Role of ATP Hydrolysis in the Antirecombinase Function of Saccharomyces cerevisiae Srs2 Protein*

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Mutants of the Saccharomyces cerevisiae SRS2 gene are hyperrecombinogenic and sensitive to genotoxic agents, and they exhibit a synthetic lethality with mutations that compromise DNA repair or other chromosomal processes. In addition, srs2 mutants fail to adapt or recover from DNA damage checkpoint-imposed G2/M arrest. These phenotypic consequences of ablating SRS2 function are effectively overcome by deleting genes of the RAD52 epistasis group that promote homologous recombination, implicating an untimely recombination as the underlying cause of the srs2 mutant phenotypes. The SRS2-encoded protein has a single-stranded (ss) DNA-dependent ATPase activity, a DNA helicase activity, and an ability to disassemble the Rad51-ssDNA nucleoprotein filament, which is the key catalytic intermediate in Rad51-mediated recombination reactions. To address the role of ATP hydrolysis in Srs2 protein function, we have constructed two mutant variants that are altered in the Walker type A sequence involved in the binding and hydrolysis of ATP. The srs2 K41A and srs2 K41R mutant proteins are both devoid of ATPase and helicase activities and the ability to displace Rad51 from ssDNA. Accordingly, yeast strains harboring these srs2 mutations are hyperrecombinogenic and sensitive to methylmethane sulfonate, and they become inviable upon introducing either the sgs1 or rad54A mutation. These results highlight the importance of the ATP hydrolysis-fueled DNA motor activity in SRS2 functions.

DNA helicases perform important functions in various chromosomal transactions, including replication, repair, recombination, and transcription (1, 2). These proteins utilize the chemical energy from the hydrolysis of a nucleoside triphosphate to dissociate DNA structures and nucleoprotein complexes. Interestingly, mutations in several DNA helicases are involved in the pathogenesis of human diseases. For instance, mutations in the XPD and XPD helicases, which constitute subunits of the transcription factor TFIIH that has a dual role in nucleotide excision repair, lead to the cancer-prone syndrome xeroderma pigmentosum (3). Furthermore, mutations in the BLM, WRN, and RecQ4 proteins, members of the RecQ helicase family, cause the cancer-prone Bloom, Werner, and Rothmund-Thomson syndromes, respectively (4, 5).

We are interested in the biology of various DNA helicases that influence homologous recombination and DNA repair processes. One such helicase is encoded by the Saccharomyces cerevisiae SRS2 gene, altered forms of which were first described as either suppressors of the DNA damage sensitivity of rad6 and rad18 mutants (6) or as hyperrecombination mutants (7). Detailed genetic analyses have shown that a major function of SRS2 is to attenuate homologous recombination activity to allow for the channeling of certain DNA lesions into the RAD6/RAD18-mediated postreplication repair pathway (8, 9). Accordingly, srs2 mutants are sensitive to DNA damaging agents and show a hyperrecombination phenotype. Genetic deletion of the RAD51 or RAD52, key members of the RAD52 epistasis group functioning in homologous recombination, alleviates the DNA damage sensitivity and hyperrecombination phenotypes of srs2 mutants (8), implicating untimely recombination events as the progenitor of these srs2 phenotypes. Srs2 mutations are lethal when combined with mutations in a variety of genes needed for DNA repair and other chromosomal processes, e.g. with mutations in the DNA repair and recombination gene RAD54 and also with mutations in SGS1, which codes for the sole RecQ helicase in S. cerevisiae (10, 11). The synthetic lethality encountered in the srs2 sgs1 and srs2 rad54 double mutants is suppressed by inactivating key recombination genes (11). The available genetic evidence therefore implicates Srs2 protein in the attenuation of recombination events that produce toxic DNA structures or nucleoprotein intermediates (12, 13).

