Communication

Nuclease Activities in a Complex of Human Recombination and DNA Repair Factors Rad50, Mre11, and p95*

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Genetic studies in yeast have indicated a role of the RAD50 and MRE11 genes in homologous recombination, telomere length maintenance, and DNA repair processes. Here, we purify from nuclear extract of Raji cells a complex consisting of human Rad50, Mre11, and another protein factor with a size of about 95 kDa (p95), which is likely to be Nibrin, the protein encoded by the gene mutated in Nijmegen breakage syndrome. We show that the Rad50-Mre11-p95 complex possesses manganese-dependent single-stranded DNA endonuclease and 3’ to 5’ exonuclease activities. These nuclease activities are likely to be important for recombination, repair, and genomic stability.

Genetic studies on Saccharomyces cerevisiae mutants sensitive to ionizing radiation and to other agents that cause DNA double-stranded breaks have identified a large number of genetic loci required for the repair of such breaks. Many of these genes, including RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, RDH54, MRE11, and XR2, show epistasis and are collectively known as the RAD52 epistasis group. Mutants of the RAD52 group also have defects of varying degrees in mitotic and meiotic recombination, which are initiated via DNA double-stranded break formation. Because meiotic recombination is essential for the proper segregation of homologous chromosomal pairs during meiosis I, the RAD52 group mutants often exhibit severe meiotic abnormalities, including inviability (see Refs. 1 and 2 for discussions and references).

Extensive genetic evidence in yeast indicates that DNA double-stranded breaks are processed exonucleolytically, yielding 3’ overhanging single-stranded (ss) tails of about 600 bases in length (3, 4). According to the double-stranded break repair model for recombination (5), the 3’ ssDNA tails formed as a result of break processing are bound by recombination proteins, which then mediate a search for the chromosomal homolog and heteroduplex DNA formation with the homolog (5). The RAD52 group genes may be divided into two categories. The first class consists of the RAD50, MRE11, and XR2 genes, whose protein products are thought to be involved in the nuclease-activating DNA double-stranded breaks (6). Consistent with this classification, the Rad50 and Mre11 proteins have been shown to be homologous to the Escherichia coli SbcC and SbcD proteins, which combine to form a complex with endonuclease and exonuclease activities (7). The second category of the RAD52 group genes includes RAD51, RAD52, RAD54, RAD55, RAD57, and RDH54, whose products nucleate onto the ssDNA tails generated from break processing and then mediate the formation of heteroduplex DNA between the recombining chromosomes (1, 2). Whether the Rad59 protein, which is homologous to Rad52 (8), also has a role in heteroduplex DNA formation remains to be established.

Important insights concerning the mechanism by which the RAD52 group proteins form heteroduplex DNA have been garnered through biochemical studies of purified human and yeast proteins (9–11). However, no information as to the biochemical functions of the RAD50 and MRE11 encoded proteins is currently available. The Rad50 and Mre11 proteins are of special interest because genetic studies in yeast have indicated that they are also indispensable for nonhomologous DNA end joining (12) and for the maintenance of telomere length (13). The human homologs of RAD50 and MRE11 genes have been identified (14, 15). Here, we purify a complex of human Rad50, Mre11, and a protein species with an apparent size of 95 kDa from nuclear extract of Raji cells. This 95-kDa protein, or p95, is most likely the same protein found to co-immunoprecipitate with Rad50 and Mre11 proteins from HeLa cell extract (14) and recently identified to be Nibrin, the product of the gene mutated in Nijmegen breakage syndrome (16, 17). Our biochemical studies now reveal that the Rad50-Mre11-p95 complex possesses an endonuclease activity and a 3’ to 5’ exonuclease activity.

MATERIALS AND METHODS

Antisera—The cDNAs encoding a portion of the human Rad50 protein (amino acid residues 518–881) and a portion of the human Mre11 protein (amino acid residues 1–320) were obtained from a human B cell library using the polymerase chain reaction. These cDNAs were fused in frame to glutathione S-transferase (GST), and the GST-Rad50 and GST-Mre11 fusion proteins were expressed in E. coli XL1 strain. The GST fusion proteins were purified by affinity chromatography on glutathione-Sepharose and used as antigens to raise polyclonal antisera in mice.

