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MRE11/RAD50 Cleaves DNA in the AID/UNG-Dependent Pathway of Immunoglobulin Gene Diversification

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Summary

MRE11/RAD50/NBS1 (MRN) is a ubiquitous complex that participates in the response to DNA damage and in immunoglobulin (Ig) gene diversification. Ig gene diversification is initiated by deamination of cytosine to uracil, followed by removal of uracil to create an abasic (AP) site. We find that MRE11 associates specifically with rearranged Ig genes in hypermutating B cells, whereas APE1, the major AP-endonuclease in faithful base excision repair, does not. We show that purified, recombinant MRE11/RAD50 can cleave DNA at AP sites and that this AP-lyase activity is conserved from humans to Archaea. MRE11/RAD50 cleaves at AP sites within single-stranded regions of DNA, suggesting that at transcribed Ig genes, cleavage may be coordinated with deamination by AID and deglycosylation by UNG2 to produce single-strand breaks (SSBs) that undergo subsequent mutagenic repair and recombination. These results identify MRN with DNA cleavage in the AID-initiated pathway of Ig gene diversification.

Introduction

Somatic hypermutation, class switch recombination, and gene conversion alter the sequence and structure of Ig genes in B lymphocytes to diversify the antibody repertoire and increase the specificity and affinity of antibody for antigen (Maizels, 2005). Somatic hypermutation produces single base changes in Ig variable (V) regions and is coupled with selection to increase the affinity of antibody for antigen. Class switch recombination deletes an extended region of chromosomal DNA to juxtapose a new constant region to the expressed V region, thereby changing efficacy of antigen clearance. Gene conversion produces templated changes in a rearranged and expressed V region, using germline pseudo-V regions as donors, and this is the sole mechanism for achieving a diverse antibody repertoire in some organisms, such as chickens, which have only one or a few functional V regions and therefore lack the potential for combinatorial diversification. These processes of Ig gene diversification are absolutely essential to the immune response, and mutations in genes that regulate or execute Ig gene diversification can result in immunodeficiencies.

Ig gene diversification is initiated by the B cell-specific enzyme activation-induced deaminase (Muramatsu et al., 2000; Revy et al., 2000). AID deaminates C to U in single-stranded DNA (Bransteitter et al., 2003; Chaudhuri et al., 2003; Dickerson et al., 2003; Ramiro et al., 2003; Sohail et al., 2003). Uracil in DNA can be faithfully repaired by a highly conserved pathway in which uracil DNA glycosylase removes uracil, apurinic/apyrimidinic endonuclease (APE1) cleaves the abasic site, and pol β carries out high-fidelity repair synthesis (Barnes and Lindahl, 2004). However, at the Ig loci of diversifying B cells, uracil in DNA can be processed by mutagenic rather than faithful repair pathways, dependent on either *Ung* or *Msh2* (Rada et al., 2004). In the *Ung*-dependent pathway, UNG2 removes U to create an AP site. UNG2 is the nuclear isoform of the enzyme encoded by the *Ung* gene, and it preferentially deglycosylates U residing on single-stranded DNA (Kavli et al., 2005). Subsequent processing of the AP site creates the DNA breaks necessary for class switch recombination and gene conversion and produces a substrate for mutagenic repair in somatic hypermutation. AID is the only B cell-specific factor required (Okazaki et al., 2002; Yoshikawa et al., 2002), so ubiquitous proteins must cleave DNA. The factor that carries out DNA cleavage has not been identified.

MRN is a multifunctional and essential complex that participates in DNA repair, cell-cycle checkpoint control, telomere maintenance, and Ig gene diversification. MRE11 and RAD50 form the core, with DNA binding activity vested in RAD50 and both endo- and exonucleolytic activities in MRE11 (Paull and Gellert, 1998; Hopfner et al., 2001; Trujillo and Sung, 2001). The regulatory subunit NBS1 interacts with MR to govern nuclear localization and activity, and responds to and regulates ATM kinase (Kobayashi et al., 2004; Stracker et al., 2004). Mutation of any of the genes encoding the MRN complex is embryonic lethal in mice (Xiao and Weaver, 1997; Luo et al., 1999; Zhu et al., 2001). MRE11 is essential in vertebrate cells, and genomic instability is evident even in cell lines expressing reduced levels of this polypeptide (Yamaguchi-Iwai et al., 1999).

MRN participates in all three processes that diversify Ig genes. MRN undergoes AID-dependent localization to nuclear foci at the Ig heavy chain locus in B cells activated for switch recombination (Petersen et al., 2001). The human genetic diseases Nijmegen break syndrome (NBS) and ataxia-telangiectasia-like disorder (ATLD), which result from hypomorphic mutations of *NBS* and *MRE11*, respectively, are characterized by reduced titers of switched serum isotypes and altered switch junctions (Pan et al., 2002; Lahdesmaki et al., 2004). Impaired switch recombination and pronounced genomic instability result from conditional ablation of the gene encoding NBS1 in murine models (Kracker et al., 2005; Reina-San-Martin et al., 2005). Both hypermutation in human B cells and gene conversion in chicken B cells are accelerated by ectopic NBS1 expression (Yabuki et al., 2005). Acceleration depends upon MRN complex formation and alters the hypermutation spectrum to increase transversion mutations and the fraction of single base changes at C. Increased mutagenesis at C suggested that the MRN complex might stimulate either

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cleavage or mutagenic processing at AID-initiated DNA damage (Yabuki et al., 2005).

Here, we demonstrate function for MRN in DNA cleavage at diversifying Ig genes. We show that MRE11 associates with the rearranged Ig heavy chain variable region (V_H) in the hypermutating human B cell line Ramos, consistent with participation in the hypermutation mechanism, and that purified, recombinant MRE/RAD50 (MR) can cleave DNA at abasic sites, the predicted products of successive attack by AID and UNG. The MR AP-cleavage activity is conserved from humans to Archaea, vested in the MRE11 subunit, and can be distinguished from the MRE11 3′–5′ exonuclease activity by targeted mutagenesis. Unlike APE1, the major AP-endonuclease, which cleaves AP sites in DNA to initiate faithful repair, MR preferentially cleaves AP sites within single-stranded regions of DNA, and it generates a DNA end that cannot be directly extended by DNA polymerase. We propose that MRN cleaves at AP sites produced by the combined action of AID and UNG2 in activated B cells and thereby promotes subsequent processing by mutagenic rather than faithful pathways.

