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# Stimulation of Dmc1-mediated DNA strand exchange by the human Rad54B protein

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## ABSTRACT

**The process of homologous recombination is indispensable for both meiotic and mitotic cell division, and is one of the major pathways for double-strand break (DSB) repair. The human Rad54B protein, which belongs to the SWI2/SNF2 protein family, plays a role in homologous recombination, and may function with the Dmc1 recombinase, a meiosis-specific Rad51 homolog. In the present study, we found that Rad54B enhanced the DNA strand-exchange activity of Dmc1 by stabilizing the Dmc1–single-stranded DNA (ssDNA) complex. Therefore, Rad54B may stimulate the Dmc1-mediated DNA strand exchange by stabilizing the nucleoprotein filament, which is formed on the ssDNA tails produced at DSB sites during homologous recombination.**

## INTRODUCTION

Chromosomal DNA is exposed to various DNA-damaging agents and sustains damage that induces genomic instability. A double-strand break (DSB) is caused by ionizing radiation, cross-linking reagents, oxidative stress and DNA replication failure. If the DSB is left unrepaired, then cell death occurs (1–4). Homologous recombination is one of the major DSB repair pathways. This repair pathway is essentially error-free, since a homologous region of the undamaged sister chromatid is used as the template for repair. In contrast to the mitotic DSB repair pathway, meiotic cell division involves homologous recombination between homologous chromosomes, but not between sister chromatids. This preferential recombination between homologous chromosomes is initiated by the formation of a programmed DSB and ensures correct chromosomal segregation at meiosis I through the formation of chiasmata, which physically connect homologous chromosomes (5,6). Thus, homologous recombination is important to maintain the integrity of the chromosome in both mitotic and meiotic cells.

In homologous recombination, a single-stranded DNA (ssDNA) tail, produced at the DSB site, is incorporated into a nucleoprotein complex called the presynaptic filament. This presynaptic filament catalyzes homologous pairing and strand exchange with an intact homologous region of the double-stranded DNA (dsDNA) molecule. The bacterial RecA protein is known to form helical presynaptic filaments and to play central roles in homologous recombination (7–12). In eukaryotes, two homologs of RecA, the Rad51 and Dmc1 proteins, which are conserved from yeast to human, are assumed to fulfill this role. Rad51 is expressed in both meiotic and mitotic cells, whereas the expression of Dmc1 is restricted to meiotic cells (13–16). Although previous biochemical studies demonstrated that Rad51 and Dmc1 have recombinational activities similar to those of RecA (17–22), other studies have also revealed that many ancillary factors, such as replication protein A (RPA), Rad52 and Rad54, significantly affect the activities of Rad51 and Dmc1 (23,24).

Rad54 is a member of the SWI2/SNF2 family of proteins, which have DNA-dependent ATPase activities and are involved in chromatin remodeling (25–31). Genetic studies revealed that Rad54-deficient cells are sensitive to DNA-damaging agents, such as ionizing radiation, methyl methanesulfonate (MMS) and mitomycin C (32). Rad54 utilizes the free energy from ATP hydrolysis to generate superhelical

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torsion into dsDNA by translocating on the DNA (33,34). Furthermore, yeast Rad54 promotes the assembly and disassembly of the Rad51 nucleoprotein filament, and both yeast and human Rad54 stimulate the Rad51-mediated homologous pairing activity by directly binding to Rad51 (35–37).

Recent studies showed that the human Rad54B protein, a homolog of Rad54, interacts with the human Rad51and Dmc1 proteins, and stimulates the homologous pairing activity mediated by these proteins (22,38). Similar to Rad54, human Rad54B is a DNA-dependent ATPase (39) and is expressed in both mitotic and meiotic cells (40). However, genetic studies revealed that human Rad54B-deficient cells are not overly sensitive to ionizing radiation, MMS and cisplatin (41). Furthermore, the human Rad54B-deficient cells are also proficient in mitotic sister chromatid exchange (41). On the other hand, a severe reduction in targeted integration frequency was detected in the Rad54B-deficient cells (41). These characteristics are different from those of Rad54, indicating that Rad54B may have a unique role in homologous recombination.

To understand the function of Rad54B in homologous recombination, in the present study, we purified the human Rad54B protein, which was overexpressed in insect cells and biochemically characterized it. The purified Rad54B protein bound to the ATPase domain of Dmc1. Furthermore, Rad54B stimulated the DNA strand exchange mediated by Dmc1 and stabilized the Dmc1–ssDNA complex. Therefore, Rad54B may stimulate the Dmc1-mediated strand exchange by stabilizing the Dmc1–ssDNA nucleoprotein filament during homologous recombination.

