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The EMBO Journal

Manuscript EMBO-2015-91520

Structural basis for processivity and antiviral drug toxicity in human mitochondrial DNA replicase

Michal R. Szymanski, Vladmir B. Kuznetsov, Christie Shumate, Qingchao Meng, Young-Sam Lee, Gayatri Patel, Smita Patel and Y. Whitney Yin

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Editor: Hartmut Vodermaier

Transaction Report:

(Nota: 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 10 April 2015

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below. As the referees generally appreciate the importance and potential interest of the presented work and its results, we would in principle be happy to consider a revised manuscript further for publication, pending satisfactory revision of the concerns raised in all three reports. As you will see, the majority of these point relates to issues of improved presentation and discussion, but there are also requests for a limited number of further experimentation (see esp. referee 2), which I would like to ask you to address in order to strengthen the appeal and impact of this work.

I would therefore like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

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REFEREE REPORTS:

Referee #1:

General Summary: This manuscript describes crystal structures of the catalytic and accessory subunits of human DNA polymerase gamma bound to a template-primer and with either dCTP or ddCTP at the polymerase active site. When compared to the previously published structure of the polymerase without DNA or a dNTP, these "ternary" complexes provide novel and important information needed to deduce conformational changes and protein-protein and protein-substrate interactions that are likely to be relevant to correct and highly processive DNA synthesis.

Opinion. Mutations in the genes encoding the two mitochondrial replication proteins studied here are responsible for several human diseases, a fact almost certainly related to mitochondrial dysfunction stemming from defects in processive mitochondrial DNA replication. The toxicity associated with treatment of HIV-1 with ddC is due to mitochondrial dysfunction resulting from chain termination due to efficient ddC incorporation by DNA polymerase gamma during mitochondrial DNA replication. Understanding these outcomes at atomic level resolution has been limited by the fact that previous structures of the two proteins examined here did not contain the relevant DNA and dNTP substrates. This study substantially overcomes that limitation. Mainly for that reason, I believe the manuscript will be of widespread interest to basic and clinical research scientists alike. Regarding the latter, it would be useful to add images showing where disease-associated mutations in the catalytic subunit map on the ternary complex structure, i.e., beyond what is already shown in Figure 8. There are now so many different mutations that this might require multiple panels, but I believe the efforts would be justified by the appeal to the biomedical community. Typical of earlier structural work from the Yin group, this study is experimentally sound, and the structural images and their descriptions are clear.

Concerns. I do think the manuscript could be improved by revisions based on the following points.

The fact that ddCTP and dCTP bind to the active site in a similar manner is consistent with incorporation of the chain terminator during mitochondrial replication to yield toxicity. However, a more interesting point that is not discussed here is whether this effect is selective for mitochondrial replication as compared to other DNA synthesis reactions in a human cell, e.g., nuclear DNA replication. It would be useful to discuss whether the new results here offer any insights into "selective" inhibition of mitochondrial replication, possibly by comparing the new ddC structure to structures of other DNA polymerase ternary complexes with a dideoxynucleotide in the active site. The absence of any insight on this point would reduce the ddCTP structure presented here to but one more example of such structures in the literature, rather than something unique to the mitochondrial replicase.

The manuscript would be improved by closer attention to distinguishing what is known from what the authors believe may be the case. Interpretations exceed facts in two categories. (1) Compared to structural work on several other polymerases where multiple snapshots of the catalytic cycle have been solved, only two are available here, the apo structure and the ternary complex. Absent (at least) a binary complex of protein and template-primer, one cannot yet distinguish whether the conformation changes described here and the interactions that result are associated with DNA binding or nucleotide binding (e.g., page 7, Heading at bottom and line 3 from bottom). I do not suggest that a binary complex is required for publication, rather that interpretations be tempered by lack of such a structure. (ii) A few statements in the manuscript do not adequately distinguish between what is actually known and what may be true. Page 6, line 5 from bottom - Is this a prediction, or is there experimental evidence for this statement in the literature? Page 6, last line - same question. Page 12, line 10-11 - same question.

Lesser points:

The first sentence in the abstract does not take into account recent evidence that PrimPol is in the mitochondrion.

Given great interest in the polymerization field regarding the catalytic mechanism and the roles of
(now as many as four) metal ions in the catalytic cycle and their identity, it would be useful to discuss this issue a bit more, e.g., in the context of Figure 2 and the text on pages 5 and 6.

