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Formation of the yeast Mre11–Rad50–Xrs2 complex is correlated with DNA repair and telomere maintenance

Mahmood Chamankhah and Wei Xiao*

Department of Microbiology and Immunology, University of Saskatchewan, 107 Wiggins Road, Saskatoon, SK S7N 5E5, Canada

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ABSTRACT

The yeast Mre11 is a multi-functional protein and is known to form a protein complex with Rad50 and Xrs2. In order to elucidate the relationship between Mre11 complex formation and its mitotic functions, and to determine domain(s) required for Mre11 protein interactions, we performed yeast two-hybrid and functional analyses with respect to Mre11 DNA repair and telomere maintenance. Evidence presented in this study indicates that the N-terminal region of Mre11 constitutes the core homo-dimerization and hetero-dimerization domain and is sufficient for Mre11 DNA repair and maintaining the wild-type telomere length. In contrast, a stretch of 134 amino acids from the extreme C-terminus, although essential for achieving a full level of self-association, is not required for the aforementioned Mre11 mitotic functions. Interestingly, deletion of these same 134 amino acids enhanced the interaction of Mre11 with Rad50 and Xrs2, which is consistent with the notion that this region is specific for meiotic functions. While Mre11 self-association alone is insufficient to provide the above mitotic activities, our results are consistent with a strong correlation between Mre11–Rad50–Xrs2 complex formation, mitotic DNA repair and telomere maintenance. This correlation was further strengthened by analyzing two *mre11* phosphoesterase motif mutants (*mre11-2* and *rad58S*), which are defective in DNA repair, telomere maintenance and protein interactions, and a *rad50S* mutant, which is normal in both complex formation and mitotic functions. Together, these results support and extend a current model regarding Mre11 structure and functions in mitosis and meiosis.

INTRODUCTION

Genes in the *Saccharomyces cerevisiae RAD52* epistasis group are required for the repair of DNA double-strand breaks (DSBs) and alkylation damage (1,2). Recently, several studies have demonstrated that a member of this group, *MRE11* (3), plays a role in crucial steps of meiotic recombination and mitotic DNA repair (4), illegitimate recombination (5,6) and end-joining (7), as well as telomere maintenance (8–10).

Physical interactions between members of the *RAD52* epistasis group have been detected genetically and biochemically, as well as *in vivo* through a yeast two-hybrid system (11–16). These interactions indicate that the formation of high-order complexes are important for meiotic recombination and mitotic DNA repair. During meiosis, *MRE11*, *RAD50* and *XRS2* act at the stage where DSBs are formed and also seem to be important for the subsequent step where DNA ends are resected to produce single-stranded tails. Mre11 has been shown to physically interact with itself, Rad50 and Xrs2 in a yeast two-hybrid assay (4) and by protein co-immunoprecipitation (17). Similarly, the human Mre11 homolog (hMre11) interacts with hRad50 and Nibrin (18), whose mutations were found to be responsible for Nijmegen breakage syndrome (19,20). Purified yeast Rad50 was also found to form a homodimer (21). However, despite intensive research in the field, several issues remain to be addressed. First, little is known about the biological significance of Mre11 dimerization and interactions with other proteins with regard to Mre11-mediated meiotic recombination and mitotic repair activities. Second, it was recently demonstrated that purified hMre11 alone exhibits a 3′–5′ exonuclease activity, which is only enhanced 4-fold in the presence of hRad50 (22); if the Mre11 3′–5′ exonuclease activity is responsible for its DNA repair function, this observation may call into question the role of complex formation in DNA repair. Third, previous studies by Usui *et al*. (17) and ourselves (23) identified a stretch of 136 or 134 amino acids at the extreme C-terminus of Mre11 to be non-essential for its DNA repair function. Since this region contains an Asp heptad repeat that was initially thought to be important for Mre11 interactions (4), we wanted to know if it is required for protein interactions. Fourth, a number of null and partially functional *rad50* and *mre11* mutations were previously identified; however, whether or not these mutations affect complex formation has not been determined. Finally, several recent reports (9,10,24) appear to establish a correlation between *MRE11* DNA repair and telomere maintenance. Hence, it is of great interest to know if any of the previously mentioned functions are directly related to the formation of Mre11–Rad50–Xrs2 complex.

We have independently isolated the *MRE11* gene by functional complementation of the yeast *ngs1-1* mutant (23). In this study, using a yeast two-hybrid system, we attempted to map domains required for Mre11 self-interaction and interactions with Rad50 and Xrs2, as well as Rad50 domain(s) required for the interaction with Mre11. Meanwhile, various *MRE11* and *RAD50* deletion and point mutations were analyzed for their effects on mitotic DNA repair and telomere maintenance. Our results show that the ability of Mre11 to form a complex with Rad50 and Xrs2

*To whom correspondence should be addressed. Tel: +1 306 966 4308; Fax: +1 306 966 4311; Email: xiaow@sask.usask.ca

correlates well with its DNA repair function, as well as its ability to maintain a wild-type level of telomere length. We were able to demonstrate that the Mre11 C-terminus is required for achieving a full level of self-association but is not essential for the above DNA repair or telomere maintenance, nor is it required for interaction with Rad50 and Xrs2. Furthermore, analysis of several *mre11* mutants carrying specific point mutations allows us to conclude that the four proposed phosphoesterase signature motifs can affect Mre11 protein interactions. Finally, we found that in contrast to Mre11S, which is deficient in both DNA repair and protein interactions, Rad50S is proficient in DNA repair, as well as being able to interact with Mre11. These results lend support to a general model of Mre11 structure and functions proposed in several recent studies (9,10,17,25).