## MATERIALS AND METHODS

### Protein purification

The human Rad54B cDNA was subcloned from pFastBac HTC (Invitrogen) into pFastBac 1 (Invitrogen), and a recombinant human Rad54B baculovirus was generated as described previously (39). High Five insect cells were infected with human Rad54B at a multiplicity of infection of 1, and were harvested after 72 h. The cells were resuspended in buffer A (pH 7.5), containing 50 mM Tris–HCl, 0.6 M KCl, 2 mM 2-mercaptoethanol (2ME), 10% sucrose and 10 mM EDTA, and were disrupted by sonication. The cell debris was removed by centrifugation for 40 min at 100 000 *g*, and the lysate was treated with ammonium sulfate (0.21 g/ml). The protein precipitate was redissolved in 50 ml of buffer B (pH 7.5), containing 20 mM HEPES–KOH, 0.2 M KCl, 0.5 mM EDTA, 2 mM 2ME, 10% glycerol and 0.01% NP-40. The protein solution was mixed with 8 ml of phosphocellulose column matrix at 4°C for 1 h, and then the mixture was packed into an Econo-column (Bio-Rad). After washing with 20 CV of buffer B, the Rad54B protein was eluted by a 20 CV linear gradient from 0.2 to 1.0 M KCl. The Rad54B protein was dialyzed against buffer C (pH 7.4), containing 20 mM potassium phosphate, 0.15 M KCl, 2 mM 2ME, 10% glycerol and 0.01% NP-40, and then was mixed with 6 ml of Q-Sepharose (GE Healthcare) column matrix at 4°C for 1 h. The proteins in the Q-Sepharose flow-through fraction were mixed with 6 ml of SP–Sepharose (GE Healthcare) column matrix at 4°C for 1 h, and then the mixture was packed into an Econo-column. The column was washed with 20 CV of buffer C and was eluted with a 20 CV linear gradient from 0.15 to 1.0 M KCl. The peak fractions were dialyzed against buffer D (pH 7.4), containing 20 mM potassium phosphate, 0.2 M KCl, 2 mM 2ME, 10% glycerol and 0.01% NP-40, and were loaded on to a 1 ml hydroxyapatite column. The column was washed with 20 CV of buffer D and was eluted with a 20 CV linear gradient from 20 to 300 mM potassium phosphate. The peak fractions of the Rad54B proteins were collected, dialyzed against buffer B and stored at −80°C.

The human Rad51 protein was purified as described previously (42), with the inclusion of a spermidine precipitation purification step. Briefly, after the removal of the $His_6$-tag, the Rad51 protein was dialyzed at 4°C against buffer E (pH 7.5), containing 100 mM Tris–acetate, 7 mM spermidine HCl and 5% glycerol. The Rad51 precipitate was collected by centrifugation and resuspended in buffer F (pH 7.0), which contained 100 mM potassium phosphate, 0.15 M NaCl, 1 mM EDTA, 2 mM 2ME and 10% glycerol, and was purified on a 1 ml Mono Q column (GE Healthcare) as described previously.

The human Dmc1, Dmc182–340 and RPA proteins were purified as described previously (43–45). The concentrations of the purified proteins were determined with a Bio-Rad protein assay kit, using BSA as the standard.

### DNA substrates

The ϕX174 circular ssDNA (5386 bases) and replicative form I DNA were purchased from New England Biolabs and Life Technologies. For the assays of strand exchange and protein transfer between DNA molecules, the replicative form I DNA was linearized with ApaL I. To perform the gel mobility shift assay for the protein transfer between ssDNA molecules, the ϕX174 circular ssDNA was cut to ∼500 base fragments by an incubation at 98°C. The 120mer oligonucleotide, called SAT-120 (5′-ATTTC TTCAT TTCAT GCTAG ACAGA AGAAT TCTCA GTAAC TTCTT TGTGC TGTGT GTATT CAACT CACAG AGTGG AACGT CCCTT TGCAC AGAGC AGATT TGAAA CACTC TTTTT GTAGT-3′), was used in the pull down assay for the protein transfer between ssDNA molecules. All DNA concentrations are expressed as molar nucleotide concentrations.

### Protein–protein binding assay

Rad51, Dmc1, Dmc182–340 and BSA were covalently conjugated to Affi-Gel 15 beads (100 µl; Bio-Rad), according to the manufacturer's instructions. The unbound proteins were removed by washing the beads for five times with binding buffer G (pH 7.5), which contained 20 mM HEPES–KOH, 0.15 M KCl, 0.5 mM EDTA, 2 mM 2ME, 10% glycerol and 0.05% Triton X-100. To block the residual active ester sites, ethanolamine (pH 8.0) was added to a final concentration of 100 mM, and the resin was incubated at 4°C for 1 h. After washing the resin three times with 500 µl of buffer G, the Affi-Gel 15-protein matrices were adjusted to 50% slurries with buffer G and were stored at 4°C.

For the binding assay, the Affi-Gel 15-protein slurry (20 µl) was mixed with 20 µg of Rad54B at room temperature for 2 h. The Affi-Gel 15-protein beads were then washed five

times with 500 µl of buffer G. SDS–PAGE sample buffer (2-fold) was mixed directly with the washed beads. After heating the mixture at 98°C for 5 min, the proteins were fractionated by 4–20% gradient SDS–PAGE. Bands were visualized by Coomassie brilliant blue staining.

