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An essential DNA strand exchange activity is conserved in the divergent N-termini of BLM orthologs

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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 04 February 2010

Dear Dr. Brill,

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three expert reviewers, whose comments are copied below. As you will see, all of them consider your identification of a new catalytic domain in BLM & Sgs1 helicases interesting and potentially important, and are thus in principle supportive of publication in The EMBO Journal pending appropriate revision of a number of specific issues. In addition to minor editorial issues, these concerns mostly pertain to the strand annealing assays, and to the effects of deletions/mutations on other BLM/Sgs1 functions and within the context of full-length proteins.

Should you be able to adequately address these various points, we would be happy to consider a revised manuscript for publication, and I am therefore inviting you to prepare such a revision along the lines suggested by the reviewers. Please be reminded that it is EMBO Journal policy to allow a single round of major revision only, and that it will thus be important that you diligently answer to all the various experimental and editorial points raised at this stage. When preparing your letter of response, please also bear in mind that this will form part of the Review Process File, and will therefore be available online to the community in the case of publication (for more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html). In any case, please do not hesitate to get back to us should you need feedback on any issue regarding your revision.

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

Yours sincerely,

Hartmut Vodermaier, PhD
Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

IMPORTANT: When you send the revision, we will require
- a word file with a detailed description of the changes made in response to the referees. (Please do not include any figures - supporting data not intended to be published should be uploaded as separate 'referee-only' supplementary material).
- a word file of the manuscript text, which is less than 55,000 characters with spaces (excluding references, tables and supplementary material)
- one separate file for each figure
- one separate file for supplementary material

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Rossner M, Yamada KM (2004) What's in a picture? The temptation of image manipulation.J Cell Biol 166: 11-15

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These forms must be signed and returned to the journal office. We prefer it if you scan the signed forms and upload them along with your revised manuscript. Alternatively, you may return them via fax.

The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 5th May 2010.

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Referee #1 (Remarks to the Author):

Chen and Brill EMBO J

Members of the RecQ helicase family are important for maintenance of genome integrity and cancer avoidance in humans. The authors have identified a novel conserved domain of Sgs1/BLM, referred to as the ST domain, which has ssDNA binding, strand annealing and strand transfer activities. Importantly, when the region encoding this domain is deleted from the full-length gene and expressed in yeast, it complements the growth defect and the MMS sensitivity of the sgs1 null mutant, but not the hyper-recombination phenotype or synthetic lethality with slx4 or slx5. Substitution of this domain of Sgs1 with the equivalent domain of BLM results in complementation of the sgs1 null phenotype in most assays. Strand annealing and transfer activities have been reported previously for the WRN and BLM helicases, but the domain responsible was not identified, and the biological significance was unknown. Ideally, the full-length protein lacking the ST domain should be tested for strand annealing activity to ensure this domain is functional in the full-length protein, and to ensure there is no defect in the ATPase activity. Given the advertised difficulty of purifying full-length Sgs1 this might be difficult to do, but should be possible for BLM.

I have three main concerns with the in vitro strand annealing and transfer assays. First, according to the Methods the reactions contain 1 mM EDTA and no divalent cations. Is strand annealing or transfer still observed using more physiologically relevant conditons, i.e., in the presence of Mg2+? This is a particular concern for the strand transfer assays because spontaneous branch migration is inhibited in the presence of divalent cations; thus, the conditions are optimized for spontaneous strand transfer and a single mismatch is known to block spontaneous branch migration. The second concern is the extremely high concentration of protein to substrate for the strand transfer reactions (100 x protein to substrate). Third, some in the field consider ability to anneal RPA-coated ssDNA as the gold standard for a true annealing function. Although the data presented in Fig. 5 suggest Sgs1 ST domain can compete with RPA, the oligos should be incubated first with RPA and then with Sgs1 to see if Sgs1 can overcome the RPA inhibition to strand annealing.

