A Single-stranded DNA-binding Protein Is Needed for Efficient Presynaptic Complex Formation by the *Saccharomyces cerevisiae* Rad51 Protein*

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Protein-promoted DNA strand exchange requires formation of an active presynaptic complex between the DNA-pairing protein and single-stranded DNA (ssDNA). Formation of such a contiguous filament is stimulated by a ssDNA-binding protein. Here, the effects of replication protein A (RPA) on presynaptic complex formation and DNA strand exchange activities of Rad51 protein were examined. Presynaptic complex formation was assessed by measuring ATP hydrolysis. With ϕX174 ssDNA, the ATPase activity of Rad51 protein is stimulated ~1.4-fold by RPA, provided that Rad51 protein is in excess of the ssDNA concentration; otherwise, RPA inhibits ATPase activity. In contrast, with ssDNA devoid of secondary structure (poly(dT), poly(dA), poly(dI), and etheno-M13 DNA), RPA does not stimulate the already elevated ATPase activity of Rad51 protein, but inhibits activity at low Rad51 protein concentrations. These results suggest that Rad51 protein and RPA exclude one another from ssDNA by competing for the same binding sites and that RPA exerts its effect on presynaptic complex formation by eliminating secondary structure to which Rad51 protein is bound nonproductively. DNA strand exchange catalyzed by Rad51 protein is also greatly stimulated by RPA. The optimal stoichiometry for stimulation is ~20–30 nucleotides of ssDNA/RPA heterotrimer. The ssDNA-binding protein of *Escherichia coli* can substitute for RPA, showing that the role of RPA is not specific. We conclude that RPA affects both presynaptic complex formation and DNA strand exchange via changes in DNA structure, employing the same mechanism used by the ssDNA-binding protein to effect change in *E. coli* RecA protein activity.

The RAD51 gene of *Saccharomyces cerevisiae*, a member of the RAD52 epistasis group, is required for mitotic and meiotic recombination (for a review, see Ref. 1). Cells deficient in *RAD51* function are sensitive to x-ray irradiation or DNA-alkylating agents, suggesting that this gene is required for repair of double-strand DNA breaks (2). Since formation of meiosis-specific double-strand DNA breaks is not inhibited in rad51 cells, *RAD51* seems to function after formation of the break in meiotic recombination. The *RAD51* sequence is conserved in a wide variety of eucaryotic organisms, suggesting that it is important to cellular function in eucaryotes (3). Rad51 protein has homology to *Escherichia coli* RecA protein (2, 4, 5). Furthermore, image reconstruction from electron micrographs of complexes of Rad51 protein and double-stranded DNA (dsDNA) confirmed this similarity and showed that the three-dimensional structure of the Rad51 protein-DNA filament is similar to the equivalent RecA protein-dsDNA complex (6). Finally, Rad51 protein from *S. cerevisiae* has single-stranded DNA (ssDNA)-dependent ATPase activity and promotes ATP-dependent DNA strand exchange (7, 8).

RecA protein plays a central role in genetic recombination in *E. coli* (for reviews, see Refs. 9–13). *In vitro* analyses have revealed that in the presence of ATP, RecA protein binds to ssDNA to form a nucleoprotein filament, referred to as the presynaptic complex, which is the active species in homologous DNA pairing. Pairing between the presynaptic filament and homologous dsDNA results in formation of the synaptic complex and is followed by DNA strand exchange, the process whereby one strand in the dsDNA is replaced by the homologous ssDNA (postsynaptic stage). The single-stranded DNA-binding protein (SSB protein) of *E. coli* stimulates RecA protein-mediated DNA strand exchange by acting at both the presynaptic and postsynaptic stages. At the presynaptic step, SSB protein disrupts secondary structure within the ssDNA to facilitate formation of a continuous RecA protein-ssDNA nucleoprotein filament. At the postsynaptic step, SSB protein facilitates DNA strand exchange by binding to the displaced ssDNA strand produced by DNA heteroduplex formation (14, 15). In both steps, physical interaction between RecA and SSB proteins is not required for stimulation (16–18).

