Function of Yeast Rad52 Protein as a Mediator between Replication Protein A and the Rad51 Recombinase*

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The **RAD51** and **RAD52** genes of *Saccharomyces cerevisiae* are key members of the **RAD52** epistasis group required for genetic recombination and the repair of DNA double-stranded breaks. The **RAD51** encoded product mediates the DNA strand exchange reaction. Efficient strand exchange is contingent upon the addition of the heterotrimeric single-stranded DNA binding factor replication protein A (RPA) after Rad51 has nucleated onto the single-stranded DNA. However, if the single-stranded DNA is incubated with Rad51 and RPA simultaneously to mimic what may be expected to occur *in vivo*, the efficiency of strand exchange decreases dramatically, revealing an inhibitory effect of RPA that is distinct from its stimulatory function. Interestingly, the inclusion of Rad52 protein, which has been purified in this study from yeast cells, restores the efficiency of strand exchange. Thus, Rad52 functions as a co-factor for the Rad51 recombinase, acting specifically to overcome the apparent competition by RPA for binding to single-stranded DNA.

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**Saccharomyces cerevisiae** genes of the **RAD52** epistasis group, including **RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, MRE11,** and **XR52,** function in genetic recombination and the recombinational repair of DNA double-stranded breaks induced by ionizing radiation. Because meiotic recombination is required for the proper disjunction of chromosomal homologs during meiosis I, mutants of the **RAD52** group often also exhibit severe meiotic abnormalities, including a failure to sporulate and low spore viability (1).

The **RAD51** encoded product is structurally related to the *Escherichia coli* recombination protein RecA (2). Like RecA, Rad51 protein mediates the homologous DNA pairing and strand exchange reaction (3). The efficiency of the **RAD51**-mediated strand exchange reaction is markedly stimulated by the heterotrimeric ssDNA-binding factor RPA (3–5). However, the manner in which RPA is incorporated is critical for obtaining maximal stimulation, such that if the ssDNA substrate is incubated with Rad51 and RPA simultaneously, the extent of ensuing strand exchange is only a fraction of what is seen when the ssDNA is first incubated with Rad51 prior to the incorporation of RPA (6). These results suggest that, although it is an important accessory factor for the **RAD51** recombinase activity, RPA can also compete with Rad51 for binding sites on the ssDNA and thus reduce the efficiency of strand exchange (6).

That RPA competes with Rad51 protein for sites on ssDNA has also been inferred from the observation that an excess of RPA inhibits the ssDNA-dependent ATPase activity of **RAD51** protein (5).

Here Rad52 protein is expressed in yeast and purified to near homogeneity. It is demonstrated that inclusion of Rad52 protein in the strand exchange reaction alleviates the inhibition by RPA, providing evidence for a co-factor function of **RAD52** protein in the **RAD51**-catalyzed DNA strand exchange reaction.

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**MATERIALS AND METHODS**

**Polyclonal Antibodies**—The portion of **RAD52** protein encompassing amino acid residues 168–456 was expressed as a fusion protein with the *E. coli* transcriptional terminator rho (ρ). The insoluble p-**RAD52** fusion protein was purified by preparative SDS-polyacrylamide gel electrophoresis and used for polyclonal antiserum production in rabbits. Antibodies were affinity-purified from the antiserum as described (7).

**Antibodies**—Antibodies against Rad51 protein were prepared as described (3), and the preparation of anti-Rad14 antibodies (8) was kindly provided by Dr. Sami Guzder.

**Immunoprecipitation**—For immunoprecipitation, extracts were prepared in cell breakage buffer (50 mM Tris-HCl, **pH** 7.5, 10% sucrose, 2 mM EDTA, 300 mM KCl, 2 mM DTT) with protease inhibitors at 2 ml of buffer/gram of cells using a French press (6). The extract was clarified by ultracentrifugation (100,000 × g, 90 min) and dialyzed against buffer I (25 mM Tris-HCl, **pH** 7.5, 10% glycerol, 0.2 mM EDTA, and 1 mM DTT) containing 150 mM KCl and protease inhibitors. After centrifugation (100,000 × g, 90 min), the clarified dialysate (0.5 ml) was mixed at 4 °C for 4 h with 10 μl of protein A beads bearing affinity-purified anti-Rad14 antibodies, anti-Rad51 antibodies, and anti-Rad52 antibodies, all at 2 mg antibodies/ml matrix. The beads were washed once each with 300 μl of buffer I containing 150 mM KCl, buffer I containing 250 mM KCl, and buffer I alone and then incubated with 30 μl of 3% SDS at 37 °C for 10 min to elute bound proteins. An aliquot of the eluates (5 μl) was subjected to immunoblot analysis to determine their content of the Rad51 and **RAD52** proteins.