Page 7, line 15 - what is meant by "larger ribonucleotides"?

Page 9, line 2 - what is "%A"?

Referee #2:

Manuscript 91520 from Szymanski et al. provides the first crystal structure of a form of the pol G holoenzyme complexed with primer-template and incoming nucleotides, extending previous work from this group on the structure of the apo-enzyme. The structure has intrinsic medical significance since mutations in polGA frequently contribute to human diseases. However, the structures have lower resolution than many other published DNA polymerase structures and do not provide a great deal of novel insight into polymerase mechanisms or nucleotide discrimination. The larger number of higher resolution structures in the paper cited by Wang et al (2012) is a more comprehensive study of this aspect, albeit in a prokaryotic polymerase.

The greatest novel contribution of this study is the extensive interaction revealed between polymerase domains, including the "spacer," with a rather extensive stretch of the primer-template. This is supported by mutagenesis of lysine residues in this region, which is shown to result in reduced polymerization efficiency. The results in Figure 6 are a bit confusing since filled circles are used to represent results with both the wild type and one mutant enzyme. This result might imply that the polymerase rate might vary significantly with the length of the primer-template stem, perhaps making the use of short primers inefficient. Additional functional experiments would enhance a manuscript that is currently a structural study of interest to a rather limited audience.

Some of the figures are less helpful to the reader than they might be. Figure 1 is clearly important, but has crowded features that do not illustrate the proximity of primer-template to protein behind the DNA. A transparent space-filling shell and rotation may be useful. In addition, the movement of the K-tract on DNA binding in Fig. 4 is difficult to appreciate.

Minor points:
1. Both in the abstract and the test the authors describe ddC as an inhibitor designed to inhibit HIV reverse transcriptase. This is incorrect as this compound was widely used in DNA polymerase research prior to its application as a therapeutic agent.
2. The primer-template sequence in Fig. 1 is written with a proportionally-spaced font that sets the strands out of register. This should be corrected.
3. On page 6 the authors state the thumb "intercalates" into the minor groove. The term intercalate implies stacking of a hydrophobic residue between adjacent DNA bases and this is not the case. This should be referred to as a simple binding event.
4. On page 7, the text "resulting exclusion of ddNTP in a similarly fashion shown" is awkward. This should be revised as "resulting in exclusion of ddNTP in a fashion similar to that shown"
5. On page 10, "hairpin tip contains of two adjacent arginine" is incorrect "of" should be deleted

There are several other instances of poor working that should be corrected by careful proofreading.

Referee #3:

The manuscript by Szymanski et al reports crystal structures of ternary complexes of human DNA polymerase gamma ( ), DNA substrate and dCTP or ddCTP, which is the nucleotide (active form) of anti-HIV prodrug Zalcitabine (nucleoside form). The structures have been long awaited since the report of the crystal structure of apo-protein of DNA pol gamma in 2009 by the same group. This is the first crystal structure of a human DNA polymerase with all its accessory subunits and DNA substrate. Despite the medium resolution (3.3 to 3.46\(=\)), these structures reveal how the polymerase achieves high processivity with the help of accessory subunits (two copies of Pol B) and accommodates anti-HIV drugs in the active site. The two Pol B subunits undergo conformational change upon DNA binding, particularly the distal subunit. Although neither of the Pol B subunits is in direct contact with DNA substrate, both enhance substrate binding and the catalytic rate of Pol A.
Many structures of bacterial homologs of Pol (known as Pol I) have been studied, and it is gratifying to learn that the basics of the finger domain opening-and-closing motions (known as induced-fit) are conserved throughout the A family DNA polymerases. The structure and mutational studies also reveal a positively charged K-tract in Pol A that increases its DNA binding and catalytic efficiency. The analysis of why Zalcitabine (ddCTP) causes toxic side effects when used to treat HIV patients is informative. Because ddCTP is accommodated in the active site as well as dCTP opposite a dG template, it will be interesting to find out whether the primer strand terminated with a dideoxynucleotide is resistant to exo proofreading. The structures have been determined expertly, and the structural analysis is thorough and thoughtful. With attention paid to the following details, the manuscript is suitable for publication in EMBO J.

