Rad52 promotes second-end DNA capture in double-stranded break repair to form complement-stabilized joint molecules

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Saccharomyces cerevisiae Rad52 performs multiple functions during the recombinational repair of double-stranded DNA (dsDNA) breaks (DSBs). It mediates assembly of Rad51 onto single-stranded DNA (ssDNA) that is complexed with replication protein A (RPA); the resulting nucleoprotein filament pairs with homologous dsDNA to form joint molecules. Rad52 also catalyzes the annealing of complementary strands of ssDNA, even when they are complexed with RPA. Both Rad51 and Rad52 can be envisioned to promote “second-end capture,” a step that pairs the ssDNA generated by processing of the second end of a DSB to the joint molecule formed by invasion of the target dsDNA by the first processed end. Here, we show that Rad52 promotes annealing of complementary ssDNA that is complexed with RPA to the displaced strand of a joint molecule, to form a complement-stabilized joint molecule. RecO, a prokaryotic homolog of Rad52, cannot form complement-stabilized joint molecules with RPA–ssDNA complexes, nor can Rad52 promote second-end capture when the ssDNA is bound with either human RPA or the prokaryotic ssDNA-binding protein, SSB, indicating a species-specific process. We conclude that Rad52 participates in second-end capture by annealing a resected DNA break, complexed with RPA, to the joint molecule product of single-end invasion event. These studies support a role for Rad52–promoted annealing in the formation of Holliday junctions in DSB repair.

DNA annealing | DNA renaturation | Rad51 | Rad54 | replication protein A

Double-stranded DNA breaks (DSBs) can arrest cell growth, lead to loss of genetic integrity, and if unrepaired, cause cell death (1). Repairing such DSBs is a function of homologous recombination. In Saccharomyces cerevisiae, homologous recombination is mediated by members of the Rad52 epistasis group (2, 3). In the classical DSB repair (DSBR) model (4, 5), repair first involves processing of the broken dsDNA ends to generate 3′-ended single-stranded DNA (ssDNA) overhangs (6), which are initially bound by replication protein A (RPA) (7, 8). RPA not only protects ssDNA from nucleases, but also prevents formation of DNA secondary structures. The binding of RPA to ssDNA is, however, nonproductive with regard to recombinational DNA repair because it blocks the binding of Rad51 protein onto the ssDNA (8). Rad51 needs to assemble on the resected ssDNA to form a nucleoprotein filament that has the capability both to find homology within an intact double-stranded (dsDNA) template and mediate the exchange of DNA strands to form a joint molecule (also referred to as a D-loop) (2, 3, 7–9). At this stage, Rad52 protein plays an important role by mediating the displacement of RPA from ssDNA by Rad51 to generate the nucleoprotein filament (10–12). Subsequently, Rad54 associates with the Rad51 nucleoprotein filament, stabilizing it and enhancing its ability to form joint molecules (13–15). This interaction with the Rad51 nucleoprotein delivers Rad54 to the dsDNA target, where the translocation capability of Rad54 extends the DNA heteroduplex (16–19). Joint molecule formation results in displacement of a DNA strand from the dsDNA target, which is then bound by RPA (8, 20).

At this point, the second end of the broken DNA molecule needs to be engaged in the repair process. The simplest possibility is that the ssDNA, formed by processing at the other end of the DSB, invades the dsDNA target to form two separate joint molecules. Rad51 would next be removed from the DNA heteroduplex by the action of Rad54 to expose the 3′ ends of both invading strands (21). The invading strands can then prime DNA synthesis to extend the length of the DNA heteroduplex (22, 23), ultimately forming two Holliday junctions (4, 24).

Alternatively, it is equally plausible that instead of a second independent invasion event, DNA synthesis extends the first joint molecule before engagement of the second end of the DSB. This would have the effect of enlarging the length of the displaced DNA strand or migrating the position of the displaced strand (“bubble-migration” (25)) toward the sequences that are homologous to the second processed DNA end. In this case, the next step in the repair process would require annealing of this displaced strand to the ssDNA tail created by resection at the other DNA end of the break (4, 26). Rad52 is a protein that is both essential to recombinational DNA repair (27) and has ssDNA-annealing activity (28). The annealing activity of Rad52 is unique among the many proteins that promote DNA annealing and renaturation because it can anneal ssDNA that is complexed with RPA (29–31). Functional homologs of Rad52 can be found in bacteria and phage, and, interestingly, annealing of protein–ssDNA complexes by these proteins displays the unique requirement for the cognate ssDNA-binding protein (SSB) (32). These facts suggested to us that ssDNA annealing was a common universal step in recombination and that this capability could be used for “second-end capture” (32, 33). Subsequent DNA synthesis from this captured second end would generate two four-way structures composed of DNA strands base paired with old and new partners simultaneously. This intermediate structure comprises a double Holliday junction (dHJ) structure (4, 24), which can then be resolved to produce either cross-over or non-cross-over products, thereby completing the repair of the DNA break.

