Single Strand DNA Binding and Annealing Activities in the Yeast Recombination Factor Rad59

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Saccharomyces cerevisiae RAD59 gene is required for homologous recombination processes and normal level of resistance to ionizing radiation. To study the biochemical functions of Rad59, it was overproduced in yeast and purified to near homogeneity. Rad59 binds DNA, showing much higher affinity for ssDNA than dsDNA. Rad59 also anneals complementary DNA strands, and order of addition experiments indicate that maximal annealing efficiency is achieved when both complementary DNA strands are present upon addition of Rad59. Thus, Rad59 resembles its homolog Rad52 in being able to bind ssDNA and anneal complementary DNA strands. However, unlike Rad52, DNA annealing by Rad59 is not accelerated by the ssDNA binding factor RPA. DNA binding and strand annealing are likely to be important for the biological functions of Rad59 in general recombination and in the single-strand annealing pathway of recombination.

Saccharomyces cerevisiae RAD52 epistasis group genes, are required for mitotic and meiotic recombination. Because of their involvement in DNA double strand break repair, mutational inactivation of these genes also leads to greatly elevated susceptibility of cells to the cytotoxic and genotoxic effects of ionizing radiation (reviewed in Ref. 1). Interestingly, RAD52 and RAD59 of this group also function in the single strand annealing (SSA) pathway of recombination (1, 16).

In the DNA double strand break repair model for recombination, subsequent to break formation, the ends of the break are processed in an exonucleolytic fashion to yield long ssDNA tails, to be utilized by other recombination factors for the formation of heteroduplex DNA with a DNA homolog. Results from genetic and biochemical studies have implicated a complex of Rad50, Mre11, and Xrs2 in the DNA end-processing reaction (1). It is thought that Rad51, the eukaryotic homolog of bacterial RecA recombinase (2), nucleates onto the ssDNA tails generated during end-processing to form a highly ordered nucleoprotein filament (3, 4) and mediates the homologous DNA pairing and strand exchange reaction that yields heteroduplex DNA joints with a DNA homolog (5). Biochemical studies have indicated that the efficiency of Rad51-mediated homologous DNA pairing and strand exchange is very much dependent on a number of accessory factors including Rad52 (6–8), Rad54 (9), and the Rad55-Rad57 complex (10).

Here we show that Rad59 binds ssDNA and mediates the annealing of complementary DNA strands, activities that are likely to be germane for the recombination functions of Rad59.

MATERIALS AND METHODS

Rad59 Expression in Yeast—RAD59 gene was placed under the control of the yeast PGK promoter (11) in the vector pPM230 (2µ, LEU2-d, PGK) to give pR59.1, which was introduced into the protease-deficient strain LP2749-9B (5).

Nucleic Acids—dsX 174 (+) strand was purchased from New England Biolabs and the replicative form (≥90% supercoiled) was from Life Technologies, Inc. pBluescript SK(+) and SK(−) were made in Escherichia coli strain XLI-Blue (Stratagene). The SK(−) strand was linearized by hybridizing an oligonucleotide complementary to positions 1458–1481 of the DNA to create an ApaLI site followed by digestion. DNA Mobility Shift—Rad59 was incubated with either dsX(+) strand or the replicative form linearized with ApaLI in buffer R (50 mM KMPBS, pH 7.2, 100 µg/ml bovine serum albumin, 25 mM KCl, and 1 mM dithiothreitol). After incubation at 37 °C, reaction products were resolved in 9% agarose gels and visualized by staining with ethidium bromide.

Annealing of Complementary DNA Strands—Linear dsX as DNA was incubated at 100 °C for 2.5 min and chilled immediately on ice. DNA annealing reaction mixtures (10 µl final volume) containing the indicated amounts of Rad59, RPA, and dsX complementary DNA strands were incubated in buffer R with 5 mM MgCl2 at 37 °C. At the end of incubation, SDS and protease K were added to 0.5% and 500 µg/ml, respectively, followed by a 2-min incubation at 37 °C to deproteinize the reaction mixtures. Products were separated in 0.9% agarose gels in TAE buffer. In time course experiments, the reaction mixtures were scaled up appropriately, and 10-µl portions were withdrawn. The pBluescript SK(+) and SK(−) single strands were used in the DNA annealing experiments shown in Fig. 5.