MATERIALS AND METHODS

Yeast strains, cell culture and transformation

Saccharomyces cerevisiae strains used in this study are listed in Table 1. The *mre11* deletion mutants were created by one-step targeted gene disruption (26). To avoid potential plasmid–chromosome recombination that could restore the wild-type *MRE11* sequence and display false positive results, we created a complete *MRE11* deletion cassette in plasmid pMCY77, by replacing the *Afl*II–*Nru*I fragment of pTZ18-NGS1 (which contains the entire *MRE11* gene within a 4.1 kb *Bam*HI–*Hin*dIII fragment) with *LEU2*. The *mre11*Δ*::LEU2* cassette was released by digesting plasmid pMCY77 with *Nde*I–*Hin*dIII. Chromosomal deletion of the entire *MRE11* open reading frame (ORF) in strain MCY27 was verified by Southern hybridization prior to phenotypic characterization. Yeast cells were grown in either complete YPD medium or minimal synthetic dextrose (SD) medium with nutrients (27) at 30°C. Genetic manipulations were as described (27). Transformation of yeast cells was performed by a modified DMSO protocol (28). For targeted gene disruption, plasmid DNA was digested with restriction enzymes and ethanol precipitated prior to transformation.

Cell killing and gradient plate assays

Liquid killing experiments were carried out as follows. Yeast cells were grown in 5 ml of selective media overnight, and a 200–500 µl aliquot was transferred to 5 ml of fresh media. Cultures were incubated until the cell titer was ~2–5 × 10^7 cells/ml. MMS at the given concentration was added and samples were taken every 20 min during incubation, treated with fresh solutions of sodium thiosulfate (5% w/v) to neutralize MMS and washed once with sterile distilled water. Cells were resuspended in sterile distilled water and serial dilutions were made and plated on YPD plates. Colonies were scored after 3–5 days of incubation at 30°C. Alternatively, transformants were grown to stationary phase in selective media, diluted and plated on YPD plates containing different concentrations of MMS. Gradient plate assay was performed as previously described (29).

Two-hybrid plasmids

Restriction endonucleases and modifying enzymes were purchased from New England Biolabs or Gibco BRL and used as instructed. *Escherichia coli* strains used for plasmid manipulation were DH5α and DH10B. MMS was purchased from Aldrich Co. All plasmids were constructed by standard procedures (30). Nucleotide sequences were determined by the dideoxy-chain termination method using a T7 DNA Polymerase Sequencing kit (Pharmacia LKB). Two different two-hybrid systems were employed to assess protein–protein interactions. In one system, the Gal4 DNA-binding domain (BD) is in one vector, such as pGBT9, pAS1, pAS2 or their derivatives (31); another set of vectors contain the Gal4 DNA-activation domain (AD), namely pGAD424, pGAD10, pGAD-C1-3, pACTII or their derivatives (31,32). In order to clone target genes with different reading frames, pAS1 and pACTII were treated with *Nco*I, followed by Klenow treatment and self-ligation to form pAS1-Nc and pACTII-Nc, respectively. Vectors carrying the Gal4 DNA BD allow selection on SD–Trp media and those carrying the Gal4 DNA AD on SD–Leu media; double transformants were selected on SD–Trp–Leu media. The second two-hybrid system takes advantage of the DNA binding activity of the *E.coli* LexA repressor. We used pBTM116 (a gift from Dr S. Fields, University of Washington, Seattle, WA), where the LexA sequence serves as the DNA binding partner, and the same set of $Gal4_{AD}$ constructs. Yeast strains Y190 and L40 (Table 1) were used for the first and second systems, respectively.

Table 1. *Saccharomyces cerevisiae* strains

Strain	Genotype	Source/reference
DBY747	*MATa his3-Δ1 leu2-3,112 trp1-289 ura3-52*	D.Botstein
WXY9221	DBY747 but *rad50Δ::hisG-URA3-hisG*	(29)
MCY27	DBY747 but *mre11Δ::LEU2*	This study
K504	*MATa his4 leu2 can1 cyh2 ura3 ade2 mre11-2*	H.Ogawa
MCY44	K504 but *mre11Δ::URA3*	This study
MCY45	K504 but *MRE11*	This study
L40	*MATa his3-Δ200 trp1-901 leu2-3,112 ade2 LYS::(LexA op)$_4$-HIS3 URA3::(LexA op)$_8$-LacZ-GAL4 gal4 gal80*	N.Hollingsworth
Y190	*MATa gal4 gal80 his3 trp1 ade2-101 ura3 leu2::URA3 GAL1-LacZ::Lys2::GAL1-HIS3*	D.Gietz
32D-3032	*MATa ura3-160,188 lys1 his7*	V.Korolev
32D-3063	32D-3032 but *rad58-4*(*mre11–58*)	V.Korolev
MCY46	32D-3032 but *mre11Δ::URA3*	This study
EI417	*MATa leu2-3,112 trp1-289 ura3-5 lys1-1 his7-2*	J.Haber
EI425	EI417 but *xrs2-1*	J.Haber