Detailed comments:
1. The “prime” for DNA ends (5¥ or 3¥) should be " ¥ " and not a half quote.
2. After Abstract, Introduction and the first sentence in Results, Zalcitabine is not mentioned again. It would be nice to label ddCTP as "ddCTP (Zalcitabine)" in Fig. 2B at least.
3. The IP and AID subdomains of Pol A are mentioned in the main text but not clearly shown or labeled in any of the main figures. The domain diagram of residue ranges including for IP and AID may be moved from Fig. E1 to Fig. 1. In Fig. 1, the spacer domain may be colored in a different shade from the rest of Pol A. In Fig. 4A legend, the AID subdomain may be mentioned. In Fig. 3, IP and AID may be labeled. In Fig. E1, it will be nice to label IP and AID subdomains.
4. There is a typo on p. 9 line 2, "(Figure 4A and %A)".
5. In Figure 6, the symbols for Pol A and Pol A K498C are indistinguishable. Both look like filled circles. One may be changed to an open circle.
6. In Fig. 7B, the nascent base pair (between the incoming ddCTP and templating dG) seems not to form hydrogen bonds. The configuration is more like a GT wobble.
7. According to the Materials and Methods, WT Pol is used in crystallization. Is the dideoxy-terminated DNA resistant to the exonuclease activity?
8. It should be proximal or distal Pol B "subunit" and not "monomer".

Point-by-point responses to the reviews
(Please note the referees’ comments are the blue italic text, and our responses are in black)

Referee #1:
Opinion Mutations in the genes encoding the two mitochondrial replication proteins studied here are responsible for several human diseases, a fact almost certainly related to mitochondrial dysfunction stemming from defects in processive mitochondrial DNA replication. The toxicity associated with treatment of HIV-1 with ddC is due to mitochondrial dysfunction resulting from chain termination due to efficient ddC incorporation by DNA polymerase gamma during mitochondrial DNA replication. Understanding these outcomes at atomic level resolution has been limited by the fact that previous structures of the two proteins examined here did not contain the relevant DNA and dNTP substrates. This study substantially overcomes that limitation. Mainly for that reason, I believe the manuscript will be of widespread interest to basic and clinical research scientists alike. Regarding the latter, it would be useful to add images showing where disease-associated mutations in the catalytic subunit map on the ternary complex structure, i.e., beyond what is already shown in Figure 8. There are now so many different mutations that this might require multiple panels, but I believe the efforts would be justified by the appeal to the biomedical community. Typical of earlier structural work from the Yin group, this study is experimentally sound, and the structural images and their descriptions are clear.

We completely agree with the referee that we should use the Pol γ ternary structure to analyze disease-associated mutations. We did so with the Apo Pol γ structure after modeling in
DNA (Lee et al., Cell, 2009). L. Kaguni has also published two reviews on the topic, again after modeling DNA into our Apo structure. In this report we therefore confined our discussion to those mutations that were not explained by the Apo structure because of DNA-binding-induced conformational changes. There are indeed many more clinically discovered mutations but most have not yet been characterized biochemically. In collaboration with our clinical colleagues in Houston, we are in the process of conducting detailed structural-functional correlation studies with several of these mutants. We think that we should complete these studies and then publish a more comprehensive, and thus more useful, analysis.

Concerns

The fact that ddCTP and dCTP bind to the active site in a similar manner is consistent with incorporation of the chain terminator during mitochondrial replication to yield toxicity. However, a more interesting point that is not discussed here is whether this effect is selective for mitochondrial replication as compared to other DNA synthesis reactions in a human cell, e.g., nuclear DNA replication. It would be useful to discuss whether the new results here offer any insights into "selective" inhibition of mitochondrial replication, possibly by comparing the new ddC structure to structures of other DNA polymerase ternary complexes with a dideoxynucleotide in the active site. The absence of any insight on this point would reduce the ddCTP structure presented here to but one more example of such structures in the literature, rather than something unique to the mitochondrial replicase.

It would be not only interesting but also extremely useful to understand how nuclear human DNA replicating polymerases discriminate against NRTIs more efficiently than mitochondrial DNA polymerase. This is a primary reason for this study. However, our structure represents the first human replicating DNA polymerase complex with deoxy- or dideoxynucleotide in the active site. Lacking structures of nuclear DNA replicases, an in-depth structural analysis, beyond simple biochemical characterizations, is not possible. We do offer limited discussion on the issue, albeit on related prokaryotic Pol I family members on p.7 lines 16-23.

