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Coordination of multiple enzyme activities by a single PCNA in archaeal Okazaki fragment maturation

Thomas Beattie, Stephen Bell

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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 21 October 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. We have now received the reports from three expert reviewers, which are copied below. Given that at least two of these reviewers are generally supportive of publication pending satisfactory revision, I would like to give you the opportunity to address the various issues and comments of all three referees by way of a revised manuscript. In this respect, please be reminded that it is our policy to allow a single round of major revision only, and that it will therefore be important to diligently and comprehensively answer to all the specific points raised at this stage in the process. Should you have any questions or concerns with regard to the referee requests, please do not hesitate to get back to me for further consultations.

We generally allow three months as standard revision time, and it is our policy that competing manuscripts published during this period will have no negative impact on our final assessment of your revised 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.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

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

Yours sincerely,
Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Beattie and Bell reconstituted lagging strand DNA synthesis with purified recombinant proteins from Sulfolobus solfataricus. The interesting fact is their original finding in 2003 (Dionne et al. Mol Cell) that the PCNA is a heterotrimer in which the three different PCNA parts interact with the DNA polymerase, the Fen1 endonuclease and DNA ligase, respectively. In this paper they now give more data on the way these proteins work together to synthesize an Okazaki fragment.

There are some problems that are inherent in the data presented:

1. Only activity data are presented, what about the order of the interactions of the DNA polymerase, Fen1 and Ligase. Pull down or IP experiments could give more insight.

2. All the components must be titrated in detail.

3. No quantifications are shown. Statements such as "significant reduction or increase" are not very meaningful. The data should be quantified.

4. The data with SSB are negative and can be omitted.

5. Moreover, it is difficult to see the Dpo4 in connection of the coordination of the Okazaki fragment protein machinery.

In summary the data presented here do not add a significant amount of novelty in our understanding how the lagging strand is coordinated. The model system of Sulfolobus solfataricus is excellent to give insight in the coordination of these enzymes and the fact that post translational modifications are not necessary would make it as an attractive model to see mechanistic details.

Referee #2 (Remarks to the Author):

The authors of this manuscript reconstitute the Okazaki fragment maturation process in Archea. This has previously been accomplished in other model organisms, but Archea offers a unique opportunity in that the clamp is composed of three different subunits. The subunits differ in the preference for either the replicative DNA polymerase, PolB1, the flap-endonuclease, Fen1 or the ligase, Lig1. The authors link the three subunits into a single polypeptide, thereby excluding the possibility that the purified clamp may have different composition (stoichiometry of the subunits). After this they successfully demonstrate the dependence of PolB1, Fen1 and Lig1 in filling a short gap, removing a downstream RNA primer and sealing the final nick with Lig1. They demonstrate the dependence of the PIP-box in Fen1 and Lig1. They also show that specific mutations in the hydrophobic pocket of the individual subunits of the clamp, specifically lower the efficiency of either PolB1, Fen1 or Lig1. Mixing of all mutants cannot rescue the deficiency supporting the model that PolB1, Fen1 and Lig1 must functionally interact with the same clamp during the maturation process.
This last observation is of great value since it has long been discussed if the polymerase, the flap-endonuclease and the ligase simultaneously bind to the same clamp. This manuscript provides direct evidence for this to be the case in Archea.

Specific points:

1. The authors frequently use the term "client protein" instead of naming the three proteins, PolB1, Fen1 and Lig1. I am not thrilled over that all proteins that associate with PCNA are referred to as client proteins. I would instead like to see that the actual proteins are listed, since they are only three (plus Dpo4 at the end of the manuscript). This is important for clarity throughout the manuscript and in particular for the clarity of the abstract.

2. The authors claim in the first paragraph of the Results that the single polypeptide version of PCNA is fully functional. I agree that it seems to be fully functional in the biochemical assay. However, the manuscript would be improved if the authors clarify whether the single polypeptide version is fully functional in vivo.

3. Page 6, last paragraph, the importance of the PIP-box in Fen1 is discussed and there are two references listed. I think that it would be fair to also add Gomes and Burgers (2000) Two modes of FEN1 binding to PCNA regulated by DNA, EMBO J. 19(14):3811-21, where it was first shown that the PIP-box was required for the PCNA directed activity of Fen1.

