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Processing of DNA structures via DNA unwinding and branch migration by the *S. cerevisiae* Mph1 protein

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Abstract

The budding yeast Mph1 protein, the putative ortholog of human FANCM, possesses a 3' to 5' DNA helicase activity and is capable of disrupting the D-loop structure to suppress chromosome arm crossovers in mitotic homologous recombination. Similar to FANCM, genetic studies have implicated Mph1 in DNA replication fork repair. Consistent with this genetic finding, we show here that Mph1 is able to mediate replication fork reversal, and to process the Holliday junction via DNA branch migration. Moreover, Mph1 unwinds 3' and 5' DNA Flap structures that bear key features of the D-loop. These biochemical results not only provide validation for a role of Mph1 in the repair of damaged replication forks, but they also offer mechanistic insights as to its ability to efficiently disrupt the D-loop intermediate.

Keywords

Mph1; helicase; recombination; D-loop; replication fork; fork regression; branch migration

1. Introduction

During DNA replication, the replication fork encounters a variety of lesions and structural hindrance that block its progression. The stalled replication fork is a fragile structure prone to collapse or giving rise to double-stranded breaks, thus it must be properly stabilized and restarted to avoid incomplete replication, chromosome rearrangements, and cell death [1].

The prevalent model for the restart of the stalled replication fork entails the conversion of the fork into a Holliday junction, formed via the annealing of the leading and lagging strands [2]. This process, termed fork regression, allows the fork branch point to migrate away from the lesion, thus permitting the restart of DNA synthesis by template switching, lesion bypass, and homologous recombination [2]. Several eukaryotic ATP-dependent DNA helicases and translocases that function in replication fork preservation and repair are able to catalyze the regression of DNA replication forks [1]. These include the RecQ-like helicase

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BLM [3], the Swi2/Snf2-like DNA motor protein Rad5/HLTF [4,5], and FANCM, the protein mutated in the cancer-prone disease Fanconi anemia (FA), of complementation group M [6,7].

FANCM is structurally related to the archeal protein Hef, which can dissociate DNA structures that resemble the replication fork or contain the Holliday junction [8]. Interestingly, Hef also possesses a structure-specific endonuclease activity that is stimulated by ATP hydrolysis [8]. Subsequently, FANCM was found to dissociate similar DNA structures via DNA branch migration [7]. However, FANCM harbors neither a nuclease nor a canonical helicase activity [9,10]. Putative orthologs of FANCM have been described in other organisms, such as the *S. cerevisiae* Mph1 (mutator phenotype 1) protein [11] and the *S. pombe* Fml1 (FANCM like 1) protein [12].

MPH1 was first identified based on the spontaneous mutator phenotype of a deletion mutant [13]. Mutant cells are also sensitive to various genotoxic agents including ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), 4-nitroquinoline 1-oxide (4NQO), and camptothecin [14]. The *mph1* mutator phenotype is suppressed by the *rev3* mutation, suggesting that mutations in *mph1* cells stem from the translesion bypass of pre-mutagenic lesions by DNA polymerase ζ that harbors Rev3 protein as the catalytic subunit [13]. Mph1 likely promotes the error-free repair of DNA lesions via homologous recombination (HR), since the *mph1* mutation is epistatic to mutations in genes of the *RAD52* epistasis group required for HR [14]. Interestingly, genetic analysis has revealed that *MPH1* regulates HR to favor the formation of non-crossovers. This HR regulatory role of *MPH1* occurs independently of other crossover suppression mechanisms that are mediated by the *SGS1* and *SRS2* genes [15]. In addition, several interactors that likely up- or down-regulate the activities of Mph1 have been identified. Specifically, the histone-fold proteins Mhf1 and Mhf2 appear to co-operate with Mph1 in DNA damage and replication fork repair in cells and are expected to up-regulate Mph1's activity [16,17]. Other studies have found a role of the Smc5-Smc6 complex, involved in different aspects of chromosome metabolism including damage repair, in the negative regulation of the Mph1 protein function [18].

