DNA Repair Factor MRE11/RAD50 Cleaves 3'-Phosphotyrosyl Bonds and Resects DNA to Repair Damage Caused by Topoisomerase 1 Poisons

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Background: The DNA repair factor MRE11-RAD50 is a multifunctional DNA complex with nuclease, DNA binding, and ATPase activity.

Results: MRE11-RAD50 cleaves 3'-phosphotyrosyl bonds, such as those in trapped topoisomerase 1-DNA complexes, and then resects, generating a DNA end that can prime repair synthesis.

Conclusion: MRE11-RAD50 is involved in the repair of trapped topoisomerase 1-DNA complexes.

Significance: MRE11-RAD50 may be a useful biomarker for tumor sensitivity to topoisomerase 1 poisons.

MRE11-RAD50 is a highly conserved multifunctional DNA repair factor. Here, we show that MRE11-RAD50 cleaves the covalent 3'-phosphotyrosyl-DNA bonds that join topoisomerase 1 (Top1) to the DNA backbone and that are the hallmark of damage caused by Top1 poisons such as camptothecin. Cleavage generates a 3'-phosphate DNA end that MRE11-RAD50 can resect in an ATP-regulated reaction, to produce a 3'-hydroxyl that can prime repair synthesis. The 3'-phosphotyrosyl cleavage activity maps to the MRE11 active site. These results define a new activity of MRE11 and distinguish MRE11-RAD50 functions in repair of Top1-DNA complexes and double-strand breaks.

Topoisomerase 1 (Top1) regulates DNA superhelicity by iteratively nicking and religating the DNA backbone in an isoenergetic reaction (1, 2). The intermediate in this reaction is a covalent complex between a tyrosine in the enzyme and a 3'-phosphate in the DNA backbone. The half-life of the Top1-DNA complex is selectively prolonged by the naturally occurring compound camptothecin (CPT), and Top1 poisons related to CPT, such as irinotecan and topotecan, are widely used in cancer chemotherapy. The hallmark of CPT-induced DNA damage is a nick adjacent to a 3'-phosphotyrosyl bond between Top1 and the DNA backbone. If replication encounters this nick, a double-strand break (DSB) will form. DSBs are thought to be a key source of cytotoxicity due to CPT treatment, and a number of repair factors and pathways implicated by genetic analyses in the response to CPT are involved in DSB repair (3). In addition, specific factors have been shown to release trapped Top1 complexes in vitro, including the structure-specific nuclease Tdp1 (4), Rad1-Rad10 (human ERCC1-XPF) (5), and Mms4-Mus81 (6). Severe CPT sensitivity results from deletion of MRE11 in Saccharomyces cerevisiae (7–9) or its homolog (rad32) in Schizosaccharomyces pombe (10). MRE11 is a multifunctional nuclease, active as a 3'–5' double-stranded DNA exonuclease, a single-stranded DNA endonuclease, and an AP lyase in vitro (11–14). It forms a complex with RAD50, which contains an ATP-binding cassette ATPase and ATP binding by RAD50 regulates the activity of the MRE11 nuclease (13, 15–18). A third factor, NBS1 (Xrs2 in S. cerevisiae), regulates the activity of MRE11-RAD50 in DNA repair and damage signaling (19, 20). MRE11 and RAD50 are highly conserved and found in all three living kingdoms, archaea, eubacteria, and eukaryotes. MRE11-RAD50-NBS1 functions in pathways, that include DNA repair, telomere maintenance, and immunoglobulin gene diversification (21–24). In DSB repair, MRE11-RAD50 (M-R) excises and bridges broken ends, and this may account in part for the function of MRE11 in the CPT response. However, the mechanism of M-R function in the response to CPT has not been clearly defined biochemically. It is of particular interest because M-R levels are reduced in some tumors, including a subset of the very common colorectal tumors (25–27). A clear picture of M-R function might provide guidance in the use of CPT derivatives in the treatment of these tumors.

