Yeast Xrs2 Binds DNA and Helps Target Rad50 and Mre11 to DNA Ends*

Received for publication, September 5, 2003, and in revised form, September 24, 2003
Published, JBC Papers in Press, September 30, 2003, DOI 10.1074/jbc.M309877200

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Saccharomyces cerevisiae  Rad50, Mre11, and Xrs2 proteins are involved in homologous recombination, non-homologous end-joining, DNA damage checkpoint signaling, and telomere maintenance. These proteins form a stable complex that has nuclease, DNA binding, and DNA end recognition activities. Of the components of the Rad50-Mre11-Xrs2 complex, Xrs2 is the least characterized. The available evidence is consistent with the idea that Xrs2 recruits other protein factors in reactions that pertain to the biological functions of the Rad50-Mre11-Xrs2 complex. Here we present biochemical evidence that Xrs2 has an associated DNA-binding activity that is specific for DNA structures. We also define the contributions of Xrs2 to the activities of the Rad50-Mre11-Xrs2 complex. Importantly, we demonstrate that Xrs2 is critical for targeting of Rad50 and Mre11 to DNA ends. Thus, Xrs2 likely plays a direct role in the engagement of DNA substrates by the Rad50-Mre11-Xrs2 complex in various biological processes.

Mutations in the Saccharomyces cerevisiae RAD50, MRE11, and XRS2 genes render cells sensitive to DNA-damaging agents and defective in meiotic recombination. Cells lacking these genes also have shortened telomeres and defects in DNA damage checkpoint signaling and in DNA double strand break repair by non-homologous end joining (NHEJ). In addition, these genes are needed for a pathway of long tract gene conversion called break-induced DNA replication or BIR and for the telomerase-independent lengthening of telomere, possibly by a BIR-like mechanism. Yeast two-hybrid and biochemical studies have revealed that the products of these genes form a stable complex, referred to as the RMX complex. The formation of the RMX complex is mediated by simultaneous interactions of Mre11 with Rad50 and Xrs2 (1–3).

The equivalents of the aforementioned protein trio in humans, namely, hRad50, hMre11, and Nbs1, also combine to form a stable complex, called the RMN complex. Again, hMre11 plays a central role in complex assembly by binding hRad50 and Nbs1 (1, 3). Like the RMX complex, the RMN complex functions in DNA damage checkpoint signaling and is needed for mitotic recombination, telomere maintenance, and resistance to DNA-damaging agents (1, 4), whereas the involvement of the human complex in meiotic recombination and NHEJ has not yet been established. Importantly, mutations in hMre11 and Nbs1 lead to the cancer prone syndromes ataxia telangiectasia-like disorder and Nijmegen breakage syndrome (NBS), respectively (1, 5–7). Mouse strains deleted for Rad50 or Nbs1 suffer early embryonic lethality (8, 9) and mice harboring hypomorphic Rad50 and NBS1 alleles are hypersensitive to genotoxic stresses and cancer prone (10–12). These latter findings reveal the involvement of the RMN complex in the maintenance of genome stability and cancer avoidance in mammals, and they aptly underscore the importance of genetic and biochemical studies on this complex and its yeast equivalent.

Rad50 belongs to the structural maintenance of chromosomes (SMC) family and, like other members of this family, contains Walker-type nucleotide binding motifs and a long coiled-coil domain (13, 14). A zinc binding motif, referred to as a zinc hook, is located at the base of the Rad50 coiled-coil and mediates Rad50 dimerization via the chelation of a zinc ion (14). Rad50 binds DNA in an ATP-dependent manner (15). Mre11 has a 3’ to 5’ exonuclease activity and incises certain DNA structures endonucleolytically (1, 16–18). As expected, Mre11 binds both ssDNA and dsDNA (1). The Rad50 dimer combines with two Mre11 molecules to form a stable tetrameric complex (19, 20). As a result of complex formation with Rad50, the nuclease activities of Mre11 are enhanced (16–18). In studies that employed atomic force microscopy (also called scanning force microscopy), a large number of hRad50-hMre11 complexes were seen to tether linear duplex DNA molecules via their ends (13).