**Rad52 Protein Purification**—**RAD52** gene from nine nucleotides upstream of the first translation initiating ATG codon until 600 nucleotides downstream of the TGA translation stop codon was cloned under the control of the phosphoglycerate kinase (*PGK*) promoter, yielding plasmid pR521.2 (2 μg, **PGK-RAD52**). This plasmid was introduced into yeast strain **LP2749-9B** harboring the **RAD51** overexpressing plasmid pR51.1 (3). For the purification of Rad52 protein, extract was prepared from 400 g of frozen yeast paste (7) and subjected to the purification scheme described in Fig. 2B. The yield of Rad52 protein was about 100 μg, and the full purification details will be described elsewhere.

**Other Proteins**—Rad51 protein was purified from yeast strain **LP2749-9B** harboring plasmid pR51.3 (2 μg, **PGK-RAD51**) as described (9). RPA was purified from strain **LP2749-9B** as described (6).

**Preparation of Affi-Gel Rad51**—Rad51 protein (2 mg) and bovine serum albumin (BSA, 3 mg) in 1 ml of coupling buffer (0.1 M potassium phosphate, pH 7.0, 0.1 M NaCl, 0.005% sodium azide) were coupled to Affi-Gel 1000 (Bio-Rad Laboratories, Hercules, CA) as described (6). After coupling, the beads were washed with 20 mM Tris-HCl, 200 mM KCl, 1 mM EDTA, and 0.01% sodium azide (pH 8.0) until no Rad51 protein was detected by SDS-PAGE. The amount of Rad51 coupled to the Affi-Gel 1000 beads was determined by SDS-PAGE. The beads were stored in 0.1 M potassium phosphate, 0.1 M NaCl, and 0.005% sodium azide (pH 8.0) at 4 °C. The purity of the coupled Rad51 protein was determined by SDS-PAGE (10% acrylamide) and immunoblot analysis (11).

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2 The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; RPA, replication protein A; DTT, dithiothreitol; BSA, bovine serum albumin; MOPS, 3-N-morpholino)propanesulfonic acid.

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MOPS, pH 7.5) were mixed with 0.5 ml of Affi-Gel 15 beads (Bio-Rad) at 25 °C for 45 min. The beads were then spun down in a microcentrifuge and washed at 4 °C once with 150 ml each of buffer T containing 150 mM KCl, buffer T containing 300 mM KCl, and buffer T containing 250 mM KCl and 520 ng of BSA or Rad51 at 25 °C for 45 min. The beads were washed with 150 mM KCl and 250 mM KCl and then treated with SDS to elute bound Rad52 protein. The output material (OP), the supernatant that contained unbound Rad52 protein (S), the two KCl washes (W1 and W2), and the SDS eluate (E) were probed for the Rad52 protein.

**RESULTS AND DISCUSSION**

As seen in immunoblot analyses, Rad52 protein in yeast extract consists of a number of closely spaced bands with sizes ranging from 61 to 63 kDa (Figs. 1 and 2). The abundance of these Rad52 protein species increases with elevated expression of RAD52, as when extract from yeast cells harboring the overproducing plasmid pR52.1 (2μ, PGK-RAD52) was analyzed (see Fig. 2A). The gel mobility of these Rad52 species was not altered by treatment with calf intestinal alkaline phosphatase, suggesting that the difference in gel mobility of the Rad52 species is due to phosphorylation. The Rad52 protein coding frame within the first 40 amino acid residues contains five potential translation initiating ATG codons; it is possible that the multiple Rad52 species are the result of alternate ATG codons being used in the translation of the RAD52 encoded message.

