Rad51 Protein Stimulates the Branch Migration Activity of Rad54 Protein*\(]^{\text{S}}\)

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The Rad51 and Rad54 proteins play important roles during homologous recombination in eukaryotes. Rad51 forms a nucleoprotein filament on single-stranded DNA and performs the initial steps of double strand break repair. Rad54 belongs to the Swi2/Snf2 family of ATP-dependent DNA translocases. We previously showed that Rad54 promotes branch migration of Holliday junctions. Here we find that human Rad51 (hRad51) significantly stimulates the branch migration activity of hRad54. The stimulation appears to be evolutionarily conserved, as yeast Rad51 also stimulates the branch migration activity of yeast Rad54. We further investigated the mechanism of this stimulation. Our results demonstrate that the stimulation of hRad54-promoted branch migration by hRad51 is driven by specific protein-protein interactions, and the active form of the hRad51 filament is more stimulatory than the inactive one. The current results support the hypothesis that the hRad51 conformation state has a strong effect on interaction with hRad54 and ultimately on the function of hRad54 in homologous recombination.

Homologous recombination (HR)\(^{\text{3}}\) is the process responsible for repairing double strand DNA breaks, exchanging genetic material, and promoting faithful segregation of homologous chromosomes (1). In humans, deficiencies in HR dramatically increase the incidents of cancer, premature aging diseases, and genome instability (2, 3). In eukaryotes, HR is initiated at the site of DNA double-stranded breaks (Fig. 1A). Then enzymes process the broken dsDNA ends into DNA duplexes with protruding 3’-ssDNA tails. Next, Rad51 protein forms a helical nucleoprotein filament on the ssDNA tails. The filament promotes the initial steps of HR, including the search for homologous dsDNA and DNA strand exchange, which lead to formation of D-loops, a key intermediate of HR. D-loops provide both the template and the primer for DNA polymerase to retrieve the information lost at the site of DNA breaks and create an overhang required for annealing of the broken DNA ends (4, 5). After primer extension, D-loops are processed further through one of the two principal mechanisms (Fig. 1B). In the first mechanism, synthesis-dependent strand annealing (SDSA), D-loops dissociate from the intact chromosome followed by re-joining of DNA ends. In the second mechanism, double strand break repair (DSBR), D-loops are processed through the capture of the second processed DNA end to produce double Holliday junctions, which are later resolved by structure-specific endonucleases (6). The factors that determine a choice between these major pathways of HR have yet to be determined.

Rad54 plays an important role in HR. The Rad54 protein is a member of the Swi2/Snf2 family of DNA-dependent ATPases that are known for their chromatin remodeling activity (7, 8). Indeed, several Rad54 orthologs were shown to possess chromatin remodeling activity in vitro (8–11). In addition, Rad54 has a number of activities that are involved in different stages of HR (12, 13). Yeast and human Rad54 proteins form a ternary complex with their cognate Rad51 proteins (14, 15). In this complex, Rad54 stabilizes the Rad51 nucleoprotein filament and stimulates DNA strand exchange activity (16–18). Reciprocally, Rad51 was shown to stimulate the dsDNA-dependent ATP hydrolysis and chromatin remodeling action of Rad54 (8, 19, 20).