We have carried out biochemical analysis directed toward defining functions of M-R that could contribute to repair of CPT-induced DNA damage by analyzing the activities of recombinant enzyme from the thermophilic archaeabacterium Pyrococcus furiosus. Here, we show that either the P. furiosus M-R complex or P. furiosus MRE11 alone can cleave a 3'-phosphotyrosyl bond to generate a product carrying a 3'-phosphate end. The M-R complex resects this DNA end to generate a 3'-hydroxyl end that can prime repair synthesis in an ATP-
regulated reaction. The ability to carry out both repair steps distinguishes M-R from other structure-specific enzymes that act at covalent protein-DNA bonds. These activities of M-R may be employed to process abortive intermediates in DNA relaxation during normal cell proliferation or to repair damage caused by Top1 poisons. These results predict that tumors expressing reduced levels of M-R will be especially sensitive to treatment with Top1 poisons and suggest that MRE11 and/or RAD50 may be a useful biomarker for tumor stratification.

EXPERIMENTAL PROCEDURES

DNAs and DNA Substrates—Phage φX174 single-stranded circular DNA was purchased from New England Biolabs. Oligonucleotides carrying a 3’-phosphothyrosine were purchased from Midland Certified Reagent Co. (Midland, TX), and other oligonucleotides were purchased from either Fisher/Operon or Integrated DNA Technologies. Oligonucleotides were gel-purified and 5’-32P-end-labeled using T4 polynucleotide kinase or polynucleotide kinase lacking 3’-phosphatase activity (New England Biolabs) and [γ-32P]ATP (PerkinElmer Life Sciences) according to the manufacturer’s instructions, and labeled DNAs were isolated on illustra MicroSpin G-50 columns (GE Healthcare). Oligonucleotide sequences and additional information on generating substrates are provided under supplemental “Materials and Methods.” Assays of 3’-phosphothyrosine cleavage activity were routinely carried out using ES22 (5’-GTAGAGGATCTAAAAGACTT-(P)-Tyr-3’). Similar enzymatic activity was observed with other DNA sequences bearing a 3’-phosphothyrosine.

Enzymes—P. furiosus MRE11 or M-R was expressed or coexpressed from Escherichia coli BL21(DE3) cells and purified as described previously (13, 14). Briefly, cells were grown at 37 °C to an OD600 of 0.6–0.8, and expression was induced with 1 mM isopropyl β-D-thiogalactopyranoside for 4 h. Cells were harvested by centrifugation and lysed by sonication. Cell debris was pelleted, the cleared supernatant was heat-treated at 75 °C for 20 min to denature endogenous E. coli nuclease activities, the debris was pelleted, and proteins were purified from the supernatant by nickel-nitrioltriacetic acid (Qiagen) affinity chromatography and dialyzed overnight. MRE11 mutants were generated by site-directed mutagenesis (Stratagene) and verified by sequencing. Oligonucleotides used for mutagenesis are shown in supplemental Table 1.

Enzymatic Assays—Unless indicated otherwise, M-R and MRE11 3’-phosphothyrosyl cleavage assays were performed in 20-μl reactions containing 50 mM MOPS (pH 6.5), 60 mM NaCl, 5 mM MnCl2, 1 mM DTT, and 0.5% (v/v) PEG 8000 with 200 nM DNA substrate and 50 nM enzyme and incubated for 1 h at 55 °C. This reaction temperature, which is suboptimal for thermophilic enzymes, was used to preserve DNA integrity based on Tm and native gel electrophoresis. Exonuclease and endonuclease reactions were performed as described previously (14). Reactions with human Tdp1 were performed as described (28) using purified human Tdp1 generously provided by Dr. James Champoux (University of Washington School of Medicine). Reactions were stopped by the addition of 0.5 volume of formamide containing 20 mM EDTA, and products were resolved on 8 M urea-20% acrylamide gels and analyzed using a PhosphorImager (Molecular Dynamics).