Mph1 protein has been purified by our research group to near homogeneity from yeast cells tailored to overexpress the protein [19]. Our biochemical studies have revealed that Mph1 possesses a DNA-dependent ATPase activity and a DNA helicase activity with a 3' to 5' polarity with regard to the direction of Mph1 translocation on ssDNA [19]. The DNA helicase activity distinguishes Mph1 from FANCM, which is devoid of such an activity. Moreover, Mph1 dissociates D-loops within the context of Rad51-mediated homologous pairing reactions, an attribute that is likely germane for its role in the suppression of crossover formation during HR [15]. Herein, we describe our studies that reveal the ability of Mph1 to process various DNA structures via its helicase function or by DNA branch migration. The results should form the basis for further defining the multi-faceted role of Mph1 in chromosome metabolism and the manner by which the various activities of Mph1 are subject to regulation by other protein factors.

2. Materials and Methods

2.1 Purification of proteins

Mph1 and the ATPase-deficient D209N mutant were expressed in yeast cells and purified to near homogeneity, as described [15,19]. Srs2 and Sgs1 were expressed in *E. coli* and insect cells, respectively, and purified to near homogeneity, as described [20,21].

2.2 DNA substrates

Substrates that resemble the DNA replication fork, Holliday junction (HJ), and the Flap structure were prepared by hybridizing oligonucleotides (Integrated DNA Technology), as described [7]. The oligonucleotides used in substrate preparation are listed in Table 1. The plasmid sized replication fork was prepared from plasmids pG46 and pG68, as described [3, 4]. The nicking enzymes Nt. BbvCI and Nb. BbvCI used in substrate preparation were from New England Biolabs.

2.3 Assay to monitor processing of Flap structures

The indicated concentration of Mph1 was incubated with 5 nM of the radiolabeled DNA substrate in 10 μ l buffer A (25 mM Tris-HCl pH 7.5, 1 mM DTT, 100 μ g/ml bovine serum albumin, 30 mM KCl, 5 mM MgCl₂, 2 mM ATP, 15 mM phosphocreatine and 30 units/ml of creatine phosphokinase) at either 30°C (Fig. 1 and Fig. 2) or 20°C (Fig. 3). The reaction was terminated at the indicated times by treatment with 0.5% SDS and 0.5 mg/ml Proteinase K for 5 min at 37°C. The reaction mixtures were resolved in a 10% native polyacrylamide gel in TAE buffer (40 mM Tris-acetate, pH 7.4, 0.5 mM EDTA) at 4°C. Gels were dried onto Whatman DE81 paper (Whatman International Limited) and then analyzed in a Personal Molecular Imager FX PhosphorImager (Bio-Rad).

2.4 Fork regression and branch migration assay

The indicated concentration of Mph1, Srs2, or Sgs1 was incubated at 30°C with 5 nM of the radiolabeled DNA substrate in 10 μ l buffer A with 0.5 mM of MgCl₂. The reaction was terminated at the indicated times and resolved in an 8% native polyacrylamide gel in TBE buffer (45 mM Tris-borate, pH 8.0, 1 mM EDTA) at 4°C as above. The gels were dried and analyzed as above.

2.5 Regression and branch migration of the plasmid-based sigma substrate

The indicated concentration of Mph1 or mph1 D209N was incubated with 0.75 nM of the radiolabeled substrate in 10 μ l of buffer A with 0.5mM MgCl₂ at 30°C. The reaction was quenched by treatment with 10 mM AMP-PNP, and subsequently treated with the indicated restriction enzyme at 37°C for 60 minutes, deproteinized and resolved in an 8% native polyacrylamide gel in TBE buffer at 4°C. Alternatively, fork regression reactions were deproteinized by SDS/PK and resolved in a 0.8% agarose gel containing 0.5 μ g/ml ethidium bromide at 25°C. The gels were dried and analyzed as above.

3. Results

3.1 Mph1 helicase activity processes DNA Flap structures

Mph1 can unwind D-loop substrates that either possess homology in the triple-stranded D-loop region or not [15]. To gain further insights into the mechanism that underlies this Mph1 attribute, we asked whether Mph1 would process Flap structures that bear resemblance to the D-loop. Given that no homology is present in these 3' and 5' Flap structures, their dissociation could only be mediated by the helicase function of Mph1.