Immunoprecipitation experiments using protein A-agarose beads covalently conjugated anti-Rad51 and anti-Rad52 antibodies were carried out to determine whether Rad52 protein in cell extract is physically associated with Rad51 protein. As shown in Fig. 1A, anti-Rad51 immunobeads precipitated, in addition to Rad51, also Rad52 protein, and likewise, anti-Rad52 immunobeads co-precipitated Rad52 protein. The amount of Rad52 protein that co-precipitated with Rad51 protein was very similar to that directly precipitated by its cognate antibodies, suggesting that a substantial portion of the cellular Rad52 protein exists as a complex with Rad51 protein. Interestingly, the amount of Rad51 protein that co-precipitated with Rad52 protein was less than 10% of that precipitated by anti-Rad51 immunobeads, suggesting that Rad51 is present in considerable excess over Rad52 protein in yeast cells. Consistent with this deduction, the amount of Rad51 protein that co-precipitated with Rad52 increased with overexpression of the Rad52 protein, as when extract from LP2749-9B harboring pR52.1 (2μ, PGK-RAD52) was used for immunoprecipitation. As a result of a 20-fold overproduction of Rad52 protein, approximately 40% of the Rad51 protein in cell extract became associated with the Rad52 protein (Fig. 1A). The association of Rad52 with Rad51 in cell extract was likely due to a direct interaction between the two proteins, because purified Rad52 bound to purified Rad51 immobilized on Affi-gel 15 beads (Fig. 1B).

Interestingly, the level of Rad52 protein in strain LP2749-9B harboring pR52.1 was enhanced about 2.5-fold upon the introduction of the RAD51 overexpressing plasmid pR51.1 (2μ, ADC1-RAD51; Ref. 3). Because Rad51 and Rad52 proteins interact (Refs. 2 and 10 and see Fig. 1), it appears that interaction of Rad52 with Rad51 results in stabilization of the former in yeast cells. For the purification of Rad52 protein, extract from strain LP2749-9B co-harboring pR51.1 and pR52.1 was subjected to the chromatographic procedure outlined in Fig. 2B. Rad52 protein from the last step of purification in Mono Q was nearly homogeneous (Fig. 2C) and was used in the studies described below.

In the earliest or the presynaptic phase of the in vitro homologous pairing and strand exchange reaction (Refs. 11 and 12 and Fig. 3A), Rad51 protein polymerizes on the ssDNA substrate to form a nucleoprotein filament, with the confines of which the ensuing pairing and strand exchange occur (4). Previous work has indicated that the most efficient pairing and
strand exchange is effected by incubating ssDNA during the presynaptic phase, first with Rad51 protein alone for a few minutes before incorporating RPA (3, 4). In this standard strand exchange reaction, about 60% (11% joint molecules and 49% nicked circular duplex) and 87% (12% joint molecules and 75% nicked circular duplex) of the input linear dsDNA had been converted into strand exchange products after 36 and 72 min of incubation, respectively. As reported previously (3, 5) and reiterated in Fig. 3 (lane 3), the amount of strand exchange products was 56% (44% nicked circular duplex) and 87% (12% joint molecules and 87% nicked circular duplex) of the input linear dsDNA when Rad51 protein was preincubated with the viral (+) strand before incorporation of RPA into the reaction (1° Rad51, 2° RPA, lanes 4–10) is shown. In lanes 1–3, RPA was omitted from the reaction. C, co-addition of RPA with Rad51 reduces the efficiency of strand exchange. In the time course experiment in lanes 1–6, the ssDNA was incubated simultaneously with Rad51 protein and RPA. For comparison, the levels of products obtained with preincubation of ssDNA with Rad51 before the incorporation of RPA (Std) are shown (lanes 7–9). Abbreviations in B and C are: ds, linear duplex; jm, joint molecules; nc, nicked circular duplex; ss, the faster migrating form is the input viral (+) strand and the slower migrating form is the displaced (+) strand. D, graphical representation of the results in B and C. The sum of joint molecules and nicked circular duplex (% Products) was plotted for each time point: △, results from lanes 4–10 of B; □, results from lanes 1–3 of B; ■, results from lanes 1–6 of C.