The purity of ATP, ADP, and AMP-PNP (all purchased from Sigma) was verified by thin layer chromatography. Extension reactions were performed on the partial DNA duplex substrate (200 nM), cleaved with 50 nM M-R in the presence of 0.5 mM ATP in a volume of 40 μl. Reactions were stopped, and DNA was purified by phenol/chloroform extraction and concentrated by ethanol precipitation. Pellets were resuspended in 20 μl of Klenow buffer (NEBuffer 2, New England Biolabs) and then split in half. For DNA extensions, exonuclease-deficient Klenow polymerase (1 unit; New England Biolabs) and dNTPs (0.5 mM; Fermentas) were added and reactions were incubated at 37 °C for 30 min. Reactions were then stopped by the addition of 0.5 volume of formamide containing 20 mM EDTA, and products were resolved on 8 M urea-20% acrylamide gels and analyzed using a PhosphorImager.

RESULTS

MRE11-RAD50 Specifically Cleaves a 3’-Phosphothyrosine, Generating a 3’-Phosphate DNA End—The activity of P. furiosus M-R was assayed on a labeled synthetic DNA substrate carrying a 3’-phosphothyrosine (Fig. 1A). This substrate recapitulates the hallmark damage formed by CPT treatment and is similar to the substrates that have been routinely used to assay activities of other structure-specific enzymes, such as Tdp1.
**P. furiosus** M-R (0–100 nM) or MRE11 was incubated with the DNA substrate (200 nM), and products were resolved by gel electrophoresis and imaged. Both M-R and MRE11 alone cleaved the DNA substrate bearing a 3’-phosphotyrosine to generate a faster migrating fragment (Fig. 1B). Cleavage of a 3’-phosphotyrosine depended upon the presence of Mn$^{2+}$ (5 mM MnCl$_2$) but was not inhibited by Mg$^{2+}$ (supplemental Fig. S1). Mn$^{2+}$ is similarly required for M-R 3’–5’ exonucleolytic, single-stranded DNA endonucleolytic, and AP lyase activities (13, 14), although not for 5’–3’ exonucleolytic activity (19). Quantitation of activities in three separate experiments showed that M-R and MRE11 were comparably active, with 50% cleavage supported by 5–10 nM enzyme (Fig. 1C). M-R and MRE11 also exhibited comparable kinetics over the course of a 90-min reaction (supplemental Fig. S2). Cleavage by M-R affected only the DNA strand carrying the 3’-phosphotyrosine, and the opposite strand remained intact (supplemental Fig. S3). We conclude that either MRE11 or the M-R complex can cleave a 3’-phosphotyrosyl bond.

To define the DNA end produced upon M-R cleavage of a 3’-phosphotyrosine, we compared mobilities of the Tdp1 cleavage product, shown to be a 3’-phosphate (4), the M-R cleavage product and synthetic oligonucleotides bearing a 3’-phosphate or a 3’-hydroxyl. The Tdp1 and M-R cleavage products co-migrated with each other and with the synthetic oligonucleotide marker bearing a 3’-phosphate and slightly faster than a marker oligonucleotide bearing a 3’-hydroxyl (Fig. 1D). Thus, based on product mobility, M-R cleavage of a 3’-phosphotyrosine appears to generate a 3’-phosphate.

Tdp1, first identified by virtue of its ability to cleave a 3’-phosphotyrosine, can also cleave a 3’-phosphobiotin as well as a variety of other covalent 3’-phospho-adducts (29). Assays of cleavage of a partial duplex substrate carrying a 3’-phosphobiotin showed that, as expected, Tdp1 cleaved the 3’-phosphobiotin substrate; however, neither *P. furiosus* M-R nor MRE11 cleaved this substrate (Fig. 1E). We conclude that M-R cleavage of a 3’-phosphotyrosyl bond does not reflect an indiscriminate activity of the enzyme.

**M-R Cleaves 3’-Phosphotyrosyl Bonds in Nicked and Gapped Substrates**—Top1 insertion into the DNA backbone creates a DNA nick adjacent to the covalent phosphotyrosine-DNA bond. We asked if M-R could cleave phosphotyrosine bonds in synthetic DNA substrates bearing nicks or gaps (Fig. 2A). M-R cleavage at a 3’-phosphotyrosine in a nicked substrate was only slightly less efficient than in a gapped (1–5 nucleotides) or partial duplex substrate (Fig. 2B and C). Thus, M-R is predicted to be active at 3’-phosphotyrosines at DNA nicks, the likely physiological context for repair of covalent Top1-DNA complexes.