Examination of the 5' Flap structure in which either the bottom or the short top DNA strand is ³²P-labeled revealed the efficient removal of 5' overhanging "Flap" DNA strand to generate a partial duplex with a 5' overhang that is, as expected from our published study [19], resistant to Mph1 action (Fig. 1A, B). That substrate processing is dependent on ATP hydrolysis was verified by substituting ATP with a non-hydrolyzable analog (ATP- γ -S or AMP-PNP) and by replacing Mph1 with the ATP hydrolysis defective mph1 D209N mutant (Fig. 1C). We note that, given the known 3' to 5' polarity of translocation of Mph1 on

ssDNA, the removal of the 5' Flap strand was unexpected and may help explain the versatility of Mph1 in the disruption of D-loops that harbor different invading DNA strands (see Discussion).

In reactions containing the 3' Flap substrate that was labeled on the short strand, Mph1 removed the Flap strand to generate the radiolabeled partial duplex and also the free short strand (Fig. 2A). The appearance of the partial duplex was as expected, based on Mph1's ability to unwind DNA with a 3'-5' polarity [19]. Interestingly, accumulation of the free short strand occurred faster than that of the partial duplex, suggesting that Mph1 can directly remove it from the Flap substrate. To test this premise further, we labeled the bottom strand of the Flap structure instead. In this case, Mph1 yielded three radiolabeled products - the Y fork, partial duplex, and the free bottom strand (Fig. 2B). Importantly, the appearance of the Y fork as the earliest product (Fig. 2B) confirmed that Mph1 is able to efficiently displace the short strand from the Flap structure. Again, we found that ATP hydrolysis is needed for substrate processing (Fig. 2C).

In the above experiments, it was possible that the removal of the 5' Flap strand (Fig. 1) and of the short strand from the 3' Flap (Fig. 2) owed to binding of Mph1 to a short DNA gap at the branch point in the substrates arising from thermal fraying, followed by displacement of the DNA strands via the 3' to 5' DNA helicase activity of Mph1. However, decreasing the reaction temperature from 30°C to 20°C did not prevent the removal of the 5' Flap strand (Fig. 3A), and the preferential dissociation of the short strand in the 3' Flap substrate was just as prevalent at the lower temperature (Fig. 3B). Thus, the results are consistent with the premise that Mph1 employs a functional attribute distinct from its 3' to 5' DNA helicase in the processing of Flap structures. Nonetheless, it remains an open question whether thermal fraying of the DNA branch point in these structures could enable Mph1 to process the structures via its 3' to 5' helicase activity. Interestingly, the addition of a 10-nt 5' tail to the short strand in the 3' Flap significantly slowed its release from the substrate (Fig. 3C). This indicates that a free branch point in the 3' Flap substrate facilitates the removal of the short strand, which is suggestive of an ability of Mph1 to recognize such a branch point.

3.2 Processing of replication fork by Mph1 through DNA branch migration

Based on our previous work showing a 3'-5' helicase activity in Mph1 [19], we expected Mph1 to unwind a Y, or splayed arm, structure. Indeed, Mph1 dissociated this structure into single strands (Fig. 4A). We also wanted to examine whether Mph1 would process a DNA structure that resembles a replication fork. Such a replication fork substrate, termed movable replication fork (MRF), was constructed with 60-mer oligonucleotides as described previously [7]. Owing to the presence of DNA homology in the two arms of the DNA fork, regression of the fork-like structure is possible, to yield two linear dsDNA duplex products, including one that is radiolabeled and easily detected by phosphorimaging analysis after gel electrophoresis (Fig. 4B). We found that Mph1 efficiently processes the MRF into linear duplex products (Fig. 4B). No product was seen when we substituted the MRF with an equivalent structure, static replication fork (SRF), that lacks DNA homology in the two arms of the fork (Fig. 4D). In fact, the SRF is refractory to much higher concentrations of Mph1 (Fig. 4D). We verified that ATP hydrolysis is needed for the processing of the MRF (Fig. 4C). The above results thus provide evidence for an ability of Mph1 to promote replication fork regression, and the dependence of this reaction on DNA homology indicates that Mph1 does so by branch migration, rather than by a DNA strand unwinding and re-annealing mechanism.