### Immunoprecipitation experiments

The human Dmc1 and Rad54B cDNA were subcloned into the same pFastBac Dual vector (Invitrogen), and a recombinant baculovirus containing both the Dmc1 and Rad54B genes was generated as described previously. Sf9 insect cells were infected with this baculovirus, and were harvested after 48 h. The cells were then resuspended in buffer G, and were disrupted by sonication. The resulting cell lysate was incubated with 10 µl of anti-Dmc1 or Rad54B antibody conjugated to rProtein A–Sepharose Fast Flow (GE Healthcare) at 4°C for 1 h. The beads were washed five times with 500 µl of phosphate-buffered saline containing 1% NP-40, and were eluted by the addition of SDS–PAGE sample buffer (2-fold). The eluted fractions were separated by 4–20% gradient SDS–PAGE, and were blotted on to a polyvinylidene fluoride membrane. The proteins transferred on the membrane were analyzed by immunoblotting. The rabbit anti-Dmc1 antibody was prepared in this study, and the preparation of the rabbit anti-Rad54B antibody was described previously (46).

### DNA strand exchange assay

All of the experiments were performed in a final volume of 10 µl of buffer H, containing 35 mM Tris–HCl (pH 7.8), 1 mM DTT, 2 mM ATP, 2.5 mM $MgCl_2$, 20 mM creatine phosphate and 75 µg/ml creatine kinase. The reactions were started by incubating 7.5 µM Dmc1 with 30 µM ϕX174 circular ssDNA at 37°C for 5 min. Then, 2 µM RPA and 200 mM KCl were added, and after a 5 min incubation at 37°C, 22 µM ϕX174 linear dsDNA and 0.025–1.6 µM Rad54B were incorporated to initiate the reaction. After incubations at 37°C for the indicated times, the reactions were terminated by the addition of 0.5% SDS and 700 µg/ml proteinase K, followed by an incubation at 37°C for 20 min. After 10-fold loading dye was added, the products were resolved by 1% agarose gel electrophoresis in TAE buffer at 3.3 V/cm for 2.5 h, and were visualized by staining with ethidium bromide.

### Protein transfer assay (1): using biotinylated-ssDNA molecules and streptavidin beads

Dmc1 (5 µM) was incubated for 5 min at 37°C with 20 µM SAT-120 (120mer) labeled with biotin at the 5′ end, in 80 µl of buffer I, containing 35 mM Tris–HCl (pH 7.8), 1 mM DTT, 2 mM ATP and 2.5 mM $MgCl_2$. The reaction mixture was then divided in two, and a 5 µl aliquot of Rad54B (200 nM) or buffer B was added. After an incubation at 37°C for 5 min, a 5 µl aliquot of ϕX174 circular ssDNA (2 mM) was added, and the reaction was incubated at 37°C for 2 h. The DNA–protein complexes were captured with 50 µl of ImmunoPure Immobilized Streptavidin Gel (PIERCE) equilibrated with buffer I with 0.1% Triton X-100. After an incubation at 4°C for 1 h, the reaction mixture was divided into the beads and the supernatant by centrifugation. After adding 2-fold SDS–PAGE sample buffer and heating the mixture at 98°C for 5 min, the supernatant was fractionated by 4–20% gradient SDS–PAGE. Bands were visualized by Coomassie brilliant blue staining.

### Protein transfer assay (2): resolving protein–DNA complexes on agarose gels

For the assay, 10 µM Dmc1 was incubated with 20 µM ϕX174 circular ssDNA at 37°C for 5 min, in 80 µl of buffer H. The reaction mixture was then divided in two, and a 5 µl aliquot of Rad54B (200 nM) or buffer B was added. After an incubation at 37°C for 5 min, these mixtures were incubated with various concentrations of ϕX174 ssDNA fragments (0–200 µM) at 37°C for 2 h. After 10-fold loading dye was added, the products were resolved by 1% agarose gel electrophoresis in TAE buffer at 3.3 V/cm for 2.5 h and were visualized by staining with ethidium bromide.

### Electron microscopic analysis

Dmc1 (10 µM) was incubated with 15 µM SAT-120 (120mer) at 37°C for 5 min, in 10 µl of buffer J, containing 25 mM Tris–HCl (pH 7.5), 1 mM DTT, 2 mM ATP and 5 mM $MgCl_2$. Then, 200 mM KCl was added, and after a 5 min incubation at 37°C, 0.4 µM Rad54B was incorporated. The protein–DNA complexes were fixed with 0.2% glutaraldehyde at 37°C for 10 min. After a 100-fold dilution with buffer J, the reaction mixture was negatively stained with 2% uranyl acetate. The complexes were observed with a JEOL JEM 2000FX electron microscope.

## RESULTS

### Purification of Rad54B and its interactions with Rad51 and Dmc1

First, we examined whether Rad54B physically interacts with Rad51 and Dmc1, because recent studies revealed that Rad54B interacts with these proteins (22,38), in contrast to our previous results (39). To do so, we newly subcloned Rad54B into pFastBac1, which lacks a $His_6$-tag site, and employed an improved Rad54B purification method. Rad54B was expressed in baculovirus-infected High Five insect cells. The cell lysate was treated with ammonium sulfate (Figure 1A, lane 2). The precipitate was redissolved and further purified by phosphocellulose column chromatography (Figure 1A, lane 3), Q-Sepharose column chromatography (Figure 1A, lane 4), SP–Sepharose column chromatography (Figure 1A, lane 5) and hydroxyapatite column chromatography (Figure 1A, lane 6). About 1 mg of purified Rad54B was obtained from 3 liters of High Five insect cell suspension culture.

