Published in final edited form as:

J Mol Biol. 2007 November 30; 374(3): 618–640.

Rad54 oligomers translocate and cross-bridge double-stranded DNA to stimulate synapsis

Piero R. Bianco^{1,*}, Justin J. Bradfield¹, Lauren R. Castanza², and Andrea N. Donnelly¹

¹ Department of Microbiology and Immunology, Centre for Single Molecule Biophysics, 321 Cary Hall, University at Buffalo, SUNY, Buffalo, NY 14214

² Deceased

Abstract

Rad54 is a key component of the eukaryotic recombination machinery. Its presence in DNA strand exchange reactions *in vitro* results in a significant stimulation in the overall reaction rate. Using untagged Rad54, we show that this stimulation can be attributed to enhancement of the formation of a key reaction intermediate known as DNA networks. Using a novel, single DNA molecule, dual-optical tweezers approach we show how Rad54 stimulates DNA network formation. We discovered that Rad54 oligomers possess a unique ability to cross-bridge or bind dsDNA molecules positioned in close proximity. Further, Rad54 oligomers rapidly translocate dsDNA while simultaneously inducing topological loops in the DNA at the locus of the oligomer. The combination of the cross-bridging and dsDNA translocation activities of Rad54 stimulates the formation of DNA networks, leading to rapid and efficient DNA strand exchange by Rad51.

Introduction

The efficient repair of DNA double-strand breaks (DSBs) is critical to the maintenance of genome stability and cell survival. DSBs can result from exposure to exogenous sources such as ionizing radiation or from events leading to replication fork collapse ^{1–3}. In eukaryotes, DSBs can be repaired by two distinct pathways: non-homologous end joining or homologous recombination ³. These pathways differ in their requirement for a homologous DNA template and the fidelity of the repair reaction. As homologous recombination uses an intact, sister duplex as the template for repair, the reaction occurs with high fidelity ⁴.

Advances of our understanding of the biochemical mechanism of homologous recombination *in vitro* have been made possible through the DNA strand exchange reaction ⁵. Studies with *E. coli* RecA demonstrate that this reaction can be divided into three kinetically distinguishable phases: presynapsis, synapsis, and post synapsis (Figure 1, reviewed in ⁶). During presynapsis, RecA binds to single-stranded DNA (ssDNA) to form a helical nucleoprotein filament. In synapsis, the filament binds to double-stranded DNA (dsDNA) and a rapid and efficient search for homology occurs within the dsDNA recipient resulting in the formation of a joint molecule. Once homology is located, RecA catalyzes the exchange of strands and branch migration ensues to yield nicked circle products. Critical to efficient pairing is the formation of homology-independent, three-dimensional DNA networks or coaggregates which are key intermediates

*Corresponding Author: Department of Microbiology and Immunology, Centre for Single Molecule Biophysics, 321 Cary Hall, University at Buffalo, SUNY, Buffalo, NY 14214, E-mail: pbianco@buffalo.edu, Telephone: 716-829-2599, Fax: 716-829-2158.

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that form directly on the reaction pathway⁷. These coaggregates form faster than joint molecules, include the majority of DNA present in reaction mixtures (*i.e.*, >90%) and yield products an order of magnitude faster than they exchange with one another or with free molecules in solution^{7; 8}.

In contrast to RecA, the structurally related eukaryotic homologue Rad51, is less efficient in performing DNA strand exchange^{9–11}. The inability of Rad51 to efficiently drive this reaction may result from very low rates of ATP hydrolysis producing less dynamic filaments⁶, extreme sensitivity to small heterologous sequences¹² and, an inability to stimulate the formation of DNA networks as only 10% of DNA is found in coaggregates¹³. However, Rad51-mediated DNA strand exchange becomes efficient and robust with rates increasing >20-fold in the presence of mediator proteins such as Rad54^{13–17}.