Results

MRE11 Associates with the Rearranged V_H Region in Hypermutating Human B Cells

Factors that participate in the mechanism of Ig gene diversification are predicted to associate specifically with the actively hypermutating V_H region in Ramos, a human B cell line (Sale and Neuberger, 1998). We used chromatin immunoprecipitation (ChIP) to assay enrichment at the rearranged V_H region of two proteins, MRE11 and APE1. Previous results from our laboratory raised the possibility that MRN might promote DNA cleavage at diversifying Ig genes (Yabuki et al., 2005). APE1 has also been considered as a candidate for cleavage at AP sites generated by successive action of AID and UNG2, because it is the major abasic endonuclease active in base excision repair (Barnes and Lindahl, 2004). ChIP with anti-MRE11 antibodies resulted in a 2.3-fold enrichment of the rearranged V_H amplicon relative to the control amplicon triosephosphate isomerase (TPI; Figure 1A, left). In contrast, ChIP with anti-APE1 antibodies showed that there is not significant enrichment of APE1 at the rearranged V_H region (Figure 1A, right). No enrichment of MRE11 was evident at two control loci: the allelic unrearranged V_H region, which is not transcribed and does not hypermutate, and *SMC1L2*, a gene which encodes a protein involved in chromosome condensation (Figure 1B). MRE11 is therefore specifically enriched at the hypermutating V_H region. The level of MRE11 enrichment is comparable to the 2- to 3-fold enrichment reported for other recombination factors at target loci in yeast or mammalian cells (e.g., Wolner et al. [2003] and Larson et al. [2005]). Association of MRE11 with the rearranged V_H locus in a constitutively hypermutating B cell line is consistent with a model wherein MRN participates in the mechanism of repair of AID-initiated DNA damage.

MR Is a Conserved AP-Lyase

To ask if MR can cleave at abasic sites, we adopted a strategy designed to minimize potential artifacts and

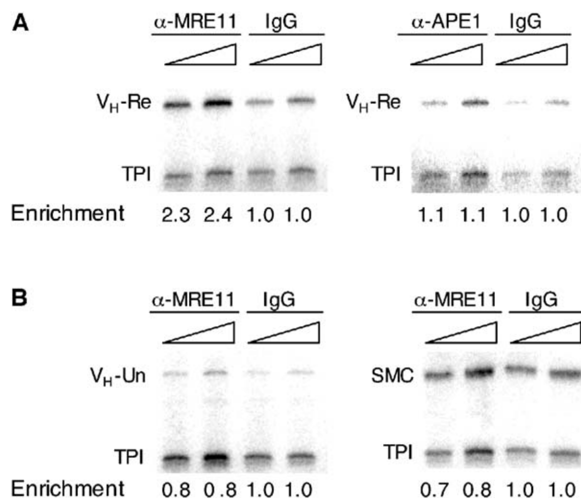

Figure 1. MRE11 Associates with the Rearranged V_H Region in Hypermutating Human B Cells

(A) Native gel electrophoresis of duplex PCR products amplified from chromatin after IP with anti-MRE11 (α-MRE11), anti-APE1 (α-APE1), or polyspecific IgG antibodies. Amplicons represent the rearranged IgH variable region (V_H-Re) and the *TPI* gene (TPI). Parallel reactions amplified a 2-fold dilution of template (triangles) for each precipitation. Enrichment relative to TPI and normalized to the IgG lane was calculated as described in the Experimental Procedures and is shown below each lane.

(B) Assay of enrichment of the unrearranged IgH variable allele (V_H-Un) and the *SMC1L2* locus (SMC) relative to the TPI amplicon after IP with anti-MRE11 antibodies.

to take advantage of the extraordinary conservation of this factor. Abasic sites are highly mutagenic (Loeb and Preston, 1986), and all cells contain multiple activities that can cleave at abasic sites, including lyases, which attack at the C-1′ of the deoxyribose, and phosphodiesterases, which break the DNA backbone. Moreover, APE1, the major AP-endonuclease, is abundant and stable, and it can confound biochemical identification of other activities that cleave at abasic sites. We therefore characterized biochemical activity of purified recombinant MRE11/RAD50 from two sources: the cloned human cDNAs (hMR), expressed in insect cells (Paull and Gellert, 1998), and genes cloned from the thermophilic archaebacterium *Pyrococcus furiosus* (Pf MR), expressed in *E. coli* (Hopfner et al., 2000). We focused on the core MR complex, because functional homologs of NBS1 are restricted to eukaryotes (Aravind et al., 1999). The first step in purification of recombinant Pf MR was a 25 min incubation at 75ºC to inactivate endogenous *E. coli* abasic cleavage activities (Hopfner et al., 2001). Activities of both hMR and Pf MR were assayed on DNA substrates carrying lesions that represent predicted intermediates in the AID-catalyzed and *Ung*-dependent pathway of Ig gene diversification. Duplex substrates contained either a U·G mismatch, predicted product of AID-catalyzed deamination, or an abasic site opposite G, produced by treating a U·G mismatch duplex with uracil DNA glycosylase (Figure 2A). We also assayed cleavage of a duplex carrying a single tetrahydrofuran (THF) substitution. This abasic-site analog lacks a 1′-hydroxyl, and DNA containing this substitution cannot be cleaved by AP-lyases, which cleave AP sites via nucleophilic attack at C-1′, but can be cleaved

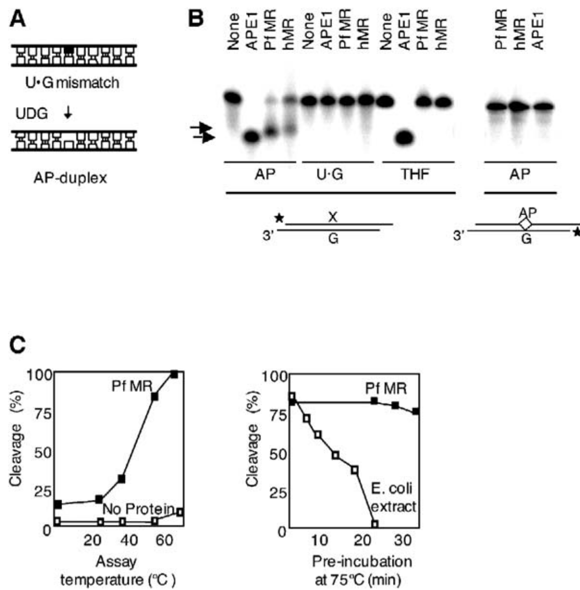

Figure 2. Conserved AP-Lyase Activity of MRE11/RAD50

(A) A DNA duplex carrying a U·G mismatch (U, filled box) and the product of deglycosylation by UNG, which removes the uracil base to produce an abasic site.