**3’-Phosphotyrosyl Cleavage Activity Maps to MRE11 Active Site**—To identify MRE11 active site residues essential for cleavage of a 3’-phosphotyrosine, we created a panel of 11 mutants modified at conserved positions in the MRE11 active site phosphodiesterase motifs (Fig. 3A). The residues mutated included those involved in both metal ion coordination and catalysis (30). Mutant MRE11 proteins were coexpressed with wild-type RAD50, and the complexes were purified and assayed for 3’-phosphotyrosyl cleavage activity. One mutant enzyme, M(H52A)-R, retained some 3’-phosphotyrosine cleavage activity, but none of the others were active on this substrate, even following an extended incubation (6 h) (Fig. 3B).
We compared the endonucleolytic and 3′–5′ double-stranded DNA exonucleolytic activities of wild-type M-R and mutants M(H52A)-R and M(H85A)-R (mutated at the catalytic histidine) (30, 31) and mutant M(H173Y)-R (which corresponds to an allele that severely impairs MRE11 function in *S. cerevisiae*) (31). M(H52A)-R, but not M(H85A)-R or M(H173Y)-R, exhibited endonuclease activity on a dX174 single-stranded DNA substrate (Fig. 3C). M(H52A)-R, M(H85A)-R, and M(H173Y)-R all exhibited impaired exonucleolytic cleavage of a blunt 45-bp DNA duplex substrate relative to wild-type M-R (Fig. 3D). Similar results have been obtained in assays of the endonucleolytic and 3′–5′ exonuclease activities of *P. furiosus* MRE11 with mutations to serine instead of alanine at His-52 and His-85 (20). MRE11 3′-phosphotyrosine cleavage activity thus maps to the conserved phosphoester motifs of the MRE11 active site.

**M-R Resects 3′-Phosphorylated Ends in ATP-regulated Reaction**—The 3′-phosphate produced upon M-R cleavage at a 3′-phosphotyrosine must be removed to enable repair synthesis. M-R can function as 3′–5′ double-stranded DNA exonuclease, but it has not been shown to resect 3′-phosphorylated ends, and no resection was evident under our standard reaction conditions on partial duplex substrates carrying either a 3′-phosphate or a 3′-hydroxyl (Fig. 4A, left). ATP binding by RAD50 can promote M-R 3′–5′ double-stranded DNA exonucleolytic activity at 3′-ends protected by 5′-overhangs (19). Consistent with this, we found that resection was stimulated by low concentrations of ATP (0.5 mM) but was inhibited by high concentrations (2.5 mM). There was no evidence of 3′-phosphotyrosine cleavage and poorly stimulated resection at all concentrations. These results suggest that ATP-dependent regulation depends upon a mechanism that stabilizes the resulting DNA end.

We compared the effects of ATP, the non-hydrolyzable analog AMP-PNP, and ADP on 3′-phosphotyrosine cleavage and resection (Fig. 4B). ATP inhibited cleavage at all concentrations, stimulated resection at low concentrations, and inhibited resection at higher concentrations. AMP-PNP inhibited 3′-phosphotyrosine cleavage but supported resection at all concentrations tested (0.5–2.5 mM). ADP inhibited cleavage and poorly stimulated resection at all concentrations. These results suggest that ATP-dependent regulation depends upon a conformation change promoted by ATP binding rather than nucleotide hydrolysis and release.

**M-R Cleaves and Resects at 3′-Phosphotyrosine to Produce Substrate for Repair Synthesis**—The evidence that M-R can both cleave and resect a substrate bearing a 3′-phosphotyrosine raised the possibility that M-R may be able to act alone to process CPT-induced damage to generate a substrate for DNA repair synthesis. To test this, we investigated whether incubation of M-R with a partial duplex substrate bearing a 3′-phosphotyrosine in the presence of ATP generated products that could be extended by exonuclease-deficient Klenow DNA polymerase (lacking both 5′–3′ and 3′–5′ exonuclease activities), as diagrammed in Fig. 5A. Extension of a substrate bearing a 3′-phosphotyrosine was dependent upon incubation with M-R and ATP and occurred very efficiently (95% of the M-R cleavage product was extended) (Fig. 5B). These results show that M-R can act as a “one-stop shop” for repair of CPT-induced DNA damage, both cleaving the covalent protein-DNA bond and resecting to enable subsequent repair synthesis. Control reactions showed that a substrate bearing a 3′-phosphate was not extended, whereas a substrate bearing a 3′-hydroxyl was very efficiently extended (Fig. 5B, right).

