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Analysis of substrate specificity of Schizosaccharomyces pombe Mag1 alkylpurine DNA glycosylase

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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.)

Editorial Decision 26 July 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. I am sorry for the substantial delay in its evaluation, owing largely to the unavailability of many potential referees at this time of the year. We have finally received three sets of comments, and we also had a chance to consider and discuss them within our team. The conclusions from this is, I am afraid to say, that we cannot consider the study a good candidate for publication in The EMBO Journal. As you will see from the reports copied below, all referees appreciate the technical quality of the study and acknowledge the intriguing finding of substrate preference switching through a single amino acid change. However, they at the same time all remain unconvinced that the study has also offered major new insights into the molecular and mechanistic basis underlying this phenomenon, nor more generally into lesion recognition and substrate selection by Mag1.

I am afraid that these criticisms in our view preclude publication as a full-length article in The EMBO Journal at this stage. Given that the main finding of the study may however nicely constitute a self-contained short report without major extensions of the experimental analysis, the manuscript in the present form might potentially be a good candidate for our sister journal, EMBO reports (see www.emboreports.org for more information). In this case, my colleagues would be happy to take a decision based on the transferred EMBO J reports and the additional arbitrating opinion of an

EMBO reports Editorial Board Member or experienced referee. I would not be able to anticipate any conclusion on their part, but the study in its present form would certainly appear to be within their overall scope and in any case, evaluation on the basis of the present report should be greatly expedited.

Should you be interested in this opportunity, please simply use the 'manuscript transfer' hyperlink given below to transfer this submission (including referee reports) directly to EMBO reports, mentioning this recommendation. For publication in The EMBO Journal, however, I am sorry to have to disappoint you on this occasion - in any case I hope you will find our referees' comments helpful and once more please accept my apologies for the delay in this evaluation.

Sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

Adhikary and Eichman report a structural and biochemical characterization of Mag1 glycosylase with the aim of elucidating the mechanism of its substrate specificity. The paper addresses an important issue, since the DNA damage recognition lies at the heart of DNA repair processes. It is a particularly interesting and significant problem for glycosylases which have to locate very minor deviations from the regular structure of nucleobases among long stretches of unmodified DNA.

The manuscript is well written and the figures very clearly illustrate the points discussed in the text. The first part of the manuscript describes the determination of the structure of S. pombe Mag1 (spMag1). Based on Table I, the crystallography is very solid. Given the relatively high resolution of the structure (2.3 Å) and the high quality of the electron density maps shown in Fig S1, the structural information should be very reliable. The structure does not provide fundamentally new insights in the structure and mechanism of glycosylases from helix-hairpin-helix superfamily - it is very similar to the previously determined structure of AlkA determined by Hollis et al. (Fig 1D). However, the manuscript does not focus on the structure itself but rather uses it as a starting point for comparative analyses and for the design of amino acid substitutions used in activity assays.

The biochemical part of the manuscript shows that spMag1 has lower activity on εA substrates compared its S. cerevisiae counterpart (scMag1), while the activity of both enzymes on 7mG substrate is very similar. In a thorough analysis the authors demonstrate that the small differences in the composition of the pocket binding the flipped out base are not responsible for the substrate specificity difference between spMag1 and scMag1. Instead, the authors identify a histidine residue (H64) that is present in spMag1 and interacts with the minor grove of the substrate. It is replaced by a serine in scMag1. The His to Ser and Ser to His exchange in the two enzymes reverses the substrate specificity towards εA substrate which is a striking result. However, what is lacking in my opinion is the explanation of the exact role the histidine residue in the recognition process. Such role is not obvious - based on what is shown in figure 4C, H64 cannot interact with the modified base or its pairing partner. Instead, it interacts with a base one nucleotide away from the modification site on the damaged strand and two nucleotides away from the modification on the non-damaged strand. Since the histidine is located at a terminus of a conserved α-helix, it is unlikely to dramatically change its position to allow direct contact with the modified base pair. Moreover, εA modification is present on the major groove side of the DNA and the interaction with the hisitidine is on the minor groove side, so a direct detection of the modification site is not possible. To fully understand the role of H64 more structural data would be needed. For example structures could be determined for the substrate state. This is obviously not straightforward and further complicated by the fact that the nucleobase-binding pocket is occupied by a terminal thymine residue of the neighboring complex molecule in the crystal. Have the authors checked whether crystals can be grown with a shortened substrate lacking this thymine residue which would free up the binding pocket?