MRE11 constructs

A 2.3 kb *Eco*RI–*Bam*HI fragment from pLexA-MRE11NN (a gift from Dr H. Ogawa, Osaka University, Japan) was cloned into the same sites of pGBT9 and pGAD424 to form pGBT9-MRE11 and pGAD-MRE11, respectively. To obtain plasmids pLexA-M11(1–559) and pGAD-M11(1–559), the 1.7 kb *Eco*RI–*Bst*YI fragment of pGBT9-MRE11 was cloned in the *Eco*RI–*Bam*HI sites of pBTM116 and pGAD424, respectively. The 1.55 kb *Eco*RI–*Eco*47III fragment was cloned in the *Eco*RI–*Sma*I sites of pBTM116 and pGAD424 to give pLexA-M11(1–515) and pGAD-M11(1–515). Plasmids pGBT-M11(1–412), pGBT-M11(1–318) and pGBT-M11(Δ318–393) were made by cleavage of pGBT9-MRE11 with *Bam*HI–*Bst*EII, *Bam*HI–*Stu*I and *Cla*I–*Stu*I, respectively, followed by Klenow treatment and self-ligation. The $Gal4_{AD}$ counterparts of these plasmids were made by cloning the *Eco*RI–*Pst*I fragment of each of the above plasmids into the same sites of pGAD424 to give pGAD-M11(1–412), pGAD-M11(1–318) and pGAD-M11(Δ318–393).

pLexA-M11(1–412) was obtained by digesting pLexA-MRE11NN with *Bam*HI–*Bst*EII, Klenow treatment and ligation. A 0.3 kb *Eco*RI–*Hin*cII fragment of pLexA-MRE11NN was cloned into the *Eco*RI–*Sma*I site of pGBT9 to form plasmid pGBT-M11(1–106). The *Eco*RI–*Bam*HI fragment of this plasmid was cloned into pGAD424 to give pGAD-M11(1–106). To clone the far N-terminal domain of Mre11, pGAD-MRE11 was digested with *Bam*HI and *Pml*I, Klenow treated and ligated to obtain pGAD-M11(1–59). To clone the far C-terminal domain of Mre11, the 0.46 kb *Bst*YI–*Bam*HI fragment of pGBT9-MRE11 was cloned into the *Bam*HI site of pAS1-Nd and pGAD-C1, resulting in pAS1Nd-M11(559–693) and pGADC1-M11(559–693), respectively. In pGBT9 and pGAD424, *Sma*I is not in frame with the coding sequence of Gal4. Thus the 1.8 kb *Hin*cII–*Bam*HI fragment of pGBT9-MRE11 was cloned in-frame into the *Sma*I–*Bam*HI sites of pAS1-Nc to give pAS1-M11(106–693). Plasmid pGAD-M11(Δ59–318) was made by double digestion of pGAD-MRE11 with *Pml*I–*Stu*I, Klenow treatment and ligation. The *Eco*RI–*Bam*HI fragment of this construct was cloned into the same restriction sites of pGBT9 to give pGBT-M11(Δ59–318). pAS1-M11(106–693) was digested with *Bam*HI–*Bst*EII, treated with Klenow and self-ligated to give pAS1-M11(106–412). Deletion of pLexA-M11(1–559) was made by *Cla*I–*Stu*I cleavage, Klenow treatment and ligation to result in pLexA-M11(1–559, Δ318–393). In YCp-M11(1–559), the *Bst*EII–*Hin*dIII fragment of our previous clone (23) was replaced with the *Bst*EII–*Hin*dIII fragment of pLexA-M11(1–559) to include an *ADH1* termination sequence.

RAD50 and *XRS2* constructs

To clone the *RAD50* gene into two-hybrid vectors, we took advantage of the *Eco*47III site at the third codon of *RAD50*. A 4.0 kb *Eco*47III–*Sal*I fragment of pNKY74 (33, a gift from Dr N. Kleckner, Harvard University, MA) was cloned into the *Sma*I–*Xho*I sites of plasmids pAS1-Nc and pACTII-Nc to give pAS1-RAD50 and pAD-RAD50, respectively. To clone the C-terminally truncated *RAD50* up to the *Nde*I site, the 3.8 kb *Nde*I fragment of pAD-RAD50 was cloned into the *Nde*I site of pAS1 in the correct orientation to form pAS1-R50(3–1279). Further C-terminal deletion of *RAD50* to remove the second heptad repeat region was achieved by double-digestion of pAD-RAD50 with *Pvu*II–*Bsiw*I, Klenow treatment and self-ligation. The resulting construct was named pAD-R50(3–685). pLexA-RAD50NN (4) was a gift from Dr H. Ogawa.

To clone the *XRS2* ORF in frame with the $Gal4_{AD}$ in pGAD424, plasmid pEI43 (34, a gift from Dr J. Habor, Brandeis University) containing the *XRS2* wild-type sequence was linearized with *Bst*EII, Klenow treated and, after addition of a *Bam*HI linker, religated to give pIE43B. The 3.5 kb *Bam*HI fragment of pEI43B was cloned into the *Bam*HI–*Bgl*II sites of pGAD424 to give pGAD-XRS2-B1. Next, the 827 bp *Bam*HI–*Stu*I fragment of pGAD-XRS2-B1 was replaced with the 73 bp *Bam*HI–*Stu*I PCR fragment to produce pGAD-XRS2. To make the pGBT9-XRS2 and pLexA-XRS2, the *Bss*HII site in pGAD-XRS2 was converted to *Bam*HI. Next, the 2.6 kb *Bam*HI fragment was cloned in the same site of pGBT9 and pBTM116 to produce pGBT-XRS2 and pLexA-XRS2. Both were able to complement the MMS sensitivity of the *xrs2* mutant.