(B) Cleavage activities on duplex DNA substrates. ^{32}P end-labeled duplexes carrying an abasic site (AP), uracil opposite G (U·G), or tetrahydrofuran (THF) were incubated with APE1 (10 nM), *P. furiosus* MR (Pf MR, 100 nM), or human MR (hMR, 2 nM) under standard conditions, and cleavage products were resolved by denaturing gel electrophoresis and quantified by phosphorimager. Arrows indicate mobility of products of MR (above) and APE1 (below) cleavage. Duplex substrates are diagrammed below the image (asterisks denote labeled 5′ ends). Lower left, the X represents position of the lesion; lower right, the diamond represents the AP site.

(C) Temperature dependence and thermostability of Pf MR activity. Left, graph of cleavage of a 5′ labeled AP-duplex in reactions containing no protein or Pf MR (50 nM), incubated at indicated assay temperatures. Right, graph of cleavage of a 5′ labeled AP-duplex by purified Pf MR and *E. coli* extract. Proteins were incubated at 75ºC for indicated times, prior to assaying cleavage of an AP-duplex under standard conditions.

by AP-endonucleases, like APE1, which attack the phosphodiester backbone. Synthetic duplexes used for these assays carried 3′ overhangs to render them resistant to the 3′–5′ exonuclease activity of MRE11 (Paull and Gellert, 1998).

Both hMR and Pf MR cleaved AP-duplexes (Figure 2B, left). Recombinant human APE1 also cleaved these substrates, as predicted, although the MR and APE1 cleavage products differed slightly in gel mobility (arrows). AP sites are intrinsically labile, and short basic peptides and polyamines have been reported to promote cleavage of substrates containing AP sites (Lindahl and Andersson, 1972; Behmoaras et al., 1981) but at concentrations three to six orders of magnitude higher than concentrations of MR used in the assays shown. Cleavage by MR produced only a single labeled product (Figure 2B, upper arrow), suggesting that the cleavage product is not subject to MR 3′–5′ exonuclease activity. Neither MR nor APE1 cleaved the corresponding duplex carrying a U·G mismatch (Figure 2B, center). Moreover, MR did not cleave the duplex substrate carrying a THF substitution, although this substrate was cleaved by APE1 (Figure 2B, right). MR thus appears to be an AP-lyase, rather than an AP-endonuclease; this probably explains the small difference in mobility between the products of cleavage by MR and APE1, a bona fide AP-endonuclease (Figure 2B, left). Cleavage was targeted to the DNA strand carrying the AP site, and the complementary strand remained intact (Figure 2B, right).

To confirm that the observed AP cleavage activity was due to MR and not a contaminant, we assayed cleavage activity at temperatures from 37ºC to 65ºC. Consistent with the thermophilic origin of Pf MR, cleavage of AP-duplex substrates dramatically increased at assay temperatures above 37ºC (Figure 2C, left). The AP site became slightly labile with increasing temperatures (Figure 2C, left). In addition, pretreatment at 75ºC destroyed AP-duplex cleavage activities endogenous to *E. coli* extracts, whereas the Pf MR cleavage activity was stable to heating (Figure 2C, right). We therefore conclude that MR is a highly conserved AP-lyase.

MRE11 AP-Lyase and Exonuclease Activities Are Separable

MRE11 contains conserved phosphodiesterase motifs that are responsible for the exonuclease activity of the MRN complex. To ask if the AP-lyase activity maps to one of the MRE11 phosphodiesterase motifs, we carried out site-directed mutagenesis, focusing on Pf MRE11 because heat treatment can be used to inactivate contaminants that might otherwise complicate activity assays. Structural biochemistry of Pf MRE11 has modeled H206 within the active site where it coordinates one of two Mn^{2+} ions required for the exonuclease activity (Hopfner et al., 2001); mutation at the corresponding position in *S. cerevisiae* MRE11 impairs processing of double-strand breaks (DSBs) (Bressan et al., 1999). We therefore generated one mutant of Pf MRE11, M^{H206A}, that carried the substitution H206A. Sequence motifs critical for AP-lyase activity are not clearly defined, but AP-lyases typically depend on lysine residues to form covalent Schiff base intermediates with the DNA (Ide and Kotera, 2004). We therefore targeted for mutagenesis the basic region at residues 110–113, replacing R110, K111, and K113 with alanine, to generate the M^{3A} mutant.

The Pf MRE11 mutant derivatives were coexpressed with RAD50 in *E. coli* and purified after heat treatment. Activities were assayed on two substrates, an AP-duplex and single-stranded circular phage DNA, a standard substrate for assays of the MR 3′–5′ exonuclease activity (Hopfner et al., 2000). Wild-type (wt) Pf MR and $M^{H206A}R$ were comparably active on the AP-duplex substrate, whereas the $M^{3A}R$ mutant displayed impaired activity on this substrate (Figure 3A). This further confirms that the AP-lyase activity is a property of MRE11. Wt Pf MR digested single-stranded circles, by virtue of its documented exonuclease activity (Hopfner et al., 2000), and this activity was retained in the $M^{3A}R$ mutant but impaired in the $M^{H206A}R$ mutant (Figure 3B).