The manuscript would be improved by closer attention to distinguishing what is known from what the authors believe may be the case. Interpretations exceed facts in two categories. (1) Compared to structural work on several other polymerases where multiple snapshots of the catalytic cycle have been solved, only two are available here, the apo structure and the ternary complex. Absent (at least) a binary complex of protein and template-primer, one cannot yet distinguish whether the conformation changes described here and the interactions that result are associated with DNA binding or nucleotide binding (e.g., page 7, Heading at bottom and line 3 from bottom). I do not suggest that a binary complex is required for publication, rather that interpretations be tempered by lack of such a structure.

We agree with the referee, and have made corrections from ‘DNA binding induced conformational change’ to ‘formation of the ternary complex with DNA and nucleotide induced conformational changes’ (p2, line 9). We also agree with the referee that the binary structure would be informative. However, despite our best efforts, we have not succeeded in crystallizing the binary Pol γ complex. We have made appropriate corrections in the manuscript.

(ii) A few statements in the manuscript do not adequately distinguish between what is actually known and what may be true. Page 6, line 5 from bottom - Is this a prediction, or is there experimental evidence for this statement in the literature? Page 6, last line - same question. Page 12, line 10-11 - same question.

We added clarification to the statement on p6 line 19-23, and p12 line 18-20, respectively.

Lesser points:
The first sentence in the abstract does not take into account recent evidence that PrimPol is in the mitochondrion.

We have changed it to ‘Pol γ is responsible for DNA replication in mitochondria’ (p2, line 2).
Given great interest in the polymerization field regarding the catalytic mechanism and the roles of (now as many as four) metal ions in the catalytic cycle and their identity, it would be useful to discuss this issue a bit more, e.g., in the context of Figure 2 and the text on pages 5 and 6.

We added a statement of the mechanistic role of divalent metal ions in the phosphotransfer reaction (p6, line 1-4). The reaction was originally proposed to use two metal ions, one for nucleophilic attack during phosphodiester bond formation, and one for stabilizing the leaving group PPi. Recent studies suggest that a third metal ion can participate the reaction by displacing an arginine residue that coordinates the b-phosphate of dNTP in the product complex. Since our structure represents substrate complex, only two metal ions are present. Unfortunately, we did not find reference structure of DNA polymerase with four metal ions, therefore, could not comment on it.

Page 7, line 15 - what is meant by "larger ribonucleotides"?

It refers to the structures of ribonucleotides in comparison to deoxyribonucleotides, as ribonucleotides contain an additional 3'OH, therefore are larger in size. We added clarification to the text (p7, line 18-20)

Page 9, line 2 - what is "%A"?

We apologize for the typo; it has been changed to “5A”.

Referee #2:

The greatest novel contribution of this study is the extensive interaction revealed between polymerase domains, including the "spacer," with a rather extensive stretch of the primer-template. This is supported by mutagenesis of lysine residues in this region, which is shown to result in reduced polymerization efficiency. The results in Figure 6 are a bit confusing since filled circles are used to represent results with both the wild type and one mutant enzyme. This result might imply that the polymerase rate might vary significantly with the length of the primer-template stem, perhaps making the use of short primers inefficient. Additional functional experiments would enhance a manuscript that is currently a structural study of interest to a rather limited audience.

We apologize for the confusion, which resulted from converting a color figure to black and white. The symbols in Figure 6 have been made distinct.

We interpret the referee comments as whether short primers (less than the 25-nt that we used in our studies) would make synthesis less efficient. Both others and we have assayed Pol γ activity on shorter primer/templates. A published pre-steady state kinetic study reported that Pol γ indeed has reduced synthesis rate on shorter primers, e.g., the rate using an 18-nt primer is 7-fold slower than a 24-nt primer, at 3.2 nt/s versus 22 nt/s (Murakami et al., J. Biol. Chem. 278: 36403, 2003), conforming our structural results. We have added this information to the text (p9, line 11-13). We also tested Pol γ synthesis rates on primers longer than 25 nt (up to 37 nt), and found they are comparable to that on 25-nt primer. Therefore, 25 nt is the shortest primer that enables maximum reaction rate of Pol γ. We added this information to the text (p9, lines 17-21).

Some of the figures are less helpful to the reader than they might be. Figure 1 is clearly important, but has crowded features that do not illustrate the proximity of primer-template to protein behind the DNA. A transparent space-filling shell and rotation may be useful. In addition, the movement of the K-tract on DNA binding in Fig. 4 is difficult to appreciate.