Mutant alleles of *MRE11* and *RAD50, mre11-2, rad58* and *rad50S*

Localization of *mre11* mutant alleles was performed using a gap repair technique as reported elsewhere (23). To clone *mre11-2* and *rad58* mutations, the 1.2 kb *Msc*I–*Bst*EII fragment of pGBT-MRE11 was replaced with the same fragments containing *mre11-2* and *rad58* mutations. The 2.3 kb *Eco*RI–*Bam*HI fragment of pGBT-rad58 was then cloned in the same sites of pBTM116 and pGAD424 to give pLexA-rad58 and pGAD-rad58, respectively. A two-step cloning procedure was followed to clone the *rad50S* mutant allele (35) in two-hybrid constructs. First, the 1.3 kb *Eco*47III–*Bgl*II fragment of pNKY349 (a gift from Dr N. Kleckner containing the *rad50S* mutant allele *KI81*) was cloned into the *Sma*I–*Bam*HI sites of pACTII-Nc to give pADrad50S-Bg. In the next step, the 0.2 kb *Stu*I–*Xho*I fragment of pADrad50S-Bg was replaced with the 2.9 kb *Stu*I–*Sal*I fragment of pNKY74 (containing the wild-type *RAD50* sequence) to give pADrad50S. The resulting construct was confirmed by sequencing.

In vivo assay of protein interaction using yeast two-hybrid system

A filter assay was employed to determine the β-galactosidase (β-gal) activity (36). Briefly, 5–10 independent co-transformants with both DNA BD and AD fusion constructs were grown on selective plates for 1–2 days. Cells were transferred to Whatman No. 1 filter paper, immersed in liquid nitrogen for 10 s to permeabilize cells, and placed on top of another filter which was presoaked in a mixture of 1.8 ml Z-buffer containing 5 µl β-mercaptoethanol and 45 µl of 20 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) in *N*,*N*-dimethylformamide (37). Plates were sealed with parafilm and incubated at 30°C. Color development was monitored at 4, 8, 16 and 24 h after incubation. Y190 and L40 cells transformed with vectors alone were used as negative controls. The β-gal activity was quantitated as follows. Briefly, Y190 or L40 double transformants from a single clone were grown overnight in selective media until later log phase (OD_{600nm} = 0.7–1.0). The culture (2 ml) was centrifuged and resuspended in Z-buffer (36); 50 µl of chloroform and 50 µl of 0.1% SDS were added and the culture was vortexed at top speed for 20 s. 200 µl of *O*-nitrophenylgalactoside (ONPG, 4 mg/ml) were added as a substrate for β-gal and the

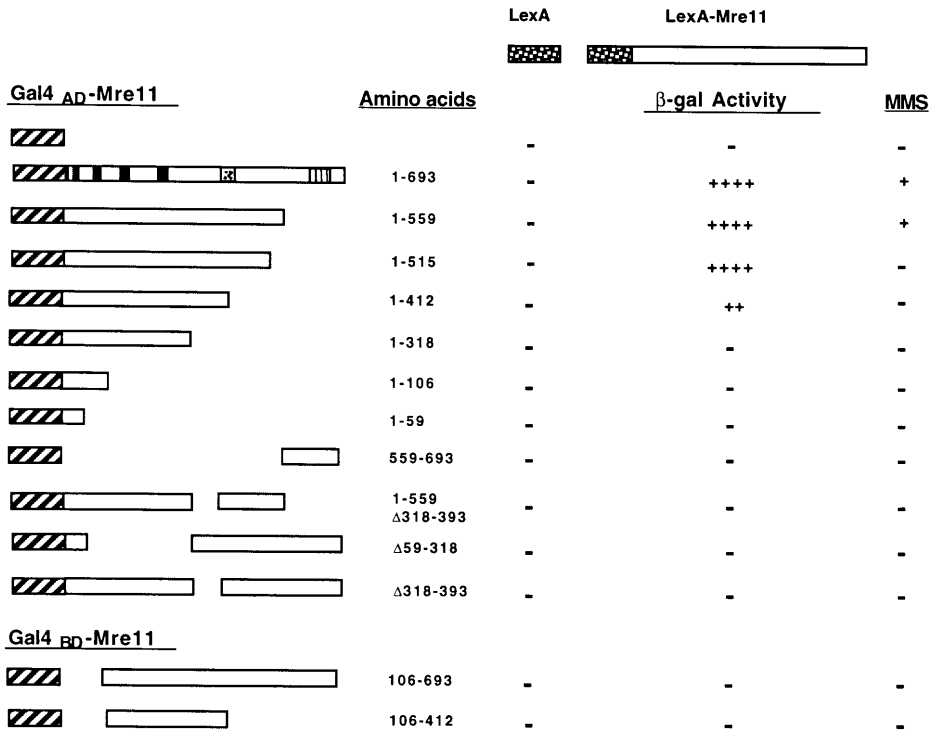

Figure 1. Two-hybrid analysis of truncated Mre11 with the full-length Mre11. β-gal assays were performed on L40 cells co-transformed with pLexA-MRE11 and various pGAD-M11 deletion constructs. In the case of constructs containing amino acids 106–693 and 106–412, pAS1-based plasmids were co-transformed with pGAD-MRE11 into Y190 strain. The β-gal activity was determined by a filter assay as described (36). Color development is indicated as follows: ++++, 15 min–1 h; +++, 1–4 h; ++, 4–8 h; +, 8–12 h; ±, 12–24 h. Colonies remaining white after 24 h were considered negative. Open bars in each construct represent the expected Mre11 polypeptides after deletion and numbers denote the amino acids remaining in the polypeptide, or (Δ) deleted from the polypeptide. The four solid boxes represent proposed phosphoesterase motifs, a speckled box represents the basic domain, and a striped box represents the Asp heptad repeats. MMS resistant (+) or sensitive (–) phenotypes of *mre11*Δ mutant transformed with *MRE11* deletion constructs are recorded on the far right column.