The MRE11 exonuclease activity is dependent upon Mn^{2+} and does not function in the presence of Mg^{2+} (Paull and Gellert, 1998). Because the H206A mutation impaired exonuclease, but not AP-lyase, activity (Figures 3A and 3B) and H206 is within a conserved

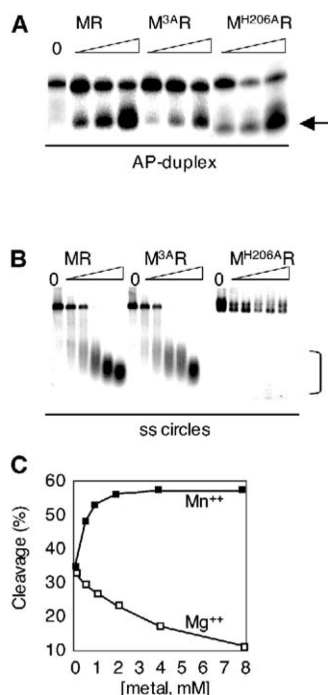

Figure 3. Separation of AP-Lyase and Exonuclease Functions by MRE11 Mutation

(A) Denaturing gel electrophoresis of cleavage products generated upon incubation of a labeled AP-duplex with Pf MR; $M^{3A}R$, an AP-lyase mutant; and $M^{H206A}R$, an exonuclease mutant. Assays contained no protein (left) or 30 nM of protein and were incubated for 10, 30, or 60 min (triangle). Arrow indicates cleavage product.

(B) Native gel electrophoresis of cleavage products generated upon incubation of single-stranded circular phage DNA with Pf MR; $M^{3A}R$, an AP-lyase mutant; and $M^{H206A}R$, an exonuclease mutant. Assays contained 50 nM wt or mutant MR and were incubated for 0, 15, 30, 60, 120, and 180 min (triangle). Products were resolved by agarose gel electrophoresis and visualized by ethidium-bromide staining. Bracket indicates exonucleolytic digesion products.

(C) Quantification of cleavage of abasic oligonucleotide by the AP lyase activity of Pf MR in the presence of increasing concentrations of Mn^{2+} or Mg^{2+}.

phosphodiesterase motif and predicted to coordinate Mn^{2+} at the active site (Hopfner et al., 2001), it was of interest to ask if the AP-lyase activity is dependent upon Mn^{2+}. Assays of Pf MR AP-lyase activity in the presence of Mn^{2+} or Mg^{2+} showed that AP-lyase activity, like exonuclease activity, depends upon Mn^{2+} and is inhibited by Mg^{2+} (Figure 3C).

These results show that the AP-lyase activities and exonuclease activities of MR are separable by mutation. They also suggest that the unusual dependence of both activities on Mn^{2+} may reflect the ability of this ion to stabilize protein conformation, consistent with the fact that two bound Mn^{2+} ions were prominent features of the crystallized MRE11 polypeptide (Hopfner et al., 2001). The region mutated in the $M^{3A}R$ mutant maps far from the exonuclease active site predicted by the crystal structure, and it may either be important for catalysis or for maintaining charge-dependent interactions with the DNA substrate. Structural and mutational analysis together will ultimately be required to identify the catalytic residues and mechanism used by MRE11 to cleave AP sites.

The Structure of the MR Cleavage Product May Promote Mutagenic Repair

To learn whether the structure of the DNA end produced upon MR cleavage may contribute to mutagenic repair, we further characterized the MR cleavage product. APE1 hydrolyzes DNA 5′ of the abasic site to leave a free 3′-hydroxyl that can prime faithful repair synthesis, whereas cleavage by AP-lyases typically leaves a 3′ phospho-α, β-unsaturated aldehyde, which cannot be directly extended by a DNA polymerase but must be removed prior to subsequent repair (Mosbaugh and Linn, 1980; Warner et al., 1980). Taq polymerase carries out primer extension quite efficiently, but this enzyme has no 3′–5′ exonuclease and therefore will not remove the 3′ end produced upon DNA cleavage by an AP-lyase. AP-endonucleases can remove 3′ moieties left by AP-lyases, and this can be an important step in repair at abasic sites (Mosbaugh and Linn, 1980; Demple et al., 1986). The MR cleavage product was not extended by Taq polymerase, although the APE1 cleavage product, which carries a 3′-hydroxyl, was efficiently extended to the end of the complementary strand (Figure 4A). Successive treatment of an AP-duplex with Pf MR and then APE1 produced a product that was identical in mobility to the APE1 cleavage product and that was readily extended by Taq polymerase (Figure 4A). Thus, APE1 can remove the 3′ moiety left by MR cleavage and thereby convert the MR cleavage product to an effective primer for polymerase extension.

One characterized nuclease, *E. coli* Endo V, cleaves the second phosphodiester bond 3′ of an AP site (Yao and Kow, 1995). We eliminated the formal possibility that MR might carry out a similar cleavage reaction by examining the product of cleavage of an AP-duplex 47 bp in length, carrying a single internal ^{32}P label at position 37, just 3′ of the AP site, and with blunt ends (Figure 4B). After cleavage of this substrate by either β elimination (NaOH or *E. coli* EndoIII) or hydrolysis (APE1), the ^{32}P label was associated with the smaller 3′ DNA fragment as predicted, and a labeled product of this same size was produced after cleavage by Pf MR or the Pf MR exonuclease ($PfM^{H206A}R$) mutant (Figure 4B). Note that cleavage of the blunt duplex by wt Pf MR was accompanied by some degradation at the 3′ end of the cleavage product, which was not evident after cleavage by the Pf MR exonuclease (Pf $M^{H206A}R$) mutant (compare Pf MR and $M^{H206A}R$, Figure 4B). We conclude that MR cleaves within the abasic deoxyribose moiety and that the DNA end generated by MR cleavage must be further processed to prime extension by DNA polymerase.