In the interaction experiment shown in Fig. 6A, the full-length tagged Sgs1 protein should be included to compare protein levels and amount of IP'ed Top3 and Rmi1. It is interesting that the sgs1-ST allele is lethal with slx4 and slx5, is it also lethal with srs2 or mus81? It would be interesting to know because the latter two synthetic lethalities with sgs1 are suppressed by rad51, whereas the slx4 sgs1 and slx5 sgs1 are not. This might give some clue about the role of the ST domain.

Minor comments:
Figure 2A. It would be helpful to indicate the protein concentrations in the figure because at first glance the 103-322 fragment appears to work better than the 1-322 fragment, but in the legend we discover different amounts of protein were used.
Figure 4/5. Were the GST or His tagged versions of the proteins used for the ST assays?
p. 15. The suggestion that proximity of the ST domain to the Top3 interaction domain in the primary structure might be important does not make much sense without knowing the tertiary structure of the full-length protein.

Referee #2 (Remarks to the Author):

Chen and Brill identified a novel catalytic domain (ST) residing between 100-300a.a. of the N-terminus of the yeast Sgs1 and its orthologs in vertebrates. The ST domains in both Sgs1 and BLM exhibit similar ssDNA binding, strand-annealing and strand-exchange activities, despite the fact this region is not conserved at the amino acid level among the orthologs. It is also quite unusual for the ATP-independent ST reaction to show a polarity preference. Genetic studies using sgs1-deltaST mutant further show that the ST domain is required for suppressing recombination/crossover frequency and for the synergistic function of Sgs1 with Top3. One of the most interesting observations is the function of the ST domain in sensing mistmatch base-pairing during strand exchange, suggesting that Sgs1 may function to discourage recombination between homologous chromosomes in favor of sister chromatids.

Overall, the conclusion is well supported by both biochemical and genetic data and provides new insight into the function of the N-terminus of Sgs1 and BLM orthologs. This novel catalytic activity

is mostly likely responsible for some of the recombination steps, which are previously thought to be contributed by the conserved helicase domain.

Additional comments:
(1) While the manuscript is well written, the figures should be made more concise to eliminate redundancy. Especially for the first 5 figures, some of the sub-figures are not essential figures and should probably be included as part of the supplementary materials (i.e. Fig 1D, 2F, 5A, 5C).

(2) Fig. 5C and D: It is not surprising that RPA and SSB inhibit the SA reaction more efficiently than the ST reaction. First, in the SA reaction, both substrates are in single-stranded form and can be bound by SSB/RPA. Therefore, it should be easier for SSB/RPA to quench the annealing reaction. Second, Sgs1 is present in significantly higher concentration in the ST reaction than that in the SA reaction and therefore is expected to compete better with SSB/RPA in the ST reaction. The suggestion that ST reaction is more physiological relevant than SA reaction based on the observation with SSB/RPA is overstated.

Referee #3 (Remarks to the Author):

Chen and Brill: An essential DNA strand-transfer activity is conserved in the divergent N-termini of BLM homologs

The authors describe and characterize ssDNA binding, strand annealing, and strand transfer (ST) activities specific to a portion of the N-terminal regions of Sgs1, human and Drosophila BLM. The biochemistry is very thorough and well controlled. Importantly, the ST domain can bind to a D-loop and the strand transfer activity is inhibited by a single mismatch in the middle of a 32 nt oligo. The ST domain is required for suppression of slow growth of top3del, hyper-rec, synthetic lethality, and heteroduplex rejection phenotypes of sgs1 mutants. The human BLM ST domain can complement these defects. Assignment of biochemical activities to the N-terminal regions of BLM homologs is very interesting.

