Yeast Rad51 Recombinase Mediates Polar DNA Strand Exchange in the Absence of ATP Hydrolysis*

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Saccharomyces cerevisiae RAD51 gene is required for genetic recombination and recombinational repair of DNA strand breaks. Rad51 protein has a DNA-dependent ATPase activity, and it catalyzes ATP-dependent pairing and strand exchange between homologous DNA molecules. We show here that the rad51 Arg-191 protein, which is devoid of ATPase activity, mediates the pairing and strand exchange reaction upon binding ATP. In addition, the wild type Rad51 protein can catalyze pairing and strand exchange in the presence of the nonhydrolyzable ATP analogues adenyl-imidodiphosphate and adenosine 5’-O-thiotriphosphate. Thus, homologous pairing and the unidirectional transfer of greater than 5 kilobases of DNA can occur efficiently without the need for nucleotide hydrolysis. Consistent with the results from the biochemical analyses, expression of the rad51 Arg-191 protein in a rad51 null mutant confers normal cellular resistance to the DNA damaging agent methylmethanesulfonate, suggesting that nucleotide binding by Rad51 is sufficient for biological function.

Saccharomyces cerevisiae RAD51 gene is a member of the RAD52 epistasis group that is required for meiotic and mitotic recombination and for the recombinational repair of DNA double-stranded breaks (reviewed in Ref. 1). The RAD51 structure and function have been conserved to a remarkable degree among eukaryotes including humans. The RAD51 encoded product and its counterparts from other eukaryotes are structurally and functionally related to the Escherichia coli recombination protein RecA (reviewed in Ref. 2).

A number of biochemical activities have been identified in RecA protein including a DNA-dependent ATPase activity and an ability to catalyze the formation of heteroduplex DNA between homologous ssDNA and dsDNA molecules in a reaction termed homologous pairing and strand exchange (reviewed in Refs. 2–5). In the presence of ATP, RecA polymerizes on both ssDNA and dsDNA to form nucleoprotein filaments in which the DNA is stretched to ∼150% the length of uncoated DNA. A substantial body of evidence indicates that pairing and strand exchange occur within the confines of the RecA-ssDNA nucleoprotein filament, with the formation of heteroduplex DNA initiating from the 3’ end of the DNA strand in the duplex molecule that is complementary to the ssDNA bound in the RecA filament (2–5). A central but as yet unresolved question concerns the molecular role of ATP in the RecA catalyzed pairing and strand exchange reactions (see “Discussion”). Earlier work on RecA protein had suggested that although homologous pairing occurs efficiently in the presence of the nonhydrolyzable analogue ATP-S (6, 7), the branch migration or strand exchange phase of the reaction requires continuous ATP hydrolysis (6). More recently, it was found that under certain reaction conditions, nucleotide binding alone can in fact enable RecA protein to carry out a limited amount of strand exchange (8–10). The function of ATP hydrolysis in RecA-mediated strand exchange remains a subject of intense debate (see “Discussion”).

Rad51 protein has been overproduced in yeast and purified to near homogeneity in our laboratory. Like RecA, Rad51 protein exhibits a DNA-dependent ATPase activity and catalyzes the pairing and strand exchange reaction (11). The efficiency of Rad51 catalyzed pairing and strand exchange is stimulated strongly by the heterotrimeric ssDNA binding factor replication protein A (RPA; Ref. 11). Rad51 forms filaments on both ssDNA (12) and dsDNA (13) in an ATP-dependent manner, and the DNA molecule in these Rad51 filaments is extended by about the same magnitude as in the RecA-ssDNA filaments (12, 13). Pairing and strand exchange again occur within the confines of the Rad51-ssDNA nucleoprotein filament (12). However, strand exchange by Rad51 commences from the 5’ end of the complementary strand in the duplex molecule, a reaction polarity opposite to that of RecA (12).

Here, we examine the role of ATP binding and hydrolysis in Rad51 function. Our results indicate that Rad51 protein can carry out extensive and complete strand exchange upon binding nucleotide alone and that the ATPase defective rad51 Arg-191 protein is in fact biologically functional. These findings suggest important differences between Rad51 and RecA in the manner ATP is utilized in promoting the strand exchange reaction.