An alternative to the DSBR pathway proposes that, after DNA synthesis, the joint molecule is disrupted to liberate the polymerase-extended invading strand that then anneals with the ssDNA formed by processing of the other end of the DSB; this pathway is termed synthesis-dependent strand-annealing (SDSA) (34). Genetic studies established that Rad52 function was essential for SDSA (2, 3). Because these ssDNA intermediates would be bound by RPA protein, the annealing of ssDNA in the SDSA pathway could also be mediated by Rad52 (31, 32).


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and an in vitro reaction that established key biochemical elements of this process was reported (33). DSBR via SDSA produces only non-cross-over products.

In the DSBR pathway, the notion that assimilation of the processed second DNA end is sequential rather than simultaneous with invasion of the first end (4, 5, 35) and that annealing proteins could be involved in second-end capture (32, 33, 36) prompted us to evaluate the role of Rad52 in more detail. Specifically, models of second-end capture by Rad52 require that it has the ability to anneal an RPA–ssDNA complex to the displaced stand of a joint molecule made by Rad51 and Rad54. In this work, we establish an in vitro reaction by using Rad51, Rad52, Rad54, and RPA that establishes the capacity of Rad52 to mediate annealing of an RPA–ssDNA complex to the displaced strand of the D-loop formed by Rad51 and Rad54. The product of this reaction is a distinct homologously paired joint molecule known as a complement stabilized D-loop (cs-D-loop) (37, 38). This activity provides further evidence for a pivotal role of Rad52 in homologous recombination.

Results

Rad51 and Rad54 Generate Complement-Stabilized D-Loops. To mimic second-end capture, we devised an experimental system (Fig. 1A) based on the described properties of cs-D-loops (37, 38). A cs-D-loop is formed when two complementary ssDNA molecules pair with the two strands of a dsDNA molecule; being complementary strands, the pairing is at the same site. Unlike single D-loops, the resulting cs-D-loops are stable upon linearization of supercoiled DNA (scDNA), which allows convenient discrimination between single and double D-loops. In our experiments, Rad51 and Rad54 catalyze single D-loop formation between an ssDNA 90-mer (oligonucleotide 1) and pUC19 scDNA (Fig. 1B, lane 1). As expected, these D-loops dissociate completely upon restriction with HindIII (Fig. 1B, lane 2), a restriction endonuclease that cleaves 24 bp away from the region of pairing. The dissociation of D-loops upon deproteinization is driven by the release of superhelical energy within the negatively supercoiled DNA (39).

When a second ssDNA 90-mer (oligonucleotide 2) that is complementary to the displaced strand of the single D-loop is added, a joint molecule is generated whose mobility is the same as the single D-loop (Fig. 1B, lane 3). However, in contrast to the single D-loops, a fraction of the resultant joint molecules are now stable upon cleavage with HindIII (Fig. 1B, lane 4). Based on this characteristic, we conclude that the pairing of oligonucleotide 2 to a joint molecule formed with oligonucleotide 1 and scDNA results in the formation of a cs-D-loop. Furthermore, in agreement with previous observations (37, 38), the linearized cs-D-loops possess the same mobility as linear pUC19 DNA (39).

Fig. 1. Rad51 and Rad54 generate complement-stabilized D-loops. (A) Schematic representation of cs-D-loop formation. (I) Rad51 and Rad54 promote D-loop formation. (II) The single D-loop can be stabilized by pairing the displaced strand in the D-loop with a complementary oligonucleotide. (IV) This complement-stabilized D-loop is stable upon restriction. Red star indicates position of 32P label on the 5’ ends of ssDNA. (B) D-loops and cs-D-loops were formed as described in Materials and Methods and analyzed by electrophoresis. Lane 1, single D-loops; lane 2, single D-loops treated with HindIII; lane 3, cs-D-loops; lane 4, cs-D-loops treated with HindIII. The positions of D-loops, cs-D-loops, free oligonucleotides, and molecular size markers (kbp) are indicated. (C) The order of oligonucleotide addition was interchanged; lanes are as in B. The amounts of cs-D-loops formed in lane 4 of B and C were 3 ± 0.5% and 1.2 ± 0.3%, respectively, expressed relative to the scDNA concentration that is limiting; error is the SEM.
RPA Inhibits Complement-Stabilized D-Loop Formation. Initially, the DNA-pairing experiments were conducted in the absence of RPA because it is not essential for D-loop formation (15, 43). However, we found that when the second oligonucleotide was added to single D-loop that was formed with oligonucleotide 1, it was used as the ssDNA for assembly of presynaptic complexes in all subsequent pairing reactions.