Replication protein A (RPA), also referred to as replication factor A, is a single-stranded DNA-binding protein of *S. cerevisiae* (19–21). RPA is a heterotrimeric protein, consisting of polypeptides with molecular masses of 70.4, 29.9, and 13.8 kDa, and each subunit is essential for cell viability. RPA enhances DNA strand exchange promoted by Rad51 protein (7). Due to the superficial similarities of Rad51 and RPA to RecA and SSB proteins, respectively, it is reasonable to hypothesize that the role of RPA in DNA strand exchange catalyzed by Rad51 protein is similar to that of SSB protein in RecA protein-catalyzed DNA strand exchange. However, it is equally possible that RPA functions differently from SSB protein, to accommodate specific requirements of the eucaryotic recombination process. To address these issues, we measured presynaptic complex and DNA strand exchange activities of Rad51 protein and assessed the effects of both RPA and SSB protein on these reactions. While we find many parallels between the eucaryotic

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1 The abbreviations used are: dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; SSB protein, single-stranded DNA-binding protein; RPA, replication protein A.
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and procaryotic systems, significant differences exist. In particular, active presynaptic complex formation by Rad51 protein has a more stringent requirement for a ssDNA-binding protein. We suggest that this requirement stems from the stable and nonproductive binding of Rad51 protein to regions of dsDNA (secondary structure) within native ssDNA.

**Experimental Procedures**

**DNA**—Both replicative form and viral (+)-strand dX174 DNAs were purchased from New England Biolabs Inc. For DNA strand exchange, the replicative form of dX174 DNA was linearized with PstI. Etheno-M13 DNA was prepared as described (22). Poly(dT) and poly(dA) were purchased from Pharmacia Biotech Inc. Poly(dT) was purchased from P-L Biochemicals. The concentrations of dX174 de dsDNA, dX174 ssDNA, poly(dT), and poly(dA) were determined using molar (nucleotide) extinction coefficients of 6500, 8125, 7300, and 8600 at 260 nm, respectively, and the concentration of poly(dT) was determined using a molar (nucleotide) extinction coefficient of 9400 at 260 nm. DNA was stored in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA. All DNA concentrations are expressed in nucleotides unless otherwise noted.

**Proteins**—The three subunits of RPA, cloned on three separate plasmids (a generous gift from Dr. Richard Kolodner), were coinduced in yeast, and the RPA heterotrimer was prepared as described (23). Rad51 protein was overproduced in yeast using the plasmid and strain kindly provided by Dr. Patrick Sung and was purified as described (7), except that Cibacron blue column fractionation was used as the first step. SSB protein was overproduced and purified as described (24). The concentrations of RPA, Rad51 protein, and SSB protein were determined using extinction coefficients (determined, for Rad51 protein and RPA, from amino acid composition) of 9.8 × 10³, 1.29 × 10³, and 3.0 × 10³ at 280 nm, respectively. Pyruvate kinase and lactate dehydrogenase were purchased from Sigma. The restriction endonuclease PstI was purchased from New England Biolabs Inc.

**ATP Hydrolysis Assays**—ATPase activity was measured at 37 °C essentially as described (25). Rad51 protein and a single-stranded DNA-binding protein (when indicated) were added in the indicated order to 120 μl of buffer containing 2.5 mM ATP, 50 μM pyruvate kinase, 10 units/ml lactate dehydrogenase, 0.3 mM phosphoenolpyruvate, 256 μM NADH, 50 μg/ml bovine serum albumin, 1 mM dithiothreitol, 20 mM magnesium acetate, 50 mM KCl, and 30 mM Tris acetate (pH 7.5). The oxidation of NADH resulted in a decrease in absorbance at 340 nm, which was continuously monitored by a Hewlett-Packard Model 8452A diode array spectrophotometer. The rate of ATP hydrolysis was calculated from the rate of change in absorbance using the following formula: rate of Δ absorbance (s⁻¹) = Δ A absorbance (s⁻¹) = rate of ATP hydrolysis (μmol/min).