In congruence with the genetic data, biochemical assays have shown that the Srs2 protein strongly suppresses the recombination activity of Rad51. Interestingly, although Srs2 has the ability to unwind DNA (14, 15) and had been predicted to dissociate DNA intermediates in recombination reactions, its antirecombinase function can be attributed to an ability to disassemble the Rad51-single-stranded DNA (ssDNA)/nucleoprotein filament (14, 15), the key catalytic intermediate in recombination reactions (16). Likewise, the failure of srs2 mu-

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1 The abbreviations used are: ssDNA, single-stranded DNA; MMS, methylmethane sulfonate; BSA, bovine serum albumin; dsDNA, double-stranded DNA; RPA, replicative protein A.
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As summarized above, Srs2 appears to have a multifunctional role in nuclear processes including an ability to unwind DNA and disassemble the Rad51-ssDNA nucleoprotein filament. Here, we address the role of ATP in Srs2 protein functions by mutating the highly conserved lysine residue in the Walker type A motif expected to be involved in ATP binding and hydrolysis. We show that the resulting srs2 K41A and srs2 K41R mutant proteins are devoid of ATPase and helicase activities and are unable to dislodge Rad51 from DNA. Genetic analyses reveal that the srs2 K41A and srs2 K41R mutations cause hyperrecombination, sensitivity to MMS, and synthetic lethality with the sgs1Δ or rad54Δ mutation. Our results thus reveal a requirement for the Srs2 DNA motor activity in recombination attenuation. However, the srs2 K41A and srs2 K41R mutants are less sensitive to MMS than srs2 null cells and exhibit a more pronounced hyperrecombinational phenotype than the latter. It therefore seems possible that Srs2 has additional functions that are not strictly linked to ATP hydrolysis and Rad51 removal from DNA.

MATERIALS AND METHODS

Yeast Media and Strains—Yeast extract-peptone-dextrose (YPD) medium, synthetic complete (SC) medium, and synthetic complete media without leucine (SC-Leu), without uracil (SC-Ura), and without both leucine and uracil (SC-Leu-Ura) were prepared as described (21). Media containing 5-fluoro-orotic acid were prepared as described (21). Except where noted, all strains are RAD5 derivatives of W303 (22). The re-
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FIG. 4. Interaction of mutant srs2 proteins with Rad51. Srs2, srs2 K41A (K/A), and srs2 K41R (K/R) proteins were mixed with Affi-Rad51 beads (A) and Affi-BSA beads (B). The starting material (I), supernatant that contained unbound Srs2, srs2 K41A, or srs2 K41R (S), and the SDS eluate (E) were resolved by SDS-PAGE in a 10% gel and then stained with Coomassie Blue.

Plasmid Construction—To generate the SRS2::URA3::leu2-k and his3-513::TRP1::his3-537 recombination reporters (7).

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FIG. 5. Mutant srs2 proteins fail to attenuate Rad51/Rad54/ RPA-mediated D-loop reaction. A, D-loop reaction scheme. The radiolabeled 90-mer oligonucleotide D1 is paired with homologous plasmid form I DNA to yield a D-loop. B, in lanes 2–9, radiolabeled D1 was incubated with Rad51, Rad54, RPA, and with or without Srs2 (40, 50, and 80 nM in lanes 3–5), srs2 K41A (K/A, 60 and 90 nM in lanes 6 and 7), or srs2 K41R (K/R, 60 and 90 nM in lanes 8 and 9) and then pBluescript form I DNA was incorporated. The reaction (lane 2) without Srs2 or mutant srs2 is designated as Std. Lane 1 contained the DNA substrates but no protein (Bf). The reaction mixtures were incubated for 4 min, deproteinized, and then subject to electrophoresis in a 1% agarose gel. The gel was dried and analyzed in the PhosphorImager. C, the data points from phosphorimaging analysis of the gel in B are plotted.

maining strains were derived from HKY344-27C and carry leu2-112::URA3::leu2-k and his3-513::TRP1::his3-537 recombination reporters (7).

Rad51 molecules displaced by Srs2 can be trapped on immobilized DNA duplex attached to streptavidin magnetic beads. Rad51 associated with the magnetic bead-bound duplex is eluted by SDS followed by SDS-PAGE analysis. B, pre-assembled Rad51-ssDNA nucleoprotein filaments (lanes 2–8) were incubated with Srs2 (WT, 60 and 90 nM in lanes 3 and 4), srs2 K41A (K/A, 90 and 180 nM in lanes 5 and 6), or srs2 K41R (K/R, 90 and 180 nM in lanes 7 and 8), and the reaction mixtures with streptavidin magnetic beads containing biotinylated dsDNA (lanes 3–8). In lane 1, free Rad51 was mixed with streptavidin magnetic beads that contained biotinylated dsDNA, and in lane 2, free Rad51 was mixed with streptavidin magnetic beads that did not contain any dsDNA. The supernatant (Super, panel I) and bead-bound (Beads, panel II) fractions were subjected to SDS-PAGE in a 7.5% gel and stained with Coomassie Blue. CK denotes creatine kinase in the buffer.
Dependence of Srs2 Functions on ATP Hydrolysis

**Table I**

<table>
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<th>Genotype</th>
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<th>His + Trp + rate</th>
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**Table II**

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<tr>
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site that is not present in the wild-type sequence. PCR products were tested for the Sty1 site by restriction digestion and agarose gel electrophoresis.