MR Efficiently Cleaves AP Sites in Duplex and Single-Stranded DNA

Transcription is prerequisite for Ig gene diversification, and one result of transcription is to transiently denature DNA to provide a target for AID, which deaminates C to U in single-stranded, but not duplex, DNA (Maizels, 2005; Pham et al., 2005). UNG2, the uracil DNA glycosylase active in Ig gene diversification, has a modest preference for deglycosylation of U in single-stranded regions relative to duplex DNA (Kavli et al., 2005). This raised the question of whether MR might similarly be active at AP sites in single-stranded DNA, which we

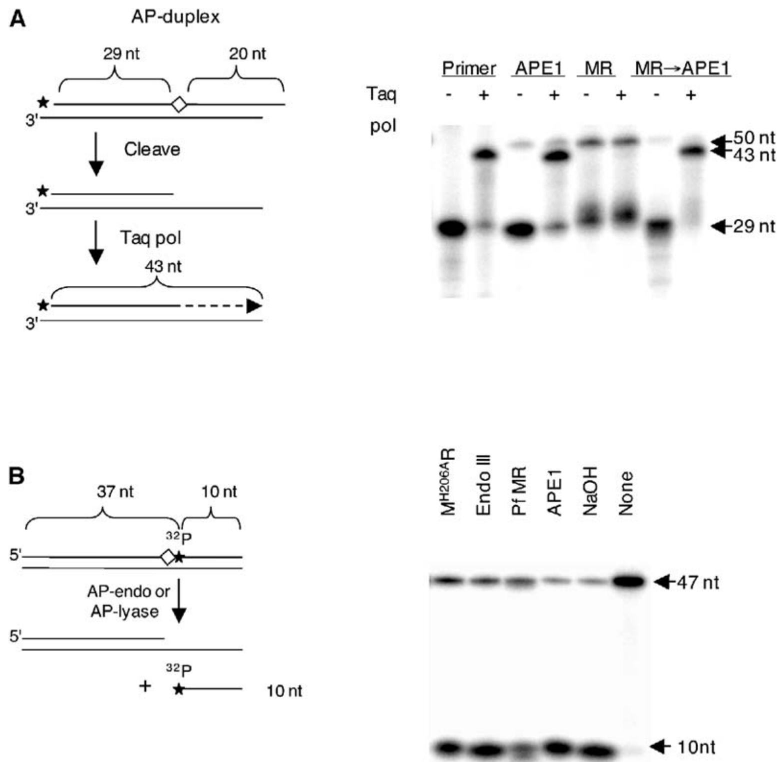

Figure 4. The MR Cleavage Product

(A) Extension of the cleavage product by Taq polymerase. As diagramed on the left, a 5′ end-labeled AP-duplex, with a single AP site (diamond) at position 30, was cleaved by Pf MR (30 nM), or APE1 (10 nM), or Pf MR followed by APE1 (MR→APE1). Cleaved DNAs were purified and resuspended in polymerase buffer and then incubated at 65°C for 10 min with (+) or without (−) 2.5 U of Taq polymerase. DNAs were then denatured and products resolved by denaturing gel electrophoresis and imaged by phosphorimager. Arrows at right indicate substrate (50 nt), cleavage product (29 nt), and extension product (43 nt).

(B) Cleavage of an AP-duplex containing a single internal ^{32}P label (asterisk) 3′ of the AP site (open diamond), as diagramed on the left. The substrate was incubated with indicated enzyme, DNA denatured, products resolved by denaturing PAGE, and imaged by phosphorimager. Arrows point to 47 nt uncleaved DNA and the 10 nt cleavage product.

addressed by comparing MR activity on duplex, bubble, and single-stranded substrates carrying single AP sites. The AP-bubble substrates carried a single AP site within a 10 nt unpaired region, with a synthetic RNA-DNA hybrid on the opposite strand (Figure 5A). Assays with end-labeled substrates showed that 50% cleavage of AP-duplex, AP-bubble, and AP-ss substrates required 13 nM, 21 nM, and 7 nM Pf MR, respectively (Figure 5A). In parallel assays with APE1, 50% cleavage of AP-duplex substrates required 0.2 nM APE1, whereas cleavage of AP-bubble or AP-ss substrates required 5 nM and 10 nM APE1, respectively. (To minimize any temperature-induced AP cleavage, Pf MR activity assays were performed at 55°C, well below the temperature optimum; and activity of Pf MR relative to APE1 may therefore be underestimated in these assays.) These comparisons showed that Pf MR was comparably active on duplex and bubble substrates, whereas APE1 was considerably more active on duplex substrates than on single-stranded or bubble substrates.

Cleavage preference was further analyzed in competition experiments in which hMR, Pf MR, or APE1 was incubated with end-labeled AP-duplex substrate in the presence of varying amounts of unlabeled competitor AP-bubble. The AP-bubble substrate competed efficiently for cleavage of a labeled AP-duplex by both Pf MR and hMR (Figure 5B). In contrast, even at 100-fold molar excess, the AP-bubble substrate did not compete for cleavage of the AP-duplex substrate by APE1. These results suggest that MR may compete very effectively with APE1 for cleavage of AP sites in single-stranded DNA—for example, within regions transiently denatured during the course of transcription.

Discussion

We have shown that the ubiquitous factor MRE11/RAD50 can cleave DNA at AP sites to generate an SSB. This cleavage activity is conserved from humans to Archaea. MR can cleave at AP sites in duplex DNA or in single-stranded regions of bubble substrates. The 3′ end of the break produced by MR cannot prime new DNA synthesis. Thus, in contrast to the APE1 cleavage product, the MR cleavage product is not an immediate target for high-fidelity repair synthesis mediated by DNA pol β. In living cells, MRN may therefore divert an AP site from the very efficient faithful repair pathway to a mutagenic repair pathway. As discussed below, considerable evidence points to MRN cleavage as a key step in diversification of Ig genes, where processing of AP sites created by successive action of AID and UNG2 leads to irreversible alterations in genomic structure and sequence.