3.3 Processing of the Holliday junction by Mph1 through DNA branch migration

Replication fork regression entails the formation of a Holliday junction (HJ) intermediate, which is often referred to as the “chicken foot” structure [2]. Since Mph1 can efficiently process the MRF (Fig. 4B), we wished to verify that it also acts on the Holliday junction. For this purpose, a pair of HJ substrates that harbor DNA homology (the movable Holliday junction or MHJ) or no homology (the static Holliday junction or SHJ) were constructed as previously described [7], and tested with purified Mph1 (Fig. 5A). Mph1 could process the MHJ efficiently to yield linear duplex, but even a much higher concentration of the protein was unable to dissociate the SHJ (Fig. 5C). The use of non-hydrolyzable ATP analogues and the mph1 D209N mutant confirmed the dependence of MHJ dissociation on ATP hydrolysis (Fig. 5B). Thus, Mph1 can process the MHJ via DNA branch migration.

3.4 Comparison of Mph1 to Srs2 and Sgs1

Like Mph1, the Srs2 and Sgs1 helicases regulate HR in favor of non-crossover formation. Srs2 prevents D-loop formation by removing Rad51 protein from the invading ssDNA strand [22,23], and Sgs1 co-operates with its partner proteins Top3 and Rmi1 to dissolve the Holliday junction to yield exclusively non-crossover products [24]. BLM, the human ortholog of Sgs1, possesses DNA helicase and branch migration activities and can mediate the regression of the replication fork [3]. Using the movable replication fork (MRF) and movable Holliday junction (MHJ) substrates, we showed that Srs2 does not act on either structure to any significant degree and that Sgs1 can mediate regression of the MRF and branch migration of the MHJ (Fig. 6). However, Mph1 appears to be more proficient than Sgs1 in the fork regression reaction (Fig. 6).

3.5 Examination of DNA replication fork regression and branch migration with a plasmid length sigma structure

The substrates used earlier to demonstrate the fork regression and DNA branch migration activities of Mph1 were constructed using oligonucleotides. We wished to test the proficiency of Mph1 to promote fork regression using a ^{32}P -labeled, plasmid DNA-based sigma-shaped substrate that bears a closer resemblance to a stalled replication fork encountered in cells [3, 4]. The use of such a substrate also allows us to examine the efficiency of DNA branch migration of up to 2.9 kb. The formation of a regressed arm on this substrate can be monitored by digestion with a number of restriction enzymes, and completion of DNA branch migration yields a 2.9 kb ^{32}P -labeled linear duplex product (Fig. 7A). As revealed by restriction digest, Mph1 mediated the regression of the fork substrate in the presence of ATP (Fig. 7B lanes 8-12), but the mph1 D209N mutant did not (Fig. 7B, lanes 14-18). Importantly, Mph1 could branch migrate the regressed fork over the 2.9 kb distance that leads to the generation of the linear duplex product (Fig. 7C). The branch migration reaction occurred efficiently, because as little as 2 nM Mph1 was able to produce a detectable amount of linear duplex product within 10 minutes (Fig. 7C). As expected, reactions containing the ATP analogs AMP-PNP and ATP γ S did not support DNA branch migration (Fig. 7D). Taken together, these results indicate that Mph1 promotes highly efficient DNA replication fork regression and DNA branch migration in an ATP hydrolysis-dependent manner.