Rad54, a member of the Swi2/Snf2 protein family, is a key component of the recombination machinery^{2; 18}. *In vitro*, Rad54 is a dsDNA-dependent ATPase using the energy from ATP hydrolysis to translocate DNA, inducing topological changes in the process^{13; 19–23}. The architecture of Rad54–DNA complexes indicates that the active form of Rad54 is an oligomer that induces negative and positive supercoils into the DNA during translocation, potentially making the target DNA more accessible to Rad51, although this is yet to be rigorously demonstrated^{23; 24}. The size of homo-multimeric Rad54 oligomers on DNA ranges in size from a dimer to particles >40 nm in diameter, containing several monomers^{23; 24}. These oligomers associate with Rad51 nucleoprotein filaments either along the length of the filament^{17; 25} or at filament ends²⁴ (Figure 1). Binding to Rad51 results in filament stabilization and the proposed role of Rad54 within the complex is to translocate or pump dsDNA past Rad51 thereby facilitating the homology search^{17; 24–26}. Rad54 also exhibits ATP-dependent chromatin remodeling activity which is proposed to function during DNA strand exchange on chromatin templates to displace nucleosomes^{27; 28}. Following the exchange of strands, Rad54 persists on the DNA and stimulates heteroduplex formation^{29; 30}, followed by the displacement of Rad51³¹. Thus, Rad54 is a multifaceted DNA motor acting during all phases of DNA strand exchange³².

We used both bulk-phase and single DNA molecule approaches to understand how Rad54 could possibly perform such a variety of roles. First, using bulk-phase assays, we show that untagged Rad54 stimulates DNA networks at least 15-fold. Second, we used a novel single molecule approach to observe DNA network formation in real-time. The results are complex, revealing dsDNA translocation, formation of topological loops in the DNA and the binding of adjacent DNA molecules in close proximity using a novel property of Rad54 we term cross-bridging. To understand each of these activities, we studied them separately on single molecules of DNA. We observed rapid and processive translocation of Rad54 oligomers on dsDNA, coincident with topological changes being introduced into the DNA. These changes are intrinsic to efficient Rad54 translocation. Dual-optical tweezers experiments allowed us to observe for the first time, the previously undiscovered cross-bridging activity of Rad54 oligomers. The combined results reveal a clear role for Rad54 in synapsis where it stimulates the formation of a key reaction intermediate: DNA networks.

Results and Discussion

Rad54 stimulates the formation of DNA networks

As Rad51 is inefficient in the formation of DNA networks¹³, we wanted to determine whether Rad54 could stimulate their formation leading to an overall increase in the DNA strand exchange reaction rate. To measure DNA networks, we used the well-established coaggregate assay (Figure 2a)^{7; 8; 33}. We reasoned that if stimulation of DNA networks is intrinsic to Rad54, a significant fraction of the input 5'-end labeled dsDNA should be recovered in pellets

or coaggregates following centrifugation at low G-forces, the hallmark of DNA network formation.

The GST-tag is widely used in Rad54 studies during protein purification and is frequently not removed thereafter. The presence of the tag results in a reduced K_m for ATP binding and a 10-fold higher k_{cat} for ATP hydrolysis for the GST-tagged protein relative to the untagged version (data not shown and^{19; 34}). Further, the GST-tag can induce unrelated, nonspecific aggregation of DNA as it is known to alter DNA binding properties of several enzymes^{35; 36}. Previous work using GST-Rad54 demonstrated a 7-fold stimulation in DNA networks relative to the Rad51 only reaction with approximately 70% of the input DNA being found in coaggregates¹³. However in the GST-Rad54 only reactions, 50% of the input DNA was recovered in the pellet fraction, versus 10% in the Rad51-only reactions. Thus the stimulation that is observed in the Rad51-Rad54 reaction is only modest relative to the Rad54 only reaction (*i.e.*, 1.4-fold). Collectively, these data, and those we provide in this work, indicate a very strong affect of the GST tag on Rad54. Therefore, it is imperative to use untagged Rad54 here (and throughout this study).

Control coaggregates assays with Rad51 or untagged Rad54 demonstrates that these proteins acting independently of one another, are inefficient in network formation with 2.5% of the input dsDNA present in coaggregates in the presence of Rad51 only, and less than 1% being observed for Rad54 only (Figure 2b). In contrast, the addition of a catalytic amount of untagged Rad54 to preformed Rad51 filaments results in a 15-fold stimulation in the amount of coaggregates being formed. Therefore, our results demonstrate that the stimulation of DNA networks is intrinsic to Rad54 protein. Further, and an important point: catalytic amounts of untagged Rad54 do not promote DNA coaggregation in the absence of Rad51, whereas GST-Rad54 does.