MRN Function in Ig Gene Diversification

In the *Ung*-dependent pathway of Ig gene diversification, deamination by AID followed by deglycosylation by UNG2 creates AP sites. We propose that MRN cleaves DNA at these AP sites to generate a substrate for subsequent mutagenic repair. This proposal is based upon both the biochemical activities of MR, which we have documented herein, and experimental observations that identify function for the MRN complex in all three processes of Ig gene diversification (Petersen et al., 2001; Pan et al., 2002; Lahdesmaki et al., 2004; Kracker et al., 2005; Reina-San-Martin et al., 2005; Yabuki et al., 2005). MRE11 is enriched at

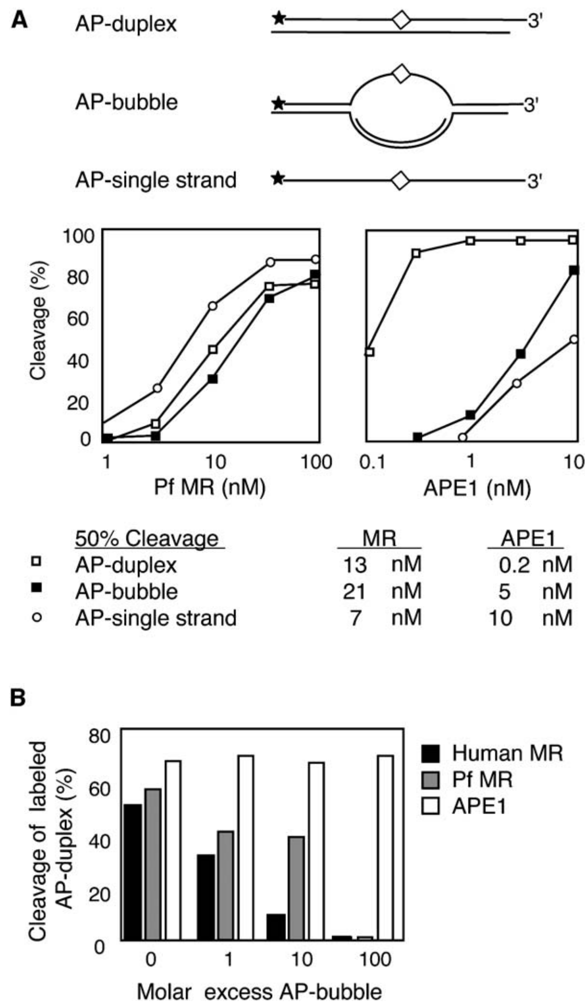

Figure 5. MR Cleaves AP Sites in Single-Stranded Regions of DNA

(A) Above, diagrams of end-labeled AP-duplex and AP-bubble substrates. AP site, open diamond. Below, quantification of cleavage of these substrates by indicated concentrations of Pf MR (left) or APE1 (right). The concentration of enzyme that produced 50% cleavage in 30 min is shown below each graph.

(B) Quantification of cleavage of end-labeled AP-duplex substrate by hMR (2 nM), Pf MR (30 nM), and APE1 (0.3 nM) in the presence of unlabeled competitor AP-bubble substrate.

rearranged and hypermutating V_H regions, but not at the allelic unrearranged V_H (Figure 1), supporting a role of this nuclease in the diversification mechanism.

Figure 6 presents a model for participation of MRN in Ig gene diversification. Transcribed Ig genes are targets for AID, which deaminates C to U within single-stranded DNA. In the *Ung*-dependent pathway, U is removed by UNG2, leaving an AP site. MR then cleaves at the AP site to produce a 3′ end that cannot be extended directly by repair polymerases. Cleavage by MR thus shifts the balance of repair from the pol β-dominated faithful pathway to mutagenic pathways. Subsequent processing of the SSB produced upon MR cleavage can give rise to the three signature outcomes of diversification at the Ig genes: gene conversion, somatic hypermutation, and class switch recombination.

MRN function in DNA cleavage places this factor just downstream of AID and UNG2 in the *Ung*-dependent Ig

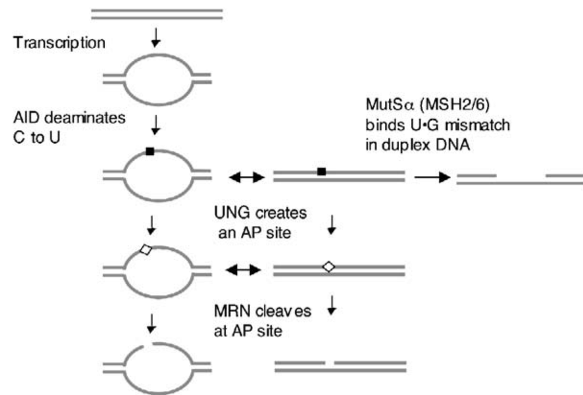

Figure 6. Model for DNA Cleavage by MRN in the AID-initiated Pathway of Ig Gene Diversification

The DNA duplex (top) is denatured by transcription. AID deaminates C to U (filed box) within a single-stranded region. In the *Msh2*-dependent pathway, after DNA renaturation, MutSα binds the U·G mismatch and promotes resection. In the *Ung*-dependent pathway, UNG2 removes U to create an AP site (open diamond). MR cleaves at this site, producing an end that cannot be directly extended by repair polymerases. DNA breaks may give rise to gene conversion, somatic hypermutation, or class switch recombination, depending on how they are repaired. Deamination, deglycosylation, and cleavage may all be targeted to single-stranded regions and coordinated to maximize mutagenesis of transcribed genes.

gene diversification pathway. Function of a ubiquitous factor in the cleavage step of this pathway was anticipated by experiments showing that AID is the only B cell-specific factor required for hypermutation or class switch recombination (Okazaki et al., 2002; Yoshikawa et al., 2002). AID deaminates only single-stranded DNA (Bransteitter et al., 2003; Chaudhuri et al., 2003; Dickerson et al., 2003; Ramiro et al., 2003; Sohail et al., 2003); and UNG2 has a slight preference for deglycosylation of uracil in single-stranded DNA (Kavli et al., 2005). The ability of MR to cleave with comparable efficiency at AP sites in duplex DNA and single-stranded regions within bubbles (Figure 5) suggests that activities of AID, UNG2, and MRN may be coordinated. DNA deamination, deglycosylation, and cleavage may therefore all occur without intervening DNA renaturation, as shown in Figure 6, left.