**Srs2 Expression and Purification—**srs2 K41A and srs2 K41R proteins were overexpressed in *E. coli* cells and purified to near homogeneity as described previously for wild-type Srs2 (14, 23). Rad51 was overexpressed in yeast and purified to near homogeneity as described (16). The concentration of the wild-type Srs2 and mutant srs2 proteins was determined by densitometric scanning of SDS-polyacrylamide gels containing multiple loadings of purified proteins against known quantities of bovine serum albumin. The concentrations of Rad51 and RPA were determined using extinction coefficients of 1.29 x 10^4 and 8.8 x 10^4 at 280 nm, respectively (24).

**DNA Substrates—**The H2 and D1 oligonucleotides used in the construction of the helicase substrate have been described (23). Oligo used in the DNA binding experiments has also been described (25). The oligonucleotides were purified from 12% polyacrylamide gels and 5’-end labeled with [γ-32P] ATP using T4 polynucleotide kinase. The unincorporated nucleotide was removed from the oligonucleotides using Spin30 columns (Bio-Rad). The DNA helicase substrate was obtained by heating equimolar amounts of H2 and radiolabeled D1 oligonucleotides to 95 °C for 10 min in buffer B (50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 100 mM KCl, 2 mM dithiothreitol, containing an ATP-regenerating system consisting of 20 mM creatine phosphate, 20 μg/ml creatine kinase) and had a final volume of 12.5 μl. The radiolabeled oligonucleotide D1 (3 μM nucleotides) was incubated with Rad51 (1 μM) for 5 min at 37 °C to assemble Rad51-ssDNA nucleoprotein filaments, followed by the incorporation of Rad54 (150 nM) and RPA (200 nM) and a 2 min incubation at 25 °C. The D-loop reaction was initiated by the addition of pllucose replicative form I DNA (50 μM base pairs). The reaction mixtures were incubated at 30 °C for 4 min, deproteinized by treatment with Sds (0.5%) and proteinase K (0.5 mg/ml) at 37 °C for 10 min, and then run in a 1% agarose gel in TAE buffer. The gel was dried and subject to phosphorimaging analysis. The percentage D-loop refers to the quantity of the replicative form I substrate that had been converted into D-loop. When present, Srs2 (40, 50, and 80 nM), srs2 K41A (60 and 90 nM), and srs2 K41R (60 and 90 nM) were added to the pre-assembled Rad51-ssDNA nucleoprotein filaments, followed by a 4 min incubation at 37 °C before Rad54 and RPA were incorporated.

**ATPase Assay—**Srs2 (35 nM) was incubated with viral ssDNA (25 μM nucleotides) in 10 μl of buffer A (30 mM Tris-HCl, pH 7.2, 2.5 mM MgCl2, 1 mM dithiothreitol, 150 mM KCl, and 100 μg/ml BSA) and 1 μl [γ-32P] ATP for the indicated times at 37 °C. The released phosphate was separated from unhydrolyzed ATP by thin layer chromatography, as described (26). The levels of hydrolysis were determined by phosphorimaging analysis of the thin layer chromatography plates in a Personal Molecular Imager FX (Bio-Rad).

**DNA Mobility Shift—**Varying amounts of Srs2, srs2 K41A or srs2 K41R (0–80 nM) was incubated with 32P-labeled oligo-1 (1.36 μM nucleotides) at 37 °C in 10 μl of buffer D (40 mM Tris-HCl, pH 7.8, 50 mM KCl, 1 mM dithiothreitol, and 100 μg/ml BSA) for 10 min. After the addition of gel loading buffer (50% glycerol, 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 0.05% orange G), the reaction mixtures were resolved by electrophoresis in a 12% non-denaturing polyacrylamide gel run in TAE buffer (40 mM Tris-HCl, pH 7.4, 0.5 mM EDTA) at 4 °C. The gel was dried onto Whatman DE81 paper and analyzed in the PhosphorImager.