reaction mixture incubated at 28°C until a yellow color appeared. To stop the reaction, 500 µl of 1 M sodium carbonate were added. The reaction mixture was centrifuged for 10 min and the absorbance of the supernatant was recorded at 420 nm. The β-gal activity was measured in Miller units (37) according to the following equation: $1000 \times [OD_{420}/(t \times V \times OD_{600})]$, where t is the time of incubation; V is volume of yeast culture used for assay; and OD_{600} is the absorbance of the yeast culture at 600 nm.

Western analysis

Y190 and L40 cells transformed with some two-hybrid constructs were analyzed for the expression of Gal4 fusion proteins using anti-Gal4 antibodies (Santa Cruz Immunologicals).

Measurement of telomere length

Genomic DNA was isolated from 5 ml saturated cultures according to the reported protocol (38). DNA (1–2 µg) was digested with *Xho*I, separated in a 0.8% agarose gel, transferred to Hybond N^+ (Amersham) membranes using an alkaline transfer method and hybridized with an [α-^{32}P]dCTP-labeled 0.8 kb telomere sequence, obtained by PCR using plasmid pYT14 (a gift from Dr T. Petes, University of North Carolina, NC) as a template and the following primers: pYT14-1: 5′- ACACACTCTCTCA-CATCTACC-3′ and pYT14-2: 5′-TTGCGTTCCATGACGAG-CGC-3′.

RESULTS

The N-terminal region of Mre11 is important for self-interaction

In order to map the Mre11 domain(s) involved in its dimerization, a series of truncated $Gal4_{AD}$–Mre11 fusion constructs were co-transformed with the full-length Mre11 ORF fused to the $Gal4_{BD}$ or LexA coding sequence. Results of two-hybrid interactions between these fusion proteins as measured by the β-gal filter assay are shown in Figure 1. Most truncated Mre11 proteins, including all N-terminal and internal deletions, were unable to interact with the full-length Mre11; the only constructs that gave a full or partial response were truncated proteins resulting from the C-terminal deletions consisting of 559, 515 and 412 amino acids. A further deletion of 94 amino acids from M11(1–412) completely abolishes β-gal activity. Western analyses suggest that the inability of truncated Mre11 fusion proteins to interact with full-length Mre11 is not due to a decreased stability of truncated proteins. Therefore, we infer that the N-terminal 412 amino acids of Mre11 contain the core dimerization domain. However, the Mre11 self-association appears to be rather complicated, as evident by the difficulty we encountered in mapping the self-interaction domain to a limited stretch of amino acids.

C-terminal domain of Mre11 is required for a full level self-interaction

Since homo-dimerization of Mre11 is what one may expect to occur in the *mre11* mutants, all deletion constructs shown in Figure 1 were subsequently self-paired for the two-hybrid analysis and β-gal activity was determined. The results from some critical constructs are summarized in Table 2. Quantitative analysis indicates that although constructs with a deletion of the C-terminal 134 and 178 amino acids are able to fully interact with full-length Mre11, their ability to homodimerize is reduced by 8-fold. It is noted that the Asp heptad repeat is present in the last 134 amino acids (Fig. 1). Thus, our results indicate that although the Mre11 C-terminal heptad acidic domain is not essential for protein interaction, it is required to achieve a wild-type level of self-interaction. Further deletion of amino acids 319–412 completely abolishes Mre11 dimerization. This region contains a basic domain recently shown to be important for DNA binding and is involved in DSB processing during meiosis (17).

Table 2. Self-association of Mre11 with different C-terminal truncation constructs

$Gal4_{AD}$ fusions	Control (pBTM116)	Interaction with full-length Mre11	Homodimerization
pGAD424	0.15[a]	1.82	NA[b]
Mre11	2.12	116.44	116.44
M11(1–559)	1.69	144.96	18.59
M11(1–515)	1.32	111.08	15.54
M11(1–412)	1.87	20.16	19.01
M11(1–318)	1.07	1.54	1.91

[a]All values were determined by a liquid β-gal activity assay and presented in Miller units. Results are the average of three independent experiments.
[b]NA, not available

Mre11 dimerization alone is not sufficient for DNA repair and telomere maintenance

In order to examine the biological relevance of Mre11 interaction to its mitotic functions, we analyzed two mitotic phenotypes, namely DNA repair and telomere maintenance, with some critical deletions made in the two-hybrid analysis. As shown in Table 2, M11(1–559) was able to form a homodimer, albeit at a reduced level compared to full-length Mre11, and is capable of DNA repair (Fig 1). This result is in agreement with a recent report (17) that an *mre11-5* mutation with a C-terminal 136 amino acid deletion can complement the MMS sensitivity of the *mre11*Δ mutant. In addition, we found that the Mre11(1–559) clone was able to rescue telomere shortening in the *mre11*Δ mutant (Fig. 2A). In contrast, clones M11(1–515) and M11(1–412), despite being proficient in homodimerization (Table 2), were neither able to complement the DNA repair deficiency (Fig. 1) nor maintain a wild-type telomere length in the *mre11*Δ mutant (Fig. 2A and data not shown). These observations collectively suggest that Mre11 dimerization may be required, but not sufficient for DNA repair and telomere maintenance.