Previously, we showed that human and yeast Rad54 binds specifically to Holliday junctions and promotes their branch migration (BM) (21). We further found that BM requires formation of a catalytically active multimeric hRad54 complexes containing 8–12 monomeric units of hRad54 protein (22). But the question remains, what is the function of Rad54 BM activity in homologous recombination? We previously suggested hRad54 BM activity can promote dissociation of D-loops, a step required for further re-joining of the broken DNA ends along the SDSA mechanism of HR. Indeed, we found that hRad54 can promote dissociation of D-loops through its BM activity (21). However, our data also indicated that hRad54 protein is only able to promote dissociation of D-loops if the filament is in the inactive ADP bound form (23). We previously showed that the hRad51-ssDNA filament conformation undergoes transition from an active ATP-bound form to an inactive ADP-bound form and that this transition can be modulated by Ca\(^{2+}\) (24).
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Here we investigated the interaction of hRad54 with model HR intermediates, in which the hRad51-ssDNA filament was maintained in the active ATP-bound form. Our results demonstrate that hRad54 can extend these native model D-loops. Moreover, our data demonstrate that the active hRad51-ssDNA filament stimulates DNA BM activity of hRad54. We also investigated the mechanism of the stimulation. Our results suggest that hRad51 stimulates BM by hRad54 through specific protein-protein interactions. Furthermore, Ca\(^{2+}\), which activates hRad51-ssDNA filament, also increases the stimulatory effect of hRad51 on hRad54. Overall, our current results support the hypothesis that the hRad51 conformation has a strong effect on the function of hRad54 BM activity by channeling HR intermediates toward either the DSBR or SDSA pathways, and thereby promoting formation of either crossover or non-crossover recombinants.

**EXPERIMENTAL PROCEDURES**

**Proteins and DNA**—Human Rad54, Rad54 K189R, Rad51, and Rad51 K133R were purified as described previously (18, 25). Human Rad51 K133A was provided by P. Sung (Yale); Saccharomyces cerevisiae Rad54 and Rad51 were provided by S. Kowalczykowski (UC Davis).

All oligonucleotides (IDT, Inc) used in this study (supplemental Table S1) were purified by electrophoresis in 6–10% polyacrylamide gels containing 50% urea. The concentrations of the purified oligonucleotides were determined spectrophotometrically using extinction coefficients provided by the manufacturer. To prepare PX-junctions, complementary ssDNA oligonucleotides were annealed as described (26) and stored at −20 °C. Oligonucleotides were labeled using \([γ-\text{P}]\)ATP and T4 polynucleotide kinase, as described previously (16).

**BM Assay**—To produce PX-junctions for the 3-stranded BM reaction, the hRad51 protein (10 μM) was incubated with ssDNA (no. 201; 1.45 μM molecules) in standard BM buffer containing 35 mM Tris-HCl, pH 7.5, 2.5 mM ATP, 1 mM DTT, 100 μg/ml BSA, an ATP-regenerating system (10 units/ml creatine phosphokinase and 15 mM creatine phosphate), 3 mM MgCl\(_2\), and 5 mM CaCl\(_2\) (unless indicated otherwise) for 15 min at 37 °C. Then \(32\text{P}\)-labeled 5′-flap DNA (no. 71/196/197/201; 1.33 μM molecules) was added, and incubation was continued for 15 min at 30 °C to form the PX-junction (no. 71/196/197/201). The total reaction volume was 20 μl. To produce PX-junctions for the 4-stranded BM reaction, the hRad51 protein (10 μM) was incubated with tailed DNA (no. 170/171; 1.45 μM, molecules) in BM buffer for 15 min at 37 °C. Then \(32\text{P}\)-labeled fork DNA (no. 71/169; 1.33 μM molecules) was added, and incubation was continued for 15 min at 30 °C forming the PX-junction (no. 71/169/170/171) in a total reaction volume of 20 μl. PX junctions were constructed in such a way (by incorporating a region of heterology; see Figs. 2A and 3A) that allowed BM only in one direction, which simulated the extension of joint molecules during HR.