RESULTS AND DISCUSSION

RAD59 gene was amplified from S. cerevisiae genomic DNA by the polymerase chain reaction and placed under the control of the yeast PGK promoter. For the purification of Rad59, extract from the protease-deficient yeast strain LP2749-9B harboring pR59.1 (2µ, PGK-RAD59) was subject to a combination of ammonium sulfate precipitation and chromatographic fractionation in columns of Q-Sepharose, SP-Sepharose, hydroxyapatite, Sephacryl S200, and Mono S. Rad59 from the last step of purification in Mono S (Fraction VII) was nearly homogeneous (Fig. 1B), and was used in all the biochemical experiments.

We used DNA mobility shift in agarose gels for examining whether Rad59 binds DNA. As shown in Fig. 2A and B, Rad59 binds both dsX174 ssDNA and dsDNA in a protein concentration dependent manner. Whereas 0.6 µg of Rad59 was sufficient to shift greater than 70% of the ssDNA (35 µM nucleotides), only about 10% of the dsDNA (35 µM nucleotides) was shifted by the same concentration of Rad59. The treatment of Rad59-ssDNA and Rad59-dsDNA nucleoprotein complexes with SDS and protease K released intact DNA, indicating that the DNA had not undergone any covalent modification (Fig. 2A, lane 9 in both panels). As shown in Fig. 2C, panel I, DNA binding is reduced appreciably by 150 mM KCl, but, as shown in panel II,
is relatively insensitive to magnesium up to 12.5 mM.

We wished to examine the relative affinities of Rad59 for ssDNA and dsDNA in greater detail by including both DNA substrates in the same binding mixture, done in the absence of magnesium or in the presence of 5 mM magnesium (Fig. 2D). It both cases, Rad59 binds specifically to the ssDNA, such that at 0.64 μM Rad59, whereas essentially all of the ssDNA (18.5 μM nucleotides) was shifted, little if any shifting of the dsDNA (12.3 μM nucleotides) occurred. There was no evidence for co-aggregation of the ssDNA and dsDNA by Rad59. Taken together, the results indicate that Rad59 binds ssDNA with a considerably higher avidity than dsDNA. Preferential binding of Rad59 to ssDNA over dsDNA was also verified using oligonucleotide-based as and ds substrates.3

Recent genetic experiments conducted in the laboratories of Haber and Symington (16)2 have indicated that Rad59 affects the efficiency of recombination by SSA. Because of this genetic observation, it was of considerable interest to examine whether Rad59 in fact has the ability to mediate the annealing of complementary DNA strands. Complementary ssX single strands were incubated with Rad59, followed by deproteinization and electrophoresis in agarose gels. As shown in Fig. 3A, incubation of the complementary ssX DNA strands with Rad59 resulted in the appearance of DNA product that migrated more slowly than the input DNA strands. The level of the new DNA species was proportional to the concentration of Rad59 (Fig. 3A, lanes 4–9). These DNA species were the product of DNA strand annealing because (i) they were completely stable to the treatment with SDS and proteinase K (Fig. 3A, lanes 4–9); (ii) no higher DNA species was observed with undenatured duplex DNA incubated with the highest amount of Rad59 (Fig. 3A, lanes 1 and 2); (iii) heating the DNA product released intact complementary DNA strands (Fig. 3C, lanes 4); and (iv) the generation of these DNA species is dependent on the presence of DNA homology in the DNA strands (Fig. 2 and see later in Fig. 5). Near-maximal DNA annealing was observed at Rad59 to ssDNA ratio of one protein monomer per 30 nucleotides (Fig. 3A, lane 7). The annealed DNA species, which failed to enter the agarose gel, very likely contained multiple complementary DNA strands annealed together, as has been shown for other recombination proteins, Rad52 for instance (13), which also have the ability to mediate DNA annealing.