In congruence with genetic data implicating the RMX complex in NHEJ, previous studies have found an ability of this complex to promote end-joining reactions mediated by the DnL4-Lf1 complex (the equivalent of mammalian DNA ligase IV-XRCC4 complex) and the Hdf1-Hdf2 complex (the equivalent of mammalian Ku heterodimer) (21). The RMX complex was seen by atomic force microscopy to engage and juxtapose DNA ends to form DNA oligomers and in biochemical experi-
ments to protect DNA ends against lambda exonuclease. In this same study, Xrs2 was shown to physically interact with Lif1. The DNA end-bridging activity of RMX is believed to be important for aligning the substrate molecules to prepare them for joining, whereas the ability of Xrs2 to bind Lif1 likely endows the RMX complex with the ability to recruit the Dnl4-Lif1 complex to DNA ends to perform the joining step (21).

Toward defining the biochemical functions of Xrs2 and its contributions to the activity repertoire of the RMX complex, we have overexpressed it in yeast cells and purified it to near homogeneity. We show that Xrs2 binds DNA and is critical for DNA end binding and juxtaposition by the RMX complex. These results suggest that Xrs2 helps target the RMX complex and associated factors to DNA substrates during various chromosomal transactions.

MATERIALS AND METHODS

Overexpression and Purification of Xrs2—The XRS2 gene was placed under the control of the galactose-inducible GAL-PGK promoter in the vector pPM231 to generate plasmid pX2.1 (2µ, GAL-PGK-XRS2, LEU2), which was introduced into the protease-deficient yeast strain BJS546. Xrs2 protein was induced by growth in medium containing galactose, as described previously (18). All the purification steps were carried out at 0–4 °C. Extract was prepared from 100 g of cell pellet and centrifuged, as described (18). The clarified extract (Fraction I; 200 ml) was treated with ammonium sulfate at 0.21 g/ml. The clarified extract (Fraction II) was applied on a Superose 6 column (250 × 2.5 cm) with 0.5 M NaCl. The peak fractions were pooled (Fraction III; 2 ml) and loaded directly onto a 6-ml Macro-Hydroxyapatite column (Bio-Rad), which was developed with a 120-ml gradient from 15 to 210 mM KCl and loaded directly onto a 6-ml Macro-Hydroxyapatite column (Bio-Rad), which was developed with a 120-ml gradient from 15 to 210 mM KCl. Xrs2 bound to SP-Sepharose was eluted with a 30-ml gradient from 100 to 400 mM KCl in T buffer. The peak fractions (Fraction IV; 3 ml and ~120 ml KCl) were loaded directly onto a 1.0-ml Mono-Q column, which was developed with a 30-ml gradient from 100 to 400 mM KCl in T buffer. The pool of Xrs2 (Fraction VI; 3 ml, ~250 mM KCl and containing 3 mg of nearly homogeneous protein) was concentrated in a Centricon-30 device (Amicon) to ~5.0 mg/ml and stored in small aliquots at –80 °C. The Xrs2 protein concentration was determined by densitometric scanning of a 7.5% SDS-PAGE gel containing multiple loadings of purified Xrs2 protein previously dephosphorylated with shrimp alkaline phosphatase against known amounts of bovine serum albumin run on the same gel. Rad50, Mre11, and Protein Complexes—Rad50 and Mre11 were overexpressed in yeast and purified as described previously (18). The Rad50-Mre11-Xrs2 complex and the Rad50-Mre11 and Mre11-Xrs2 subcomplexes were reconstituted and purified as described (21). The concentration of Rad50 and Mre11 was determined by densitometric scanning of a 7.5% SDS-PAGE gel containing multiple loadings of the two purified proteins against known amounts of bovine serum albumin run on the same gel.

DNA Substrates—The dX174 (+) strand (ss; 18 µm nucleotides; panel I), replicative form I DNA (ac; 12 µm nucleotides; panel II), Stul-linearized replicative form DNA (ds; 12 µm nucleotides; panel III), and a combination of all three DNA species (panel IV) were incubated with Xrs2 (40, 80, 150, 210, 320, 420, 520, and 1040 nM in lanes 2–9) at 23 °C for 15 min and then analyzed by electrophoresis. In lanes 10, the DNA substrates were incubated with the highest amount of Xrs2 at 23 °C for 15 min and then treated with 0.5% SDS and 0.5 mg/ml proteinase K (SDS/PK) for 5 min at 37 °C before gel analysis.