4. Discussion

The studies presented herein and earlier have uncovered ATP-dependent activities in Mph1 (see Supplemental Fig. 1 for summary) germane for understanding its multifaceted role in genome maintenance [15,19]. Aside from possessing a 3'-5' DNA helicase activity, Mph1 also efficiently unwinds D-loops within the context of Rad51-mediated homologous DNA pairing reactions [15]. Moreover, Mph1 is also adept at dissociating static D-loops in which

the displaced strand harbors no homology to the duplex region within the D-loop. The D-loop dissociative activity Mph1 is rather versatile, being capable of dismantling static D-loops and Rad51-made D-loops with no ssDNA overhang or with a 3' or 5' ssDNA overhang [15]. This attribute of Mph1 is very likely relevant for its role in the suppression of crossovers in favor of the formation of gene conversion products during DNA double-strand break repair by HR [15].

We note that Mph1 can likely utilize its 3'-5' helicase activity to disrupt D-loops that harbor a 5' invading strand (Fig. 8A; [15]). In the present study, using Flap DNA substrates that bear key features of various types of D-loop, we have provided additional insights into the versatility of Mph1 to dissociate D-loops that harbor either a 3' invading strand or no overhang. Specifically, we show that Mph1 can efficiently remove the short strand from a 3' Flap structure (Fig. 2) and also the 5' Flap strand (Fig. 1), which both resemble an invading 3' strand in a D-loop expected to be made by recombinase proteins during DSB repair in cells (Fig. 8A). The use of Flap substrates that bear only a 3' Flap strand or both 3' and 5' ssDNA extensions (Fig. 3B, C) have provided evidence that the presence of a free branch point facilitates the removal of the short DNA strand. Even though we favor the possibility that Mph1 possesses a functional attribute distinct from its 3'-5' helicase activity allowing it to remove the short strand from such a Flap structure, it remains possible that thermal fraying at the branch point leads to activation of the 3' to 5' helicase function of Mph1.

FANCM, its putative *S. pombe* ortholog Fml1, and Mph1 have been implicated in DNA replication fork repair [9,12,14,25,26]. Both FANCM and Fml1 catalyze the regression of the DNA replication fork to form a HJ-like intermediate called the “chicken foot” structure, and FANCM has been shown to mediate extensive branch migration of this structure [6, 7, 12]. Likewise, we have shown herein that Mph1 is highly adept at replication fork regression and branch migration of the “chicken foot” structure over several kilobase pairs.

In summary, our work with Mph1 helps establish replication fork regression and DNA branch migration as intrinsic properties of FANCM/Fml1/Mph1 class of DNA translocases. These activities are likely germane for the replicative bypass of DNA lesions as depicted in Figure 8B. We note, however, that unlike Mph1, neither FANCM nor Fml1 possesses a significant helicase activity [9, 12]. As we have mentioned above, it is possible that the 3'-5' helicase activity of Mph1 allows it to disrupt the D-loop intermediate formed by a 5' invading DNA strand.

The replication fork regression activity of FANCM is up-regulated by a complex of two conserved histone fold proteins MHF1 and MHF2 [16,17], and genetic studies in *S. cerevisiae* have provided evidence for a role of the Smc5-Smc6 complex in the negative regulation of Mph1. The results from our biochemical studies described earlier [15,19] and herein should provide the requisite experimental foundation for examining the functional co-operation of Mph1 with the *S. cerevisiae* Mhf1-Mhf2 complex and the regulation of Mph1 activities by the Smc5-Smc6 complex.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- DNA Flap unwinding by Mph1 explains how it dissociates D-loops.
- Mph1 proficiently regresses the DNA replication fork.
- Mph1 processes the Holliday junction by branch migration.

Figure 1. Processing of the 5' Flap structure by Mph1

(A), (B) 5' Flap substrates labeled with ^{32}P (*) on either the short (A) or bottom strand (B) were incubated with Mph1 (2 nM) for indicated times. The results were quantified and graphed. Each experiment was performed in triplicate, and mean values are shown with standard deviation.

(C) To show dependence of 5' Flap unwinding on ATP hydrolysis, the mph1 D209N mutant (2 nM) was examined and ATP was replaced by ATP- γ -S or AMP-PNP. The incubation time was 10 min.

In all the experiments, the substrate concentration was 5 nM and the reaction temperature was 30°C. Symbol: NP, minus Mph1 control.