To initiate BM, the hRad54 protein was added to the reaction mixtures containing hRad51 protein-PX-junction complexes. The final concentrations were 250 nM hRad51 protein, 100 nM of hRad54, 33 nM (molecules) of PX-junctions in standard BM buffer (unless otherwise indicated). The reactions were carried out for indicated periods of time at 30 °C in a total reaction volume of 60 μl. The same experimental conditions were used when human proteins were substituted with yRad51 and yRad54. In experiments from Fig. 5, the DNA substrates concentrations were decreased to 20 nm, and the reaction conditions were altered (temperature was decreased to 20 °C and CaCl\(_2\) concentration to 3 mM) to facilitate measurements of the reaction rates in the presence and absence of hRad51 protein. In experiments from Fig. 7, the reaction temperature was lowered to 25 °C to extinguate the difference between reaction rates in the presence of mutant hRad51 proteins. Aliquots (10 μl) were withdrawn, and DNA substrates were deproteinized by treatment with stop buffer (1.4% SDS, 960 μg/ml proteinase K, 7.5% glycerol, 0.015% bromphenol blue) for 5 min at 22 °C. DNA products were analyzed by electrophoresis in 8% polyacrylamide gels (29:1) in 1× TBE buffer (89 mM Tris borate, pH 8.3, and 1 mM EDTA) at 22 °C. The gels were dried on DE81 chromatography paper (Whatman) and quantified using a Storm 840 PhosphorImager (Molecular Dynamics).
Spectrophotometric ATPase Assay—The hydrolysis of ATP by Rad54 protein was monitored spectrophotometrically as described previously (27). The oxidation of NADH, coupled to ADP phosphorylation, resulted in a decrease in absorbance at 340 nm, which was continuously monitored by a Hewlett-Packard 8453 diode array spectrophotometer using UV-Visible ChemStation software. The rate of ATP hydrolysis was calculated from the rate of change in absorbance using the following formula: rate of $A_{340}$ decrease (s$^{-1}$) $\times$ 9880 = rate of ATP hydrolysis (μM/min). The reactions in standard buffer containing 35 mM Tris-HCl, pH 7.5, 3 mM MgCl$_2$, 5 mM CaCl$_2$, 1 mM DTT, 2.5 mM ATP, 3 mM phosphoenolpyruvate, pyruvate kinase (20 units/ml), lactate dehydrogenase (20 units/ml), and NADH (200 μg/ml), and the indicated concentrations of Rad54 protein and DNA were carried out at 30 °C.

Thin Layer Chromatography (TLC) ATPase Assay—4-Strand PX-junction complexes in the presence or absence of hRad51 were prepared as described above for the BM assay in standard buffer containing CaCl$_2$ at indicated concentrations and 20 nCi of [$\gamma$-32P]ATP. The hRad54 protein (60 nM) was incubated with the complexes formed by hRad51 (250 nM) with PX-junctions (33 nM, molecules) in BM buffer for 5 min at 30 °C. The products of ATP hydrolysis were analyzed by TLC on PEI-cellulose plates in a running buffer of 0.3M KH$_2$PO$_4$, pH 7.0. The extent of ATP hydrolysis was determined using a Storm 840 PhosphorImager.

D-loop Disruption Assay—hRad51 protein (5 μM) was incubated with 32P-labeled tailed DNA (no. 199/209; 150 nM molecules) in buffer containing 35 mM Tris-HCl, pH 7.5, 1 mM ATP, 2 mM DTT, 100 μg/ml BSA, and 1 mM CaCl$_2$ for 15 min at 37 °C. D-loop formation was initiated by the addition of pUC19 supercoiled DNA (250 μM, nucleotides) for 15 min at 37 °C. D-loops were then diluted 5-fold in buffer containing 35 mM Tris-HCl, pH 7.5, 2.5 mM ATP, 1 mM DTT, 100 μg/ml BSA, the ATP-regenerating system (10 units/ml creatine phosphokinase and 15 mM creatine phosphate), 3 mM MgCl$_2$, indicated CaCl$_2$ and incubated for 5 min at 37 °C. Disruption of D-loops (50 μM nucleotides, 9.3 nM molecules) containing hRad51 protein (1 μM) was initiated by additions of hRad54 protein (100 nM) for indicated periods of time at 37 °C. Aliquots (10 μl) were withdrawn, and DNA substrates were deproteinized by treatment with stop buffer (1.4% SDS, 960 μg/ml proteinase K, 7.5% glycerol, 0.015% bromphenol blue) for 5 min at 22 °C. The products were analyzed by electrophoresis in a 1% agarose gel. The gels were dried on DE81 chromatography paper (Whatman) and quantified using a Storm 840 PhosphorImager.