If there is a weakness in the paper, it is that the author use deletions to disable the ST domain rather than single amino acid substitutions. This approach points to a slight canard imparted by the authors, in which they state (in the Abstract and in the Introduction) that the N-terminus has only one known function, which is to bind Top3-Rmi1. The N-terminus of BLM is known to bind RPA, p53, SUMO, and UBC9. Also, UBC9-binding is required for BLM SUMOylation in vitro. Personally, I think these facts makes the biochemical activity that the authors have described even more interesting, but it does raise some questions about what deletion of the ST domain is doing, because it might affect other functions of the BLM homologs. How conserved are these aforementioned protein-protein interactions is unknown (to me at least). The authors should correct the "only known function" words, and the dissection of these different functions with specific mutations (if they can be dissected) can be future work.

I think I know what the authors mean by the word "essential" in the title (indispensable to their function), but it is a little ambiguous the way it is stated. Moreover, the authors know only that deletion of the ST domain is essential to Sgs1 function. Deletions of BLM's N-terminal regions 1-131 and 131-237 are partially suppressed for high SCEs. Consequently, there is some question whether the same in vivo rules apply to human BLM. I am unable to find the authors a better word, and leaving it off is not entirely satisfactory either.

Minor edits
1. "Bloom's Syndrome" by convention is Bloom's (or Bloom) syndrome
2. "Annealling" should be "annealing" pg 9, p13, and wherever else it is found

1st Revision - authors' response 02 March 2010

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March 2, 2010

Dr. Harmut Vodermaier
Editor
EMBO Journal

RE: EMBOJ-2010-73612 manuscript revision

Dear Dr. Vodermaier:

Thank you for transmitting the reviews of our paper and the opportunity to submit a revised manuscript. Below I address each of the reviewerís comments which were generally positive although they pointed out some weaknesses and omissions in the study. These have now been corrected.

First however, I would like to point out that the revised manuscript uses the more commonly used term DNA strand ìexchangeî in place of DNA strand ìtransferî. Originally I had intended to distinguish our activity (the ATP-independent reaction catalyzed by the BLM/Sgs1 domain) from better known ATP-dependent DNA strand-exchange reactions catalyzed by the RecA and Rad51 recombinases. Athough none of the reviewers objected to ìtransferî, this term is most often used in describing the inter- and intra-molecular strand transfer steps that occur during retroviral DNA replication. Further, there are multiple cases in which nearly identical reactions have been reported as strand ìexchangeî. These cases include exchange that occurs (1) in the absence of ATP hydrolysis by RecA and Rad51 (Rice et al., 2001), (2) in the complete absence of nucleotide cofactor by human and yeast Rad52 (Bi et al., 2004; Kumar and Gupta 2004), and (3) full-length WRN/BLM (Machwe et al., 2005, Fig. 6B). I do not wish to confuse the field by implying a difference in the BLM/Sgs1 reaction where none exists. In keeping with these authors I believe that strand ìexchangeî is most appropriate. Hereafter, I use this terminology.

References:

Rice KP, Eggler AL, Sung P, Cox MM (2001) DNA pairing and strand exchange by the Escherichia coli RecA and yeast Rad51 proteins without ATP hydrolysis: on the importance of not getting stuck. J Biol Chem 276(42): 38570-38581

Bi B, Rybalchenko N, Golub EI, Radding CM (2004) Human and yeast Rad52 proteins promote DNA strand exchange. Proc Natl Acad Sci USA 101(26): 9568-9572

Kumar JK, Gupta RC (2004) Strand exchange activity of human recombination protein Rad52. Proc Natl Acad Sci USA 101(26): 9562-9567

Machwe A, Xiao L, Groden J, Matson SW, Orren DK (2005) RecQ family members combine strand pairing and unwinding activities to catalyze strand exchange. J Biol Chem 280(24): 23397-23407

Reviewer 1

ìIdeally, the full-length protein lacking the ST domain should be tested for strand annealing activity to ensure this domain is functional in the full-length protein, and to ensure there is no defect in the ATPase activity. Given the advertised difficulty of purifying full-length Sgs1, this might be difficult to do, but should be possible for BLMî.