**D-loop Reaction—**The reactions were carried out in Buffer R (35 mM Tris-HCl, pH 7.4, 2.0 mM ATP, 2.5 mM MgCl2, 50 mM KCl, 1 mM dithiothreitol, containing an ATP-regenerating system consisting of 20 mM creatine phosphate, 20 μg/ml creatine kinase) and had a final volume of 12.5 μl. The radiolabeled oligonucleotide D1 (3 μM nucleotides) was incubated with Rad51 (1 μM) for 5 min at 37 °C to assemble Rad51-ssDNA nucleoprotein filaments, followed by the incorporation of Rad54 (150 nM) and RPA (200 nM) and a 2 min incubation at 25 °C. The D-loop reaction was initiated by the addition of plllucose replicative form I DNA (50 μM base pairs). The reaction mixtures were incubated at 30 °C for 4 min, deproteinized by treatment with Sds (0.5%) and proteinase K (0.5 mg/ml) at 37 °C for 10 min, and then run in a 1% agarose gel in TAE buffer. The gel was dried and subject to phosphorimaging analysis. The percentage D-loop refers to the quantity of the replicative form I substrate that had been converted into D-loop. When present, Srs2 (40, 50, and 80 nM), srs2 K41A (60 and 90 nM), and srs2 K41R (60 and 90 nM) were added to the pre-assembled Rad51-ssDNA nucleoprotein filaments, followed by a 4 min incubation at 37 °C before Rad54 and RPA were incorporated.

**Transfer of Rad51 to Bead-bound Biotinylated dsDNA—**M13mp18 circular (+) strand (7.2 μM nucleotides) was incubated for 5 min with Rad51 (2.4 μM) at 37 °C, followed by the addition of Srs2 (60 and 90 nM), srs2 K41A (90 and 180 nM), or srs2 K41R (90 and 180 nM) in a final volume of 20 μl of buffer R containing 0.01% igel. After 3 min at 37 °C, 4 μl of magnetic beads containing dsDNA (14) were added to the reaction mixture, followed by constant mixing for 5 min at 25 °C. The beads were captured with the Magnetic Particle Separator (Roche Molecular Biochemicals), washed twice with the same buffer, and the bound Rad51 was eluted with 20 μl of 1% Sds. The supernatant, which contained unbound Rad51, and the Sds eluate (10 μl of each) were analyzed by SDS-PAGE.

**Electron Microscopy—**The reactions were carried out in buffer R and had a final volume of 12.5 μl. To assemble the Rad51 presynaptic filament, M13mp18 (+) strand (7.2 μM nucleotides) and Rad51 (2.4 μM) were incubated at 37 °C for 5 min, followed by the addition of Rpa (300 nM) and a 3 min incubation. To test the effects of Srs2 and mutant srs2 proteins, 60 nM of these proteins were incubated with the pre-assembled Rad51-ssDNA nucleoprotein filaments at 37 °C for 3 min. For electron microscopy, 2.5 μl of each reaction mixture was applied to copper grids coated with thin carbon film, after glow-discharging the grids for 2 min. The grids were washed twice with buffer R and stained with 30% with 0.75% uranyl formate. After air-drying, the grids were examined with a Philips Tecnai 12 electron microscope under low-dose conditions. Images were recorded with a charge-coupled device camera (Gatan).
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**RESULTS**

**srs2 Variants Mutated for the Walker Type A ATP Binding Motif**—Srs2 contains canonical Walker-type ATP binding motifs. For addressing the role of ATP binding and hydrolysis in Srs2 functions, we have substituted the highly conserved lysine residue (lysine 41) in the Walker type A motif with either alanine or arginine using site-directed mutagenesis (Fig. 1A). The srs2 K41A and srs2 K41R mutant genes were sequenced to ensure that no unintended change had been introduced during the mutagenesis procedure. To express the srs2 K41A and srs2 K41R proteins, the mutant genes were placed under the isopropyl-1-thio-β-D-galactopyranoside-inducible T7 promoter in the *E. coli* expression vector pET11c, which we previously used for the expression and purification of wild-type Srs2 (14). Expression of the srs2 K41A and srs2 K41R mutant proteins in *E. coli* was verified by SDS-PAGE analysis of cell extracts (Fig. 1B) and by immunoblot analysis of these extracts with affinity-purified anti-Srs2 polyclonal antibodies (14). The srs2 K41A and srs2 K41R mutant proteins were purified to near homogeneity (Fig. 1C) using the chromatographic procedure that we have developed for wild-type Srs2 (14).