The addition of 0.5% SDS at the very beginning of the DNA

![Fig. 1. Purification of Rad59. A, extracts from yeast strain LP2947-9B harboring vector pPM230 (2μ, PGK, lane 1) or the Rad59 overexpressing plasmid pR59.1 (2μ, PGK-RAD59; lane 2) and purified Rad59 (50 ng in lane 3) were subject to immunoblot analysis with affinity-purified anti-Rad59 antibodies. B, purity analysis. Rad59, 2 μg in lane 2, was run along with size standards (lane 1) in an 11% SDS-polyacrylamide gel and then stained with Coomassie Blue.](image1)

![Fig. 2. DNA binding by Rad59. A, ssX single-stranded DNA (ss; panel I) or linear duplex (ds; panel II), each at 35 μM nucleotides, was incubated with Rad59 (0.15, 0.3, 0.45, 0.6, 0.75, 0.9, and 1.05 μM) in lanes 2–8, respectively) at 37 °C for 5 min. In lane 9, the DNA was incubated with 1.05 μM Rad59 at 37 °C for 5 min, but the reaction was treated with SDS (0.5%) and protease K (PK; 500 μg/ml) for 2 min at 37 °C prior to electrophoresis. Nucleoprotein complexes are marked as complex, and the well position of the gel is indicated. B, the gels in A were subject to image analysis and the results plotted. Symbols: ● ssDNA binding; ○, dsDNA binding. C, ssX 174(+)-strand and the linear duplex, 35 μM nucleotides, were incubated with 1 μM Rad59 as described in A but with increasing KCl (panel I) and MgCl2 (panel II). In all the reactions in panel II, KCl was present at 25 mM. Symbols: ● ssDNA binding; ○, dsDNA binding. D, ssX (+)-strand (18.5 μM nucleotides) and linear duplex (12.3 μM nucleotides) were co-incubated with increasing amounts of Rad59 (0.09, 0.18, 0.27, 0.36, 0.5, and 0.64 μM in lanes 2–7, respectively) at 37 °C for 5 min either in the absence (left panel) or presence (right panel) of 5 mM MgCl2 as indicated.](image2)

3 G. Petukhova and P. Sung, unpublished observations.
Fig. 3. Rad59 anneals complementary DNA strands. A, φX complementary strands (46 μm nucleotides, designated as ss, (+)/−), were incubated with increasing amounts of Rad59 (0.25, 0.5, 1, 1.5, 2.0, and 2.5 μm in lanes 4–9, respectively) at 37 °C for 5 min, deproteinated with SDS and proteinase K, and then analyzed. Duplex φX DNA (30 μm nucleotides) without heat denaturation (lanes 1 and 2) was also incubated with Rad59 (2.5 μm in lane 2) under the same conditions. B, results from image analysis of the gel in A (lanes 3–9). C, φX complementary strands (46 μm nucleotides), designated as ss, (+)/−, were incubated without (lane 1) or with Rad59 (2.0 μm in lanes 2–4) as described in A, except that SDS (0.5%) was added to the reaction in lane 3 at the beginning of incubation and that the reaction product in lane 4 was boiled for 2 min and chilled on ice before being analyzed. D, φX complementary strands (46 μm nucleotides), designated as ss, (+)/−, were incubated with Rad59 (2.0 μm) for the indicated times. E, results from image analysis of the gel in D.

annealing reaction completely abolished the ability of Rad59 to mediate this reaction (Fig. 3C, lane 3). Magnesium at 5 mM was included in all of the reactions shown in Fig. 3, but DNA annealing occurs just as efficiently in the absence of magnesium.3 As can be seen in Fig. 3, D and E, the DNA annealing reaction is relatively rapid, such that maximal level of annealing was accomplished after 1 min of incubation, although the size of the annealed product appeared to continue to increase until about 2 min. Lowering the reaction temperature from 37 °C to 30 °C did not affect the kinetics of DNA annealing appreciably, but further decreasing the temperature to 25 °C slowed down the annealing reaction significantly.3 Taken together, the results indicate that Rad59 promotes rapid and efficient annealing of complementary DNA strands. From time course experiments, it is clear that Rad59 does not act catalytically in DNA annealing, as the amount of annealed DNA product is largely or entirely dependent on the initial amount of Rad59.3

Rad52 binds to ssDNA preferentially and mediates annealing of complementary DNA strands (14). Interestingly, DNA annealing by Rad52 is accelerated by the heterotrimeric ssDNA-binding protein RPA (13, 15). We examined the effect of RPA on Rad59-mediated DNA annealing. From Fig. 4, it is clear that little if any effect on the kinetics of the annealing occurred at relatively low concentrations of RPA (0.2 μm), and at higher concentrations of RPA (0.6 μm and 1.2 μm), progressive inhibition of the annealing reaction was seen. We have also examined concentrations of RPA lower than 0.2 μm and intermediate between 0.2 and 0.6 μm and again found no stimulation of Rad59-mediated DNA strand annealing. Under these conditions, RPA, at the concentrations used, does not by itself mediate DNA annealing during the incubation time.