4. Page 7, first paragraph, The authors compare the cleavage products from the coupled activity of PolB1 and Fen1, with previously reported studies (Garg et al (2004), Rossi and Bambara(2006)). It would add strength to manuscript if the authors also addressed if PolB1 is able to idle when encountering the downstream primer. As shown by Garg et al 2004, not all polymerases have this ability which enhances the efficiency of the ligase reaction during the final step in the maturation process.

5. This is also relevant when challenging Fen1 with Dpo4 in the last experiment on page 12. Here the authors replace PolB1 with Dpo4 in the maturation assay and ask if Dpo4 also have a functional interaction with Fen1. This was not the case and it was concluded that this was due to competition between Dpo4 and Fen1 for the same subunit of the clamp. This may be the case, but other experiments in the manuscript do not convincingly show that Fen1 exclusively interact with the preferred subunit. There could be less efficient interactions with other subunits of the clamp if the preferred subunit is not accessible. What would the result be if PolB1 can idle (presenting an optimal substrate for Fen1) and Dpo4 cannot idle? Comparing figure 1B (lane 11) and figure 6B (lane 9), it appears that Fen1 in the presence of PolB1 efficiently process the downstream RNA-primer, while Fen1 in the presence of Dpo4 is almost unable to process the downstream primer.

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The manuscript by Beattie and Bell describes a biochemical study on the processing of Okazaki fragments in the crenarcheon Sulfolobus solfataricus. This is of general interest as DNA replication proteins and the structure of Okazaki fragments in Archaea are closely related to their counterparts in eukaryotic systems, but little is currently known regarding the maturation of Okazaki fragments in the third domain of life. This study also addresses the question how multiple interaction partners of replication clamps form temporally and spatially controlled protein complexes during DNA replication and repair processes. The latter problematic is currently actively pursued in many different experimental systems. The heterotrimeric replication clamp that carries specialized interaction sites for its distinct interaction partners provides a particularly well suited unique tool for addressing the coordination of DNA replication and repair by the replication clamp.
The experimental results presented in this well executed study provide strong biochemical support for the central conclusion that six proteins (PCNA1-3, DNA polymerase, Fen1 and Dna Ligase) are necessary and sufficient for coupled DNA synthesis, RNA primer removal and DNA ligation, thus revealing an evolutionary conserved core pathway for Okazaki fragment maturation in Archaea and Eukarya. This work also suggests that Okazaki fragments with "long" flaps, which in Eukarya are processed via the Dna2 dependent pathway, are not formed during archaeal DNA replication. Moreover, mixing experiments using mutant proteins and competing DNA polymerase Dpo4 indicate that a single replication clamp molecule is necessary for efficient coordination of the activities of all three client proteins, and that efficient coordination is achieved only when different proteins can simultaneously access PCNA. As the observed differences in experiments performed with mutant proteins are often quite small (2-3 fold), special consideration has been given to

ensuring the statistical significance of the data presented. Overall, these results provide an excellent example how fine-tuning specific interactions with the replication clamp can increase efficiency and specificity of DNA replication/repair processes.

Major comments:
1. To simplify experimental procedures, the work reported here was performed using a synthetic PCNA protein, where the three subunits are fused in a single polypeptide. No references or data are given to indicate whether all the individual subunits are actually expressed during the S-phase in this organism. It remains also unclear how stable the naturally occurring heterotrimeric replication clamp (i.e. the non-fused complex) would be under the experimental conditions used for the reconstitution experiments.

2. This work is based on the assumption that archaeal DNA ligases are not able to ligate substrates that contain ribonucleotides on the 3' side of the nick, which has been observed with some ATP-dependent DNA ligases in the past. No information is provided whether archaeal DNA ligases carry domains that have evolved to discriminate against RNA and/or DNA substrates. A useful control experiment would be to demonstrate to what extent an Sso DNA ligase is able to ligate the Okazaki fragment mimic used in these experiments to a DNA strand.

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1st Revision - authors' response 19 December 2011

Response to Referees:

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There are some problems that are inherent in the data presented:

1. *Only activity data are presented, what about the order of the interactions of the DNA polymerase, Fen1 and Ligase. Pull down or IP experiments could give more insight.*

We are unclear what the referee is asking for. We present pull down data in Supplementary figure 2 and have previously shown further interaction data (Dionne et al, 2003)

2. *All the components must be titrated in detail.*

The referee correctly surmises that extensive titrations were performed in our preliminary experiments to establish the reconstituted system. We have included some examples as a new Supplementary figure 1.

3. *No quantifications are shown. Statements such as "significant reduction or increase" are not very meaningful. The data should be quantified*.