**Biochemical Properties of srs2 K41A and srs2 K41R Mutant Proteins**—Based on studies with the equivalent Walker mutants in other DNA-dependent ATPases (28, 29), the srs2 K41A and srs2 K41R mutant proteins were expected to be defective in ATP hydrolysis. This expectation was confirmed by examining the ATPase activity of purified proteins with [α-32P] ATP and thin layer chromatography (14, 23). We showed previously that ATP hydrolysis by Srs2 occurs only in the presence of DNA with ssDNA being much more effective than dsDNA in this regard (9, 23). As summarized in Fig. 2A, although robust ATPase activity was observed with wild-type Srs2 in the presence of ssDNA (14, 23), the two srs2 mutant proteins showed less than 1% of the wild-type level of ATP hydrolysis. Likewise, no significant ATP hydrolysis by either of the srs2 mutants was seen when the ssDNA was omitted or substituted with dsDNA (data not shown). We next examined the two srs2 mutant proteins for DNA helicase activity using a [32P]-labeled substrate that contained a 40-bp duplex region adjacent to a 40-nucleotide 3′-ssDNA overhang (Fig. 2B and Ref. 23). As shown in Fig. 2B, although wild-type Srs2 at 40 nM unwound greater than 70% of the substrate after 5 min of incubation, neither of the srs2 mutants, even at the increased concentration of 80 nM, showed a significant helicase activity under the same conditions (Fig. 2B) or even after a prolonged incubation (data not shown).

Even though the results presented in Fig. 2 were consistent with the premise that the K41A and K41R mutations abolish the ATPase activity of Srs2, there existed the possible caveat that this defect had originated from a loss of DNA binding by the mutant proteins. For this reason, we compared the DNA binding ability of the two Walker mutants to that of the wild-type protein by a DNA mobility shift assay. To do this, increasing amounts of wild-type Srs2 and the two mutant proteins were incubated with a [32P]-labeled 83-mer oligonucleotide, followed by resolution of the reaction mixtures in non-denaturing polyacrylamide gels and phosphorimaging analysis of the dried gels to detect and quantify the DNA mobility shift. As shown in Fig. 3, both srs2 mutant proteins were just as proficient as wild-type Srs2 in DNA binding. Consistent with this result, using the same DNA substrate, we found that DNA binding by wild-type Srs2 and the two srs2 mutant proteins is not influenced by ATP (data not shown).

**Attenuation of Rad51-mediated Homologous DNA Pairing and Strand Exchange by Srs2 Requires ATP Hydrolysis**—Recently, we (14) and Fabre and co-workers (15) demonstrated that Srs2 is highly adept at attenuating Rad51-mediated homologous DNA pairing and strand exchange, the biochemical reaction that serves to link recombining chromosomes (30). In addition, a physical interaction between Rad51 and Srs2 was demonstrated by us (14). Before examining the srs2 K41A and srs2 K41R mutant proteins for their ability to suppress the Rad51 recombinase activity, we first verified that the srs2 mutant proteins retain the ability to interact with Rad51. For this, purified Srs2, srs2 K41A, and srs2 K41R proteins were each mixed with Affi-Gel beads that contained covalently conjugated Rad51 protein (Affi-Rad51) or Affi-Gel beads that contained conjugated bovine serum albumin (Affi-BSA). After being washed with buffer, the Affi-Rad51 and Affi-BSA beads were treated with SDS to elute bound Srs2 and srs2 mutant proteins. As shown in Fig. 4A, the two srs2 mutant proteins interacted with Rad51 just as avidly as wild-type Srs2 did. As expected, neither the wild-type Srs2 protein nor either of the srs2 mutants was retained on the Affi-BSA control beads (Fig. 4B).

![Fig. 2](image-url)
We employed the D-loop assay (Fig. 5A) for testing the proficiency of the srs2 K41A and srs2 K41R mutant proteins in recombination attenuation. As reported in our published work, the addition of a catalytic quantity of Srs2 (40–60 nM) to the D-loop reaction containing Rad51 (1 μM), Rad54 (150 nM), and RPA (200 nM) caused pronounced inhibition (Fig. 5, B and C). For instance, at 50 nM Srs2, the level of D-loop was suppressed by greater than 10-fold (Fig. 5, B and C). Importantly, as much as 90 nM of srs2 K41A and srs2 K41R did not exert any inhibitory effect on the D-loop reaction (Fig. 5, B and C), thus revealing a requirement for the Srs2 ATPase activity in the attenuation of the Rad51 recombinase activity. We independently verified this conclusion by using a homologous DNA pairing and strand exchange system that employs dX174 (+) strand DNA and linear duplex as substrates (Refs. 14 and 16, data not shown).