RESULTS

Rad51 Protein Efficiently Stimulates 3-Strand and 4-Strand BM Activity of Cognate Rad54 Protein—Previously, we showed that hRad54 can disrupt native D-loops containing hRad51 protein through its ATPase-dependent BM activity (23). However, D-loop disruption could only occur when the filament was in an inactive ADP-bound state, e.g. in the absence of calcium ion. Here, we investigated the effect of the active hRad51 filament on the BM activity of hRad54. We constructed oligonucleotide DNA substrates that mimicked the product of ssDNA invasion in homologous duplex DNA at the initial step of HR. For this purpose, we prepared PX-junctions using the annealing activity of hRad51 protein. First, the hRad51 filament was formed with either tailed dsDNA or ssDNA and then forked or 5’-flap DNA were added, respectively. Rad51-promoted annealing created two types of PX-junctions (Figs. 2A and 3A) These PX-junctions were prepared as described above for the BM assay in standard buffer containing CaCl$_2$ at indicated concentrations and 20 nCi of [$\gamma$-32P]ATP. The hRad54 protein (60 nM) was incubated with the complexes formed by hRad51 (250 nM) with PX-junctions (33 nM, molecules) in BM buffer for 5 min at 30 °C. The products of ATP hydrolysis were analyzed by TLC on PEI-cellulose plates in a running buffer of 0.3M KH$_2$PO$_4$, pH 7.0. The extent of ATP hydrolysis was determined using a Storm 840 PhosphorImager.

Effect of hRad51 Concentration on the Stimulation of hRad54 BM Activity—Here, we wish to determine the hRad51 stoichiometry that is required for stimulation of the hRad54 BM activity. Human Rad51, at varied concentrations, was mixed with tailed dsDNA- or ssDNA-forming nucleoprotein filaments. These substrates were then annealed to complementary fork or 5’-flap DNA producing PX-junctions (Figs. 2A and 3A). These substrates were used to determine the rates of BM promoted by hRad54 in 4-strand and 3-strand reactions (Fig. 4). We found that at a constant concentration of hRad54 (100 nM), maximal stimulation of the rate of hRad54-promoted BM occurred at relatively low concentrations of hRad54 (250 nM) for both the 3-strand and 4-strand reaction. Given that a hRad51 monomer binds three nucleotides of ssDNA or three base pairs of dsDNA, maximal stimulation occurs under conditions where the hRad51 filament covers approximately one-quarter the length of the ssDNA or tailed DNA substrates. Human Rad51 has comparable affinity for ss- and dsDNA, therefore hRad51 should bind randomly along the DNA substrate. Under sub-saturating concentrations of hRad51, the number and length of filaments on any given substrates may vary. Further increase in hRad51 concentration caused an inhibition of the rate (Fig. 4) and, especially, the extent (data not shown) of hRad54-promoted BM. Apparently, the higher hRad51 concentrations resulted in a population of substrates that hRad54 cannot branch migrate. Thus, the hRad51 efficiently stimulates...
hRad54 BM activity in 3-strand and 4-strand reactions at sub-stoichiometric concentrations relative to the concentrations of DNA substrates.

Effect of the hRad54 Concentration on BM in the Presence of hRad51—We previously showed that maximal BM activity requires 10 ± 2 monomers of hRad54 per molecule of DNA (22). The data suggested that during BM two hRad54 subcomplexes, 4–6 protein monomers each, bind to the two opposing DNA arms of the Holliday junction (or PX-junction) forming a catalytically active complex. Here, to better understand the mechanism of stimulation of hRad54 BM activity by hRad51 we wanted to examine whether the hRad51 that is bound to the PX-junction has an effect on the optimal hRad54 stoichiometry. The experiments were performed at 20 °C for more accurate measurements of the rate of BM, as we previously showed that the temperature has no significant affect on the hRad54 stoichiometry (22). In accordance with our previous results, in the absence of hRad51 we observed 10.5 ± 1.5 monomers of hRad54 per one PX-junction molecule were required to achieve the maximal initial rate of BM (Fig. 5, circles). However, in the presence of hRad51 a significantly lower hRad54 stoichiometry of 4.5 ± 1.5 monomers per PX-junction molecule was required for the maximal rate of BM (Fig. 5, squares). Thus, hRad51 reduces the amount of hRad54 that is required for the maximal rate of BM approximately by a factor of two.