A possible stimulation of DNA strand annealing by RPA was also examined at concentrations of Rad59 lower than that used in Fig. 4. Here also, while relatively low concentrations of RPA had little or no effect on the extent or kinetics of DNA annealing by Rad59, relatively high concentrations of RPA inhibited the annealing reaction progressively.

In prior experiments (Figs. 3 and 4), the complementary

Fig. 4. DNA annealing by Rad59 is not stimulated by RPA. A, φX complementary strands (46 μm nucleotides), designated as ss, (+)/−, were incubated with Rad59 (2.0 μm in lanes 2–17) without RPA (lanes 2–5) or with 0.2 μm RPA (lanes 6–9), 0.6 μm RPA (lanes 10–13), and 1.2 μm RPA (lanes 14–17) for the indicated times. B, results from image analysis of the gels in A were plotted. Symbols for the annealing reactions: ■, Rad59 alone; □, Rad59 with 0.2 μm RPA; Δ, Rad59 with 0.6 μm RPA; A, Rad59 with 1.2 μm RPA. φX(+) and φX(−) DNA strands were incubated simultaneously with Rad59. In order to assess the effect of precoating either or both of the complementary strands on the annealing reaction, we turned to pBluescript SK(+) and SK(−) single strands (2,960 bases) that are homologous to each other. As shown in Fig. 5A, incubation of Rad59 with a mixture of the SK(+) and SK(−) strands also resulted in annealed species (lanes 6 and 7). Incubation of Rad59 with either the SK(+) or the SK(−) strand alone did not generate any SDS/proteinase K-resistant high molecular size DNA species (Fig. 5A, lanes 2 and 4), again emphasizing the requirement for DNA homology in the formation of the annealed product. We next examined the effect of
precoating either or both of the SK single strands with Rad59 on the annealing reaction. As shown in Fig. 5, B and C, precoating of the SK(+) strand before the addition of the SK(−) strand resulted in a significant reduction of annealed product; a similar reduction in the level of annealed product was observed when the SK(−) strand was preincubated with Rad59 before the addition of the SK(+) strand. Interestingly, precoating both SK(+) and SK(−) strands with Rad59 in separate reactions before mixing resulted in further suppression of the annealing reaction (Fig. 5, B and C). These observations indicate that the highest efficiency of DNA strand annealing is achieved when both complementary strands are present at the time Rad59 is added.

The results show that Rad59 binds ssDNA with considerably higher affinity than dsDNA, both in the absence or presence of magnesium and relatively high concentrations of KCl. The affinity of Rad59 for ssDNA could be important for the utilization of recombinationogenic ssDNA substrates for homologous recombination pathways in vivo (12). Consistent with the absence of nucleotide binding motifs in Rad59, we have not detected an ATPase activity in purified Rad59, both in the absence of DNA and presence of either ssDNA or dsDNA. Also Rad59 does not promote homologous DNA pairing and strand exchange between oligonucleotide-based DNA substrates or dX 174 DNA substrates. We have examined the effect of adding Rad59 to the strand exchange reaction mediated by Rad51 and RPA, but thus far have not observed any stimulatory effect of Rad59. In the model proposed by Bai and Symington (12), Rad59 is envisioned to function in the context of a complex with Rad51 and other recombination factors. Although our studies have not yet found an obvious role of Rad59 in the Rad51-mediated strand exchange reaction, it remains possible that other recombination factors are needed to reveal an effect of Rad59 in this reaction.

Genetic evidence points to a role for RAD52 and RAD59 in the SSA mode of recombination (1, 16). Consistent with the observed genetic requirement, both Rad52 (14) and Rad59 (this study) have now been shown to mediate the annealing of complementary DNA strands. However, whereas the DNA annealing activity of Rad52 is stimulated by RPA (13, 15), Rad59 does not appear to depend on RPA for efficient single strand annealing. Whether Rad59 functionally cooperates with Rad52 in single strand annealing in vitro and in vivo remains to be determined. It also remains possible that the single strand annealing ability of Rad59 is germane for the DNA break-induced replication mode of long tract gene conversion in which a short heteroduplex joint may be formed via strand annealing to prime DNA synthesis (1).

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