RPA Inhibits Complement-Stabilized D-Loop Formation. Nimonkar et al. previously established that the displaced DNA strand in a D-loop is stabilized by an SSB or RPA proteins, which will prevent reannealing and dissociation of the nascent joint molecule (20, 44). Because Rad52 interacts with RPA to form a complex that can anneal complementary ssDNA–RPA complexes (10, 29, 30, 45), we wished to determine whether Rad52 could promote cs-D-loop formation in the presence of RPA. Consequently, cs-D-loop reactions, inhibited by RPA, were performed by using decreasing concentrations of Rad52. Oligonucleotide 2 was preincubated with both RPA (0.4 μM) and Rad52, and then the complex was added to D-loops formed by Rad51 and Rad54 between oligonucleotide 1 and pUC19. This concentration of RPA completely blocked spontaneous annealing of oligonucleotide 2 to the D-loop (Fig. 3A, lanes 1 and 5). As anticipated, despite the presence of RPA, Rad52 enabled cs-D-loop formation (Fig. 3A, lanes 1–5). The formation of cs-D-loop was a function of Rad52 concentration and time, with product formation saturating at 0.8 μM and at 15 min, respectively (Fig. 3B and Fig. S2).

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Discussion

The integrated cascade of events during DSBR that initiates with the resection of ends and culminates with recombinant DNA molecules requires the concerted action of many proteins involved in homologous recombination (2, 3). After DNA strand invasion and synthesis primed by the invading strand, the D-loop can progress via two distinct pathways. In the SDSA pathway, the extended invading arm is displaced from the D-loop and then

![Fig. 2. RPA inhibits complement-stabilized D-loop formation in the absence of Rad52.](image)
annealed to the second resected DNA end, generating non-cross-over repair products. Alternately, in the canonical DSBR pathway, the second resected end can either invade the dsDNA target or it can anneal with the displaced strand of the D-loop (4). Further extension and branch migration of this doubly paired intermediate results in the formation of dHJs. Although dHJs can potentially be resolved to generate cross-over or non-cross-over recombinants (reviewed in ref. 46), the temporal appearance of the recombinant products during meiosis suggests that dHJs are mostly resolved to form cross-overs (5). Thus, the second ends of resected DNA can participate in two biochemically distinct reactions that dictate the nature of resolution products, i.e., cross-over or non-cross-overs. It is now evident that the primary decision point as to whether a particular recombination event will generate a cross-over or a non-cross-over is not necessarily the resolution step, but an earlier step that determines whether the second-end is captured (5, 35, 36, 47). The pairing of the second processed end to the target duplex DNA leads to dHJs and to cross-overs, whereas the renaturation of the second end to the evicted invading strand after DNA synthesis (SDSA) will result in non-cross-overs.

Our data show that productive engagement of an RPA-coated strand that is complementary to the D-loop requires Rad52. Rad52 functions in presynapsis by mediating Rad51-dependent displacement of RPA (10, 11, 48). The observation that Rad52 remains at a DNA break longer than Rad51 suggested that this protein also plays a role in a step subsequent to DNA strand invasion and DNA synthesis (7, 49). Also, our work is in agreement with recent findings that demonstrated the role of Rad52 during the postinvasion steps of recombination (36, 50) and fits with the DSBR model depicted in Fig. 5. After processing of a DNA break to create a 3’-ssDNA overhang, RPA is the first protein to bind (Step I). A species-specific interaction then recruits Rad52 (Step II). Rad52 facilitates the formation of a Rad51–ssDNA nucleoprotein filament by mediating the displacement of RPA at either or both ends of the DSB (Step III) (49, 51). The newly formed Rad51–Rad54–ssDNA complex then catalyzes invasion of the homologous dsDNA target, generating a displaced strand (Step IV). The displaced ssDNA in the joint molecule is a substrate for RPA and Rad52 (Step IV) (20, 30). Subsequent translocation by Rad54 clears Rad51 from the heteroduplex DNA (21), which makes the 3’ end of the invading strand accessible to DNA polymerases (53). Elongation from the 3’ end of the invading ssDNA by DNA synthesis results in further DNA strand displacement and additional binding of RPA and Rad52 (49, 51). Once a region of ssDNA that is complementary to the other side of the DNA break is exposed, annealing between the displaced strand and the resected DNA end can
ensue because both ssDNA molecules are covered with RPA and Rad52 [Step V; the oligomeric form of Rad52 required for DNA annealing is not illustrated (54)] (32, 33). After DNA synthesis from the second end, branch migration, and subsequent ligation, a dHJ is generated (Step VI). The dHJs can then be resolved as cross-overs or non-cross-overs.