Minor points:
It would be helpful for non-expert readers to include chemical structures of nucleobase modifications studied in the manuscript in one of the figures.

The authors should discuss the discrepancy between the reported lack of activity of spMag1 on εA (Alseth, 2005) and their own result showing small but significant activity on this substrate.

Error bars are given in Fig 4D but the error values are not given in Table II.

Referee #2:

Adhikary and Eichman report the crystal structure of Schizosacharomyces pombe Mag1 (spMag1) complexed with an abasic analog DNA substrate and comparison of its substrate preference with homologous glycosylases, AlaA from E. coli (ecAlkA) and Mag from Sacharomyces cerevisiae (scMag) and bacillus halodurans (bhMag). The structure of spMag1 is highly homologous to several glycosylases that recognize alkylated bases, so the focus of this manuscript is to answer why similar enzymes have different substrate specificity. However, spMag1, sc Mag and bhMag all can cleave after 1,N6-ethenoadenine (eA) and, the enzymatic activities measured in product formation differ by only 2-3 fold. It is obvious eA is not a good substrate for any Mag tested in this manuscript since completion of the reaction takes more than 8 hours rather than the 40 minutes for 7mG (Fig. 2 and S5). It is puzzling why the authors chose the small difference of a very poor enzymatic activity to study substrate specificity of a very large pool of DNA glycosylases. Nevertheless, it is nice in a broad stroke that the small differences between spMag1 and scMag can be switched by swapping one non-conserved residue (His versus Ser) in the two enzymes. However, the conclusion of substrate specificity determined by that residue lacks experimental support. Firstly, the authors haven't distinguished whether the different catalytic activities of these glycosylases are due to substrate binding affinity, recognition kinetics, substrate orientation relative to the active site, or catalytic chemistry. How eA or m7G are recognized by Mag and how catalysis occurs remain unknown. Secondly, the substitution of S97 by His in scMag leads to a general loss of activity towards eA and 7mG rather than altering the substrate preference. Thirdly, it is hard to guess what happens to these enzymes during the 50-hour experimental time. Protein structure and enzymatic activity could change during the long period of incubation in a different way from protein stability measured by thermal melting. There are other troubling issues. For example, the DNA orientation in the structure appears to be a result of crystal lattice contact (Fig. 1 and S4). Whether DNA base flipping out occurs before, after or simultaneous with DNA bending is not known either. In summary this manuscript provides little advance to our understanding of substrate specificity of Mag.

Referee #3:

The manuscript "Altering substrate specificity of Sp alkylpurine DNA glycosylase Mag1" describes the novel structure of SpMag1 with product DNA (THF, abasic site analog) and mutational/biochemical analyses of residues that interact with DNA. Although the active site of the S. pombe enzyme is almost identical to that of isozymes in S. cereviseae and B. haldurans, the enzyme is slightly (2-3 fold) less active with εA lesions despite similar activity against 7mG lesions. Although alterations in the active site did not explain the substrate preference, mutation of a histidine side chain contacting the minor groove to a serine (conserved in other enzymes) did restore similar levels of activity. This result provides experimental evidence for the growing hypothesis that DNA repair enzymes recognize their specific lesions by testing the overall DNA structure as well as specific damage base recognition. The authors should address the following points.

Points.

1. The most interesting aspect of this study is that a minor groove interacting protein outside of the active site can alter specificity. The authors state that "one possible mechanism is that His64 directly senses a local perturbation in the duplex prior to base flipping". If mutation to serine increases activity, would it not be the serine that is more sensitive than histidine? Another possible

interpretation is that the histidine is somehow inhibiting activity, such as binding product with higher affinity (as suggested from the two potential interactions that might be occluded with the serine) or increasing end-binding activity.

2. Table S2 suggests that specificity is not in the lesion binding but in the catalysis and non-productive binding is a problem. Despite the excess protein, the SpMAG does not actually incise all the product over time. It is possible that this is due to unproductive binding that blocks the lesion from productive binding. The serine mutant no longer has this problem. DNA binding assays for end binding, εA, 7mG, and THF-containing DNA with WT and mutants may clarify this issue. (Using the catalytically impaired mutants might help with the lesion-containing DNA).