FIG. 1. Expression and purification of Xrs2. A, extracts from yeast cells harboring pX2.1 (lane 2) and the empty expression vector pPM231 (lane 1) were subjected to immunoblot analysis with anti-Xrs2 antibodies. B, purified Xrs2, 1.0 µg in lane 1, was analyzed by 7.5% SDS-PAGE and staining with Coomassie Blue. M, molecular size markers.

FIG. 2. Interactions of Xrs2 with dX DNA species. dX174 viral (+) strand (ss; 18 µm nucleotides; panel I), replicative form I DNA (ac; 12 µm nucleotides; panel II), Stul-linearized replicative form DNA (ds; 12 µm nucleotides; panel III), and a combination of all three DNA species (panel IV) were incubated with Xrs2 (40, 80, 150, 210, 320, 420, 520, and 1040 nM in lanes 2–9) at 23 °C for 15 min and then analyzed by electrophoresis. In lanes 10, the DNA substrates were incubated with the highest amount of Xrs2 at 23 °C for 15 min and then treated with 0.5% SDS and 0.5 mg/ml proteinase K (SDS/PK) for 5 min at 37 °C before gel analysis.
**Exonuclease Protection**—The exonuclease protection assay was carried out as described (21). Briefly, a 400-bp 5’-end-labeled linear DNA fragment was preincubated with individual proteins or protein complexes at 23 °C for 10 min in a total volume of 10 μl and then placed on ice. A 1.5-μl aliquot of the reaction was removed and incubated separately as control. To the remainder of the reaction, 0.1 unit of a exonuclease (Invitrogen) was added, and the incubation continued at 0 °C. At the indicated times, 1.5-μl aliquots were removed and mixed with 0.5 μl of 3% SDS. Small portions of these samples were applied to polyethyleneimine cellulose sheets (J. T. Baker) to separate the released 32P label from the DNA substrate (18). Data quantification was done by phosphorimaging analysis.

**Topological Unwinding Assay**—Individual proteins and protein complexes were incubated with topologically relaxed DNA in 10 μl of buffer G containing 2 mM ATP at 23 °C. After 10 min, 3 units of calf thymus topoisomerase I (Invitrogen) was added, and the incubation was continued for another 10 min at 37 °C. The reactions were deproteinized with 0.5% SDS and 0.5 mg/ml protease K for 5 min at 37 °C. DNA species were resolved by electrophoresis in 1% agarose gels in TAE buffer and visualized by staining with ethidium bromide.

**ATP Hydrolysis**—Individual proteins and protein complexes were incubated at 30 °C in buffer G containing 150 μM [γ-32P]ATP in a volume of 6.5 μl. At the indicated times, a 1-μl portion of the sample was removed and mixed with 0.5 μl of 1% SDS. Reaction mixtures were resolved by thin layer chromatography in polyethyleneimine cellulose sheets, followed by phosphorimaging analysis (22).

**Atomic Force Microscopy**—AFM was performed using a Nanoscope Scanning Probe system (Digital Instruments) in the tapping mode, following the published procedures (21). Briefly, a 400-bp DNA fragment with 5’ cohesive ends (10 nt of each end) was incubated at 0 °C for 10 min with 20 nM each of the RMX, RM, and MX complexes with or without ATP in a volume of 10 μl. The reaction mixtures were diluted and applied to freshly cleaved mica disks, which were washed with water and dried with a stream of air. Images were captured at a scan rate of 1.0–1.5 Hz using Nanosensors Pointprobe silicon cantilevers (type NCL-W; length = 200 μm; resonance frequency between 177 and 191 kHz).

**RESULTS**

**Expression and Purification of Xrs2**—Xrs2 was expressed in yeast cells using the galactose-inducible GAL-PGK promoter and identified by Western blotting. A number of immunoreactive protein species ranging from ~100 to 105 kDa were detected in cell extract (Fig. 1A), which are all close to the predicted size of Xrs2 (96 kDa). A procedure was devised to purify Xrs2 to near homogeneity (Fig. 1B). The cluster of Xrs2 species detected in extract co-purified. The slower migrating forms are phosphorylated species of Xrs2, because the mobility of these forms was reduced to that of the fastest migrating form after treatment with either shrimp alkaline phosphatase or lambda phosphatase (data not shown).