Purification of Rad50-Mre11-p95 Complex—Clarified nuclear extract from 80 ml of human Burkitt’s lymphoma cell pellet (Raji cells; purchased from the National Cell Culture Center in Minneapolis) obtained from 50 liters of culture was treated with ammonium sulfate at 0.28 g/ml and then subjected to fractionation in columns of Source Q, Hydroxyapatite, Sepharose 6B, Phenyl-Sepharose, Sepharose 6B, and Mini S. The full purification details will be described elsewhere.3

DNA Substrates—The dX174 circular ssDNA and replicative form DNA (95% supercoiled form) were purchased from New England Biolabs and Life Technologies, Inc., respectively. For preparing substrates for exonuclease reactions, pUC18 DNA was digested with ScaI or EcoRI to linearize the DNA. To obtain the 3’ end-labeled species, the EcoRI-linearized pUC18 DNA was treated with a mixture of $^{32}$PdATP and unlabeled dCTP, dGTP, and TTP and E. coli Klenow polymerase. For 5’ end labeling, the pUC18 DNA linearized with ScaI was first treated with calf intestinal alkaline phosphatase to remove the preexisting 5’ phosphate group, purified by phenol extraction and ethanol precipita-

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3 The abbreviations used are: ss, single-stranded; GST, glutathione S-transferase; MOPS, 4-morpholino propane sulfonic acid.

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tion, and then treated with polynucleotide kinase and [γ-32P]ATP to label the 5' end. Both the 3' and 5' end-labeled DNA substrates were purified from the labeling reaction mixtures using a GeneClean kit.

Nuclease Reactions—DNA was mixed with the indicated amounts of the Rad50-Mre11-p95 complex in reaction buffer (30 mM potassium-MOPS, pH 7.2, 1 mM dithiothreitol, 1 mM ATP, 25 mM KCl, and 2 mM MnCl2). After incubation at 37 °C, the nuclease reaction was terminated by adding 1/10 volume of 3% SDS and 2/10 volumes of loading buffer (0.1% orange G in 50 mM Tris-HCl, pH 7.5, 50% glycerol, and 1 mM EDTA). Reaction samples were run in 0.8% agarose gels at 100 mA and photographed with Polaroid type 55 films. Photographic negatives were subjected to image analysis to obtain data points for a graphical representation of the results. The filled circles represent the percentage of ssDNA incised, and the open circles represent the percentage of supercoiled DNA incised. D, nuclease activity is manganese-dependent. Rad50-Mre11-p95 complex, 60 ng (lanes 3) or 120 ng (lanes 2 and 4–6) was incubated with 100 ng of 4X ssDNA in the absence of metal (lane 5), in the presence of 2 mM Mn2+ (Mn, lanes 2–4), and in the presence of 2 mM Mg2+ (Mg, lane 6) for 60 min. SDS was added to the reaction mixture in lane 2 to 0.3% at the beginning of the incubation, and the DNA substrate was incubated in buffer alone in lane 1, kb, kilobases pairs.

RESULTS AND DISCUSSION

For identifying the Rad50 and Mre11 proteins in human cells, polyclonal antisera were raised in mice against GST-Rad50 and GST-Mre11 fusion proteins produced in and purified from E. coli. As shown in Fig. 1A, in the left panel, the anti-Rad50 serum detected in nuclear extract of Raji cells a protein band with an apparent size of about 150 kDa, which was in excellent agreement with the predicted size of 153 kDa for Rad50 protein (15). The anti-Mre11 serum detected a band with an apparent size of about 80 kDa (Fig. 1A, right panel) in the Raji nuclear extract, which was in excellent agreement with the predicted size of 81 kDa for Mre11 protein (14). The human Rad50 and Mre11 proteins are associated in a tight complex (14). To purify the Rad50-Mre11 complex, a scheme involving ammonium sulfate precipitation of nuclear extract, followed by chromatographic steps in columns of Source Q, Hydroxyapatite, Sepharose 6B, Phenyl-Superose, a second Sepharose 6B step, and Mini S was used, and the elution of the Rad50-Mre11 complex from the columns was monitored by immunoblot analysis. Interestingly, during the second gel filtration step on Sepharose 6B, we found a 95-kDa protein species eluting precisely with the Rad50 and Mre11 proteins. When the protein pool consisting of Rad50, Mre11, and p95 was further fractionated in either Mini S or Mini Q, precise coelution of p95 with Rad50 and Mre11 proteins was again observed. The final protein pool used for the biochemical studies described below contained Rad50, Mre11, and p95 in nearly homogeneous form (Fig. 1B). The yield of this complex of Rad50, Mre11, and p95 from 80 ml of packed volume of Raji cell
pellet was about 50 μg. Two independent preparations of the Rad50-Mre11-p95 complex obtained using Mini S as the final step of purification and one preparation of this complex purified using Mini Q as the final column gave the same results when used in the biochemical studies described below. We believe that p95 is identical to the protein species with the same size which in immunoprecipitation studies (14) was found to be associated with Rad50 and Mre11 proteins in HeLa cell extract. Interestingly, the p95 species appears to be substoichiometric in regard to Rad50 and Mre11 (Fig. 1 B), suggesting that either p95 is associated with a fraction of the Rad50-Mre11 complex or p95 is of a lower stoichiometry in the protein complex.