3. The hypothesis that the histidine/serine are probing the distortion raises the following questions. What is the distance of the refined histidine position to the two interacting positions? A simple addition of distances to the corresponding fig would help readers. What is the distortion, if any, of this region of the minor groove? How could the serine be involved at all in the distortion of this region? Can anything be learned by overlaying the different structures and examining this region of the helix and the position of the serine. Are all the other residues, listed in Figure 1C, invariantly conserved? For example, to alter bending or probing the DNA stability. The sequence alignment should have the DNA-interacting residues highlighted.

4. Inference to recognition of ethenoadenine would be likely deepened by examination of lesion-containing DNA alone (Leonard, GA et al., 1994). Is there any subtle distortion of the minor groove in the region of the protein-free DNA where the histidine/serine would interact. Although a structure of 7mG containing DNA has not been determined, is there a reason why the SpMAG would actually be better? The binding studies above may help clarify that question.

5. References to Fig 7C in the text seem to be more appropriately linked to Fig 7D.

Editorial Decision 17 August 2011

Thank you again for the submission of your manuscript to EMBO reports and my apologies for the delay in getting back to you. We have now received the report from the referee we asked to evaluate the comments forwarded from The EMBO Journal. As you can see below, the reviewer is very positive but some minor concerns need to be addressed prior to acceptance.

The modifications you suggest in your response to referees' comments should be added to the final version of the manuscript. We also think that a discussion of the referee #2 concern remarked in point 3, regarding the overall reduction in activity after the substitution Ser97His in ScMag, should also be added.

Browsing through the manuscript myself, I have noticed that statistical analysis is not properly described for figure 4D. Statistical analyses must be described either in the Materials and Methods section or in the legend of the figure to which they apply. Please include a definition of the error bars used and, state the statistical significance of the results and the method used to calculate it. I have also noticed that you have included a results section in the supplementary data. We only accept figures and figure legends as supplementary data. If results or discussion sections are required to understand the supplementary information, they must be included in the main text with reference to supplementary figures.

I look forward to seeing final version of your manuscript when it is ready.

Yours sincerely,

Editor
EMBO reports

REFEREE REPORT:

Referee #4:

This manuscript reports the crystal structure of the S. pombe 3-meA DNA glycosylase and provides novel insight into lesion specificity of DNA glycosylases that initiate the Base Excision Repair pathway for alkylation DNA damage. The structural work is very solid and beautifully done. The issue of how 3-meA DNA glycosylases possess such broad substrate specificity and discriminate between different types of damage is very relevant and interesting. The authors report the novel finding that a residue outside the active site of the enzyme is very important for base discrimination and consequently enzymatic activity. They do so elegantly, by using enzymatic assays that are appropriate to answer the question and routinely used in this type of analysis.

Revision - authors' response 26 August 2011

We were pleased by the overall positive responses from the reviewers, who noted the significance and quality of the work. Most of the major criticisms were very helpful and we have revised our manuscript to reflect these comments. Other issues raised were either based on the referee's misunderstanding or are beyond the scope of this work. Clarification and responses to each point raised are detailed below.

Referee #1

1. … what is lacking in my opinion is the explanation of the exact role the histidine residue in the recognition process. Such role is not obvious - based on what is shown in figure 4C, H64 cannot interact with the modified base or its pairing partner. Instead, it interacts with a base one nucleotide away from the modification site on the damaged strand and two nucleotides away from the modification on the non-damaged strand…

Response: The explanation for the role of H64 in lesion recognition is in fact discussed on p.11. As discussed in the manuscript, our structure represents the complex after base flipping and catalysis has occurred, and based on current models for glycosylase encounter of DNA damage, it is possible that the histidine contacts the lesion as the enzyme slides along the DNA during a scanning process. However, in response to the reviewer's comment, the histidine does not need to contact the lesion directly for it to be important in lesion recognition. Distortion of the DNA as a result of the lesion can be sensed by the overall protein-DNA binding mode; the distorted DNA duplex is an important factor in base excision, since the DNA must be held in a specific orientation for the flipped base to be fully recognized.

2. Moreover, εA modification is present on the major groove side of the DNA and the interaction with the histidine is on the minor groove side, so a direct detection of the modification site is not possible.

Response: Although the etheno modification itself lies on the major groove side, the modification would disrupt the Watson-Crick base pairing, resulting in a distortion of the entire base pair, and would thus be directly detectable from either the major or minor groove side of the duplex.