Domains responsible for Mre11–Rad50 and Mre11–Xrs2 interactions

The Rad50–Mre11–Xrs2 complex appears to be crucial for effective recombinational repair and meiotic recombination.

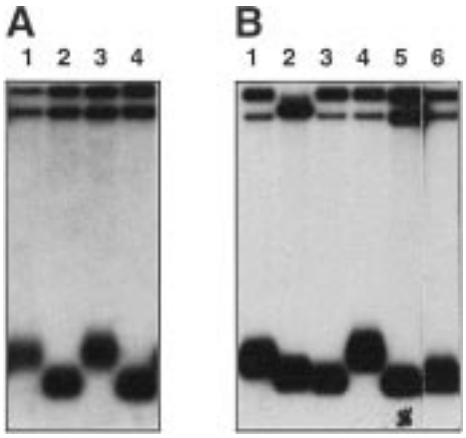

Figure 2. Analysis of telomere maintenance. (**A**) The 134 amino acid Mre11 C-terminus is not essential for telomere maintenance. *Xho*I-cleaved genomic DNA from wild-type (lane 1), *mre11*Δ (lane 2) and MCY27 transformed with a single-copy YCp-M11(1–559) (lane 3) or YCp-M11(1–515) (lane 4) was hybridized with the 0.8 kb telomere probe that recognizes a characteristic 1.3 kb fragment in wild-type cells. (**B**) *mre11-2* and *rad58* mutations are defective in telomere maintenance. The *mre11-2* (K504, lane 3), *rad58S* (32D-3063, lane 6) mutants, their corresponding isogenic wild-types (MCY45 and 32D-3032, lanes 1 and 4, respectively) and *mre11*Δ mutants (MCY44 and MCY46, lanes 2 and 5, respectively) are compared for telomere length as described in (A).

Mre11 was previously reported to interact with Rad50 and Xrs2 in a two-hybrid assay (4). Hence, we asked whether or not this complex formation is required and sufficient for DNA repair and telomere maintenance. We observed an interaction between Mre11 and Rad50, albeit at a lower level compared to Mre11 dimerization. In order to map the domain responsible for Mre11–Rad50 interaction, we examined whether any of the Mre11 deletion mutants were able to interact with Rad50. Negative results were obtained with all truncated Mre11 constructs examined in Figure 1, with the exception of the M11(1–559) construct (Fig. 3A and data not shown). Although M11(1–515) is indistinguishable from M11(1–559) with respect to Mre11 self-interaction (Table 2), it is completely defective in complex formation with Rad50 (Fig. 3A). Surprisingly, deletion of the C-terminal 134 amino acids resulted in a 7-fold increase in the Mre11 interaction with Rad50 (Fig. 3A). Furthermore, using a filter assay, we could hardly detect an interaction between the full-length Mre11 and Xrs2; however, β-gal activity was readily detected in cells harboring M11(1–559) and *XRS2* constructs (data not shown), again indicating an enhanced interaction between C-terminally truncated Mre11 protein with Rad50 and Xrs2.

Analysis of the deduced Rad50 protein revealed an ATP binding domain at the far N-terminus and two large heptad repeats (39). Two Rad50 N-terminal deletion constructs, both removing the ATP binding domain as well as the first heptad repeat, were made previously and found to abolish the interaction with Mre11 (4). The authors concluded that the N-terminal one-third of the Rad50 protein is required for protein–protein interactions. To further address this issue, a C-terminal deletion construct of Rad50 was made to remove the second heptad repeat while leaving the first heptad repeat intact. Our results show that C-terminal deletion of Rad50 heptad repeat II leads to an undetectable β-gal activity in a two-hybrid assay (Fig. 3B),