**Xrs2 Binds DNA**—Because neither Xrs2 nor Nbs1 has been...
examined for a DNA-binding activity, we were very interested in determining whether Xrs2 has such an activity. To do this, purified Xrs2 was incubated with 400 nM of Mre11 (M), Mre11-Xrs2 (MX), Rad50-Mre11 (RM), and Rad50-Mre11-Xrs2 (RMX) at 37 °C. Samples were drawn at the indicated times, deproteinized, and run in a polyacrylamide gel. Two endonucleolytic products, designated A and B, and an exonuclease product, AMP, were generated (18). B, the 3’ labeled duplex DNA substrate (7.4 μM nucleotides) was incubated with 25 nM Mre11 (M), Mre11-Xrs2 (MX), Rad50-Mre11 (RM), or Rad50-Mre11-Xrs2 (RMX) at 4 °C. Samples were drawn at the indicated times, deproteinized, and resolved by TLC. The data points from phosphorimaging analysis of the TLC plates were plotted.

**Fig. 5. Effect of Xrs2 on the nuclease activities of Mre11 and Rad50-Mre11.** A, the hairpin substrate (HP2; 120 nM nucleotides) was incubated with 400 nM of Mre11 (M), Mre11-Xrs2 (MX), Rad50-Mre11 (RM), and Rad50-Mre11-Xrs2 (RMX) at 37 °C. Samples were drawn at the indicated times, deproteinized, and run in a polyacrylamide gel. Two endonucleolytic products, designated A and B, and an exonuclease product, AMP, were generated (18). B, the 3’ labeled duplex DNA substrate (7.4 μM nucleotides) was incubated with 25 nM Mre11 (M), Mre11-Xrs2 (MX), Rad50-Mre11 (RM), or Rad50-Mre11-Xrs2 (RMX) at 4 °C. Samples were drawn at the indicated times, deproteinized, and resolved by TLC. The data points from phosphorimaging analysis of the TLC plates were plotted.

Xrs2 DNA-binding Activity Is Structure-specific—Due to its large size, the dsX ssDNA contains substantial secondary structures. It was therefore of interest to study the DNA-binding activity of Xrs2 using short substrates that are devoid of secondary structure or that have defined structures. The first set of experiments involved the use of an 83-mer oligonucleotide (OL2) with no defined secondary structure and a duplex obtained by hybridizing OL2 to its complement. These 32P-labeled DNA substrates were mixed and then incubated with Xrs2, followed by gel electrophoresis and staining as before to detect mobility shift. As shown in Fig. 2 (panel IV), shifting of the ssDNA occurred with the lowest amount of Xrs2 (40 nM; lane 2), followed by supercoiled DNA at a significantly higher Xrs2 concentration (320 nM; lane 6), but the linear duplex was not shifted until the Xrs2 concentration was increased to between 520 and 1040 nM (lane 8 and 9). Thus, the order of preference of Xrs2 for these large DNA species is ssDNA > supercoiled DNA > linear duplex.

Xrs2 Binds DNA and Targets Rad50 and Mre11
Nucleoprotein complexes were resolved by electrophoresis in polyacrylamide gels, followed by phosphorimaging analysis of the dried gels to detect DNA mobility shift. Interestingly, unlike the experiments with the long DNA substrates (Fig. 2), wherein Xrs2 bound ssDNA preferentially, Xrs2 did not show a preference for OL2 (Fig. 3).

To test the idea that Xrs2 recognizes secondary structures in the dX ssDNA molecule, we used oligonucleotides that, when subject to heating and cooling, fold to give a hairpin structure of 22 bp with either a 3’- or 5’-single-stranded overhang of 18 dT residues. These DNA substrates were used without separation from the unannealed form in binding reactions with Xrs2. As expected, the hairpin with tails was the preferred substrate (Fig. 4A). Effects of Xrs2 on the Mre11 Nuclease Activities—Previous results showed that Mre11 has a 3’- to 5’-exonuclease activity and a structure-specific endonuclease activity that can cleave a hairpin, a stem-loop structure, and a 3’-ssDNA overhang that borders a duplex DNA region. Rad50 enhances the endonuclease, but not the exonuclease activity of Mre11 and the RM subcomplex (Fig. 5, A and B). Thus, Xrs2 potentiates the exonuclease activity of Mre11 and both the exonuclease and structure-specific endonuclease activities of the Rad50-Mre11 subcomplex.