Human Rad51, but Not Yeast Rad51, Promotes Interspecies Stimulation of Rad54 BM Activity—To investigate the role of protein-protein interactions between Rad51 and Rad54 in stimulation of BM activity, we tested the ability of the yRad51 nucleoprotein filament to stimulate the BM activity of hRad54. Human Rad54 BM activity was not stimulated by the presence of yRad51 in the 3-stranded reaction (Fig. 6A). We then tested the ability of hRad51 to stimulate BM activity of yRad54. Surprisingly, we found that the initial rate of BM activity of yRad54 was stimulated 4-fold by hRad51 (Fig. 6B). Thus, the initial rate of BM for yRad54 was the same in the presence of yRad51 or hRad51 (compare Figs. 3C and 6B). However, the extent of the reaction was lower for hRad51, indicating the presence of a population of substrates containing hRad51 that yRad54 cannot branch-migrate.

We wanted to determine whether the specificities of hRad51 and yRad51 in stimulation of BM activity correlate with their specificities in stimulation of the ATPase activities of Rad54. Consistent with a previous report by Kiianitsa et al. (28), the results we obtained using a spectrophotometric ATPase assay showed that the hRad51 nucleoprotein filament formed on a PX-junction stimulates the ATPase activity of yRad54 (Fig. 6C). In contrast, we found that the yRad51 nucleoprotein filament does not stimulate the ATPase activity of hRad54. Thus, hRad51 and yRad51 showed the same specificities in stimulation of the BM and ATPase activities of hRad54 and yRad54...
Rad51 and Rad54 proteins (17, 18, 29). These data indicate that different types of Rad54–Rad51 complexes are required for DNA strand exchange and BM.

**Stimulation of hRad54 BM Activity Is Not Dependent on the ATPase Activity of hRad51**—We used two hRad51 ATPase-deficient mutants to determine the role its ATPase activity plays in stimulating the BM activity of hRad54. In these proteins, the conserved Walker type A lysine was mutated to either arginine (K133R) or alanine (K133A). Although both of these mutants lack ATPase activity, the hRad51 K133R protein retains the ability to form an active nucleoprotein filament that promotes DNA strand exchange, but the hRad51 K133A protein forms a filament that is inactive in DNA strand exchange (30).

When included in our 4-strand BM assay, both hRad51 K133R and wild-type hRad51 stimulated the BM activity of hRad54 to a similar extent both at 25 °C (Fig. 7, open circles and closed triangles) and at 30 °C (data not shown). Previously, it was shown that wild-type and ATPase-deficient Rad51K191R mutant proteins equally stimulate the ATPase activity of Rad54 (29, 31). Thus, stimulation of Rad54 BM activity by Rad51 parallels with stimulation of the ATPase activity.

However, stimulation of the rate of hRad54 BM by hRad51 K133A was only half as efficient as wild-type hRad51 (Fig. 7, open triangles). Thus, the ATPase activity of hRad51 is not required to stimulate the BM activity of hRad54, but the conformation of the filament does play a role. We further found that in BM, the ATPase activity of hRad54 does not compensate for the lack of the ATPase activity of hRad54; no BM activity of the ATPase-deficient hRad54 K189R mutant was observed in the presence of hRad51. Similarly, when a non-hydrolyzable ATP analog, AMP-PNP, was used, BM did not occur in the presence or absence of hRad51 (21). Thus, ATP hydrolysis by hRad54, but not hRad51, is required for BM.