Disassembly of Rad51-ssDNA Nucleoprotein Filament by Srs2 Requires ATP Hydrolysis—The results above have verified that the ATPase activity of Srs2 protein is indispensable for attenuating Rad51-mediated recombination reactions in vitro. We have devised previously a bead-based biochemical assay to monitor the Srs2-mediated dissociation of Rad51 from ssDNA. Briefly, Rad51 molecules displaced by Srs2 from the presynaptic filament are trapped on a biotinylated duplex DNA fragment tethered to streptavidin-conjugated magnetic beads, followed by elution of Rad51 from the beads and SDS-PAGE analysis (Fig. 6A, and Ref. 14). As reported previously, incubation of Rad51 nucleoprotein filaments with Srs2 protein (60–90 nM) resulted in the release of Rad51 from the filaments as indicated by Rad51 being trapped on the streptavidin-magnetic beads that contained duplex DNA (Fig. 6B). Importantly, neither of the srs2 mutants, even in an amount twice that of Srs2 (180 nM), was capable of releasing Rad51 protein from the nucleoprotein filaments (Fig. 6B).

We also used electron microscopy to examine the ability of the two srs2 mutant proteins to catalyze the disassembly of the presynaptic filament, following the guidelines described in Krejci et al. (14). Briefly, the Rad51 presynaptic filament, which is extended and has a striated appearance (Fig. 7A), was incubated with Srs2 or the srs2 mutant proteins in the presence of RPA, and the dissociation of the Rad51 filament was gauged by the disappearance of the filament and the concomitant appearance of RPA-ssDNA nucleoprotein complexes, which appear as compact structures with bulges of bound protein molecules (14) (Fig. 7B). As expected, incubation of the Rad51 presynaptic filament with Srs2 led to its replacement by the RPA-ssDNA complex (Fig. 7C). However, the Rad51 filament was completely stable in the presence of either srs2 K41R (Fig. 7D) or srs2 K41A (Fig. 7E). Taken together, the results from the biochemical and electron microscopy analyses (Figs. 6 and 7) clearly indicated that disassembly of the Rad51 presynaptic filament by Srs2 requires the ATPase activity of the latter.

**Genetic Characterization of the srs2 K41A and srs2 K41R Mutant Alleles**—Previous studies (8, 9) of SRS2 have highlighted the role of SRS2 in attenuating Rad51-mediated recombination. We determined the effect of loss of Srs2 ATP hydrolysis on mitotic gene conversion, using two reporters that measure intra-chromosomal gene conversion between heteroalleles. As shown in Table I, both srs2 K41A and srs2 K41R elevated the gene conversion rates with both reporters. Interestingly, the hyperrecombination phenotype of two srs2 mutants was even more pronounced than that of the srs2 deletion mutant. Thus, the results show clearly that the antirecombination function of Srs2 requires the ATPase activity of this protein. Although the rate of gene conversion is increased in the srs2 mutants, the spontaneous mutation rate measured for forward mutations at CAN1 remains unchanged (Table II).

Mutants of SRS2 are sensitive to MMS and other DNA damaging agents (8), and MMS sensitivity was actually used in our study to select transplacement segregants harboring the two srs2 Walker mutant alleles (see “Materials and Methods”). The MMS sensitivity of the srs2 K41A and srs2 K41R strains is shown in Fig. 8. Both srs2 mutants were sensitive in this assay, but the srs2Δ strain was significantly more sensitive than either of the two point mutants.

The srs2Δ mutation by itself is not lethal, but srs2Δ cells become inviable when SGS1 or RAD54 is also ablated (9, 11, 12). To determine the role of the Srs2 ATPase activity in these genetic interactions, we combined the srs2 K41A and srs2 K41R mutations with sgs1Δ or rad54Δ. The srs2 K41A sgs1Δ and srs2 K41R sgs1Δ double mutants are inviable or grow extremely
poorly (Fig. 9A), whereas the srs2 K41A rad54Δ and srs2 K41R rad54Δ double mutants are inviable (Fig. 9B). We checked the possible suppression of the srs2 rad54Δ lethality by rad51Δ, rad9Δ, or rad17Δ (8) by constructing the respective triple mutants. The lethality of srs2 rad54Δ is fully overcome by rad51Δ (Fig. 9B) and partially suppressed by rad17Δ (Fig. 9C), but rad9Δ was ineffective in this regard (Fig. 9D).