Modulation of Rad50 ATPase Activity by Mre11 and Xrs2—Rad50 contains Walker A and B motifs believed to be involved in the binding and hydrolysis of ATP. We tested for the ability of Rad50 to hydrolyze ATP and examined possible effects of Mre11 and Xrs2 on this activity. To keep the Mre11 nuclease function dormant, the experiments were done in buffer that contained magnesium instead of manganese (16, 18). Rad50 by itself exhibited negligible ATPase activity, with 

k_{cat} for ATP hydrolysis is 0.68 min^{-1} (Fig. 6A, panel I). The RM subcomplex also has a DNA-stimulated ATPase activity similar in potency to that of the RMX complex (Fig. 6A, panel II).

ATP-dependent Alteration of DNA Topology by the RMX Complex—We examined the ability of the RMX complex to change the topology of DNA. The experiments were done in buffer that contained magnesium to keep the Mre11 nuclease function dormant (16, 18). The reaction entailed incubating this protein complex with topologically relaxed DNA, ATP, and calf thymus topoisomerase I, followed by its denaturation and analysis by gel electrophoresis to look for a DNA-linking number change. As shown in Fig. 7A, RMX mediated a change in the DNA-linking number, with the degree of change being proportional to the amount of protein complex (lanes 2–4). We

Fig. 6. Effects of Mre11 and Xrs2 on the Rad50 ATPase activity. A, the Rad50-Mre11-Xrs2 (RMX) complex (1.4 μM) was incubated with [γ-32P]ATP (150 μM) at 30 °C without DNA and with dX174 (+) strand (ssDNA; 23 μM nucleotides) or linear duplex (dsDNA; 23 μM bp). Samples were drawn at the indicated times, deproteinized, and then analyzed by TLC. The data points from phosphorimaging analysis of the TLC plates were plotted. B, the Rad50-Mre11 (RM) and RMX complexes (1.2 μM each) were incubated at 30 °C with 150 μM [γ-32P]ATP with ssDNA. Samples were drawn at the indicated times, deproteinized, and then analyzed by TLC. The data points from phosphorimaging analysis of the TLC plates were plotted.
Fig. 7. RMX complex induces DNA topology change. A, the Rad50-Mre11-Xrs2 (RMX) complex (200, 400, and 800 nM in lanes 2–4) was incubated with topologically relaxed αX174 DNA (6 μM nucleotides) and calf thymus topoisomerase I in the presence of ATP at 37 °C. In lane 5, the highest amount of RMX (800 nM) was incubated with the DNA substrate in the absence of topoisomerase, and in lane 1, the DNA substrate was incubated with topoisomerase. After deproteinization treatment, the reaction mixtures were resolved in an agarose gel, and the DNA species was stained with ethidium bromide. Form U denotes negatively supercoiled DNA species generated as a result of RMX binding. At 400 nM of the RMX complex, the Form U product possessed on average of five negative superhelical turns, corresponding to σ = −0.01 (lane 3), which is significantly less than that (σ = −0.05 to −0.06) expected for plasmid molecules isolated from cells. B, the RMX complex, 800 nM, was incubated with relaxed DNA (6.0 μM nucleotides) and topoisomerase in the absence of ATP (lane 3), with ATP (lane 2), ADP (lane 5), ATP-γS (γS; lane 4), AMP-PNP (PnP; lane 6), or AMP-PCP (PCP; lane 7) and analyzed as above. In lane 1, the DNA substrate was incubated with ATP and topoisomerase. C, Rad50, Mre11, Xrs2, Rad50-Mre11 (RM), Mre11-Xrs2 (MX), and RMX, 800 nM each, were incubated with topologically relaxed DNA (6.0 μM nucleotides) and topoisomerase I in the presence of ATP and analyzed (lanes 2–7) as above. In lane 1, the DNA substrate was incubated with topoisomerase.

Xrs2 Is Needed for Protecting DNA Ends from Exonuclease Digestion—Previously, we showed the ability of the RMX complex to engage DNA ends by two different methods: biochemically, by assessing the protection of radiolabeled DNA ends from exonuclease digestion, and physically, by examining the binding of RMX to DNA ends with atomic force microscopy. In this work, we examined a possible contribution of Xrs2 to DNA end engagement by RMX using both the biochemical means (this section) and atomic force microscopy (next section).