Figure 2. Processing of the 3' Flap structure by Mph1

(A), (B) 3' Flap substrates labeled with ^{32}P (*) on either the short (A) or bottom strand (B) were incubated with Mph1 (2 nM) for the indicated times. The results were quantified and graphed. Each experiment was performed in triplicate, and mean values are shown with standard deviation.

(C) To show dependence of 3' Flap unwinding on ATP hydrolysis, the mph1 D209N mutant (2 nM) was examined and ATP was replaced by ATP- γ -S or AMP-PNP. The incubation time was 10 min.

In all the experiments, the substrate concentration was 5 nM and the reaction temperature was 30°C. Symbol: NP, minus Mph1 control

Figure 3. Relevance of the DNA branch point in the Flap substrates processing

(A) 5' Flap substrate, (B) 3' Flap substrate, and (C) 3' Flap substrate containing a 10-nt 5' tail on the short strand, all labeled with ^{32}P (*) on the bottom strand, were incubated with Mph1 (2 nM) for the indicated times. The results were quantified and graphed. Each experiment was performed in triplicate, and mean values are shown with standard deviation.

In all the experiments, the substrate concentration was 5 nM and the reaction temperature was 20°C. Symbol: NP, minus Mph1 control.

Figure 4. Replication fork regression by Mph1

(A) ^{32}P -labeled (*) Y DNA was incubated with Mph1 (2 nM) for the indicated times. The results were quantified and graphed. Each experiment was performed in triplicate, and mean values are shown with standard deviation.

(B) ^{32}P -labeled (*) movable replication fork DNA (MRF) was incubated with Mph1 (1 nM) for the indicated times. The results were quantified and graphed. Each experiment was performed in triplicate, and mean values are shown with standard deviation. Note that the substrate harbored an A/C mismatch to minimize spontaneous regression.

(C) To show dependence of replication fork regression on ATP hydrolysis, the mph1 D209N mutant (1 nM) was examined and ATP was replaced by ATP- γ -S or AMP-PNP. The incubation time was 5 min.

(D) ^{32}P -labeled static replication fork (SRF) was incubated with Mph1 (5 to 40 nM) for 5 and 10 min.

In all the experiments, the substrate concentration was 5 nM and the reaction temperature was 30°C. Symbols: NP, minus Mph1 control; HD, heat-denatured substrate.

Figure 5. Branch migration of the Holliday junction by Mph1

(A) ^{32}P -labeled (*) movable Holliday junction (MHJ) was incubated with Mph1 (1 nM) for the indicated times. The results were quantified and graphed. Each experiment was performed in triplicate, and mean values are shown with standard deviation.

Note that the indicated positions in the two right arms of the HJ harbored a different base pair (A/T or G/C) to minimize spontaneous branch migration.

(B) To show dependence of branch migration on ATP hydrolysis, the mph1 D209N mutant (1 nM) was examined and ATP was replaced by ATP- γ -S or AMP-PNP. The incubation time was 5 min.

(C) ^{32}P -labeled static Holliday junction (SHJ) was incubated with Mph1 (5 to 40 nM) for 5 and 10 min.

In all the experiments, the substrate concentration was 5 nM and the reaction temperature was 30°C. Symbols: NP, minus Mph1 control; HD, heat-denatured substrate.

Figure 6. Comparison of Mph1, Srs2, and Sgs1 for fork regression and branch migration
 ^{32}P -labeled movable replication fork (MRF) and movable Holliday junction (MHJ) were incubated with Mph1, Srs2, or Sgs1 (5 nM each) for 5 min. The results were quantified and graphed. Each experiment was performed in triplicate, and mean values are shown with standard deviation.

In all the experiments, the substrate concentration was 5 nM and the reaction temperature was 30°C. Symbols: NP, no DNA motor protein added; HD, heat-denatured substrate.

Figure 7. Fork regression and branch migration examined using a plasmid sized sigma structure (A) Schematic representation of the ^{32}P -labeled sigma substrate and the outcome of Mph1-mediated regression and branch migration. A, B, E, F, and N denote sites for the restriction endonucleases AvrI, BamHI, EcoRI, AflIII, and AlwNI, respectively. The formation of a regressed fork structure and branch migration of the four-way junction structure can be monitored by restriction digest. Branch migration of the four-way junction point over 2.9 kb yields a ^{32}P -labeled linear duplex. The asterisk denotes the ^{32}P label.