DISCUSSION

To assess the role of ATP hydrolysis in Srs2 protein functions, we have constructed variants of this protein that harbor mutations in the Walker type A motif involved in ATP binding and hydrolysis. We have overexpressed the srs2 K41A and srs2 K41R proteins in E. coli and purified them to near homogeneity. Our biochemical analyses show that both of these mutant proteins retain DNA binding activity and the ability to interact with Rad51, but they are defective in ATP hydrolysis and lack DNA helicase activity. Both srs2 mutant proteins are unable to dissociate the Rad51 presynaptic filament and, accordingly, do not exert any inhibitory effect on Rad51-mediated homologous DNA pairing and strand exchange. The biochemical studies reported here thus establish the requirement for ATP hydrolysis in Srs2-mediated DNA unwinding and disassembly of the Rad51 presynaptic filament.

The results from our genetic studies provide support for the premise that ATP hydrolysis by Srs2 is needed to prevent untimely recombination, as the srs2 K41A and srs2 K41R mutants both exhibit a hyperrecombinogenic phenotype. Interestingly, the degree of hyperrecombination (measured as intrachromosomal gene conversion between heteroalleles) is even more pronounced in the srs2 point mutants than in the srs2Δ strain. In this regard, the two srs2 Walker mutants resemble the srs2–101 mutant described previously, which harbors the amino acid change of P39L, that is also significantly more hyperrecombinogenic than the srs2Δ mutant (9). These observations (Ref. 9 and this study) suggest that when Srs2 is absent, other non-recombinational pathways are used to repair spontaneous DNA damage, but when Srs2 protein is present but defective, it can interfere with these DNA repair pathways.

Alternatively, or in addition, the srs2 Walker mutants and the srs2–101 mutant could induce DNA damage that is channeled into the homologous recombination pathway for repair, convert non-recombinogenic DNA lesions into recombinogenic ones, or enhance the activities of homologous recombination proteins. Because Srs2 is also important for the maximal activation of the S phase DNA damage checkpoint (18), it remains possible that the two srs2 Walker mutants exert a positive influence on homologous recombination efficiency through its effect on checkpoint pathways (18). Further studies are needed to distinguish among these possibilities. That the srs2 K41A and srs2 K41R mutants do not behave exactly like the srs2Δ mutant is further attested by the observation that they are less sensitive to MMS than the latter. Just as in the case of srs2Δ (8, 9), we observed synthetic lethality of both srs2 Walker mutants with either rad54Δ or sgs1Δ. We have also shown that the lethality of srs2 K41A rad54Δ and srs2 K41R rad54Δ can be overcome by deleting RAD51 or RAD17.

Recently, reports from Haber and co-workers (20) and Kupiec and co-workers (19) showed that Srs2 is also needed for the repair of a site-specific double-strand break by synthesis-dependent single-strand annealing or double-ended synthesis-dependent single-strand annealing, the major pathway of gene conversion in mitotic yeast cells. In one of these published studies, the srs2 K41A allele was found to be defective in synthesis-dependent single-strand annealing (20). Likewise, the function of Srs2 in promoting adaptation or recovery from DNA damage checkpoint-mediated G2/M arrest is reliant on its ATPase activity, as the srs2 K41A mutant is defective in this regard (17). We similarly expect the srs2 K41R mutant to be impaired in synthesis-dependent single-strand annealing and recovery/adaptation from DNA damage checkpoint-mediated G2/M arrest. However, as alluded to above, whether or not the two srs2 Walker mutants retain S phase checkpoint function (18) will have to be determined experimentally.

We have demonstrated a physical interaction between Srs2 and Rad51 by the yeast two-hybrid system and also by biochemical means with purified proteins (14). We have suggested that the physical interaction between Rad51 and Srs2 may be germane for targeting the latter to chromosomal sites, e.g. ssDNA gaps created at stalled DNA replication forks, where Rad51 molecules are bound. However, it remains possible that the physical interaction noted (14) enables Srs2 to specifically displace Rad51 from ssDNA. The isolation of Rad51 and Srs2 mutants defective in complex formation will be necessary to address this issue.

REFERENCES