To examine the interaction, Rad51 and Dmc1, separately conjugated on Affigel 15 beads (Rad51-beads and Dmc1-beads, respectively), were incubated with purified Rad54B, and the protein bound to either the Rad51 beads or the Dmc1 beads was detected by SDS–PAGE. As shown in Figure 1B, Rad54B interacted with both Dmc1 and Rad51 (lanes 3 and 4). These results are consistent with the previous data (22,38,46). Furthermore, Rad54B interacted with a Dmc1 mutant ($Dmc1_{82–340}$) lacking 81 amino acid residues

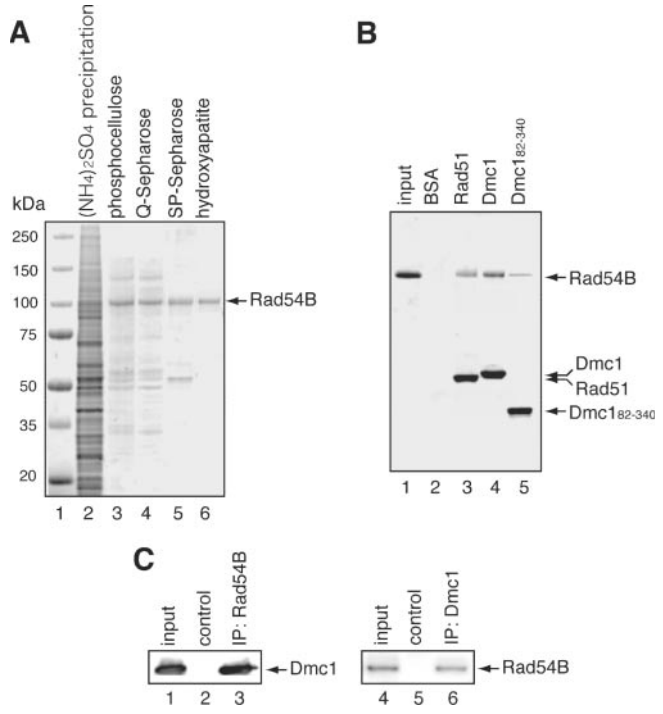


**Figure 1.** Rad54B purification and complex formation. (**A**) SDS–PAGE of the column fractions containing Rad54B. The proteins precipitated by ammonium sulfate (lane 2), the phosphocellulose peak fraction (lane 3), the Q-Sepharose flow-through (lane 4), the SP–Sepharose peak fraction (lane 5) and the hydroxyapatite peak fraction (lane 6) were fractionated on a 4–20% SDS–PAGE gel and stained with Coomassie brilliant blue. Lane 1 indicates the molecular mass markers. (**B**) Protein–protein interaction assay of Rad54B with Rad51, Dmc1 and $Dmc1_{82–340}$. Rad54B was mixed with either Rad51, Dmc1 or $Dmc1_{82–340}$ that was covalently conjugated to an Affi-Gel 15 matrix. After an incubation at room temperature, the Affi-Gel 15 matrices were washed with binding buffer, directly mixed with 2-fold SDS–PAGE sample buffer and fractionated on a 4–20% gradient SDS–PAGE gel. Lane 1 is one-tenth (2 μg) of the input proteins, lane 2 is the negative control using the Affi-Gel 15 matrix conjugated with BSA, lane 3 is the Affi-Gel 15 matrix conjugated with Rad51, lane 4 is the Affi-Gel 15 matrix conjugated with Dmc1 and lane 5 is the Affi-Gel 15 matrix conjugated with $Dmc1_{82–340}$. The bands were visualized by Coomassie brilliant blue staining. Notably, the Rad51, Dmc1 and $Dmc1_{82–340}$ proteins form multimers and thus, these proteins can indirectly associate with the Affi-Gel matrix via interactions with proteins crosslinked to the matrix. Rad51, Dmc1 and $Dmc1_{82–340}$ detected in lanes 3–5 represent fractions that were not covalently conjugated to the Affi-Gel beads. In contrast, BSA was not detected (lane 2), since BSA does not form multimers, and all proteins were covalently conjugated to the beads. (**C**) Immunoprecipitation (IP) experiments of Rad54B and Dmc1. Sf9 insect cells were infected with a baculovirus containing both the Dmc1 and Rad54B genes. The cells were lysed and subjected to immunoprecipitation with anti-Rad54B antibody (lane 3) or anti-Dmc1 antibody (lane 6). The precipitates were analyzed by immunoblotting. As negative control experiments, the cell lysate was incubated with normal IgG (lanes 2 and 5). Lanes 1 and 4 are one-tenth of the input cell lysate.

from the N-terminus (Figure 1B, lane 5). The region encompassing amino acids 82–340 of Dmc1 corresponds to the core ATPase domain (45), which is structurally conserved among the RecA-family proteins, such as eukaryotic Rad51 and Dmc1 (43,47,48), archaeal RadA (49,50) and bacterial RecA (51). Therefore, the data suggest that the core ATPase domains of Rad51 and Dmc1 are a common target for Rad54B binding. Notably, when we performed this assay using $His_6$-tagged Rad54B purified according to the present purification method, which differs from that described previously (39), the same results were observed (data not shown). Hence, our previous result was not related to the $His_6$-tag fused to the N-terminus of Rad54B. Instead, the difference was probably due to the difference in the purification methods.