3. To fully understand the role of H64 more structural data would be needed. For example

structures could be determined for the substrate state. This is obviously not straightforward and further complicated by the fact that the nucleobase-binding pocket is occupied by a terminal thymine residue of the neighboring complex molecule in the crystal. Have the authors checked whether crystals can be grown with a shortened substrate lacking this thymine residue which would free up the binding pocket?

Response: Determination of the substrate complex is beyond the scope of this work. The reviewer is correct in that this is not a straightforward experiment. We have been unsuccessful in generating crystals of either enzyme-substrate or -product complex lacking the 5′-terminal thymine T1 residue. This is not a surprise given the importance of this residue to the crystal lattice.

4. It would be helpful for non-expert readers to include chemical structures of nucleobase modifications studied in the manuscript in one of the figures.

Response: We have added these as a separate figure (Fig 1).

5. The authors should discuss the discrepancy between the reported lack of activity of spMag1 on εA (Alseth, 2005) and their own result showing small but significant activity on this substrate.

Response: We are only able to speculate on the lack of activity reported by Alseth et al since that group did not present the data for their claim (from that paper, "Mag1 showed no activity towards hypoxanthine or 1,N6-ethenoadenine (data not shown).") We assume that the discrepancy lies in differences between the experimental conditions used by the two groups, and have added this statement at the bottom of p.7. We discuss the low levels of spMag1 activity in the discussion as it relates to the aspartate mutants (p. 9), the possible role of His64 during the search process (p.11), and the differences in S. pombe and S. cerevisiae alkylation responses (p.12).

6. Error bars are given in Fig 4D but the error values are not given in Table II.

Response: The actual standard deviations were previously not shown in Table II for clarity, and the legend stated, "Values represent the average from three independent measurements, with standard deviations ≤15% for εA and ≤20% for 7mG." This table has been moved to the supplement (Table S3), and we have added the S.D. values. In addition, we now define the error bars and include *p*-values to the Fig. 5D legend and have added a description of the statistical analysis to the Supplemental Methods.

Referee #2

1. It is puzzling why the authors chose the small difference of a very poor enzymatic activity to study substrate specificity of a very large pool of DNA glycosylases.

Response: There are a large pool of DNA glycosylases that recognize a wide range of substrates (oxidized, deaminated, uracil bases, etc), but there are no systematic studies to understand specificity for alkylated bases. The rules governing specificity of an enzyme specific for alkylated bases will certainly differ from other, more stable lesions. We have chosen the HhH superfamily as a model system because of their high sequence and structural similarities but widely varying substrate preferences.

2. Firstly, the authors haven't distinguished whether the different catalytic activities of these glycosylases are due to substrate binding affinity, recognition kinetics, substrate orientation relative to the active site, or catalytic chemistry. How eA or m7G are recognized by Mag and how catalysis occurs remain unknown.

Response: These are questions we are very interested in pursuing, but clearly lie outside of the scope of this manuscript. The work required to address these questions would constitute multiple manuscripts, as has been seen, for example, in the large number of biochemical

papers that describe various aspects of the mechanism of uracil DNA glycosylase that followed the crystal structures and constituted over 10 years of work.

3. Secondly, the substitution of S97 by His in scMag leads to a general loss of activity towards eA and 7mG rather than altering the substrate preference.

Response: The S→H substitution causes less than a 2-fold reduction in 7mG activity ($p<0.05$) compared to over 5-fold reduction in εA activity ($p<0.002$). Conversely, the significant increase in εA activity by spMag1 is not observed with 7mG. These different effects on εA and 7mG activities between the two enzymes suggest that there may be subtly different modes of detection of 7mG and εA lesions, consistent with the different effects of the catalytic aspartate residues. We have added this point to the discussion on p. 11.

4. Thirdly, it is hard to guess what happens to these enzymes during the 50-hour experimental time. Protein structure and enzymatic activity could change during the long period of incubation in a different way from protein stability measured by thermal melting.

Response: We have done the necessary control to show that the enzyme is not losing activity during the long incubation time. To do this, we pre-incubated the enzyme for an extended period of time under the experimental conditions before adding the substrate, and found that the kinetics are the same as when the substrate is added to fresh enzyme. We have added the data as Supplemental Fig. S8 and added the following, which now appears in the Supplemental Methods due to length constraints, "As a control to verify that the reaction rates were not affected by changed in the protein as a result of the long reaction times, we performed control reactions in which the protein was pre-incubated for 4 hours under the reaction conditions prior to initiating the enzymatic reaction."