**DISCUSSION**

We have shown that M-R cleaves a 3′-phosphotyrosine to generate a 3′-phosphate and resects to create a DNA end that can prime repair synthesis. M-R is sufficient for both the cleavage and resection steps in this repair reaction. M-R is thereby distinct from structure-specific nucleases that can cleave a 3′-phosphotyrosine but not further process the resulting end. M-R activity at 3′-phosphotyrosines is also distinct from its
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FIGURE 5. M-R cleaves and resects at a 3′-phosphotyrosine to produce a substrate for repair synthesis. A, outline of the experiment. A 5′-end-labeled partial duplex bearing a 3′-phosphotyrosine was incubated with M-R in the presence of 0.5 mM ATP, treated with phenol/chloroform, and then extended with exonuclease-deficient Klenow polymerase (DNA pol) and dNTPs. Oligonucleotide lengths in nucleotides (nt) prior to and after extension are indicated. B, denaturing gel electrophoresis of reaction products, indicating 3′-ends of 5′-32P-end-labeled partial duplex DNA substrates and inclusion of M-R and ATP (0.5 mM) and Klenow polymerase (exo). Arrows indicate substrates and the 43-nucleotide extension product; the bracket indicates exonuclease digestion products.

activity at Spo11-DNA complexes in meiotic yeast, where M-R removes 5′-DNA oligonucleotides covalently bound to protein (32). These results identify a novel nuclease activity of M-R. They also establish a possible mechanistic role for M-R in repair of DNA damage induced by Top1 poisons independent of its role in DSB repair.

Taken together, our results lead to a working model for direct function of M-R in repair of CPT-induced DNA damage or of aberrant intermediates formed if Top1 fails to excise correctly from DNA. Quick action by M-R could eliminate a trapped protein-DNA complex prior to replication (Fig. 6, left), circumventing formation of a replicative DSB (Fig. 6, right) or cytotoxic lesions that may contribute to death of CPT-treated cells. Direct repair of CPT-induced damage by M-R would not preclude M-R also functioning in repair of replication-induced DSBs.

3′-Phosphotyrosine Cleavage Activity Maps to MRE11 Active Site—Two kinds of evidence establish that 3′-phosphotyrosine cleavage activity is vested in the MRE11 subunit of the M-R complex. First, MRE11 alone can cleave a 3′-phosphotyrosine with efficiency comparable with M-R. Second, systematic mutagenesis of highly conserved residues in the MRE11 active site identified 10 different MRE11 mutants with impaired cleavage at a 3′-phosphotyrosyl bond.

We did not identify any mutant that completely separated exonuclease and 3′-phosphotyrosylase cleavage activities. We did identify one mutant, P. furiosus MRE11(H52A), that had impaired exonuclease activity yet retained considerable 3′-phosphotyrosylase cleavage activity (Fig. 3). Structural analysis suggests that this mutation affects a histidine in phosphoesterase motif II of the MRE11 active site that is involved in phosphate rotation of the DNA backbone (20).

The mutational analysis we have carried out suggests that the same structural features of the active site may support the exonuclease and 3′-tyrosine cleavage activities of P. furiosus MRE11. A covalent 3′-DNA intermediate is formed by type 1B topoisomerases in eubacterial and eukaryotic cells and by a structurally distinct class of enzymes (type 1C topoisomerases) in archaea. A gene encoding a type 1C topoisomerase has not been identified in the P. furiosus genome. This raises the possibility that P. furiosus may not require the 3′-tyrosine cleavage activity of MRE11, at least for release of Top1-DNA intermediates. This activity of MRE11 may persist because it protects cells from other covalent protein-DNA intermediates or because its loss would impair the exonuclease activity.