**Ca$$^2+$$ Enhances the Stimulatory Effect of hRad51 on hRad54 BM Activity**—We previously showed that calcium ion is required for stimulation of the DNA strand exchange activity of hRad51 by hRad54 (18). Here, we asked whether Ca$$^2+$$ also affects the ability of hRad51 to stimulate the BM activity of hRad54. We found that in the presence of Mg$$^2+$$ alone, hRad51 produces a 4-fold stimulation of the rate of hRad54-dependent BM in the 4-strand reaction, compared with an 15-fold stimulation observed in the presence of both Ca$$^2+$$ and Mg$$^2+$$ (Fig. 8A). However, in the absence of hRad51, Ca$$^2+$$ causes inhibition of the BM activity of hRad54 (Fig. 8A, squares), paralleling the inhibitory effect of Ca$$^2+$$ on hRad54 ATPase activity (18) (Fig. 8C, circles). Ca$$^2+$$ showed a concentration-dependent effect on hRad51 and yeast Rad54, yRad51 only stimulates the activities of yRad54 and not hRad54 (compare Figs. 3C and 6A). Still, interactions between yRad54 and the hRad51 filament are apparently not specific enough to stimulate the DNA strand exchange activity of hRad51; yRad54 could not stimulate the D-loop formation activity of hRad51 under tested conditions.\(^3\)

\(^3\) M. J. Rossi and A. V. Mazin, unpublished observations.

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**FIGURE 3.** Both human and yeast Rad51 stimulate the BM activity of Rad54 in a 3-strand BM reaction. A, the scheme of the 3-strand BM. 4-base pair heterologies (denoted by AATA and CAGC) were introduced into the PX-junction to block protein-independent BM. B, kinetics of hRad54-dependent 3-strand BM. The hRad51 filaments were formed by incubation of hRad51 protein (250 nM) with ssDNA (no. 201) (36 nM, molecules) in standard buffer. The yRad51-DNA complexes were then incubated with 5'-flap DNA to produce 3-stranded PX-junctions (nos. 71/196/197/201; 33 nM, molecules). The BM was initiated by the addition of hRad54 protein (100 nM). C, the kinetics of yRad54-dependent 3-strand BM. The reaction conditions were identical to those described in B, except human proteins were replaced with their yeast counterparts. Experiments were repeated at least three times. Error bars represent S.E.

**FIGURE 4.** The effect of hRad51 concentration on stimulation of BM activity in 3- and 4-strand reactions. PX-junctions (33 nM molecules) were constructed in the presence of varying concentrations of hRad51 under standard conditions. BM was initiated by the addition of hRad54 (100 nM), and time points were taken over a 10-min interval. For each concentration of hRad51 protein, the initial rate of BM was determined using the linear part of the kinetic curve during the first 3 min of the reaction. Data are the mean of at least three experiments. Error bars represent S.E.
the ability of the hRad51 filament to stimulate the BM activity of hRad54. In the presence of hRad51 and 3 mM Mg2+/H11001, the rate of the BM activity of hRad54 doubles when the Ca2+/H11001 concentration increased from 0 to 5 mM (Fig. 8B, squares). But over the same range in the absence of hRad51, the rate of BM activity of hRad54 dropped 4-fold (Fig. 8B, circles). The stimulation was specific for Ca2+/H11001, because there was no increase in the rate of BM in the presence of hRad51 when the concentration of Mg2+/H11001 was raised to 8 mM (data not shown). Because hRad54 BM activity showed dependence on ATP hydrolysis, we also examined the effect of Ca2+/H11001 on the ATPase activity of hRad54 using a TLC ATPase assay and our oligonucleotide-derived PX-junctions as a substrate. We found that in the absence of hRad51, an increase in Ca2+/H11001 concentration from 0 to 5 mM causes ~1.5-fold inhibition in the extent of ATP hydrolysis, whereas in the presence of hRad51 we observed no significant change in ATP hydrolysis by hRad54 (Fig. 8C). These data show that the hRad51 filament causes greater stimulation of hRad54 activities or are made more resistant to inhibition when the filament is in the Ca2+/H11001-stabilized, active (ATP-bound) conformation.