**DNA Strand Exchange Assays**—DNA strand exchange was performed as follows (all concentrations are those in the final reaction mixtures). 33 μM (nucleotides) dX174 viral (+)-strand DNA was incubated with 9.8 μM Rad51 protein in a total volume of 8.5 μl of 2.5 mM ATP, 50 μg/ml bovine serum albumin, 1.0 mM dithiothreitol, 20 mM magnesium acetate, 50 mM KCl, and 30 mM Tris acetate (pH 7.5) at 37 °C. After 5 min, RPA or SSB protein was added to the indicated concentration, and incubation was continued for 30 min. PstI-linearized dX174 dsDNA (1 μl) was added to a final concentration of 33 μM (base pairs) and incubated for an additional 90 min. Samples were deproteinized for 15 min by the addition of SDS and proteinase K to final concentrations of 0.5% and 1 mg/ml, respectively. Reaction products were separated by electrophoresis through a 1.0% agarose gel run in 40 mM Tris acetate and 2 mM EDTA (pH 8.5) at 40 V for 13 h and were visualized by staining afterward with ethidium bromide.

**Results**

**RPA Stimulates the ssDNA-dependent ATPase Activity of Rad51 Protein**—We first examined the effect of RPA on the ssDNA-dependent ATPase activity of Rad51 protein (Fig. 1). ATPase activity was measured using a spectrophotometric assay that couples ADP production to oxidation of NADH, which results in a decrease in absorbance (25). In the presence of dX174 ssDNA, Rad51 protein displayed a linear rate of ATP hydrolysis. Subsequent addition of RPA increased the rate of ATP hydrolysis, showing that the ssDNA-dependent ATPase activity of Rad51 protein is stimulated by RPA. In the presence of dX174 dsDNA, ATP hydrolysis was also increased, but to only ~40% of the level achieved with ssDNA; RPA did not stimulate this activity (Fig. 2). In the absence of DNA, ATP hydrolysis by Rad51 protein was observed above the background level, but the rate was ~10–15-fold lower than in the presence of ssDNA. The addition of RPA had no effect on ATPase activity when ssDNA was absent. Also, the RPA preparation had no ATP hydrolysis activity in either the presence or absence of ssDNA (data not shown; Fig. 2).

In Rad51 protein-catalyzed DNA strand exchange, optimal product formation occurs at ~3 nucleotides of ssDNA/Rad51 protein monomer; exceeding this amount of protein results in a strong inhibition (8). To determine the optimal ratio of Rad51 protein to ssDNA for ATPase activity, a Rad51 protein titration at a fixed concentration of dX174 ssDNA (6.87 μM) was performed (Fig. 2). The ATP hydrolysis rate reached a plateau value at 4–5 μM Rad51 protein (1.4–1.7 nucleotides of ssDNA/Rad51 protein monomer), irrespective of the presence or absence of RPA; a similar plateau was obtained with dsDNA. This optimal activity requires approximately twice the amount of protein that is required for optimal DNA strand exchange. RPA (222 nM) stimulated ssDNA-dependent, but not dsDNA-dependent, ATP hydrolysis at the higher Rad51 protein concentrations; interestingly, at the lower Rad51 protein concentrations (0.5 and 1.0 μM), RPA either had no effect or slightly decreased the ATP hydrolysis rate. Similar behavior was obtained previously for RecA and SSB proteins: when RecA protein was in excess over ssDNA, SSB protein stimulated formation of the RecA protein-ssDNA complex; but when ssDNA was in excess, SSB protein inhibited complex formation (18). Our findings suggest that a comparable relationship exists for Rad51 protein and RPA.

**RPA Inhibits ATPase Activity at Low Concentrations of Rad51 Protein**—To further quantify the effects of RPA on ATPase activity, reactions containing various concentrations of Rad51 protein and a fixed concentration of ssDNA (6.87 μM) were titrated with RPA. The ATP hydrolysis rate obtained in the presence of RPA relative to that in the absence of RPA was plotted against RPA concentration (Fig. 3). When the Rad51 protein concentration was equal to or higher than 2.5 μM (~2.75 nucleotides/Rad51 protein), RPA increased the ATP hydrolysis rate as expected. When the Rad51 protein concentration was 0.5 μM (13.7 nucleotides/Rad51 protein), ATPase activity was clearly decreased by RPA in a concentration-dependent manner. Therefore, RPA can have either a stimulatory or an inhibitory effect on the ssDNA-dependent ATPase activ-
of Rad51 protein either in the presence (●) or absence (○) of 222 nt RPA. RPA was added after preincubation of Rad51 protein

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FIG. 3. Effects of RPA on presynaptic complex formation are competitive with Rad51 protein concentration. The ATP hydrolysis rates shown in Fig. 2 and other additional experiments were plotted as a function of RPA concentration. The ATP hydrolysis rates are shown relative to the rates obtained without RPA. All reactions contained a fixed amount of φX174 ssDNA (6.87 µM; nucleotides) and the following concentrations of Rad51 protein: 0.5 (●), 1.0 (▲), 2.5 (▼), 3.5 (○) 5.0 (□), and 7.0 (△) µM.