To gain further evidence that Rad54B actually interacts with Dmc1, Rad54B and Dmc1 were co-expressed in Sf9 insect cells, and immunoprecipitation was performed. When the cell lysate was mixed with anti-Rad54B antibody-conjugated beads, Dmc1 co-precipitated (Figure 1C, lane 3). Similarly, Rad54B was co-precipitated by anti-Dmc1 antibody-conjugated beads (Figure 1C, lane 6). Therefore, these results demonstrate that Rad54B and Dmc1 can interact in living cells.

### Stimulation of the Dmc1-mediated DNA strand exchange by Rad54B

We next examined whether Rad54B affects the DNA strand exchange activity of Dmc1. In this assay, ϕX174 circular ssDNA and homologous linear dsDNA were used as substrates (Figure 2A), and the reactions were conducted in the presence of RPA. Dmc1 was first incubated with ssDNA, and then dsDNA was added into the reaction mixture (standard procedure, Figure 2B, lane 2). A nicked circular duplex is generated when complete strand transfer takes place (Figure 2A). Rad54B itself did not promote strand exchange (Figure 2B, lane 7). When Rad54B was added to the reaction mixture along with the linear dsDNA substrate in the standard procedure, the Dmc1-mediated strand exchange was significantly enhanced (Figure 2B–E). In the experiments presented here, we observed that only sub-stoichiometric amounts of Rad54B (0.025 μM) were required to stimulate the Dmc1-mediated strand exchange (7.5 μM Dmc1). Therefore, Rad54B may act as a trigger in the conversion of the Dmc1–DNA complex from an inactive to an active form.

### Rad54B stabilizes the Dmc1–ssDNA complex

Mazin *et al.* (52) demonstrated that Rad51 filaments on ssDNA are stabilized by Rad54, and proposed that this is essential for the stimulation of the Rad51-mediated strand exchange. We therefore investigated whether Rad54B stabilizes the Dmc1–ssDNA complex, by monitoring the transfer of Dmc1 molecules preassembled on ssDNA to a competitor DNA, using two different experimental procedures.

First, we performed a pull down assay using biotinylated DNA and streptavidin beads. In this assay, Dmc1 was assembled on a biotinylated 120mer ssDNA (SAT-120), and was incubated in the presence or absence of Rad54B, followed by the addition of ϕX174 circular ssDNA as a competitor. After SAT-120 was immobilized on the streptavidin beads, the reaction mixture was divided into the beads and the supernatant (Figure 3A). The supernatant was fractionated on a 4–20% gradient SDS–PAGE gel (Figure 3B). When we performed this assay without competitor DNA, there was essentially no Dmc1 detected in the supernatant (data not shown), indicating that Dmc1 was bound to SAT-120. In the presence of competitor DNA, Dmc1 was detectable in the supernatant, indicating that Dmc1 transferred from the preassembled Dmc1–ssDNA complex to the competitor DNA. The amount of Dmc1 transferred in the presence of Rad54B was ~3-fold