We have added a 90-degree rotation view of the structure to Figure 1. However, a transparent space-filling shell did not yield a clearer illustration. We instead made an electrostatic surface rendition for the holoenzyme and ribbons for the nucleic acids to show the proximity of primer-template to protein behind the DNA.

We also made changes in Figure 4 by increasing the contrast between apo Pol γ and the
ternary complex to enhance the structural differences.

Minor points:

1. Both in the abstract and the text the authors describe ddC as an inhibitor designed to inhibit HIV reverse transcriptase. This is incorrect as this compound was widely used in DNA polymerase research prior to its application as a therapeutic agent.

   We thank the referee for pointing this out. We have corrected it to ‘used for inhibiting HIV reverse transcriptase’ (p2, line 5).

2. The primer-template sequence in Fig. 1 is written with a proportionally-spaced font that sets the strands out of register. This should be corrected.

   Corrected.

3. On page 6 the authors state the thumb "intercalates" into the minor groove. The term intercalate implies stacking of a hydrophobic residue between adjacent DNA bases and this is not the case. This should be referred to as a simple binding event.

   The statement has been corrected according to the referee’s suggestion.

4. On page 7, the text "resulting exclusion of ddNTP in a similarly fashion shown" is awkward. This should be revised as "resulting in exclusion of ddNTP in a fashion similar to that shown"

   Corrected as the referee suggested.

5. On page 10, "hairpin tip contains of two adjacent arginine" is incorrect "of" should be deleted.

   There are several other instances of poor working that should be corrected by careful proofreading.

   The word ‘of’ is deleted.

Referee #3:

The "prime" for DNA ends (5´ or 3´) should be " ' " and not a half quote.

All ‘prime’ for DNA ends have been corrected throughout the manuscript.

After Abstract, Introduction and the first sentence in Results, Zalcitabine is not mentioned again. It would be nice to label ddCTP as "ddCTP (Zalcitabine)" in Fig. 2B at least.

We added ‘Zalcitabine’ to Figure 2B.

The IP and AID subdomains of PolγA are mentioned in the main text but not clearly shown or labeled in any of the main figures. The domain diagram of residue ranges including for IP and AID may be moved from Fig. E1 to Fig. 1. In Fig. 1, the spacer domain may be colored in a different shade from the rest of PolγA. In Fig. 4A legend, the AID subdomain may be mentioned. In Fig. 3, IP and AID may be labeled. In Fig. E1, it will be nice to label IP and AID subdomains.

Thanks to the referee for pointing out our oversight. We clearly labeled the IP and AID subdomains in Figure S1. We also added appropriate labels and legends to Fig 3 and Fig 4A.

There is a typo on p. 9 line 2, "(Figure 4A and %A)".

It has been corrected to 5A.
In Figure 6, the symbols for PolγA and PolγA K498C are indistinguishable. Both look like filled circles. One may be changed to an open circle.

This oversight resulted from converting color to black-and-white. It has been corrected.

In Fig. 7B, the nascent base pair (between the incoming ddCTP and templating dG) seems not to form hydrogen bonds. The configuration is more like a GT wobble.

The template residue and incoming nucleotide do form a Watson-Crick base pair.

According to the Materials and Methods, WT Polγ is used in crystallization. Is the dideoxy-terminated DNA resistant to the exonuclease activity?

Dideoxy-terminated DNA is hydrolyzed at a slower rate than other NRTI-terminated primer DNA. However, it is not resistant to exonuclease activity. For crystallization we used exonuclease deficient PolγA (exo). We added additional clarification in Materials and Methods (p. 16 line 10).

It should be proximal or distal PolγB "subunit" and not "monomer".

Historically, Pol γB was always referred to as the accessory subunit for Pol γA. However, Pol γB is a homodimer, and using the term subunit in different ways is confusing. We named each monomer of the homodimer ‘proximal’ and ‘distal’, respectively, by their distance from the catalytic subunit Pol γA.

Pre-acceptance letter 30 April 2015

Thank you for submitting your revised manuscript for our consideration. I have now had a chance to look through it and to assess your responses to the comments raised by the original reviewers, and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

Before we will able to send you a formal letter of acceptance, there is one remaining editorial issue I need to ask you for:
- Please provide PDB accession codes for the newly determined structures.

After that, we should then be able to swiftly proceed with acceptance and production of the manuscript.