For the exonuclease protection experiments, a 400-bp linear DNA fragment was radiolabeled with 32P at the 5′-end and subjected to digestion by λ exonuclease in the presence of RMX, the RM and MX subcomplexes, and individual components of the full complex. To avoid possible complications, the buffer used in this series of experiments contained magnesium to keep the Mre11 nuclease function dormant (16, 18). The 32P-labeled mononucleotide released by λ exonuclease was separated from the undigested substrate by thin layer chromatography and quantified by phosphorimaging analysis. The results showed that only RMX protects the substrate from exonucleolytic attack (Fig. 8). Whereas nearly 75% of the 32P label was removed by λ exonuclease after 15 min of incubation in the absence of another protein and with the individual components of the RMX complex, the RM subcomplex, or the MX subcomplex, less than 10% of the label was lost when RMX was used (Fig. 8). Thus, it appears that Xrs2 is indispensable for the RMX complex to shield DNA ends from λ exonuclease.

Effect of Xrs2 on DNA End Bridging by RMX Complex as Defined by AFM—AFM was employed to further characterize...
Xrs2 Binds DNA and Targets Rad50 and Mre11

FIG. 9. Xrs2 is critical for targeting Rad50 and Mre11 to DNA ends. A, the DNA substrate was incubated with the Rad50-Mre11-Xrs2 (RMX; panel II), Rad50-Mre11 (RM; panel III), and Mre11-Xrs2 (MX; panel IV) complexes at 0 °C for 10 min in the absence of ATP and analyzed by AFM. The DNA substrate was also analyzed alone (panel I). Representative scans are shown. B, scans of spreads from the above series of reactions and from another series of reactions carried out in the presence of ATP were analyzed to calculate the percentage of monomeric, dimeric, and oligomeric forms of the DNA substrate in each case. RMX (−ATP), n = 1459 molecules examined; RMX (+ATP), n = 444 molecules examined; MX (−ATP), n = 1171 molecules examined; RM (−ATP), n = 928 molecules examined; RM (+ATP), n = 385 molecules examined.

the role of Xrs2 in targeting Rad50 and Mre11 to DNA ends. Reaction mixtures containing RMX, RM, or MX and a 400-bp DNA fragment were applied onto mica and examined in the atomic force microscope. The buffer used had magnesium in it, so as to keep the Mre11 nuclease function dormant (16, 18). Many DNA molecules were examined in each spread with representative images shown in Fig. 9A. Quantitative results are summarized in Fig. 9B. The DNA molecules alone were seen as monomers (Fig. 9A, panel I), but, confirming the results from the exonuclease protection experiments (Fig. 8) and our published study (21), incubation with the RMX complex resulted in the majority of the DNA fragments being converted into linear dimers and linear oligomers (Fig. 9A, panel II, and Fig. 9B). With the MX subcomplex, there was a low level (<10%) of DNA dimer formation but no detectable DNA oligomer (Fig. 9A, panel IV, and Fig. 9B). The RM subcomplex showed a somewhat higher capacity for making DNA dimers than the MX complex and a weak ability to promote DNA oligomer formation (Fig. 9A, panel III, and Fig. 9B). Because the above experiments were conducted in the absence of ATP, we next examined the effect of ATP on the interactions of the protein complexes with the substrate. It is clear that ATP does not enable the RM or MX complex to make more DNA dimers and oligomers, although it appears to slightly reduce the ability of the RMX complex to generate DNA oligomers. Overall, the results (Figs. 8 and 9) indicate that only the RMX complex possesses a significant ability to engage DNA ends and bridge DNA fragments, thus revealing a specific role of Xrs2 in the interaction of the RMX complex with DNA ends.

DISCUSSION

Xrs2 Binds DNA Structures and Influences the Activities of the RMX Complex—Rad50 and Mre11 both bind DNA (1). Using gel mobility shift assays, we have presented evidence that Xrs2 has an intrinsic DNA-binding activity also. The results show that Xrs2 recognizes duplex-single strand DNA junctions. It remains to be seen whether Xrs2 also binds other DNA structures. Xrs2 exerts a slight stimulation on the nuclelease activities of Mre11, with the exonuclease activity being enhanced with or without Rad50, whereas elevation of the endonucleolytic activity is realized only in the context of the RMX complex.