SSBs in AID-Initiated Ig Gene Diversification

The SSBs produced upon DNA cleavage by MRN would be effective precursors to each of the three distinct mutagenic outcomes of Ig gene diversification. (1) In gene conversion, sequence information is transferred when pseudo-V donors serve as templates for new DNA synthesis primed by a 3′ end of DNA from the target V gene. DNA cleavage by MRN could produce an SSB with a free 3′ end that, after subsequent processing, could serve as a primer for gene conversion. An SSB in the nontemplate strand alone would be sufficient to support the documented 5′–3′ directionality (McCormack and Thompson, 1990) of Ig gene conversion. (2) In class switch recombination, MRN cleavage at AP sites could give rise to the DSBs that are precursors to switch junctions in two ways. If cleavage occurred on both strands,

it would produce staggered breaks, which could be converted to flush-ended DSBs by repair synthesis; if cleavage occurred on a single strand, then replication of the strand carrying an SSB would generate a DSB. MRN is multifunctional, and it may also promote repair by recruiting factors in the nonhomologous end-joining pathway or by promoting DNA ligation (Rooney et al., 2004). Function in either cleavage or repair would account for the reduced levels of switched serum Ig isotypes and altered junctions evident in human diseases due to NBS1 or MRE11 deficiencies (Pan et al., 2002; Lahdesmaki et al., 2004) and in conditional knockouts of the murine gene encoding NBS1 (Kracker et al., 2005; Reina-San-Martin et al., 2005). (3) In somatic hypermutation, nontemplated single base changes are produced at transcribed V regions. Mutagenic repair at or opposite (translesion synthesis) an SSB created by MRN could contribute to nontemplated mutagenesis.

Both SSBs (Kong and Maizels, 2001; Arudchandran et al., 2004) and staggered DNA ends (Zan et al., 2003) have been identified at Ig genes in diversifying B cells and are consistent with participation of MRN in the cleavage mechanism. DSBs have also been identified at Ig loci or in reporter constructs in mammalian B cells (Bross et al., 2000; Papavasiliou and Schatz, 2000; Catalan et al., 2003; Zan et al., 2003). However, DSBs appear not to depend upon ongoing hypermutation (Bross et al., 2000) or AID expression (Bross et al., 2002; Papavasiliou and Schatz, 2002; Zan et al., 2003) and may not be relevant to AID-initiated Ig gene diversification.

Somatic hypermutation occurs in separable phases distinguished by dependence upon specific factors (reviewed in Neuberger et al. [2005]). Phase 1 produces transition mutations at C-G pairs, which are thought to arise when U·G mismatches undergo replicative repair; Phase 2 depends on MutSα (MSH2/MSH6) and EXO1 to create mutations at A-T nucleotides. Transversion mutations at C-G nucleotides constitute a distinct component of the hypermutational spectrum not fully accounted for by Phase 1 or Phase 2 but appear to reflect participation of MRN in hypermutation, because the fraction of transversions at C-G pairs is significantly higher ($p < .001$, χ^2 test) in Ramos NBS1 transfectants (Yabuki et al., 2005) than in Ramos (Sale and Neuberger, 1998). This component of the hypermutation spectrum may reflect mutagenic repair at or opposite breaks created by MRN.

MR Cleavage May Determine Repair by a Mutagenic (Rather Than Faithful) Pathway

Our results identify function for MRN in DNA cleavage at AP sites in diversifying Ig genes. In principle, the major abasic endonuclease APE1 could also attack these AP sites; however, experimental analysis thus far has not suggested a role for APE1 in Ig gene diversification. Unlike MRE11, APE1 is not enriched at hypermutating V regions as assayed by ChIP (Figure 1); we note that this observation is not sufficient to exclude any participation of APE1 in Ig gene diversification, as transitory protein-DNA associations may be difficult to assay by ChIP. MR cleaves comparably at AP sites in duplex and single-stranded regions and could therefore cleave at AP sites exposed within genes transiently denatured by transcription, whereas APE1 shows clear preference for AP sites in duplex DNA (Figure 5B). This substrate preference would allow coordinated function of MRN with AID and UNG2 at the diversifying Ig genes and could link levels of DNA cleavage to transcription levels. The DNA end produced upon MR cleavage cannot directly prime new DNA synthesis (Figure 4), but the APE1 cleavage product can, and it is used as a primer for synthesis by pol β, which carries out high-fidelity repair at sites of APE1 cleavage. This difference between the MR and APE1 cleavage products could contribute to determining a mutagenic outcome of repair of AP sites at the Ig genes of diversifying B cells.

The Conserved MR AP Lyase Activity

The MR AP-lyase activity has not been documented previously. Structural homology had been noted between the active site of the MRE11 3′–5′ exonuclease and that of the major AP-endonuclease APE1 (Hopfner et al., 2001), but this structural homology does not reflect conserved catalytic mechanism. The MRE11 AP-lyase and exonuclease activities are separable by mutagenesis (Figure 3); and the cleavage products of MRE11 and APE1 are distinct (Figure 4).

Strikingly, the AP-lyase activity of MR is conserved from humans to Archaea. AID and AID-initiated Ig gene diversification arose only in early vertebrates (Conticello et al., 2005), so evolutionary conservation in Archaea, which lack an NBS1 homolog, almost certainly reflects function of the MR AP-lyase in more general and ubiquitous pathways of DNA repair. AP sites are highly mutagenic (Loeb and Preston, 1986), and the MR AP-lyase may provide useful redundancy in repair of these lesions. In addition, MR may be especially able to initiate repair in specific contexts. For example, just as factors associated with the transcription apparatus identify lesions for the nucleotide excision repair pathways, MR may participate in detection and repair of AP sites in transcribed genes. Analysis of the biological effects of mutation of the MR AP-lyase should provide useful insights into the ubiquitous and cell type-specific functions of this activity.