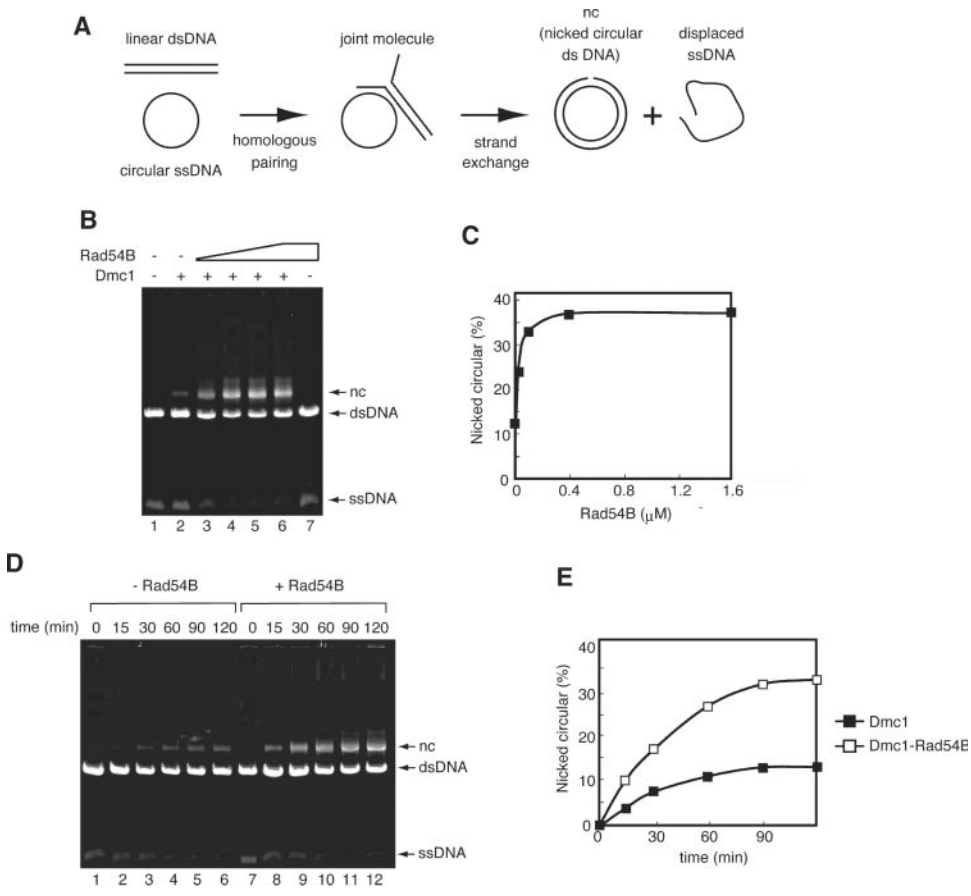


**Figure 2.** Stimulation of the Dmc1-mediated DNA strand exchange activity by Rad54B. (**A**) Schematic representation of the DNA strand exchange assay. The nucleoprotein filament is formed by incubating Dmc1 with circular ssDNA. The filament is paired with homologous linear dsDNA to yield a joint molecule. A nicked circular duplex (nc) and linear ssDNA are then generated by completing strand exchange over the length of the DNA molecules. (**B**) Dmc1-mediated DNA strand exchange activity with Rad54B. A constant amount of Dmc1 (7.5 μM) was incubated for 2 h with ϕX174 circular ssDNA (30 μM), ϕX174 linear dsDNA (22 μM), RPA (2 μM), KCl (200 mM) and increasing concentrations of Rad54B (0, 0.025, 0.1, 0.4 and 1.6 μM in lanes 2–6, respectively), in the order described for the procedure at 37°C. In lane 1, ssDNA and dsDNA were incubated in buffer with RPA and KCl, but without other recombinant proteins, and in lane 7, ssDNA and dsDNA were incubated in buffer with RPA, KCl and Rad54B (1.6 μM), but no Dmc1. The reaction mixtures were deproteinized, fractionated on a 1% agarose gel and stained with ethidium bromide. (**C**) Graphic representation of the experiments shown in (B). The amounts of nicked circular duplex are presented. (**D**) Time course experiments for DNA strand exchange. Dmc1 (7.5 μM) was incubated with ϕX174 circular ssDNA (30 μM), ϕX174 linear dsDNA (22 μM), RPA (2 μM) and KCl (200 mM), in the presence (open squares) or absence (closed squares) of Rad54B (0.4 μM), in the order described for the procedure at 37°C, for the indicated times. (**E**) Graphic representation of the experiments shown in (D). The amounts of nicked circular duplex are graphically presented.

less than that in the absence of Rad54B (Figure 3C), implying that Rad54B prevented the Dmc1–ssDNA complex from dissociating.

Second, a gel mobility shift assay was carried out to confirm that Rad54B actually stabilizes the Dmc1–ssDNA complex. Dmc1 was assembled on ϕX174 circular ssDNA, and was incubated in the presence or absence of Rad54B, followed by the addition of ϕX174 ssDNA fragments (~500 bases) as a competitor. In this assay, by monitoring the migration distance of the ϕX174 circular ssDNA, we investigated whether the transfer of Dmc1 occurred. In the absence of Rad54B, increasing the concentration of the competitor DNA resulted in the faster mobility of the Dmc1–ssDNA complex on the agarose gel (Figure 4, lanes 2–6). This result indicates that the Dmc1 had transferred from the preassembled Dmc1–ssDNA complex to the competitor DNA. In contrast, in the presence of Rad54B, the migration distances of the Dmc1–ssDNA–Rad54B complexes did not change upon titration with excess amounts of the competitor DNA, indicating that Rad54B inhibited the transfer of Dmc1 from the preassembled complex to the competitor DNA (Figure 4, lanes 7–11). Therefore, Rad54B stabilizes the Dmc1–ssDNA complex.

## Rad54B associates with the terminus of the Dmc1–ssDNA helical filament

We next used electron microscopy to examine whether the nature of the Dmc1–ssDNA complex would change by the addition of Rad54B. In both the absence and presence of Rad54B, the Dmc1–ssDNA helical filament was observed (Figure 5A and B). However, in the presence of Rad54B, we observed Dmc1–ssDNA filaments with a mass of ~20 nm associated with the termini (Figure 5C, closed arrow). We did not detect such protein–DNA complexes without Rad54B, implying that Rad54B associated with the terminus of the Dmc1–ssDNA filament. Interestingly, Rad54 preferentially associates with the terminus of the