Our apologies for using these descriptive phrases. We have replaced them with more quantitative statements.

4. *The data with SSB are negative and can be omitted.*

As neither of the other 2 referees requested these data be removed we would prefer to leave them in the manuscript.

5. *Moreover, it is difficult to see the Dpo4 in connection of the coordination of the Okazaki fragment protein machinery.*

Our apologies for not making this point clear to the referee. We have amended the introduction to this section on Page 13 to emphasize the following points. At no point do we claim Dpo4 is involved in coordinating lagging strand synthesis. Rather we employ Dpo4 as a tool to test the effect of competition between Fen1 and Dpo4 in accessing a single PCNA subunit's IDCL (both proteins bind the PCNA 1 subunit – Dionne et al, 2003; Dionne et al, 2008). Indeed the competition that we observe between these factors may have considerable physiological relevance – in preventing excessive DNA synthesis being driven by Dpo4 by inhibiting strand displacement synthesis – a point that we have commented upon at the end of this section.

In summary the data presented here do not add a significant amount of novelty in our understanding how the lagging strand is coordinated. The model system of Sulfolobus solfataricus is excellent to give insight in the coordination of these enzymes and the fact that post translational modifications are not necessary would make it as an attractive model to see mechanistic details.

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Mixing of all mutants cannot rescue the deficiency supporting the model that PolB1, Fen1 and Lig1

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We apologise for the lack of clarity and have specifically named the relevant proteins in the revised version.

2. *The authors claim in the first paragraph of the Results that the single polypeptide version of PCNA is fully functional. I agree that it seems to be fully functional in the biochemical assay. However, the manuscript would be improved if the authors clarify whether the single polypeptide version is fully functional in vivo.*

This would be a wonderful experiment however it is technically impossible at this point given the very limited tools for genetic manipulation of Sulfolobus. We also note that had it been possible to do the experiment and we had found that Sulfolobus was not viable when the only PCNA was the fusion version, we could not conclude that that was as a consequence of its role in Okazaki fragment maturation. We and others have shown that Sulfolobus PCNA, like the eukaryal protein, plays roles in DNA repair processes in addition to replication. To partly address the referee's concern we have included further biochemical data where we compare the activities of the fusion with the reconstituted heterotrimer in Supplementary figure 4.

3. *Page 6, last paragraph, the importance of the PIP-box in Fen1 is discussed and there are two references listed. I think that it would be fair to also add Gomes and Burgers (2000) Two modes of FEN1 binding to PCNA regulated by DNA, EMBO J. 19(14):3811-21, where it was first shown that the PIP-box was required for the PCNA directed activity of Fen1.*

Our apologies for omitting the reference to this important study. We have added it to the revised version.

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Many thanks indeed for this excellent suggestion. We have performed the suggested experiment and included the data in the revised manuscript as Supplementary figure 3. We find that PolB1 does indeed idle at the downstream primer and that this idling is significantly reduced in the presence of Fen1. Interestingly, the level of idling that we see is far less (almost 3 orders of magnitude) than that observed by Garg and colleagues. We comment on this in the revised manuscript.

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Dpo4 is almost unable to process the downstream primer.

Our previous biochemical and structural data has demonstrated the interaction of Fen1 to be highly specific to the PCNA1 subunit (Dionne et al, 2003 and Dore et al, 2006) and therefore we think it unlikely that interactions with other regions of PCNA could support stimulation of Fen1 activity.

Dpo4 is indeed unable to idle, however given that PolB1 idling activity itself is very inefficient, together with our demonstration that both polymerases pause at very similar sites several nucleotides into the downstream primer (Figures 2B and 7B), we propose that both polymerases are capable of generating very similar flap structures suitable for Fen1 activity. We therefore argue that it is the ability to coordinate simultaneously around PCNA that determines coupling of polymerase and Fen1 activities. We have included a discussion of these issues on page 14.