MATERIALS AND METHODS

Overproduction and Purification of Rad51 and Mutant rad51 Proteins—RAD51 gene maintained in M13 mp18 was subjected to oligonucleotide-directed mutagenesis to change lysine 191 in Rad51 protein to alanine and arginine. RAD51, rad51 Ala-191, and rad51 Arg-191 genes were placed under the control of the PGK promoter in the vector pMA91 (2 μ, PGK, Leu-2d) to yield plasmids p51.3 (PGK-RAD51), p51.4 (PGK-rad51 Ala-191), and p51.5 (PGK-rad51 Arg-191), which were introduced into the rad51Δ strain LSY411. To purify Rad51, rad51 Ala-191, and rad51 Arg-191 proteins, extracts from LSY411 harboring the various plasmids were treated with ammonium sulfate and centrifuged to collect the precipitated proteins, which were dissolved to give fraction II as described (12). Fraction II was then subjected to chromatographic fractionation in columns of Q Sephars, Bio-Rex 70, hydroxyapatite, and Mono Q to yield fraction VI Rad51, rad51 Ala-191, and rad51 Arg-191 proteins, which were judged by Coomassie Blue staining of a 10% denaturing polyacrylamide gel containing these proteins to be greater than 98% pure (see Fig. 1C).

Strand Exchange Reactions—In the experiment described in Fig. 2B, 108 μg of rad51 Arg-191 protein (2.5 nmol) or 24 μg of Rad51 protein (0.56 nmol) added in 5 μl of storage buffer (12) was incubated with 500

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1. The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; RPA, replication protein A; MOPS, 3-(N-morpholino)propanesulfonic acid; AMP-PNP, adenylyl-imidodiphosphate; AMP-PCP, β, adenosine 5’-(β,γ-methylene)diphosphonate; ATP-S, adenosine 5’-O-thiotriphosphate; MMS, methylmethane sulfonate.
ng of dX viral (+) strand (1.54 nmol of nucleotides) in 40 μl of buffer R (42 mM Tris, pH 7.2, containing 1 mM dithiothreitol, 3 mM MgCl₂, 2.5 mM ATP) for 5 min at 37 °C, and then 6 μg of RPA (50.8 pmol) was added in 3 μl of storage buffer (12), followed by a 10-min incubation at 37 °C. After the incorporation of 500 ng of double-stranded dX 174 DNA (linearized with PstI; 1.54 nmol of nucleotides) in 3 μl and 4 μl of 50 mM spermidine, the reaction mixture (50 μl) was incubated at 37 °C. At the times indicated, a 6-μl portion of the reaction was stopped with 6 μl of 1% SDS containing 1 mg/ml proteinase K and then incubated at 37 °C for 10 min. The deproteinized samples were analyzed in a 0.9% agarose gel run in TAE buffer (40 mM Tris acetate, pH 7.5, 0.5 mM EDTA). In Fig. 3, the reaction used 108 pg of Rad51 protein (2.5 nmol) and AMP-PNP instead of ATP.

Examination of Reaction Polarity—Reaction polarity was examined as described for the mutli-copy plasmids pR51.3 (Rad51 protein 0.67 pmol) or 6 μg of Rad51 protein (0.14 pmol) was incubated with 125 ng of M13 mp18 viral (+) strand (0.38 nmol of nucleotides) in 10 μl of buffer R for 5 min, followed by the addition of 1.5 μg of RPA (12.7 pmol) in 1 μl and a 10-min incubation, and then 50 ng of [γ-32P]ATP (Amersham Corp.; 3,000 Ci/mmol). After the incorporation of dSNA, the reaction mixtures were incubated for a further 120 min, and 0.5-μl samples were subjected to thin layer chromatography in a polyethyleneimine cellulose sheet using 1 M formic acid and 0.3 M LiCl as the developing solvent, as described previously (11, 14).

ATPase Assay—The reaction mixtures containing Rad51, rad51 Ala-191, and rad51 Arg-191 were assembled exactly as described for the strand exchange reaction, except that they also contained 3 μCi of [γ-32P]ATP (Amersham Corp., 3,000 Ci/mmol). After the incorporation of dSNA, the reaction mixtures were incubated for a further 120 min, and 0.5-μl samples were subjected to thin layer chromatography in a polyethyleneimine cellulose sheet using 1 M formic acid and 0.3 M LiCl as the developing solvent, as described previously (11, 14).

Examination of Cellular Sensitivity to Methylmethane Sulfonate—The procedure for examining the MMS sensitivity of various yeast strains was based on that described by Prakash and Prakash (15). Yeast strains were grown in complete synthetic medium lacking leucine. Plates were incubated at 30°C for 3–4 days before counting colonies.

RESULTS

Lysine 191 in Rad51 protein represents an invariant residue of the Walker type A motif (Fig. 1A), previously shown to be central to the binding and hydrolysis of nucleoside triphosphates in various proteins. The change of the analogous lysine residue to arginine in the RecA protein (10) and in the yeast Rad3 protein (14), both of which possess DNA-dependent ATPase and deoxy-ATPase activities, abolishes the nucleotide hydrolytic activity of these proteins without adversely affecting the ability to bind ATP (14) or deoxy-ATP (10). Thus, the change of this invariant lysine to arginine represents a useful approach for uncoupling nucleotide binding from the nucleotide hydrolysis step. To address the roles of ATP binding and hydrolysis in Rad51 biological function and DNA pairing and strand exchange activity, we altered lysine 191 in Rad51 protein to arginine (Fig. 1A). Lysine 191 was also changed to alanine (Fig. 1A), which was expected to give a mutant rad51 protein with little or no affinity for ATP.