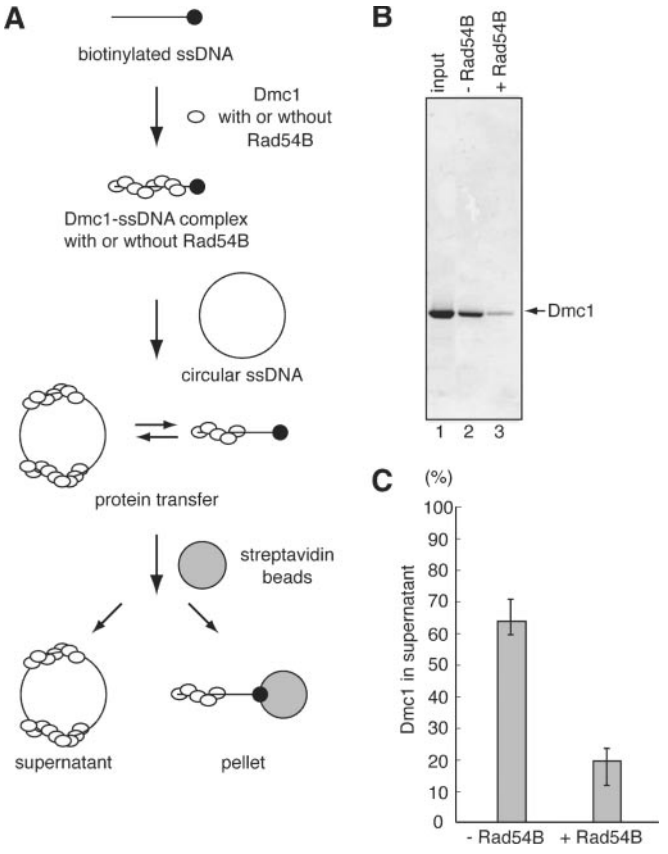


**Figure 3.** Pull down assay for the protein transfer between ssDNA molecules. (**A**) A schematic diagram of the pull down assay. Dmc1 and biotinylated DNA form a complex in the absence or presence of Rad54B. Then, circular ssDNA is added as a competitor, and protein transfer occurs. Biotinylated DNA is immobilized on streptavidin beads, and the reaction mixture is divided into the beads and supernatant. (**B**) Dmc1 (5 μM) was incubated with SAT-120 (20 μM) labeled with biotin at the 5′ end in the absence (lane 2) or presence (lane 3) of Rad54B (200 nM), followed by an incubation with ϕX174 circular ssDNA (2 mM). After immobilization on streptavidin beads for 1 h at 4°C, the reaction mixture was divided into the beads and the supernatant by centrifugation. Then, one-tenth of the supernatant was fractionated on a 4–20% gradient SDS–PAGE gel. Lane 1 is one-tenth of the input protein. (**C**) Graphic representation of the experiments shown in (B). The amounts of Dmc1 within the supernatant are presented.

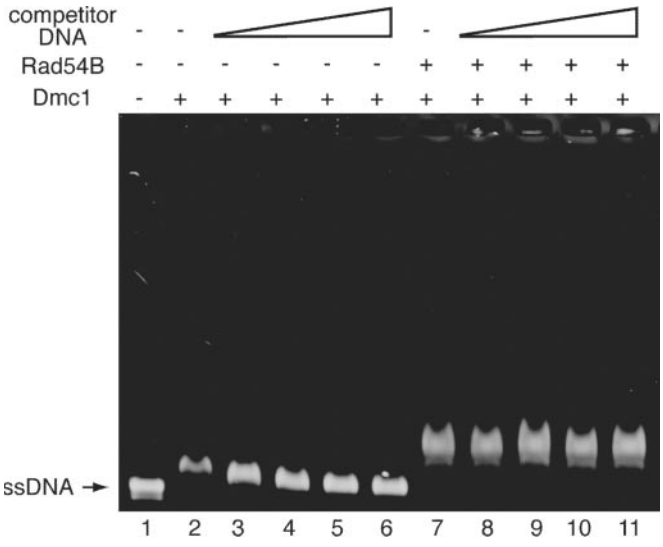


**Figure 4.** Gel mobility shift assay for the protein transfer between ssDNA molecules. A constant amount of Dmc1 (10 μM) was incubated with ϕX174 circular ssDNA (20 μM) in the absence (lanes 2–6) or presence (lanes 7–11) of Rad54B (200 nM), followed by an incubation with increasing amounts of ϕX174 ssDNA fragments (0, 25, 50, 100 and 200 μM in lanes 2–6 and lanes 7–11, respectively) at 37°C for 2 h. In lane 1, ϕX174 circular ssDNA was incubated in buffer without any recombinant proteins.

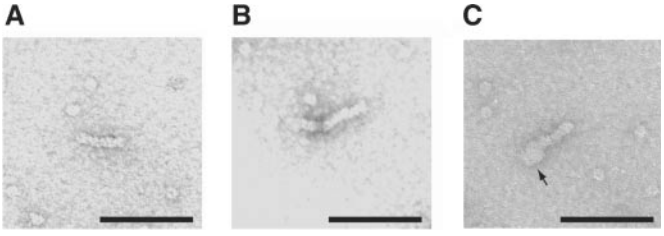


**Figure 5.** Electron microscopic images of Rad54B and Dmc1 with ssDNA. Images of the Dmc1–ssDNA helical filament in the absence (**A**) or presence (**B**) of Rad54B, and the Rad54B–Dmc1–ssDNA complex (**C**) were photographed after negative staining with uranyl acetate. The closed bar denotes 100 nm.

Rad51–dsDNA helical filament (53). This terminal association of Rad54B may stabilize the Dmc1–ssDNA filament.