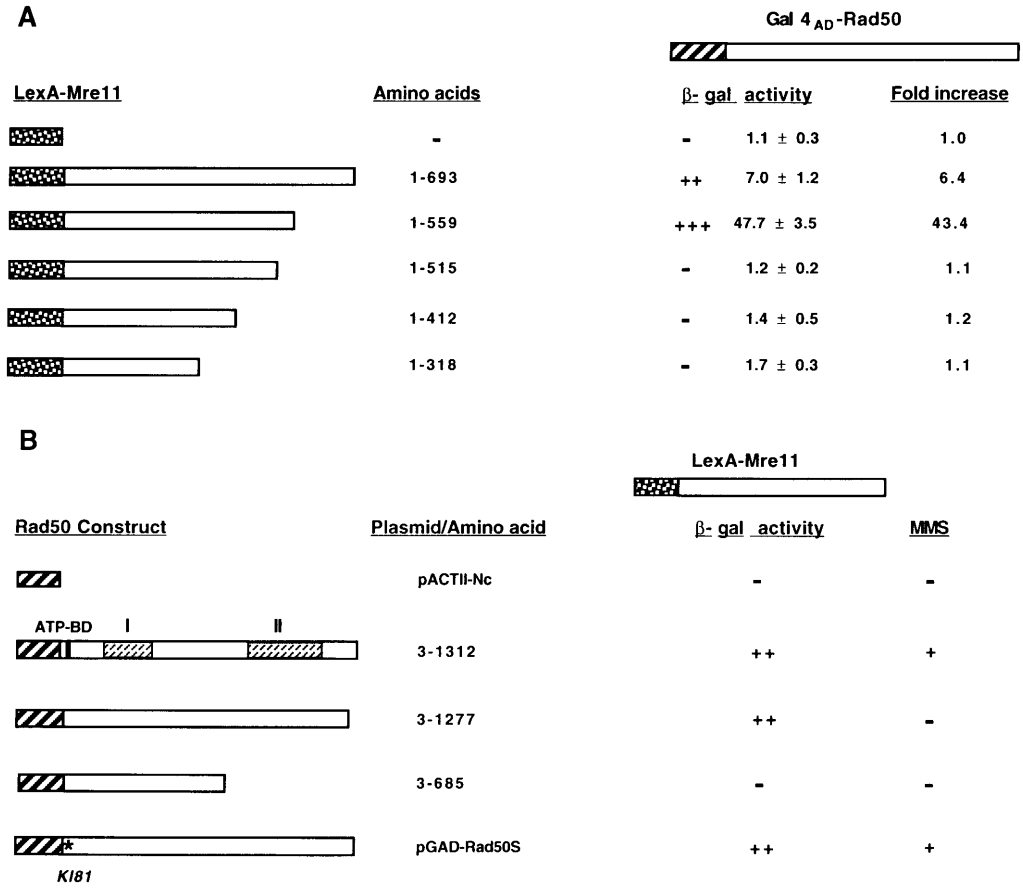

Figure 3. Mre11–Rad50 interaction domains. (**A**) The Mre11 N-terminus is required to interact with Rad50. pLexA-MRE11 and its C-terminal deletion constructs were co-transformed with pAD-RAD50 and β-gal activities were determined using both filter and liquid assays. The liquid β-gal assays are the average of at least three independent experiments, and are presented in Miller units with standard deviations. (**B**) The C-terminal heptad repeat of Rad50 is required to interact with Mre11. pAD-RAD50 and its deletion or *rad50S* mutation constructs were co-transformed with pLexA-MRE11 and the β-gal activities were determined by a filter assay. The *RAD50* constructs were also used to transform DBY747r50 (*rad50*Δ) to determine their ability to rescue MMS-induced killing. An ATP BD and two heptad repeats within Rad50 are indicated by a solid and two hatched boxes, respectively. The relative position of the KI81 *(rad50S)* mutation is indicated by an asterisk.

suggesting that the C-terminus containing heptad repeat II is also important for Mre11–Rad50 interaction. The pLexA-Rad50NN construct used in a previous study (4) lacks a C-terminal 35 amino acid coding sequence. Our results show that although the C-terminal 35 amino acids are dispensable for the interaction with Mre11, the truncated Rad50 protein is unable to complement the MMS sensitivity (Fig. 3B).

Effects of specific *mre11* and *rad50* mutations on protein interaction

The Mre11 N-terminus contains the four proposed phosphoesterase motifs believed to be important for Mre11 nuclease function (9,10,40). Our two-hybrid analyses of Mre11 deletions show that M11(1–318), which contains all four motifs, is defective in self-interaction (Table 2), interaction with full-length Mre11 (Fig. 1), as well as interaction with Rad50 (Fig. 3A), suggesting that a region containing these motifs alone is insufficient for Mre11 protein interactions. The same construct was also unable to complement the MMS sensitivity of the *mre11*Δ mutant (Fig. 1). To further examine whether or not these motifs are required for protein interactions, we took advantage of two previously determined *mre11* point mutations, *mre11-2* (23) and *rad58* (23,41), that affect the first and fourth phosphoesterase motifs, respectively. The *mre11-2* mutant (42) contains a single amino acid substitution (Gly55Asp) at the second phosphoesterase motif of Mre11 (23) and is extremely sensitive to killing by MMS, but still retains about one-tenth of MMS resistance compared to the otherwise wild-type *mre11*Δ mutant (data not shown). The *rad58* mutant (43) displays an *S* (separation of function) phenotype during meiosis (41) and contains two mutations, resulting in amino acid substitutions within (His213Tyr) and adjacent to (Leu225Ile) the fourth proposed phosphoesterase signature motif (23,41). Unlike *mre11-2*, the *rad58* mutant is as sensitive to killing by MMS and γ-radiation (41) as the *mre11*Δ mutant, suggesting that its DNA repair function is completely abolished. In both *mre11-2* and *rad58* mutant strains, telomeres are shortened to the same level as the *mre11*Δ mutant (Fig. 2B). Similarly, self-interactions of Mre11-2 and Rad58S, as well as their interactions with wild-type Mre11 and Rad50, were undetectable (Table 3). These observations together indicate that the proposed phosphoesterase signature motifs are required for Mre11 interactions, which agrees with two recent reports (17,25).

Table 3. Interactions of *mre11* mutant proteins with Mre11, Rad50 and Xrs2

$Gal4_{AD}$ fusions	LexA fusions			
	pBTM116	mre11-2	rad58	RAD50
pGAD424	–[a]	–	–	–
MRE11	–	–	–	+++
mre11-2	–	–	ND	+/–
rad58	–	ND[b]	–	+/–
RAD50	–	+/–	+/–	–
XRS2	–	–	–	–

[a]β-gal activities were determined by a filter assay and presented as described in Materials and Methods.
[b]ND, not determined.