The Rad50 and Mre11 proteins are structurally related to the SbcC-SbcD complex, which possesses a ssDNA endonuclease activity (7). For this reason, it was of great interest to test whether the Rad50-Mre11-p95 complex has a ssDNA endonuclease activity. To do this, purified Rad50-Mre11-p95 complex was incubated with dX circular ssDNA, and at different times, SDS was added to 0.3% to terminate the reaction. The reaction mixtures were run in an agarose gel and stained with ethidium bromide. As shown in Fig. 2, neither nuclease activity nor Rad50-Mre11-p95 complex was found in the remaining Mini S fractions (fractions 1–5 and fractions 14 and 15). B, Mini Q fractions 9–15 were examined for the Rad50-Mre11-p95 complex and for nuclease activity; co-elution of the nuclease activity with the Rad50-Mre11-p95 complex was again seen. Neither nuclease activity nor Rad50-Mre11-p95 complex was seen. The m5, kilobases pairs.

Fig. 3. Nuclease activity coelutes chromatographically with the Rad50-Mre11-p95 complex. A, Mini S fractions 6–13 were subjected to immunoblot analyses to determine their content of the Rad50-Mre11-p95 complex and assayed for nuclease activity using dX ssDNA as substrate. Coelution of the nuclease activity with the Rad50-Mre11-p95 complex was seen. Neither nuclease activity nor Rad50-Mre11-p95 complex was found in the remaining Mini S fractions (fractions 1–5 and fractions 14 and 15). B, Mini Q fractions 9–15 were examined for the Rad50-Mre11-p95 complex and for nuclease activity; co-elution of the nuclease activity with the Rad50-Mre11-p95 complex was again seen. Neither nuclease activity nor Rad50-Mre11-p95 complex was seen. The m5, kilobases pairs.

To examine whether the Rad50-Mre11-p95 nuclease would also act on double-stranded DNA, the nuclease reaction was repeated, substituting the dX ssDNA with the double-stranded supercoiled form. Incision of the dX supercoiled DNA by Rad50-

Fig. 4. Nuclease action generates 3' hydroxyl and 5' phosphate termini. A, DNA digested with Rad50-Mre11-p95 complex (lane 2) and DNA from the control reaction without the nuclease complex (lane 1) were run in an agarose gel and stained with ethidium bromide. B, DNA digested with Rad50-Mre11-p95 (lane 2) and DNA from the control reaction (lane 1) were treated with terminal transferase and [γ-32P]dATP, purified by GeneClean, and then run in an agarose gel. The gel was dried and subjected to autoradiography. C, DNA digested with Rad50-Mre11-p95 (lanes 2 and 4) and DNA from the control reaction (lanes 1 and 3) were treated with polynucleotide kinase and [γ-32P]ATP and then analyzed as described for B. The DNA samples in lanes 3 and 4 had been incubated with alkaline phosphatase (CIP) prior to the labeling reaction with polynucleotide kinase. The products formed as a result of Rad50-Mre11-p95 nuclease activity. The details are described under “Materials and Methods.” kb, kilobases pairs.

Fig. 5. Rad50-Mre11-p95 complex has 3' to 5' exonuclease activity. A, for examining exonuclease activity, 200 ng of the 5' and 3' end-labeled DNA species were incubated with 200 ng of the Rad50-Mre11-p95 complex in 20 μl of reaction buffer. At the times indicated, a 5-μl portion of the reaction mixture was withdrawn and mixed with an equal volume of loading buffer containing 1% SDS. The reaction samples were run in a 0.8% agarose gel, which was stained with ethidium bromide and photographed (upper panel, AUTORAD). In lanes 1 and 5, the end-labeled DNAs were incubated in buffer without Rad50-Mre11-p95. B, the 3' end-labeled DNA (60 ng) was incubated in buffer alone (lane 1) and with 60 ng of Rad50-Mre11-p95 protein complex for 60 min either without metal ion (lane 2), with 2 mM Mn2+ (Mn, lane 3), or with 2 mM Mg2+ (Mg, lane 4) as indicated.
Rad50-Mre11-p95 would generate a nicked circular duplex DNA molecule. As shown in Fig. 2B, the Rad50-Mre11-p95 complex converted some of the supercoiled DNA into the nicked circular form, but the incision of the supercoiled DNA clearly occurred at a much slower rate than the incision of ssDNA (Fig. 2C). For instance, at the reaction end point of 70 min, only about 15% of the supercoiled DNA had been incised, as compared with about 80% incision of the circular ssDNA by 30 min (Fig. 2C).

The addition of SDS at the beginning of the reaction abolished nuclease activity (Fig. 2D). Interestingly, the nuclease function was dependent on manganese, which could not be at all substituted by magnesium (Fig. 2D). In this regard, the Rad50-Mre11-p95 nuclease activity resembles the SbcC-SbcD nuclease complex, which is also specifically dependent on manganese for activity (7).