We placed the RAD51 gene and the two mutant rad51 alleles under the control of the yeast phosphoglycerate kinase (PGK) promoter in a multi-copy plasmid and transformed pMA91 (PGK-RAD51) yielding pR51.3 (PGK-RAD51), pR51.4 (PGK-rad51 Ala-191), and pR51.5 (PGK-rad51 Arg-191). These plasmids were introduced into yeast strain LS4Y11, in which the genome RAD51 gene has been deleted (16). By immunoblot analysis, we determined that the plasmid-bearing LS4Y11 cells contained a level of Rad51 and mutant rad51 proteins ~20-fold that of Rad51 protein in isogenic wild type W303–1B cells (Fig. 1B). Rad51 protein and the two rad51 mutant proteins were purified to near homogeneity (Fig. 1C) for use in the mechanistic studies described below.

For wild type Rad51 protein, the strand exchange reaction (Fig. 2A) is accompanied by preincubating Rad51 with ssDNA at the optimal ratio of 2.5–3 nucleotides/protein monomer in the presence of ATP and 4–6 mM MgCl₂, followed by the addition of RPA and a brief incubation, and then homologous linear dsDNA is incorporated (11, 12). The purified rad51 Ala-191 and rad51 Arg-191 mutant proteins were tested for the ability to catalyze DNA pairing and strand exchange under a range of reaction conditions, while keeping the order of addition of components the same as when wild type Rad51 protein is used (Refs. 11, 12; see “Materials and Methods”). The variables in these experiments were pH, rad51 Ala-191 or rad51 Arg-191 protein concentration, and MgCl₂ concentration during preincubation of the rad51 mutant protein with the ssDNA. At pH values greater than 7.0, at MgCl₂ concentrations of 2.5–5 mM, and at ssDNA to protein ratios ≤ 1 nucleotide/protein monomer, pairing and strand exchange were readily detected with the rad51 Arg-191 protein. In contrast, rad51 Ala-191 protein was inactive under all the conditions tested. A strand exchange experiment in which the ssDNA (in nucleotides) to rad51 Arg-191 protein monomer ratio was 0.6 is shown in Fig. 2B. When compared with a reaction that used wild type Rad51 at a nucleotide/protein monomer ratio of 2.7, the rate of product formation (sum of joint molecules and the complete strand exchange product) by the rad51 Arg-191 protein was about half (Fig. 2C). The rad51 Arg-191-mediated strand exchange reaction is dependent on ATP (Fig. 2B), and omission of RPA from the reaction reduces the amount of products by more than 10-fold (data not shown). We observed previously with wild type Rad51 protein that strand exchange initiates at the 5’ end of the complementary strand in the duplex partner (12). This reaction polarity is maintained in the rad51
Arg-191-mediated reaction (Fig. 2D).

To determine whether rad51 Arg-191 protein was devoid of ATPase activity, ATP hydrolysis was measured under conditions employed for the strand exchange reaction (Fig. 2) using \([\gamma-32P]\)ATP and thin layer chromatography. Although Rad51 protein hydrolyzed 55% of the input \([\gamma-32P]\)ATP in a total incubation time of 135 min, corresponding to \(k_{cat}\) of 0.73 min\(^{-1}\), hydrolysis of ATP was not seen with rad51 Arg-191 protein. Because the detection limit for ATP hydrolysis in these assays is \(\sim 1\%\), the maximum possible \(k_{cat}\) for ATP hydrolysis for the rad51 Arg-191 protein is 0.003 min\(^{-1}\) but is likely to be lower or nonexistent. ATP hydrolysis was also not detected with the rad51 Ala-191 protein under the same reaction conditions. Taken together, it can be concluded that rad51 Arg-191 carries out substantial strand exchange in the absence or near complete absence of ATP hydrolysis, whereas rad51 Ala-191 is defective in both ATP hydrolysis and strand exchange.