## DISCUSSION

The yeast and human Rad54 proteins stimulate strand exchange by Rad51 (54,55), but, thus far, the activation of Dmc1-mediated strand exchange by the Rad54 homologs has not been reported. The present findings suggest that the human Rad54B protein stimulates the Dmc1-mediated strand exchange, probably through the stabilization of the presynaptic filament formed by ssDNA and Dmc1. Recent studies have shown that Rad54B enhances D-loop formation by Dmc1 and Rad51 (22,38). Taken together, Rad54B assists Dmc1 in the initial strand invasion step (homologous pairing), as well as in the extension of the heteroduplex region (strand exchange). To stabilize the Dmc1–ssDNA complex, catalytic amounts of Rad54B were sufficient. Consistent with this result, we observed that Rad54B associated with the termini of the Dmc1–ssDNA filament, by an electron microscopic analysis. These observations suggest that Rad54B may stabilize the Dmc1–ssDNA complex by interacting with the terminal region of the Dmc1–ssDNA complex.

How could Rad54B stabilize the Dmc1–ssDNA complexes? One possibility is that Rad54B could prevent the Dmc1–ssDNA nucleoprotein filament from disassembling by binding to one end of the filament. This may lead to the unidirectional assembly of the Dmc1–ssDNA nucleoprotein filament at the DSB site. The second possibility is that by physically interacting with Dmc1, Rad54B could alter the conformation of the Dmc1–ssDNA nucleoprotein filament from an inactive form to an active form. Multiple studies have indicated that the fundamental mechanism of Dmc1-mediated recombination is the same as that of the RecA homologs, suggesting that Dmc1 forms helical filaments when

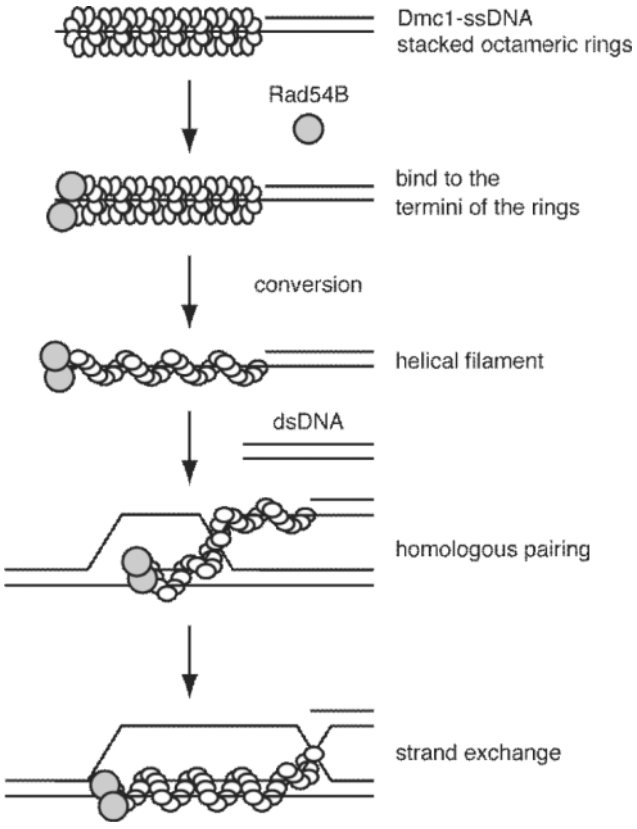


**Figure 6.** Model of Rad54B converting the Dmc1–DNA complex from the octameric ring form to the helical filament form. Rad54B interacts with terminus of the stacked octameric rings of the Dmc1–DNA complex, causing a conversion into the helical-filament form. This conversion results in the stabilization of the Dmc1–ssDNA complex, and the stimulation of the DNA strand exchange promoted by Dmc1.

performing the strand exchange reaction (22,45,48,56–58). However, Dmc1 forms octameric rings alone and on DNA (59), and the conversion from rings to filaments is probably essential for the proper function of Dmc1. A recent study indicated that ATP binding may act as a trigger in the conversion of the Dmc1 octameric ring form to the helical filament form (43). We found that Rad54B bound to the isolated ATPase domain of Dmc1, and this interaction may assist in changing in the conformation of the Dmc1 monomer to favor the formation of the helical filament, thus stabilizing the complex. Based on these possibilities, we propose the following model (Figure 6), which explains the mechanism of DNA strand exchange promoted by Rad54B and Dmc1. First, Rad54B associates with the terminus of the stacked rings, and binds to the ATPase domain of Dmc1. This process may catalytically prevent the Dmc1–ssDNA filament from disassembling or promote the conversion of the Dmc1–DNA complex from stacked octameric rings to helical filaments. The Dmc1–ssDNA nucleoprotein filament, which is probably the active form for the strand exchange reaction, then invades the homologous duplex DNA, forming a heteroduplex DNA intermediate.

Recent studies have shown that Rad54 has a potent translocase activity and stimulates the branch migration activity of Rad51 (55,60). These activities suggest that Rad54 is involved in the post-synaptic phase of recombination. Although the previous and present Rad54B results have suggested its involvement in the presynaptic phase of recombination, it is easy to imagine that Rad54B functions in the post-synaptic phase of recombination, such as Rad54. Further analyses of the effects of Rad54B on Dmc1 complexed with various DNA structures representing recombination intermediates may provide clues toward understanding the precise mechanism of the Rad54B-stimulated homologous recombination.

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