To confirm that the nuclease activity is intrinsic to the Rad50-Mre11-p95 complex, we subjected fractions from the last step of protein purification in Mini S to immunoblot analysis to determine their content of the Rad50-Mre11-p95 complex, and the same fractions were also used in nuclease assays with 32P ssDNA as substrate. As shown in Fig. 3A, the level of nuclease activity paralleled the amount of Rad50-Mre11-p95 complex in the Mini S fractions. When a Mini Q column was used instead of Mini S as the final purification step, we again observed co-elution of the nuclease activity with the Rad50-Mre11-p95 complex (Fig. 3B).

To determine the nature of the DNA termini generated as a result of Rad50-Mre11-p95 nuclease activity, 32P ssDNA was treated with the Rad50-Mre11-p95 complex, and the nucleolytic product was purified. A portion of the Rad50-Mre11-p95 digested DNA was run in an agarose gel and stained with ethidium bromide to examine the extent of nucleolytic activity (Fig. 4A), and other portions were incubated with terminal transferase and [α-32P]dATP and with polynucleotide kinase and [γ-32P]ATP, with or without prior treatment with alkaline phosphatase. Fig. 4B shows that the product of nucleolytic action was labeled readily by terminal transferase, indicating the presence of a 3' hydroxyl group. Also, labeling of the nucleolytic product by polynucleotide kinase was stimulated markedly by prior phosphatase treatment of the digested DNA, indicating the presence of a 5' phosphate (Fig. 4C). Thus, the Rad50-Mre11-p95 nuclease generates 3' hydroxyl and 5' phosphate termini.

To test whether Rad50-Mre11-p95 nuclease complex would act exonucleolytically, we labeled restriction DNA fragments at the 3' or 5' end with 32P and then treated the labeled DNA species with purified Rad50-Mre11-p95 complex, followed by electrophoresis of the reaction mixtures in an agarose gel. The gel was dried and subjected to autoradiography and PhosphorImager analysis to determine whether there was exonucleolytic digestion of the end-labeled species. As shown in Fig. 5A, we found that incubation of the 3' end-labeled species with the Rad50-Mre11-p95 complex for 10 min resulted in >70% loss of the 32P label, whereas little release of the 32P label was observed with the 5' end-labeled species even after 30 min, indicating that the Rad50-Mre11-p95 complex also acts exonucleolytically, in the 3' to 5' direction. The 3' to 5' exonuclease activity, like the endonuclease function, has a specific requirement for Mn2+ (Fig. 5B).

We have presented evidence that the Rad50-Mre11-p95 complex has Mn2+-dependent endonuclease and 3' to 5' exonuclease activities. The Rad50-Mre11-p95 nuclease generates 3' hydroxyl and 5' phosphate termini, suggesting that when mediating DNA scission in vivo, the nuclease complex creates DNA termini that are suitable for priming DNA synthesis and for ligation. The nuclease activity in this protein complex likely resides in the Mre11 protein, because the S. cerevisiae Mre11 protein alone has been found to possess endonuclease activity.2 In the previous studies (14, 16), two additional protein species with sizes of 200 and 400 kDa were found to be co-immunoprecipitating with the Rad50-Mre11-p95 complex. The 200-kDa species was identified to be fatty acid synthase, whereas the identity of the 400-kDa species remains to be established (16). These two protein species are not present in our purified preparations of the Rad50-Mre11-p95 complex (Fig. 1B).

Cells derived from patients with Nijmegen breakage syndrome, which is characterized by elevated cellular sensitivity to ionizing radiation indicative of a DNA repair defect, fail to assemble Rad50-Mre11 containing nuclear repair foci (16). p95 (Nibrin) may regulate the nuclear localization of the Rad50-Mre11 complex. In addition, it is possible that p95 affects the nuclease activities of the Rad50-Mre11-p95 complex and could also have a role in linking the DNA repair machinery to cell cycle checkpoints (16, 17).

Genetic studies in yeast have implicated the Rad50 and Mre11 proteins in homologous recombination (6), recombination repair (6), repair by nonhomologous DNA end joining (12), and in telomere length maintenance (13). Given the high degree of conservation of these proteins among eukaryotes, it seems reasonable to suggest that, in addition to DNA repair, the human Rad50-Mre11-p95 complex also plays an important role in recombination and telomere length homeostasis. In these chromosomal transactions, Rad50-Mre11-p95 nuclease activities may function alone or in combination with other novel protein factors. Among such novel protein factors, there could be a DNA helicase, which would cooperate with the Rad50-Mre11-p95 endonuclease activity to create a 3' ssDNA tail (3, 4) for the nucleation of Rad51 and other proteins that function in heteroduplex DNA formation (9–11, 18).

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