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FIG. 4. RPA does not stimulate the ATPase activity of Rad51 protein in the presence ssDNA devoid of secondary structure. A, Rad51 protein (1.3 (●), 3.9 (▲), 6.5 (□), and 9.2 (○) µM) was incubated with 8.0 µM (nucleotides) poly(dT) in reaction buffer, followed by the addition of RPA. ATP hydrolysis was analyzed as described for Fig. 3. The absolute rates of ATP hydrolysis in the absence of RPA were 0.64, 2.10, 2.61, and 2.88 µM/min for 1.3, 3.9, 6.5, and 9.2 µM Rad51 protein, respectively. B, similar experiments were conducted using 2.5 µM Rad51 protein and 6.87 µM (nucleotides) etheno-M13 DNA (εM13; □), poly(dA) (p(dA); ▲), and poly(dI) (p(dI); ▼). The absolute rates of ATP hydrolysis in the absence of RPA were 1.45, 1.23, and 1.04 µM/min for etheno-M13 DNA-, poly(dA)-, and poly(dI)-stimulated reactions, respectively.

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These results are somewhat different from the effect of SSB protein on RecA protein activity, where saturating amounts of SSB protein fully inhibit the ATPase activity of RecA protein irrespective of the RecA protein concentration (18), and suggest that the stability of the Rad51 protein-ssDNA filament relative to RPA is greater than that of the RecA protein filament relative to SSB protein.

E. coli SSB Protein Can Stimulate the ATPase Activity of Rad51 Protein—The results presented so far strongly suggest that RPA stimulates presynaptic complex formation of Rad51 protein by a mechanism that is quite similar to that developed for SSB and RecA proteins. Therefore, we examined whether SSB protein could substitute for RPA in these reactions. The addition of SSB protein to reactions containing Rad51 protein and ϕX174 ssDNA resulted in the changes shown in Fig. 5. SSB protein stimulated ATP hydrolysis when the Rad51 protein concentration was 3.5 and 5.0 μM (2.0 and 1.4 nucleotides/Rad51 protein, respectively) and inhibited it at 1.0 and 0.5 μM (6.9 and 13.7 nucleotides/Rad51 protein, respectively). These data are similar to those obtained for RPA shown in Fig. 3.

RPA and SSB Protein Can Stimulate DNA Strand Exchange by Rad51 Protein—Because SSB protein can substitute for RPA in presynaptic complex formation, we expected that SSB protein could substitute for RPA in DNA strand exchange mediated by Rad51 protein. Fig. 6A shows the effect of RPA on DNA strand exchange. RPA extensively stimulated formation of nicked circular dsDNA (OC; the final product of the DNA strand exchange reaction) and the slower migrating joint molecules (JM; intermediates of the exchange reaction), as reported (7, 8). Often we did not detect any product in the absence of RPA; but in other experiments, we could sometimes observe a trace amount (data not shown). This is in contrast to RecA protein-mediated DNA strand exchange, where products can be found in the absence of SSB protein (26). Quantitative analysis of the gel (Fig. 6C) shows that stimulation by RPA reached its optimum (~90% of the total DNA) at a ratio of ~20–30 nucleotides of starting ssDNA/RPA heterotrimer.

When SSB protein was used in place of RPA in otherwise identical reactions (Fig. 6B), SSB protein increased both joint molecule and nicked circular dsDNA formation. Stimulation by SSB protein reached its optimal level (~20% of the total DNA) at a ratio of ~7–10 nucleotides of starting ssDNA/SSB protein monomer (Fig. 6C), which is similar to that required for the RecA protein-mediated reaction (26). These results indicate that SSB protein can substitute for some of the functions of RPA required for DNA strand exchange mediated by Rad51 protein. They also suggest that no specific protein-protein interaction between Rad51 protein and RPA is required for stimulation of DNA strand exchange catalyzed by Rad51 protein alone. Although the maximum yield of the product formation supported by SSB protein appears to be lower than that sup-

![Fig. 5. E. coli SSB protein can substitute for RPA in presynapsis.](image)

Experiments were performed in a manner similar to that described for Fig. 3, except that SSB protein was added instead of RPA. SSB protein was added to reactions containing a fixed concentration of ϕX174 ssDNA (6.87 μM; nucleotides) and the following concentrations of Rad51 protein: 0.5 (△), 1.0 (▲), 3.5 (○), and 5.0 (□) μM, which were preincubated for 17–20 min.

