ATPase domain is bidirectional and might move back and forth, which is not clear if this fits with the story. Furthermore, RIG-I is also reported to harbor helicase activity in a manner dependent on ATP.
Further specific comments are required to be addressed.

We thank the referee for appreciating the significance and novelty of this study. We agree that Fig. 5C is in part speculative and therefore in the figure legend for new Fig. 5 and revised discussion on page 5, we have indicated so. The proposed model is based on the previously shown ATP-dependent translocase activity of RIG-I [1] and the data shown in this study on ATP-dependent oligomerization of RIG-I. We speculate that although a single RIG-I molecule on RNA is able to translocate in a bi-directional manner, loading of multiple RIG-I molecules on RNA may limit the bi-directional translocating movement as more molecules accumulate on the RNA and therefore the result can still be formation of an oligomer. The referee is correct in saying that RIG-I has been shown to possess helicase activity. However, the helicase activity was shown only in presence of 3'-overhang RNAs [2], which are not optimal inducers of RIG-I [3]. Furthermore, Myong et al. were not able to detect any helicase activity in FRET-based assays [1]. We agree with the referee that more mechanistic studies on how the translocase, helicase and ATPase contribute to RIG-I activation are needed to answer these questions, which are beyond the scope of this report.

Specific comments
1. The authors claim that oligomerization of RIG-I on a dsRNA is essential for strong type I IFN responses. Although the authors adjusted numbers of dsRNA for transfection, it is not appropriate to compare the length of dsRNA required for RIG-I activation. Thus, the authors should use same weight of dsRNAs with different lengths for inducing cellular activation. Since many reports have shown that relatively short (about 25 mer) dsRNAs with 5' triphosphate end are strong type I IFN inducer via RIG-I activation, it is not clear why this study is conflicted with the previous studies.

In view of this comment regarding Fig. 3A, we have replaced the data with an experiment using same weight of RNAs as opposed to same number of molecules. We see the same trend in IFN-I induction (revised Fig. 3A). The 94 bp wild-type RNA induces highest level of IFN-I activation, while the 46 bp and 25 bp RNAs show lower levels of IFN-I activation in comparison. We would like to emphasize that the WT and stem truncation RNAs still retain the 358 b loop, which forms the majority of weight on these RNAs.

We agree with the referee in that many studies have found small dsRNAs with 5'-ppp to be good inducers of IFN-I, however these studies have not compared different lengths of RNAs for IFN-I induction. The 25-bp RNA also induces IFN-I (Fig. 3A). However, the longer length 46-bp RNA and the 94-bp WT RNA are more potent inducers of IFN-I, based on higher IFN-I reporter

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activation. Thus, our data indicate that the length of dsRNA stem dictates the level of IFN-I induction. This data is in agreement with a study by Binder et al., where they also demonstrate dsRNA length-dependent activation of IFN-I although they do not test oligomerization of RIG-I on different lengths of RNA nor determine the requirements for oligomerization [4].

2. Based on EMSA data shown in Figures 3 and 4, the authors claim that RIG-I binding with 94 bp triphosphate dsRNA are oligomeric. However, the blot requires dsRNA staining, since it is not clear if the band is truly a dsRNA-RIG-I complex.

We thank the referee for this comment as it indicated to us that we did not emphasize well in our previous version the data on RNA staining that was included in Fig S3. In this figure, we used biotin-labeled RNA in the RIG-I oligomerization reactions and performed native PAGE followed by a biotin immunoblot to visualize the RNA in the native PAGE separated complexes. By performing an immunoblot for RIG-I in parallel, we found that the RNA was indeed present at the minimal as well as oligomeric high molecular weight complexes of RIG-I. To better illustrate these data we have moved our previous Fig. S3A to new Fig. 4D. In addition, we also found that digesting dsRNA with RNase V1 after RIG-I oligomerization results in loss of the RIG-I oligomeric complexes (please see response to point no. 6 of referee 1).

References:

1. Myong S et al. (2009) Cytosolic viral sensor RIG-I is a 5′-triphosphate-dependent translocase on double-stranded RNA. Science 323:1070-4.
2. Takahasi K et al. (2008) Nonself RNA-sensing mechanism of RIG-I helicase and activation of antiviral immune responses. *Mol Cell* 29:428-40.
3. Schlee M et al. (2009) Recognition of 5′ triphosphate by RIG-I helicase requires short blunt double-stranded RNA as contained in panhandle of negative-strand virus. *Immunity* 31:25-34.
4. Binder M et al. (2011) Molecular mechanism of signal perception and integration by the innate immune sensor retinoic acid-inducible gene-I (RIG-I). *J Biol Chem* 286:27278-87.

2nd Editorial Decision 18 June 2013

Thank you for your patience while we have reviewed your revised manuscript. As you will see from the reports below, the referees are now all positive about the publication of your study in EMBO reports. although referee 1 notes that several of his/her previous minor comments have not been addressed and requests that this be done.

I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once the reiterated concerns of referee 1 and other minor issues have been attended to, as follows.

I have noticed that your study seems to contain no statistical information as to how the data was obtained and analyzed, although error bars are displayed in figures 1B,C,D 2A,B,C,D, 3A,B,C, 5B, and supplementary figures 2B,C,D and 3A,C. Please go through your manuscript carefully once more and ensure that all relevant figures and supplementary figures have been generated according to proper statistical analysis procedures, and all figure legends include information on the number of independent experiments analyzed, the type of error bars used and statistical test applied to the data (in Fig. 5B).