Experimental Procedures

ChIP

Chromatin was prepared as described previously (Larson et al., 2005). Anti-human MRE11 antibody and anti-APE1 were purchased from Novus Biologicals (Littleton, CO). Amplifications were performed by using Hot-Start Taq polymerase (Promega, Madison, WI) and the following oligonucleotide primers: for the Ramos rearranged V_H region, RV_HFOR and JOH48 (Sale and Neuberger, 1998); for TPI, 5′-CTCCCTGGAGAATGCTGAGTCTG-3′ and 5′- CA CAATTCCTAACCAGTGCCCTGG-3′; for SMC1L2, 5′-CCGGCCA AAAACCTTAGTAACAGCCAG-3′ and 5′-CTAGCTCAGTACCTGCA ATAGTAGGTCC-3′; and for the Ramos unrearranged V_H allele, RV_HFOR and 5′-GGCGAGGAGACAGACCACTGAGCTGTTGAG-3′. PCR products were quantified by using ImageQuant software (Amersham). Enrichment was calculated as the ratio of the amplicon of interest to the TPI amplicon, normalized to the ratio of products from IP with polyspecific IgG antibodies, e.g.: Enrichment V_H = (anti-MRE11 [V_H / TPI]) /(IgG [V_H / TPI]). Enrichment was compared at two template concentrations to confirm that assays were within the linear range of PCR.

Protein Expression and Purification

Recombinant hMR was expressed by coinfecting Sf9 insect cells with baculovirus vectors encoding His-tagged MRE11 and RAD50, a gift from Martin Gellert (NIH, Bethesda, MD), and purified essentially as described (Paull and Gellert, 1998). hMR was estimated to be greater than 90% pure by SDS-PAGE, and staining with Coomassie. Recombinant *P. furiosus* MR was expressed in *E. coli* BL21 Star (DE3) (Invitrogen, Carlsbad, CA) carrying a Pf MR expression construct (Hopfner et al., 2000) provided by James Carney (University of Maryland, Baltimore, MD). Frozen cell pellets were resuspended in 10 ml lysis buffer (20 mM Tris [pH 8], 300 mM NaCl, 5% glycerol, 1 mM imidazole, and 4 mM BME) containing one protease inhibitor minitablet (Roche, Mannheim, Germany) and sonicated for three pulses of 20 s each. Extracts were heated at 75ºC for 25 min and clarified by centrifugation for 30 min at 25,000 × g. The soluble fraction was loaded on 0.5 ml His-Select affinity gel (Sigma, St. Louis, MO), which was washed successively with 5 ml lysis buffer, lysis buffer containing 5 M guanidine, and lysis buffer. Pf MR was eluted in 2.5 ml lysis buffer containing 10 mM NaCl and 0.2 M imidazole and further purified by MonoQ chromatography (Amersham, Piscataway, NJ). Pf MR was estimated to be greater than 90% pure by SDS-PAGE and staining with Coomassie. Exonuclease and AP-lyase mutants of Pf MRE11 were made by QuikChange mutagenesis (Stratagene, La Jolla, CA) using 5′-TATCTCTACTACGCCTTAGGCG CAATTCACAAAAGATACGAGACAAG-3′ primer for $M^{H206A}R$ and 5′-GGATTAGTTTATGTAATAGGAATGGCAGCAGAAGCAGTTGAG AATGAATACTTAACGAG-3′ for $M^{3A}R$ and the corresponding complements. Assays of thermostability of AP cleavage activities in *E. coli* used clarified extracts from cells carrying the expression vector pET200DLacZ (Invitrogen). Recombinant human APE1 was expressed in *E. coli* by using an expression construct provided by Bruce Demple, Harvard Medical School, Boston, MA (Demple et al., 1991); nickel chromatography was carried out as described for Pf MR, omitting guanidine wash of the affinity resin.

DNA Substrates and Cleavage Assays

Oligonucleotides were synthesized by Operon Biotechnology (Huntsville, AL), end-labeled with T4 polynucleotide kinase (New England Biolabs, Beverly, MA), and purified by centrifugation through prepacked Sephadex G-50 spin columns (Amersham). Complementary strands were annealed in 25 mM MOPS (pH 6.8) and 50 mM NaCl. Most AP-duplexes carried 3′ overhangs to protect the DNA from degradation by the MR 3′–5′ exonuclease activity. The duplex DNA substrate carrying a U·G mispair was generated by annealing oligonucleotides 5′-CAGAAAGGGAAAGTATACAACAAAAA GCA<u>U</u>CTCAAGTCTTGGAGAGAACA-3′ and 5′-CTCCAAGACTTGAG GTGCTTTTTGTTGTATACTTTCCCTTTCTGTGACCT-3′. In the THF oligonucleotide, a single THF (dSpacer) replaced the U (underlined). Artificial transcription bubble substrates were created by annealing the uracil-containing oligonucleotide above with 5′-TTCTCTCCAAG ACTACTCGACGAATTTGTTGTATACTTTCCCTTTCTGTG-3′ and its partial RNA complement 5′-UUCGUCGAGACCAA-3′. An AP-duplex substrate carrying an internal ^{32}P label 3′ of the AP site was generated by annealing 5′-GCCTACTGATCCTAAATAGCTCGCTGAGGC TGAGGAU-3′ and 5′-TTTAAATTTGATCCTCAGCCTCAGCGAGCT ATTTAGGATCAGTAGGC-3′, extending with Taq polymerase (New England Biolabs) in a reaction containing dATP, dTTP, and α-^{32}P-dCTP, blunting by treatment with Mung-Bean nuclease (New England Biolabs), and purification. AP sites were generated by treatment of substrates carrying a uracil with 1 U uracil glycosylase (New England Biolabs) for 5 min at 37ºC.

Cleavage by MR and APE1 was assayed in 20 μl reactions containing 50 mM MOPS (pH 7.0), 25–100 mM NaCl, 5 mM $MnCl_2$, 0.1% Tween-20, and 50 fmol radiolabeled DNA substrate. Assays contained 400 fmol of hAPE1 or 40 fmol hMR or 600 fmol Pf MR heterotetramers (Hopfner et al., 2001). Reactions were incubated at 37ºC for 30 min and terminated by addition of stop mix to produce final concentrations of 0.25% SDS, 10 mM EDTA, and 100 μg/ml Proteinase K (Promega, Madison, WI), and cleavage products were resolved on 7.5 M urea 15% polyacrylamide gels and analyzed by phosphorimager. Exonuclease assays were performed as described (Hopfner et al., 2000; Hopfner et al., 2001). *E. coli* EndoIII (New England Biolabs) was used according to the manufacturer's instructions.

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