In parallel, we found that the increase in Ca2+ concentration causes a gradual increase in the ability of hRad51 to protect D-loops against disruption by hRad54 (Fig. 9). Thus, Ca2+ both increases the stimulatory effect of hRad51 on hRad54 BM activity and inhibits disruption of the D-loops by hRad54.

DISCUSSION

Synergistic interaction of Rad51 and Rad54 proteins is critically important for efficient HR. During the early stages of DNA double strand break repair, the hRad51 filament forms a nucleoprotein filament on ssDNA that promotes a critical invasion step resulting in the D-loop formation (Fig. 1A). Rad54 greatly stimulates DNA pairing activity of Rad51. After D-loop extension by DNA polymerase, these HR intermediates are thought to proceed via two pathways, SDSA or DSBR (Fig. 1B). The SDSA involves dissociation of the D-loops and restoration of the broken DNA molecule by annealing of tailed dsDNA molecules (6). The DSBR requires extension of the D-loops by BM,
our current results show that both active and inactive filament protects joint molecules from dissociation by hRad54, through its own BM activity.

of hRad51, which would be expected if hRad51 contributes to the stimulation of BM does not depend on the ATPase activity in the presence of an ATPase-deficient hRad54. Third, our experiments (21). Second, hRad51 does not show any BM conditions in the absence of hRad54 within the time frame of (32), hRad51 does not show BM activity under our reaction although BM activity of hRad51 has recently been reported an additive effect of BM activities of hRad51 and hRad54. First, D-loops is in an inactive (ADP-bound) form. Here, we modeled the invasion event promoted by hRad51-ssDNA filament using synthetic DNA substrates and investigated the effect of an active (ATP bound) and an inactive hRad51 filament on the BM activity of hRad54 (Figs. 2A and 3A).

Here, we found that the hRad51 filament stimulates the BM activity of hRad54. The stimulation was observed in both 4-stranded and 3-stranded BM reactions and showed no dependence on a particular branched DNA structure, e.g. X-junctions or PX-junctions. Stimulation of Rad54 BM by Rad51 appeared to be evolutionarily conserved, as yRad51 also stimulates BM activity of yRad54. These results indicate that the role of the active hRad51 filament is not limited to protection of joint molecules against dissociation by hRad54 or BLM (23). By stimulating the hRad54 BM activity the active hRad51-ssDNA filament directs it toward an extension of joint molecules. Thus, the conformation of the hRad51 may affect the choice of the HR pathways, e.g. promoting the DSB or channel recombination intermediates instead of the SDSA.

The observed increase in the BM activity could not be due to an additive effect of BM activities of hRad51 and hRad54. First, although BM activity of hRad51 has recently been reported (32), hRad51 does not show BM activity under our reaction conditions in the absence of hRad54 within the time frame of our experiments (21). Second, hRad51 does not show any BM activity in the presence of an ATPase-deficient hRad54. Third, the stimulation of BM does not depend on the ATPase activity of hRad51, which would be expected if hRad51 contributes through its own BM activity.

Whereas we found previously that only an active hRad51 filament protects joint molecules from dissociation by hRad54, our current results show that both active and inactive filament can stimulate BM activity of hRad54. Still, the stimulation was greater in the presence of an active hRad51 filament. The hRad51 K133R mutant protein, which forms an active nucleoprotein filament, was ~2-fold more efficient in stimulation of the rate of hRad54 BM than hRad51 K133A mutant, which forms an inactive filament. Similarly, stimulation of hRad54 BM by wild-type hRad51 increased ~2-fold when the reaction mixture was supplemented with Ca^{2+}, which promotes formation of an active hRad51 filament (24). Thus, greater stimula-
Rad54 and Rad51 form different complexes during DNA strand exchange and BM.