5. … the DNA orientation in the structure appears to be a result of crystal lattice contact (Fig. 1 and S4).

Response: In virtually all DNA glycosylase crystal structures determined to date, the ends of the DNA form crystal lattice contacts. Slight deviation in the DNA bend angle as a result of lattice contacts at the ends of the duplex would not change our conclusions whatsoever.

6. …Whether DNA base flipping out occurs before, after or simultaneous with DNA bending is not known either.

Response: This is an interesting question that we address in the Discussion of the current manuscript. Determination of this experimentally lies outside of the scope of the current work.

Referee #3

1. The most interesting aspect of this study is that a minor groove interacting protein outside of the active site can alter specificity. The authors state that "one possible mechanism is that His64 directly senses a local perturbation in the duplex prior to base flipping". If mutation to serine increases activity, would it not be the serine that is more sensitive than histidine? Another possible interpretation is that the histidine is somehow inhibiting activity, such as binding product with higher affinity (as suggested from the two potential interactions that might be occluded with the serine) or increasing end-binding activity.

Response: We agree with the reviewer, and in fact this comment is not contradictory to our discussion of the mechanism of how the His/Ser at this position can sense damage. We have incorporated these ideas in the revised manuscript.

2. Table S2 suggests that specificity is not in the lesion binding but in the catalysis and nonproductive binding is a problem. Despite the excess protein, the SpMAG does not actually incise

all the product over time. It is possible that this is due to unproductive binding that blocks the lesion from productive binding. The serine mutant no longer has this problem. DNA binding assays for end binding, εA, 7mG, and THF-containing DNA with WT and mutants may clarify this issue. (Using the catalytically impaired mutants might help with the lesion-containing DNA).

Response: Dissociation constants for THF, unmodified DNA, and 5'-overhang DNA for wildtype were previously shown in Table S4. We have also measured the Kd values for εA and THF for both wild-type and an H64S D170N double-mutant, and find that the mutation has no effect on affinity for substrate or product. We have updated Table S4 to include these values

3a. What is the distance of the refined histidine position to the two interacting positions? A simple addition of distances to the corresponding fig would help readers.

Response: 3.0 Å (G19) and 3.1 Å (A6). These have been added to Fig 5C.

3b. What is the distortion, if any, of this region of the minor groove?

Response: There is no apparent distortion to this region of the DNA from canonical B-DNA.

3c. How could the serine be involved at all in the distortion of this region? Can anything be learned by overlaying the different structures and examining this region of the helix and the position of the serine.

Response: The structure of AlkA bound to 1-azaribose-DNA shows the serine to be within van der Waals contact to the minor groove, which would be sufficient to probe for local alterations in the helix that resulted from various modified bases. Similarly, overlay of the unliganded bhMag structure on spMag1/DNA complex suggests vdW contacts would exist in the Mag orthologs as well.

3d. Are all the other residues, listed in Figure 1C, invariantly conserved? The sequence alignment should have the DNA-interacting residues highlighted.

Response: We have highlighted the DNA interacting residues in Fig S2 sequence alignment. Those residues for which the side chain is contacting the DNA are highly conserved or invariant. The other residues shown in Fig 1C are contacting the DNA from the main chain atoms, so they are not conserved in all cases.

4. Is there any subtle distortion of the minor groove in the region of the protein-free DNA where the histidine/serine would interact. Although a structure of 7mG containing DNA has not been determined, is there a reason why the SpMAG would actually be better? The binding studies above may help clarify that question.

Response: This is an excellent question, and we are unable to detect a significant distortion in the εA-DNA in the flanking base pairs where the His/Ser would interact. We comment on why spMag1 is better at recognition of 7mG in the discussion. In short, the specificity for *N*3- or *N*7-substituted purines likely has to do with the decreased stability of the positively charged bases relative to uncharged εA. A glycosylase with less catalytic power would be more specific for less-stable residues.

5. References to Fig 7C in the text seem to be more appropriately linked to Fig 7D.

Response: We appreciate the reviewer catching this mistake, and assume that the reference is to Fig 5. These have been corrected in the revised manuscript.

Editorial Decision 29 August 2011

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

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

Yours sincerely,

Editor
EMBO Reports