![Fig. 6. RPA and SSB protein stimulate DNA strand exchange promoted by Rad51 protein.](image)
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The potential target for interaction with RPA. Thus, by virtue of an absence of a specific need for RPA in the reactions described here with Rad51 protein alone, our studies both support the conclusion and suggest further that RPA acts primarily via interaction with a different protein(s) of the RAD52 group, minimally, Rad52 protein (28).

For all the similarities, there exist some notable differences between the two systems. One obvious difference is the overall rate of reaction. In our experiments, the $k_{cat}$ for φX174 ssDNA-dependent ATP hydrolysis by Rad51 protein is 0.40–0.44 min$^{-1}$ under optimal conditions and ~0.5 min$^{-1}$ for poly(dT). This value is ~50-fold lower than for RecA protein (18). Also, DNA strand exchange promoted by Rad51 protein is 20–30-fold slower than for RecA protein, and the yield is reduced (26). This difference may simply reflect an intrinsically slower rate for eucaryotic recombination relative to procaryotic recombination; alternatively, it may reflect the need for other stimulatory factor(s) in presynaptic complex formation and/or a later step of DNA strand exchange.

Another difference is the rather stringent requirement for a ssDNA-binding protein in the DNA strand exchange reaction. Rad51 protein catalyzes DNA strand exchange very poorly when a ssDNA-binding protein is absent. In contrast, RecA protein catalyzes the same reaction in the absence of SSB protein at approximately half the efficiency of the reaction in the presence of SSB protein (26). A possible explanation for this severe requirement for a ssDNA-binding protein may stem from the fact that Rad51 protein can bind to dsDNA as well as to ssDNA (2). In the absence of a ssDNA-binding protein, Rad51 protein will bind to both the single- and double-stranded regions of native ssDNA (Fig. 7a). The Rad51 protein filament formed on the base-paired regions of the DNA might inhibit DNA strand exchange. Therefore, removal of Rad51 protein from the double-stranded regions by RPA and its replacement by a uniform Rad51 protein-ssDNA complex might be critical for efficient DNA strand exchange in vitro. Thus, the nonproductive binding of Rad51 protein to these regions of DNA secondary structure, the stabilization of these dsDNA regions, or an instability of the resultant discontinuous presynaptic filament could explain the poor DNA strand exchange activity in the absence of a ssDNA-binding protein.

Our results also suggest a potential second function for RPA. Maximal ATPase activity (reflecting optimal presynaptic complex formation) required approximately one RPA heterotrimer/70 nucleotides of ssDNA; however, optimal DNA strand exchange requires twice as much RPA (one RPA heterotrimer/20–30 nucleotides of ssDNA). This suggests that there is another role of RPA in DNA strand exchange other than to enhance formation of the presynaptic complex. This proposed additional function is unknown, but it might be a postsynaptic role similar to that established for SSB protein in RecA protein-promoted DNA strand exchange (14, 15). In this second capacity, SSB protein binds to and prevents reinvasion of the displaced DNA strand in RecA protein-catalyzed DNA strand exchange.

Alani et al. (23) reported that the DNA binding stoichiometry of RPA to ssDNA (or “site size”) is 80–100 nucleotides/RPA heterotrimer. Based on this value, more RPA than is needed to saturate the ssDNA is required for optimal stimulation of both ATPase and DNA strand exchange activities. We also analyzed the ssDNA binding characteristics of our RPA preparation by fluorescence quenching, under the conditions of both our DNA

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3 T. Sugiyama, N. Kantake, and S. C. Kowalczykowski, unpublished observations.

4 A. Shinohara, M. Shinohara, and T. Ogawa, submitted for publication.

5 E. M. Zaitseva and S. C. Kowalczykowski, unpublished observations.
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