The reviewer has two points here: (1) to compare Sgs1 and Sgs1- ST proteins to confirm that the activities we have identified are present in the full-length protein, and (2) to show that the mutant

protein is not simply inactivating the RecQ helicase ATPase. With respect to the first idea, we agree that it is important to study SA and SE activities in the context of the full-length protein and, indeed, the multimeric BLM/Sgs1 complex. However, as implied by the modifier ìideallyî, it is impossible for us to do this in time for the revision. First, as mentioned by the reviewer, we have been unable to purify full-length Sgs1 protein. Second, should we be able to create the suggested full-length BLM proteins quickly, there is no guarantee that BLM- SE will behave differently from wt BLM in the annealling assay since the RecQ domain of BLM exhibits its own annealing activity. At best we would hope to see a quantitative reduction in annealing activity in the BLM- SE protein. The more diagnostic experiment is to assay strand exchange activity as part of the full-length protein because ATP-independent SE is not an activity known to be shared with the helicase domain. This goal is also more relevant since our data argue that SE, not annealing, is what is biologically important for this domain (i.e., SE is mismatch sensitive). Still, the urgency for this experiment is mitigated by the fact that ATP-independent SE has already been observed in full-length BLM. Although we predict that the SE domain is responsible for this activity in BLM, the merits of our study should not dependent on confirming this hypothesis.
With respect to the reviewerís second point, we agree that it would be beneficial to have biochemical confirmation that the sgs1- SE allele does not affect other BLM/Sgs1 functions like ATPase. However, we have already demonstrated that, in vivo, there is no apparent defect in the DNA helicase activity of Sgs1- SE since the sgs1- SE allele does not display the slow-growth or MMS-sensitive phenotypes expected of the sgs1-hd (helicase defective) allele. While we cannot exclude the possibility that the sgs1- SE allele has a subtle effect on DNA helicase activity, and that this is responsible for its phenotypic defects, it is unreasonable to suggest that the human domain with only 13% sequence identity is able to complement these phenotypes by restoring heterologous ATPase activity. The only reasonable interpretation of the data, including inter-species complementation, is that both species possess SE activities and that SE activity is biologically important.

Other comments:
1. We have performed the requested experiment to allay the concern that the lack of Mg2+ played a role in the SE reaction. The new data, presented in Fig. S5A & B, demonstrate that SE occurs in the presence of 1 ñ 3 mM Mg2+ (where 1 mM is generally considered to be the concentration of free Mg2+ in the cell) and that the mismatch sensitivity is not affected by the presence or absence of Mg2+.
SA is also unaffected by 1 mM Mg2+. This is shown in our nucleotide-dependence assays which had included 1 mM Mg2+ (Fig. 3D). The legend corrects this omission by stating that this set of assays include Mg2+. Thus, Mg2+ is not a factor in either assay and this is described in the text of the Results (pg 9, para 3).

2. To address the reviewerís concerns about the ìextremely high concentrationî of enzyme used in our SE reactions, we have quantitated new reactions and determined the stoichiometry needed to observe SE activity. This data is presented in Fig. S5C. Because ATP-independent strand exchange is known to be catalyzed by the yeast and human Rad52 proteins, the experiment in Fig. S5C employs higher substrate concentrations that approximate the conditions used by Radding (PNAS 101, p9568-9572, 2004). The data indicate that 40% strand exchange occurs at a ratio of one molecule of Sgs1-SE protein per 7 nts of ssDNA. For comparison, optimal exchange by hsRad52 (~38%) occurred at a ratio of one molecule per 15-20 nts of ssDNA. It should be noted that we did not use the preferred 3í-tailed substrate in this experiment, nor did we pre-incubate the enzyme with ssDNA as in the Rad52 protocol. Thus, the efficiency of our enzyme is not extremely low. A description of these results is now in the text (pg 9, para 3).