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Moreover, mixing experiments using mutant proteins and competing DNA polymerase Dpo4 indicate that a single replication clamp molecule is necessary for efficient coordination of the activities of all three client proteins, and that efficient coordination is achieved only when different proteins can simultaneously access PCNA. As the observed differences in experiments performed with mutant proteins are often quite s
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(2-3 fold), special consideration has been given to ensuring the statistical significance of the data presented. Overall, these results provide an excellent example how fine-tuning specific interactions with the replication clamp can increase efficiency and specificity of DNA replication/repair processes.
Major comments:

1. *To simplify experimental procedures, the work reported here was performed using a synthetic PCNA protein, where the three subunits are fused in a single polypeptide. No references or data are given to indicate whether all the individual subunits are actually expressed during the S-phase in this organism. It remains also unclear how stable the naturally occurring heterotrimeric replication clamp (i.e. the non-fused complex) would be under the experimental conditions used for the reconstitution experiments.*

Before addressing these important points, we would like to emphasize that we used the fused heterotrimeric PCNA in these assays to ensure that subunit exchange could not be occurring between heterotrimers. This is of pivotal importance in the experiments where we mutate the IDCL in individual interfaces.

We have addressed these concerns as follows. First we performed a synchronization experiment and analysed PCNA subunit expression during S phase by western blotting. We confirm that all three subunits are indeed expressed in S-phase This data has been added as a new Figure 1. Regarding the stability of the reconstituted heterotrimer we have added explicit reference to the work of

Ellenberger and colleagues (Pascal Mol. Cell, 2006) who showed that it is possible to purify the heterotrimeric assembly by virtue of its ability to withstand 85 °C for 25 minutes. In contrast, the individual PCNA 1 and PCNA3 subunits are denatured under these conditions. Finally, we use heterotrimeric PCNA assembled from individual PCNA1, PCNA2 and PCNA3 in the maturation assay and reveal that is active in the assay (Supplementary figure 4).

2. *This work is based on the assumption that archaeal DNA ligases are not able to ligate substrates that contain ribonucleotides on the 3' side of the nick, which has been observed with some ATP-dependent DNA ligases in the past. No information is provided whether archaeal DNA ligases carry domains that have evolved to discriminate against RNA and/or DNA substrates. A useful control experiment would be to demonstrate to what extent an Sso DNA ligase is able to ligate the Okazaki fragment mimic used in these experiments to a DNA strand.*

We have added this important control to our manuscript (new Figure 4B) and confirm that Sulfolobus Lig1 is indeed incapable of ligating DNA to RNA.

3. *In the Figure 1C, relatively weak degradation of the substrate is observed in the absence of Fen1, PCNA and PolB1, which has not been sufficiently described in the text. Was this background subtracted during the quantitative data analyses?*

The background signal was subtracted during data analysis and the figure legend has been revised to clarify this fact.

4. *Including a schematic model for the processing of Okazaki fragments in archaeal systems would help a general audience to appreciate the uniqueness of the obtained results more clearly.*

Thanks for this suggestion, we have added a model as a new Figure 8 to the manuscript.

Minor comments:

1. *Figure 1 would be easier to understand if the position of the radioactive label within the marked oligonucleotide would be indicated.*

We have added this information

2. *More details could be given to describe the strand displacement activity of Sso DNA polymerases. Has this activity been described prior to this study?*

We are not aware of any study that has described this activity previously for Sulfolobus PolB1. We have added a statement to this effect on Page 6.

3. *The use of the abbreviation "PIP" in many figures is somewhat confusing. In particular, without referring to the main text, it is often unclear whether "PIP" refers to a functional or non-functional PIP-motif. This should be better clarified.*

Our apologies for not clarifying this issue – we have amended the figure legends accordingly.

4. *It is not clear why the so-called double flap substrate, not present in the Okazaki fragment mimic, was used in the figure 5B for Fen1 activity tests.*

The double flap substrate has previously been described as the optimal substrate for Fen1 activity (Kao et al, 2002). Therefore, in these control experiments which simply required a sensitive measurement of Fen1 activity, rather than a direct relevance to Okazaki fragment maturation, we used the best substrate available. We have revised the main text and figure legend to clarify this.

Acceptance letter 09 January 2012

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by two of the original referees (see comments below), and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

You shall receive a formal letter of acceptance shortly.

Yours sincerely,

Editor
The EMBO Journal

Referee #2

(Remarks to the Author)
I am satisfied with the author's responses to my specific criticism.

Referee #3

(Remarks to the Author)
By adding new data and addressing previous critic, the authors have improved the manuscript on reconstitution of lagging strand DNA synthesis with purified archaeal proteins. This work has provided strong biochemical evidence for the fact that the polymerase, the flap-endonuclease and the ligase simultaneously bind to the same clamp molecule thus coordinating the action of individual proteins. Considering the uniqueness and clarity of the presented results, this manuscript is now suitable for publication.