To gain insight into how a translocating motor could stimulate DNA networks, we set out to visualize coaggregate formation in real-time. This was achieved by manipulating fluorescently-labeled, DNA-bead complexes trapped side-by-side, using dual optical tweezers in the first channel of a two-channel, micro-machined flow cell positioned on the stage of an inverted microscope (Figure 3a). Reaction buffer containing preformed, Rad51-Rad54 nucleoprotein filaments and ATP was introduced into the second channel under conditions of laminar flow, creating a situation where the two solutions flowed parallel to one another with minimal mixing. Once DNA-bead complexes were trapped side-by-side in separate optical traps, the stage was moved so that these complexes would relocate from the sample to the reaction side of the flow cell to initiate pairing (Figure 3a, left inset). In this experiment, to mimic the concurrent interactions that occur between multiple nucleoprotein filaments and several dsDNA molecules in the bulk-phase coaggregate assay, optically trapped DNA-bead complexes contained 3 to 7 DNA molecules per bead. To visualize the optically trapped DNA, the fluorescent dye YOYO-1 was bound at an average of 1 dye per 30 bp of DNA. This DNA:dye ratio does not inhibit the activity of Rad54 in bulk-phase ATPase assays (Supplementary Figure 1).

Not surprisingly, and as anticipated, the visualization of DNA networks in real-time reveals that these are complex structures and which we propose are equivalent to those obtained in the coaggregate assay (Fig. 3b). The formation of the networks results from at least the combination of the following series of events. The first invasion event of an optically trapped DNA molecule by a nucleoprotein filament (arrow 1), causes the DNA in the trap to twist (beads are free to rotate in the trap), bringing the DNA end distal to the bead into proximity with the neighboring DNA molecules in the adjacent trap. These DNA molecules subsequently adhere *via* a Rad54-dependent structure we call a cross-bridge (arrow 2) and the fluorescence intensity at the cross-bridge increases as additional DNA is translocated into its position. Additional invasion events occur (arrows 3 and 4), and subsequent dsDNA translocation by Rad54 contributes to the

formation of the collapsed, heterogeneous structure or DNA network. Additional invasion events, accompanied by translocation, results in dislodging of the DNA network from the traps. Although the resulting structure is complex, it is typical and representative of what we observe under these reaction conditions. It also provides visual evidence of two key activities of Rad54 at work in this process: dsDNA translocation and dsDNA cross-bridging.

To determine whether formation of these complexes requires Rad54, experiments were repeated using Rad51 nucleoprotein filaments only. Consistent with the bulk-phase coaggregate assay (Figure 2b), networks were not observed in the absence of Rad54 (data not shown; n=24). To show that these filaments were active for pairing, the solution containing them was removed from the syringe, a catalytic amount of Rad54 added and allowed to bind. Following the introduction of these Rad51-Rad54 filaments into the flow cell, DNA networks were again observed (data not shown; n=6). Therefore, DNA networks observed in both bulk-phase and single molecule assays are Rad54-dependent. As DNA networks are complex and to more clearly understand the Rad54 activities responsible for their formation, namely dsDNA translocation and cross-bridging, a separate study of each activity in the absence of Rad51 was conducted. The results are presented below.

Rad54 oligomers translocate dsDNA

Visualization of dsDNA translocation was achieved by manipulating fluorescent-labeled, single DNA-bead complexes using optical tweezers and the actions of Rad54 detected (Figure 3a; right inset)³⁷. Here, DNA-bead complexes containing 1 DNA per bead, were introduced into the first channel of the flow cell and translated into the second channel containing reaction buffer, Rad54 and ATP to initiate the reaction. After several seconds, a Rad54-dependent bright spot forms on each DNA molecule at a position distal to the bead in the flow. The bright spot then moves along that same DNA molecule towards the bead, concomitant with a decrease in the length of the polymer (Figure 4a). The bright spot consists of a Rad54 oligomer and dsDNA which is being translocated into the position of the oligomer resulting in protein-constrained DNA loops and is concomitant with displacement of an unknown fraction of the fluorescent dye (Figure 4a, inset). Evidence demonstrating oligomer and DNA loop formation is presented below.