We found that BM on branched DNA substrates fully saturated with hRad51 was inefficient. Maximal stimulation of the rate of BM activity occurred under subsaturating concentrations of hRad51; ~1 hRad51 monomer per 12 nucleotides of ssDNA or tailed DNA substrate. An increase in hRad51 concentration caused inhibition of the extent of BM and, at higher hRad51 concentrations, of the initial rate of BM. Taken together, these data suggest a model in which efficient stimulation of BM by hRad54 results from binding of hRad54 to the end of the invading hRad51 filament and promotion of BM toward the DNA region that is not covered with hRad51. It was recently reported that yRad54 shows preference for association with the terminal part of the filament formed on linear dsDNA (34). This preference may be greatly enhanced at the DNA junction created by the filament invasion, to which Rad54 has ~200-fold greater binding affinity compared with linear dsDNA.

Our results show that while stimulating the BM activity of hRad54, hRad51 also has a profound effect on the stoichiometry of hRad54 in BM. Previously, we found that the maximal initial BM rate for hRad54 was observed at a stoichiometry of 10.5 ± 1.5 hRad54 monomers per one PX-junction molecule. We suggested that in BM two multimeric (tetra- or hexameric) complexes of hRad54 bind symmetrically to the opposite dsDNA arms flanking the Holliday junction. Our data indicated that the formation of these multimeric BM complexes is initiated by binding of hRad54 dimer to the HJ (22). This parallels with recent observation by Muzzolini et al. (35), which related different activities of RECQ1 helicase with different quaternary forms of the protein. Our current results show that in the presence of hRad51 the apparent stoichiometry of hRad54 was reduced by a factor of approximately two, yielding 4.5 ± 1.5 hRad54 monomers per PX-junction molecule. We propose the following interpretation of this result: when PX-junctions (or D-loops) are formed through invasion of the hRad51-ssDNA filament, hRad51 protein bound to one arm of DNA flanking the junction may support and stabilize initial binding of a hRad54 monomer via protein-protein interactions, providing the nucleation for assembly of a single hRad54 multimeric (tetra- or hexameric) complex at the junction.

Our current studies demonstrate that Rad51 protein stimulates the BM activity of Rad54. Thus, BM activity of Rad54 is added to the ATPase and chromatin remodeling activities of Rad54, which stimulation by hRad51 has been reported earlier. It was recently shown in S. cerevisiae that interaction between Rad51 and Rad54 can be regulated by a meiosis-specific mediator protein called Hed1 (36). Hed1 appears to block the binding of yRad54 to yRad51; thereby shutting HR intermediates through a Dmc1-dependent pathway that ensures the proper level of crossover products occur between homologous chromosomes during meiosis. We found that the conformation of the hRad51 filament also plays an important role in interactions of the filament with hRad54, which may lead to either dissociation or expansion of joint molecules and thereby contribute to the choice between the SDSA and DSBR pathways during HR, thereby providing the cell with another mechanism of regulation.
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REFERENCES

1. Krogh, B. O., and Symington, L. S. (2004) *Annu. Rev. Genet.* **38**, 233–271
2. Wyman, C., Ristic, D., and Kanaar, R. (2004) *DNA Repair (Amst.*) **3**, 827–833
3. West, S. C. (2003) *Nat. Rev. Mol. Cell Biol.* **4**, 349–404
4. Thoma, N. H., Czyzewski, B. K., Alexeev, A. A., Mazin, A. V., Kowalczykowski, S. C., and Pavletich, N. P. (2005) *Nat. Struct. Mol. Biol.* **12**, 350–356
5. Alexiadis, V., Lusser, A., and Kadonaga, J. T. (2004) *J. Biol. Chem.* **279**, 27824–27829
6. Zhang, Z., Fan, H. Y., Goldman, J. A., and Kingston, R. E. (2007) *Nat. Struct. Mol. Biol.* **35**, 4124–4140
7. Muzzolini, L., Beuron, F., Patwardhan, A., Popuri, V., Cui, S., Niccolini, B., Rappas, M., Freemont, P. S., and Vindigni, A. (2007) *PLoS Biol.* **5**, e20
8. Busygina, V., Sehorn, M. G., Shi, I. Y., Tsoubouchi, H., Roeder, G. S., and Sung, P. (2008) *Genes Dev.* **22**, 786–795