compared with about 80% conversion into products after the same incubation time in the standard reaction (Fig. 3, B and D). These results indicated that RPA can also exert a negative effect on DNA strand exchange and suggested that other RAD52 group proteins may function to neutralize the inhibition by RPA. I therefore examined whether purified Rad52 protein was capable of alleviating the inhibition by RPA. Because the results from the immunoprecipitation experiments summarized earlier (Fig. 1A) revealed that Rad52 protein is of a much lower cellular abundance than Rad51 protein, initially, a molar amount of Rad52 protein about one-tenth that of Rad51 protein was used in the strand exchange reaction. Interestingly, the inclusion of Rad52 protein (1.25 μM) with Rad51 (11.6 μM) and RPA (1.35 μM) resulted in marked stimulation (Fig. 4, A and B), restoring strand exchange to a level comparable with that seen in the standard reaction (Fig. 3, B and D). For instance, at the mid-point of the reaction (36 min of incubation), the amount of strand exchange products was 56% (44% nicked circular duplex) of the input linear dsDNA when Rad52
Functional Interaction among Rad51, Rad52, and RPA

Rad52 proteins was essentially the same as the extremely low level seen with Rad51 alone (Fig. 3C), which suggested that Rad52 does not function to replace RPA. Strand exchange enhancement was also examined as a function of Rad52 protein concentration, and it was found, in reactions containing 11.6 μM Rad51 and 1.35 μM RPA, that the maximal level of stimulation occurred at about 1.0 μM Rad52 protein (Fig. 4C).

The possibility that Rad52 might have pairing and strand exchange activity was also examined. However, in the absence of Rad51 protein, there was no evidence of homologous pairing, even at a Rad52 protein concentration (5 μM) much higher than those used in the experiments shown in Fig. 4 and regardless of whether or not RPA was added to the reaction during or after incubation of Rad52 with the ssDNA (data not shown).

In its role as a co-factor for the Rad51 recombinase, Rad52 protein resembles the heterodimer of the Rad55 and Rad57 proteins, which is also capable of overcoming the inhibition by RPA (6). Interestingly, the recombination defects in rad55 and rad57 mutants can be suppressed partially by introducing a multicyclic plasmid that contains the RAD52 gene (13), suggesting that a common biochemical function exists in the Rad55-Rad57 heterodimer and Rad52 protein. Our results now provide evidence that this common function may be an ability of these protein factors to facilitate Rad51-ssDNA nucleoprotein assembly in the presence of RPA. The function of Rad52 protein and the Rad55-Rad57 heterodimer in the Rad51-mediated strand exchange reaction is reminiscent of that described for the bacteriophage T4 UvsY protein (Refs. 14 and 15 and references therein) and the combination of the E. coli RecO and RecR proteins (16), which act to allow their cognate recombinases UvsX protein and RecA protein to efficiently utilize ssDNA coated with T4 gene 32 protein and E. coli SSB protein for pairing and strand exchange. Both Rad52 protein and the Rad55-Rad57 heterodimer bind ssDNA (6, 17) and interact physically with Rad51 protein (2, 6, 10, 13, 18), suggesting a mechanism by which ssDNA-bound Rad52 or Rad55-Rad57 heterodimer actively recruits Rad51 to the ssDNA substrate.

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was present (Fig. 4, A and B), whereas only 7% of the input dsDNA (3% nicked circular duplex) had been converted to products in its absence (Fig. 3, C and D).

As shown in Fig. 4A (lanes 1–3), in the absence of RPA, the level of pairing and strand exchange obtained with Rad51 and Rad52 proteins was essentially the same as the extremely low level seen with Rad51 alone (Fig. 3C), which suggested that Rad52 does not function to replace RPA. Strand exchange enhancement was also examined as a function of Rad52 protein concentration, and it was found, in reactions containing 11.6 μM Rad51 and 1.35 μM RPA, that the maximal level of stimulation occurred at about 1.0 μM Rad52 protein (Fig. 4C).

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