The material and methods section is quite succinct. Basic Materials and Methods required for understanding the experiments performed must remain in the main text, although additional detailed information may be included as Supplementary Material. Given the size of the final figures, I think your text could be expanded to a maximum of 30,000 characters (including spaces) to accommodate any changes to the text you deem necessary.

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As a standard procedure, we edit the title and abstract of manuscripts to make them more accessible to a general readership. Please find an edited version of your abstract (which already deals with a couple of outstanding concerns of reviewer 1) at the end of this email, and let me know if you do NOT agree with any of the changes.

Lastly, we encourage the publication of original source data -particularly for electrophoretic gels and blots- with the aim of making primary data more accessible and transparent to the reader. If you agree, you would need to provide one PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figures. The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files and should be uploaded when you submit your final version. If you have any questions regarding this please contact me.

After all remaining corrections have been attended to, you will receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports.

REFEREE REPORTS:

Referee #1:

Patel et al. have addressed the reviewers' comments and supported their findings with additional data. A few minor details still need to be corrected in the text, including:

1. In the abstract: the authors should refrain from referring to the RIG-I translocase activity when this has not been explicitly investigated, as previously pointed out.
2. In the abstract: the use of "Sendai virus DI RNA as a model ligand" is misleading as they have used in vitro transcribed RNA, as previously pointed out. It should be rephrased.
3. In the Results & Discussion 1st paragraph: the fact that any RNA sequence with a 5' triphosphate and a panhandle structure should in theory be capable of inducing the same response as SeV DI IVT-RNA should be clearly stated, as was previously pointed out.
4. In the Results & Discussion 1st paragraph: the sentence "We found that these mutants... because these RNAs do not bind..." should be rephrased as the data does not reflect causality but correlation.
5. In the Results & Discussion 1st paragraph: there is a typo in "requirements", as previously pointed out.

Referee #2:

The revised manuscript is substantially improved. Now this paper is ready for publication.

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Edited abstract

The cytosolic pathogen sensor RIG-I is activated by RNAs with exposed 5'-triphosphate (5'-ppp) and terminal double-stranded structures, such as those that are generated during viral infection. RIG-I has been shown to translocate on dsRNA in an ATP-dependent manner. However, the precise role of the ATPase activity in RIG-I activation remains unclear. Using in vitro-transcribed Sendai virus defective interfering RNA as a model ligand, we show that RIG-I oligomerizes on 5'-ppp dsRNA in an ATP hydrolysis-dependent and dsRNA length-dependent manner, which correlates with the

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strength of type-I interferon (IFN-I) activation. These results establish a clear role for the ligand-induced ATPase activity of RIG-I in the stimulation of the IFN response.
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2nd Revision - authors' response 18 June 2013

This is regarding our manuscript titled, "ATPase-driven oligomerization of RIG-I on RNA allows optimal activation of type-I interferon" by Jenish R. Patel, Ankur Jain, Yi-ying Chou, Alina Baum, Taekjip Ha, and Adolfo García-Sastre submitted to *EMBO Reports* (EMBOR-2013-37536V2).
We thank the editor and the referees for a positive review of the manuscript and we are happy to hear that our revised manuscript will be accepted for publication in *EMBO Reports*.
In the revised version of the manuscript included, we have now added statistical information on the experiments and data where appropriate in the figure legends of main and supplemental figures. We have also corrected the text as suggested by referee 1 (please see page 2 of manuscript file). The edited abstract reads well, and we have included it in the revised manuscript. As requested, we have added more details in the methods section. We have also included annotated original scans as source data for the key figures.
Please let us know if you require further information.

3rd Editorial Decision 20 June 2013

In going through your study before final acceptance, I am afraid one last issue has come up. In the legend of Sup Fig 3, the number of times the experiments were performed has not been included. Importantly, in all other instances, experiments have been performed 2-3 times or only 2 independent times in the case of figure 5.

However, from a mathematical point of view, it is incorrect to calculate errors if an experiment has been performed less than three independent times. Please refer to Cumming et al. JCB 2007 and Vaux DL et al EMBO rep, 2012 for guidance. As this seems to affect most of the data in the manuscript, I feel it would considerably weaken the study to simply take the errors bars and statistical testing out. Rather, the number of independent experiments would have to be increased to be able to provide proper statistical analysis. When your manuscript is ready, please upload the files through our online system.

I look forward to receiving the final version of your study when it is ready.

3rd Revision - authors' response 21 June 2013

This is regarding our manuscript titled, "ATPase-driven oligomerization of RIG-I on RNA allows optimal activation of type-I interferon" by Jenish R. Patel, Ankur Jain, Yi-ying Chou, Alina Baum, Taekjip Ha, and Adolfo García-Sastre submitted to *EMBO Reports* (EMBOR-2013-37536V3).
We regret that there was an issue with some of the statistical analyses. We would like to point out that representative data shown in all figures have error-bars calculated from at least 3 independent samples from experiments done at different times. Our replicates are not readings of the same samples but independently setup reactions. Due to the nature of *in vitro* experiments, we thought they could be regarded as independent samples for the purpose of statistical analyses. However, in case there is still an issue, we have included data from

additional experiments for figure 1D and the ATPase assays in figures 2C and 3C, where previously the data represented independent samples obtained from experiments performed only two independent times. We have also added new data to figure 5B and recalculated the error-bars and statistical significance tests. Lastly, we have added the missing information in the legend of supplemental figure 3. Importantly, we find that adding new data has not changed any of our conclusions.
We hope that relieves your concerns. Please let us know if you require further information.

4th Editorial Decision 24 June 2013

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.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

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