Genetic observations strongly suggest that the interaction of Rad50 with ATP is critical for its biological functions (1, 23). The RM and RMX complexes exhibit an ATPase activity that is stimulated by either ssDNA or dsDNA, and incubating the RMX complex with topologically relaxed DNA and calf thymus topoisomerase I results in a DNA linking number change (this work). Pauli and Gellert (17) have shown an ability of the human RMN complex to melt a short duplex. The DNA unwinding activity of the RMN complex is stimulated by ATP but not by non-hydrolyzable analogues of ATP. Given this, it seems reasonable to suggest that the alteration of DNA topology induced by the yeast RMX complex also stems from an ability to separate DNA strands. The omission of ATP or its substitution with non-hydrolyzable analogues reduces the magnitude of the DNA linking number change induced by RMX, suggesting that ATP hydrolysis is needed for the expression of the DNA unwinding activity. It is important to note that even though the RM complex hydrolyzes as much ATP as the RMX complex, topological unwinding of DNA is seen only with the latter, indicating that Xrs2 plays an important role in the DNA unwinding reaction.

Previously, we reported that the RMX complex binds DNA ends and bridges linear dsDNA molecules via their ends (21). Here we have presented biochemical and physical evidence that only the RMX complex is capable of protecting DNA ends from exonuclease digestion and bridging DNA fragments. Interestingly, the DNA end binding and bridging activities of the RMX is independent of ATP. In fact, a reduced number of DNA oligomers were seen upon inclusion of ATP. At present, we do not know whether ATP attenuates the ability of the RMX complex to juxtapose DNA or it accelerates the turnover of the protein complex from DNA ends to result in disassembly of the DNA oligomers, or both. We note that the end-bridging function of the RMX complex is distinct from its endonucleolytic and DNA unwinding activities, in that the latter activities are stimulated, rather than attenuated, by ATP (Ref. 18 and this study).

Functional Implications—RMX enhances the end-joining efficiency of Dnl4/Lif1, and it does so by juxtaposing linear DNA fragments via their ends and recruiting Dnl4/Lif1 through an interaction between Xrs2 and Lif1 (21). Here we have demonstrated that Xrs2 is in fact also critical for targeting Rad50 and Mre11 to DNA ends and for the DNA end-bridging activity of the RMX complex. It seems reasonable to consider the possibility that the DNA-binding activity of Xrs2 contributes to the specific interactions of the RMX complex with DNA ends, perhaps by conferring end-binding specificity to this complex and stabilizing the nucleoprotein complex that contains RMX and DNA ends.
RNX is needed for BIR. During BIR, a ssDNA tail invades a homologous sequence to form a primer template junction for initiating DNA synthesis, which continues until reaching the end of the donor chromosome. BIR also requires Rad52, Rad59, and Rdh54/Tid1 (24, 25). It seems reasonable to suggest that RMX co-operates with these other factors to make the DNA joint needed for priming DNA synthesis. In this regard, the ability of RMX (and its human equivalent RMN) to melt duplex DNA may be germane for DNA joint formation and DNA synthesis in BIR.

RMX is indispensable for the formation of meiotic DNA double strand breaks catalyzed by Spo11, a topoisomerase II-like protein (26). The precise role of RMX in this reaction is not yet defined, but it is possible that it helps target the Spo11 complex to the sites of meiotic break formation and activates the topoisomerase function of Spo11. The DNA-binding activity of Xrs2 and possible interactions of Xrs2 with Spo11 or its associated factors may be critical for establishing a nucleoprotein structure conducive for the cleavage reaction. Xrs2 can likewise contribute to the DNA damage checkpoint functions of the RMX complex by interacting with the checkpoint kinases or other components of the checkpoint machineries and by targeting RMX and associated checkpoint proteins to the damage sites. In fact, a recent study has found physical interaction between Xrs2 and Tel1, the *Saccharomyces cerevisiae* equivalent of ATM kinase in mammals, and Xrs2-dependent targeting of Tel1 to a DNA double strand break (27).

Even though Nbs1 has only limited homology to Xrs2, it is clearly the functional equivalent of the latter. Nbs1 is indispensable for the functional integrity of the intra-S DNA damage checkpoint by virtue of its ability to bind ATM and serve as a substrate for the ATM kinase activity (1). In addition, Nbs1 is important for the expression of the full activity repertoire of the RMN complex (1, 17). In light of our results with Xrs2, it will be of considerable interest to test for a DNA-binding function in Nbs1.

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*J. Biol. Chem. 2003, 278:48957-48964.*
*doi: 10.1074/jbc.M309877200 originally published online September 30, 2003*

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