Based on the results with the rad51 Arg-191 protein, we reasoned that perhaps wild type Rad51 protein could utilize nonhydrolyzable ATP analogues for promoting pairing and strand exchange. We therefore examined whether Rad51 would mediate pairing and strand exchange in the presence of ADP, AMP-PCP, AMP-PNP, and ATP\(_S\). Indeed, we found that Rad51 protein carried out extensive pairing and strand exchange with either AMP-PNP (Fig. 3) or ATP\(_S\) (data not shown) as co-factor, although ADP and AMP-PCP were inactive. AMP-PNP and ATP\(_S\) are equally effective in this reaction (data not shown), which also requires that the pH be above 7.0, Mg\(^{2+}\) be in the range of 2.5–5 mm, and the ssDNA to Rad51 protein ratio be \(\sim 1\) nucleotide/protein monomer. Here also, strand exchange initiates at the 5′ end of the homologous strand in the linear duplex and is dependent on RPA (data not shown).

The biochemical experiments described thus far indicate that nucleotide binding is the key determinant in Rad51-mediated pairing and strand exchange. It was of considerable importance to determine whether or not the rad51 Arg-191 mutant is biologically active. We therefore examined the sensitivity of the rad51\(\Delta\) strain LS411 harboring plasmids expressing either Rad51 protein or mutant rad51 proteins at \(-20\)-fold of the wild type Rad51 level (Fig. 1B) to the alkylating agent MMS, which induces DNA damage that is repaired via the RAD52 recombination pathway (1). As expected, plasmid pR51.3 (PGK-RAD51; □), pR51.4 (PGK-rad51 Ala-191; △), or pR51.5 (PGK-rad51 Arg-191; ○) were examined for resistance to MMS.

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ATP (6–9) and strand exchange (8, 9, 18, 19) in the presence of considerable debate (8–10, 18, 19) has centered on the role of Rad51 pairing and strand exchange activity is required for recombinational processes.

RecA shares ~30% amino acid sequence identity with Rad51 in the middle portion spanning about 220 residues (2, 17). Considerable debate (8–10, 18, 19) has centered on the role of ATP binding and its hydrolysis in the RecA-mediated strand exchange reaction. Although RecA protein promotes pairing (6–9) and strand exchange (8, 9, 18, 19) in the presence of ATPγS, and the recA K72R mutant protein (analogous to rad51 Arg-191) is also active in these reactions (10, 18), strand exchange in these instances is limited (8–10, 18, 19) and is apparently bidirectional (18, 19). In one view, ATP hydrolysis is seen as being important for the interconversion of high affinity and low affinity DNA binding states of RecA and for turnover of the low affinity ADP-bound form from DNA (8–10). In an alternate view, it is suggested that the free energy derived from ATP hydrolysis is utilized for rotation of the incoming DNA molecule relative to the axis of the RecA-DNA nucleoprotein filament (18) and also for imparting directionality (18, 19). In contrast to RecA, the strand exchange reaction catalyzed by Rad51 protein in the absence of nucleotide hydrolysis displays the same polarity as when ATP is hydrolyzed and it proceeds fairly efficiently, and unlike the recA K72R mutant (20), the rad51 Arg-191 mutant retains biological activity. The above noted differences would suggest that RecA and Rad51 differ in their dependence upon ATP hydrolysis for their action.

Genetic studies in yeast have indicated that recombinational processes initiated from DNA double-stranded breaks involve 5’ to 3’ exonucleolytic processing of the breaks, giving rise to a 3’ ssDNA region (21, 22). It may be expected that nucleation of Rad51, RPA, and possibly other recombination proteins on such ssDNA regions would render these regions recombinogenic, leading to pairing with the homologous chromosomal partner and an exchange of genetic information between the recombining chromosomes. Because in normal yeast cells Rad51 protein will hydrolyze ATP upon binding the ssDNA region that results from double-stranded break processing, the question concerning the biological role of ATP hydrolysis remains. The biochemical data reported here indicate that pairing and strand exchange in the absence of ATP hydrolysis require relatively larger amounts of Rad51 or rad51 Arg-191 protein. Because the formation of a Rad51-ssDNA nucleoprotein filament is a prerequisite to pairing and strand exchange, our results are consistent with the speculation that the assembly of catalytically active Rad51-ssDNA nucleoprotein filaments may be facilitated by ATP hydrolysis. Future studies will address whether ATP hydrolysis helps promote the removal of secondary structure in ssDNA that may present a kinetic barrier to filament assembly and whether it enables Rad51 protein to adopt a conformation conducive for filament assembly.

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DISCUSSION

Although ATP hydrolysis by Rad51 protein might be expected to be indispensable for homologous pairing and strand exchange, results from our present study indicate that nucleotide binding alone is sufficient not only for pairing but also for complete strand exchange. In addition, we have determined that when expressed at the optimal level, rad51 Arg-191 protein is defective in pairing and strand exchange and has no biological activity in vivo, providing direct evidence that the Rad51 pairing and strand exchange activity is required for recombinational processes.

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