Using an in vitro assay, we have provided direct biochemical evidence for the ability of Rad52 to mediate second-end capture in the presence of RPA is specific because neither E. coli SSB nor hRPA can substitute in the pairing reaction. In addition, our observation that E. coli RecO mediates second-end capture only in the presence of RecA and SSB (E. Valencia-Morales and S.C.K., unpublished results), but not in the presence of Rad51, Rad54, and RPA further illustrates the specificity and general conservation of this process. This work and recent related work (33, 36, 50) demonstrate the importance of Rad52 in second-end capture and establish an alternative to direct DNA strand invasion by the second processed end in DNA break repair.

Materials and Methods

Enzymes and Reagents. T4 polynucleotide kinase and HindIII were purchased from New England Biolabs. ATP (disodium salt), phosphoethanolpyruvate, and pyruvate kinase were purchased from Sigma. [γ-32P]ATP (6,000 Ci/mmol) was purchased from NEN. Proteinase K was purchased from Roche.

DNA. Covalently closed supercoiled plasmid DNA (pUC19) was purified from E. coli DH5α cells by detergent lysis followed by equilibrium centrifugation in a CsCl-ethidium bromide gradient (55). Its concentration was determined by using an extinction coefficient of 6.6 × 10^4 M^−1 cm^−1 at 260 nm. Two complementary 90-mer oligonucleotides, 1 (5′-CGGGTGTCGGGGCTGGCTGATATGCGGTGTGAAATACCCGACGATGCGT-3′) and 2 (5′-ACCGCATCTGGCAGATTTCGCCATATGCAAGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGT-3′) were synthesized and gel-purified by Sigma–Genosys. The two oligonucleotides have the same sequence as residues 471–560 of pUC19. The concentrations of the oligonucleotides were determined spectrophotometrically by using molar (nucleotide) extinction coefficients of 9.7 × 10^3 and 9.3 × 10^3 M^−1 cm^−1 at 260 nm, respectively. Oligonucleotides were 5′-32P-labeled with T4 polynucleotide kinase and purified by using MicroSpin G-25 columns (GE Healthcare). DNA concentrations are expressed in moles of nucleotide as well as molecules.

Proteins. S. cerevisiae Rad51 (56, Rad52 (11), RPA (57), Rad54 (17), E. coli RecO (58), E. coli SSB (59), and hRPA (A. Carreira and S.C.K., unpublished data) were purified as described. Rad54 was purified as described in ref. 17 except for two modifications: the ammonium sulfate precipitation step was omitted, and a Sephacyr S300HR column was used instead of Sephacyr S400. Protein concentrations were determined by using the extinction coefficients provided in each reference; the concentration of SSB is given as a monomer.

Joint Molecule Formation. Unless stated otherwise, Rad51 (0.12 μM) and Rad54 (0.12 μM) were preincubated with 5′-32P-labeled oligonucleotide 1 (0.9 M μm, 10 nM molecules) in a buffer containing 25 mM Tris acetate (pH 7.5), 10 mM magnesium acetate, 1 mM DTT, 100 μg/mL BSA, 2 mM ATP, 3 mM phosphoethanolpyruvate, and 20 units/ml pyruvate kinase for 15 min at 30 °C. The concentration of DNA contributed by the Rad51 and Rad54 storage buffers were 2 mM and 66 mM, respectively. Unless indicated otherwise, when present, the RPA and Rad52 storage buffers added 3 mM and 40 mM, respectively, to the reaction buffer. D-loop formation was initiated by the addition of pUC19 (26 μM nt, 5 nM molecules), and incubation was continued at 30 °C for 15 min. For cs-D-loop formation, 5′-32P-labeled oligonucleotide 2 (0.9 M μm, 10 nM molecules) was added, and incubation was continued at 30 °C for 15 min. Unless stated otherwise, oligonucleotide 2 was preincubated with 5′-32P-labeled oligonucleotide 1. Rad52 was added, and incubation was continued at 30 °C for 15 min. Finally, reactions were stopped by the addition of termination buffer (final concentration, 7 M urea, 100 mM MgCl2, 0.12 M EDTA, and 2% SDS), with further incubation at 37 °C for 30 min. The reaction products were analyzed by 1% agarose gel electrophoresis (9 V/cm for 1 h 15 min). After electrophoresis, the gels were dried on DE81 paper (Whatman) and quantified by storage phosphor analysis using a Molecular Dynamics Storm 860 PhosphorImager (GE Healthcare). Errors are reported as the standard error of the mean (SEM) and were determined by using GraphPad Prism v.5.

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