(B) The sigma substrate was incubated with Mph1 or mph1 D209N (2 nM) for 20 min and then treated with the indicated restriction enzymes (see (A) for definition).

(C) The sigma substrate was incubated with Mph1 (2 nM) for the indicated times and then subjected to agarose gel electrophoresis and phosphorimaging analysis.*** The results were quantified and graphed. The experiment was performed in triplicate, and mean values are shown with standard deviation.

(D) To show dependence of fork regression and branch migration on ATP hydrolysis, the mph1 D209N mutant (2 nM) was examined and ATP was replaced by ATP- γ -S or AMP-PNP. The incubation time was 10 min.

In all the experiments, the substrate concentration was 0.75 nM and the reaction temperature was 30°C. Symbols: S, I, P1, and P2 represent the sigma substrate, intermediates harboring the regressed fork, product 1, and product 2, respectively; NP, minus Mph1 control. In (C) and (D), free ^{32}P -labeled pG46B plasmid used in substrate construction is denoted by **, and the species resulting from the annealing of linearized pG46B and pG68A is denoted by ***.

Figure 8. Functional attributes of Mph1 germane for biological functions

(A) Dissociation of D-loop structures by Mph1 in HR regulation. Our results suggest that Mph1 utilizes its structure-specific unwinding activity to disrupt D-loops that harbor a 3' invading strand but its 3'-5' helicase activity to dissociate D-loops that harbor a 5' invading strand.

(B) Relevance of replication fork regression and branch migration activities of Mph1 in replication fork restart. To restart the replication fork, the “chicken foot” structure in step (4) can be dissociated by reverse branch migration or used as the substrate to initiate homologous recombination. For discussions see the review by Atkinson and McGlynn [2].

Table 1

Oligonucleotides used in this study

Name	Length	Sequence (5' to 3')
H1	40	ATTAAGCTCTAAGCCATGAATTCAAATGACCTCTTATCAA
H1-10	50	GTGTACATCCATTAAGCTCTAAGCCATGAATTCAAATGACC TCTTATCAA
H3	80	TTGATAAGAGGTCATTTGAATTCATGGCTTAGAGCTTAATTG CTGAATCTGGTGCTGGGATCCAACATGTTTTAAATATG
H5	80	CATATTTAAAACATGTTGGATCCCAGCACCAGATTCAGCAT ACGTTACCGATCGTACGTTTCGATGCTGGCTACTGCTAGC
H6	40	GCTAGCAGTAGCCAGCATCGAACGTACGATCGGTAACGTA
X01	61	GACGCTGCCGAATTCTACCAAGTGCCTTGCTAGGACATCTTTG CCCACCTGCAGGTTACCC
X02	62	TGGGTGAACCTGCAGGTGGGCAAAGATGTCCATCTGTTGTA ATCGTCAAGCTTTATGCCGTT
X03	63	GAACGGCATAAAGCTTGACGATTACAACAGATCATGGAGCT GTCTAGAGGATCCGACTATCGA
X04	62	ATCGATAGTCGGATCCTCTAGACAGCTCCATGTAGCAAGGC ACTGGTAGAATTCGGCAGCGT
FM	61	GGGTGAACCTGCAGGTGGGCAAAGATGTCCCAGCAAGGCA CTGGTAGAATTCGGCAGCGTC
XM3	61	GGGTGAACCTGCAGGTGGGCAAAAATGTCCTAGCAAGGCA CTGGTAGAATTCGGCAGCGTC
XM4	62	GAACGGCATAAAGCTTGACGATTACAACAGATGGACATTTT TGCCACCTGCAGGTTACCC
DS1	30	GGACATCTTTGCCCACCTGCAGGTTACCC
DS2	31	TGGGTGAACCTGCAGGTGGGCAAAGATGTCC
DS3	31	CATGGAGCTGTCTAGAGGATCCGACTATCGA