3. The reviewer points out that the SA reaction might be more sensitive to RPA (Fig. 5C) if the RPA were pre-assembled on ssDNA. Pre-assembly did not result in a significant increase in inhibition (see data for referees), presumably because the reactions in Figure 5 were assembled on ice prior to incubation at 37C and RPA is known to bind ssDNA under such conditions. It should be noted that we are not claiming that SA or SE is resistant to RPA, just that SA and SE respond differently to the two SSBs. We have stated this more clearly and make the point that high levels of both SSBs inhibit SA and SE. The debate about whether this sensitivity violates the gold standard of SA proteins will require a side-by-side comparison with such proteins. Future studies should also address whether additional domains of Sgs1 provide ìmediatorî function to relieve the inhibition by excess RPA.

4. To address concerns about whether Sgs1- SE binds Top3-Rmi1 as well as wt Sgs1, we have included the side-by-side comparison in a new panel (Fig. 6B). We state in the text (pg 10, para 1) that wt Sgs1 and Sgs1- SE co-immunoprecipitate approximately equal amounts of Top3 and Rmi1.

5. The reviewer notes that we did not test the sgs1- SE allele in the mus81 or srs2 backgrounds. Indeed, we have not tested the allele in all backgrounds so we cannot comment on whether there is a correlation between the need for SE and rad51-suppressible synthetic-lethalities. Our choice of using SLX4 and SLX5 as tester strains was simply due to the fact that these null mutants show an absolute requirement for the Sgs1 N-terminal 652 aa. In our hands mus81 cells display background growth with the Sgs1654-1447 protein which lacks both the SE and TR domains.

Minor comments:
1. Fig. 2A: The figure now uses differently sized triangles to emphasize that different titrations were used.
2. Fig. 4/5: The figure legends now include this statement: Throughout, all proteins are His6-tagged.
3. We agree that, in the absence of structural data, it is possible that other domains of Sgs1/BLM may be closer to the TR domain than the SE domain is. However, given that a peptide bond is not elastic, the SE domain cannot be distal to the TR domain. We have let our statement stand.

Reviewer 2.
1. Four panels of data were cited by the referee as redundant. We have moved one of these to the supplementary data as suggested. Specifically,

Fig. 1D: This panel characterizes the specific length of ssDNA bound by the SE domain in an EMSA. This finding is not shown elsewhere in Figure 1 or the rest of the manuscript. We believe it is diagnostic for this class of SE proteins and is therefore worthy of its position in the main body of the paper.

Fig. 2F: Panel is now Fig. S2.

Fig. 5A: The titrations shown here are novel because they compare proteins purified without the GST tag. Based on this data we are able to conclude that the activities of the three SE domains are equal. The pairing of this data with the kinetic analysis in Fig. 5B is the minimum required for a proper biochemical characterization. The two are not redundant, they are complementary.

Fig. 5C: The effect of RPA is not redundant with Fig. 5D because they examine two different assays (SA vs SE). The results are quantitatively different.

2. As mentioned above, in response to comments by Reviewers 1 and 2 we have modified the text and removed any interpretation as to the physiological relevance of the patterns of RPA inhibition.

Reviewer 3.
1. The reviewerís comment about N-terminal protein-protein interactions is well taken. We have re-phrased our description of the N-terminus and now say that it has ìno known catalytic activityî in both the Abstract and the Introduction.

2. We appreciate the reviewerís comments as to the meaning of the term ìessentialî and the fact that deletions of the SE domain may generate different responses in BLM and Sgs1. Obviously, our use of the term is based on the yeast phenotype. However, because a precise SE deletion has not yet been examined in BLM, there is the possibility that it may recapitulate the yeast phenotype. One can also take a different view of the effect of deletions within BLM and say that because the BLM mutant lacking residues 131-237 has a partial defect in suppressing SCEs, it illustrates some role for the SE domain. We have therefore retained ìessentialî in the title.

Minor edits:
The spelling of syndrome and annealing have been corrected.

I thank the reviewers for their insightful comments. The above changes have improved the paper, which I trust meets your approval.

Sincerely yours,

Steven J. Brill
Professor

Acceptance letter 15 March 2010

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by the original referee #1, 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