In S. cerevisiae, mutations predicted to affect MRE11 exonuclease activity are known to cause CPT sensitivity (9, 33, 34). This has been interpreted as reflecting a role of M-R in resolution of ends of replicative DSBs to promote their homology-dependent repair. However, these mutants may also affect either cleavage or resection of a 3′-phosphotyrosine. In this context, it is interesting that the MRE11(H52A) mutant affects the same active site histidine as an S. cerevisiae mutant (mre11-H59A) that is sensitive to hydroxyurea, which induces DSBs, but is relatively resistant to CPT (35). This is the phenotype predicted for a mutation that has a more severe effect on exonuclease activity essential to DSB repair than on 3′-phosphotyrosylase cleavage activity. Similarly, mutation at the corresponding position of the S. pombe MRE11 homolog (rad32-H68S) does not render cells sensitive to CPT (20).

FIGURE 6. Working model for M-R functions in repair of CPT-induced DNA damage. The model outlines direct repair of damage by M-R (left) or repair following formation of a replicative DSB (right). M-R carries out two steps in the direct repair pathway. First, M-R cleaves the phosphotyrosyl bond to generate a 3′-phosphate DNA end. Next, M-R resects to generate a 3′-hydroxyl in an ATP-regulated reaction. Resection produces a gap, which can be repaired by DNA synthesis and ligation or by homology-dependent mechanisms. M-R may also function in repair of a replicative DSB (right).

ATP-regulated Resection by M-R in CPT Response and Other Pathways—The ability of M-R not only to cleave covalent 3′-phosphotyrosyl bonds but also to resect and generate a DNA end that can prime DNA synthesis distinguishes it from other
activities that cleave a 3’-phosphotyrosyl bond. For example, Tdp1 releases trapped Top1 to leave a 3’-phosphate, which must be further processed prior to repair synthesis (4). Phosphatases may remove the 3’-phosphate, but exonucleolytic resection by M-R of DNA ends created by other structure-specific nucleases might also generate the 3’-hydroxyl required to initiate repair synthesis.

ATP regulates both 3’-phosphotyrosine cleavage and resection by M-R. Regulation probably reflects conformational changes caused by ATP binding to the RAD50 subunit of the M-R complex, as suggested by structural analysis documenting the close relationship between the RAD50 ATPase domain and the MRE11 nuclease domain (36–38). Upon ATP binding, RAD50 dimerizes to form a functional ATPase domain by interaction of its N-terminal Walker A and C-terminal Walker B motifs, causing MRE11 to undergo conformational changes and reorient itself within the M-R complex (36, 37, 39). RAD50 is not only an ATP-binding cassette ATPase but may also have -phosphatase activity (Fig. 3), and at least one B motifs, causing MRE11 to undergo conformational changes caused by ATP binding to the RAD50 subunit of the ADP were not concentration-dependent (over the 0.5–2.5 mM 3

M-R lacks 3’-phosphatase activity (Fig. 3), and at least one nucleotide must be released to generate an end bearing a 3’-hydroxyl. The evidence that DNA resection depends upon ATP but is inhibited by ADP (Fig. 5) raises the possibility that ATP hydrolysis may regulate the extent of resection and thus the length of the resulting gap. A minimal resection product, bearing a 1-nucleotide gap, would resemble an intermediate typically associated with base excision repair and may be repaired by activities associated with that pathway, such as polymerase β. Longer gaps may be repaired by other polymerases and other pathways.

Implications for Stratification of Tumors for Cancer Chemotherapy—The results we have presented form a biochemical foundation that predicts that M-R deficiency may contribute to tumor sensitivity to Top1 poisons related to CPT, such as irinotecan and topotecan, commonly used in chemotherapy. Both MRE11 and RAD50 carry intronic mononucleotide repeats known to contract in mismatch repair-deficient tumors and diminish protein expression (25–27). This suggests a strategy for stratification of tumors for chemotherapy based on MRE11 and/or RAD50 status in addition to mismatch repair status. This may provide a powerful method of identifying patients who will benefit especially from treatment with CPT derivatives, with the potential to affect a considerable patient population, as defects in the mismatch repair pathway characterize 10–15% of colorectal cancers, one of the most common tumors, with 150,000 new cases a year in the United States alone (41).

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