The fact that Rad58S failed to interact with itself and Rad50 prompted us to investigate whether or not Rad50S also affects protein interactions. The *rad50S* mutants display extremely poor spore viability in meiosis, yet are nearly normal in their ability to repair DNA damage (35) and in telomere maintenance (24) during vegetative growth. We found that Rad50S is capable of interaction with Mre11 at a level indistinguishable from wild-type Rad50 (Fig. 3B). Therefore, the phenotype of *rad50S* mutants during meiosis does not appear to be due to an impaired interaction with Mre11.

DISCUSSION

Characterization of Mre11–Rad50–Xrs2 complex formation is an important step toward understanding the early steps of meiotic recombination and recombinational repair of DNA damage. The objective of the present study was to establish a correlation between Mre11–Rad50–Xrs2 complex formation and its mitotic functions. During the course of this study and preparation of the manuscript, several laboratories reported their findings on the structure and functions of Mre11 (9,10,17,25) that were relevant to our studies. Our results and conclusions are consistent with these findings.

Our inability to delineate a specific interacting domain suggests that Mre11 interactions probably require multiple contacts in order for proper assembly to occur. Alternatively, a specific interacting domain may rely on proper folding of other regions to be presented on the surface; a deletion in various regions may lead to disruption of such a conformation. The observation that to achieve a full level of self-association, at least one Mre11 monomer should contain the C-terminus suggests that Mre11 dimerization is not likely through C–C- or N–N-terminal contact. Two simplistic models would accommodate the above observations. First, Mre11 may dimerize through an inter-molecular N–C-terminal contact, and a minimal N-terminal region (1–559) may be required to form a proper conformation. Alternatively, an intra-molecular N–C contact can be an important step for Mre11 to present its dimerization domain(s).

We demonstrated that Mre11 dimerization alone is insufficient for Mre11–Rad50–Xrs2 complex formation, DNA repair and telomere maintenance, since several deletion constructs, although capable of self-interaction, were unable to interact with Rad50 and Xrs2, nor did they complement the MMS sensitivity and telomere shortening of the *mre11*Δ mutant. In this regard, Mre11 amino acid residues 516–558 appear to be critical for these functions.

Our observations also support the hypothesis that the C-terminus of Mre11 is only involved in Mre11 self-association and not the interaction with Rad50 or Xrs2. The fact that the M11(1–559) construct severely reduces homodimerization indicates that deletion of the C-terminus may enhance its ability to interact with Rad50 and Xrs2. Alternatively, the extreme C-terminal end of Mre11 may be involved in interaction(s) with other unknown targets, such as DNA or proteins other than Rad50 and Xrs2, resulting in a sequestering of cellular Mre11 proteins. Indeed, the extreme C-terminal 50 amino acids of Mre11 were recently shown to possess a DNA binding activity essential for DSB formation during meiosis (9). The C-terminal region is also required to interact with three meiosis-specific proteins (17).

Our observation that two Mre11 variants with specific amino acid substitutions within the proposed phosphoesterase motifs severely affect self-interaction and interaction with Rad50 clearly demonstrates that these motifs are also important for Mre11–Mre11 and Mre11–Rad50–Xrs2 complex formation. These motifs were initially identified based upon the high degree of conservation of these regions between *S.cerevisiae* Mre11, *Schizosaccharomyces pombe* Rad32, *E.coli* SbcD and bacteriophage T4 gp47 (40). Recently, the SbcC and SbcD polypeptides were purified and shown to form a large complex that functions as an ATP-dependent double-strand DNA exonuclease and an ATP-independent single-strand endonuclease (44,45). Although Mre11 (11) and SbcD (46) display similar enzymatic activities, it is not known if these motifs merely constitute a catalytic domain (40), are involved in DNA binding (4) and/or are also required for self-interaction and complex formation with other proteins (4,44,45). The involvement of phosphoesterase motifs in protein interactions may be due to involvement of these motifs in the interaction with metal ions, which in turn could affect the secondary structure of the protein and its interaction with other proteins (47).

The yeast (39), mouse (48) and human (18) Rad50 belongs to the SMC family of proteins and is a homolog of *E.coli* SbcC and T4gp46 (35,39,40). Members of this family possess 'Walker-A' and 'Walker-B' nucleotide binding motifs (49) separated by an α-helical region. The deduced Rad50 sequence suggests that the far N-terminal portion of the protein is an ATP-BD and the remainder of the protein contains two large heptad repeats (35). Our two-hybrid analysis indicates that a long stretch within Rad50 encompassing both the ATP-BD and the two heptad repeats is essential for interaction with Mre11. The heptad repeats are postulated to allow Rad50 to adopt an α-helical coiled-coil conformation (50,51). Since the consensus region in the ATP-binding domain appears to be involved in nucleotide binding and hydrolysis, mutations in this region result in a null phenotype in terms of DNA repair and meiotic viability (39). However, all *rad50S* mutations map outside the consensus region (35) and presumably alter the interaction with ATP rather than abolishing it. Our observations confirm the proposal (17) that the complex formation between Rad50 and Mre11 is essential for DNA repair function, but may not correlate with meiotic functions. In addition, these results also suggest that a compromised Mre11–Rad50 complex formation may be sufficient for the generation of meiotic DSBs, but insufficient for the processing of these DSBs. Furthermore, although both *rad50S* (35) and *mre11S* (41,52) display a similar meiotic phenotype, they may be defective in different biochemical activities, which result in completely distinct phenotypes in DNA recombinational repair and telomere maintenance.

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