Visualization of reactions reveals that the complexes of Rad54 (*i.e.*, bright spots) form at different times following translation of the DNA into the reaction channel (Figure 4). Once formed, motion is rapid (2,882 to 4,385 bp/sec for the two DNA molecules shown) and processive (N = 28 to 34 kbp per binding event), with the observed trajectories being unique (Figure 4b). Analysis of the motion of each translocating complex reveals that the trajectory consists of periods of decreasing velocity punctuated by periods of increasing velocity (Figure 4b; lower panel). In addition, a translocation impasse lasting from 14.1 to 16.5 sec was observed for the lower complex. During this period the velocity of the complex slows, it pauses, backtracks at a rate of 500 bp/sec, then reverses direction. This process is then repeated in a more dramatic fashion with the complex then moving forward at an increasingly rapid rate, stalls again and then continues moving forward reaching a maximum velocity of 7,980 bp/sec.

The net result of the changes in velocity is an almost periodical shape to the translocation trajectory. To determine whether the almost periodical behaviour is a general property of Rad54, analysis of eleven trajectories was done. The analysis shows that each is unique and that the movement consists of periods of decreasing velocity, followed by periods of increasing velocity (Figure 4c). For each complex, changes in velocity (*i.e.*, from slow to rapid and *vice versa*) occur at distinct positions on the DNA, indicating that they are not sequence-dependent. In addition, pausing and backtracking (*i.e.*, where the instantaneous velocity is negative) is observed for 83% of the trajectories shown. Backtracking is not due to fluctuations in the buffer

flow causing the bright spot to change position, since complexes can backtrack up to 1,300 bp, well beyond the noise in our analysis (174 ± 31 bp).

The above-mentioned phenomena were observed at 112 nM protein, our standard reaction condition. To determine if protein concentration could affect the resulting trajectories and rates, experiments were repeated at three separate concentrations: 11, 45 and 225 nM protein. The data show that trajectories are unique and the velocities of each complex exhibit similar undulating behavior as that observed at 112 nM protein (Fig. 4d). Further, the complex formed at 11 nM protein exhibits two periods of backtracking similar to that observed in Figure 4b. Therefore, the variations in rate are not protein concentration-dependent. Furthermore, velocity changes and backtracking are not unique to Rad54, having been observed for several distinct DNA motors^{38–40}. It is not clear what causes the changes in velocity for Rad54; this is currently under investigation.

Bright spots contain Rad54 oligomers

Rather than containing a single monomer, we interpret the bright spot that forms on dsDNA distal to the bead in the flow, to be a Rad54 oligomer of unknown size pulling or translocating dsDNA into its position. However, the observed phenomenon, that is formation of a bright spot followed by DNA shortening, could also be interpreted as DNA condensation resulting from protein binding along the length of the DNA, with the bright spot being a toroid similar to that observed for protamine condensation of single DNA molecules⁴¹. Therefore, it is critical to demonstrate that what is observed here is ATP-dependent translocation by a Rad54 oligomer.

Because Rad54 forms large oligomers on DNA^{23; 24} and to determine if the bright spots we observe contain a Rad54 oligomer, a protein titration was done on single molecules of DNA. If the bright spots contain Rad54 oligomers, then their rates of formation should be inversely related to protein concentration but once formed, oligomers should move at comparable speeds. Furthermore, as Rad54 is a DNA motor, bright spot motion should be ATP-dependent. The results show that at Rad54 concentrations <120 nM, bright spot motion is rapid ($4,346 \pm 1,705$ bp/sec) and ATP-dependent, with no discernible reaction occurring in the absence of ATP (Figure 5a). At 377 nM Rad54, the reaction occurred at a 4-fold slower rate relative to our standard concentration of 112 nM Rad54. We attribute the reduction in rate to Rad54 bound to internal sites on the DNA (between the moving complex and the bead) inhibiting the progress of the active oligomer (data supporting this statement is provided in a subsequent section).

Having established that bright spot motion is ATP-dependent, we analyzed the lag times for the assembly of complexes. Here, we define the lag as the time required for bright spot detection once a DNA molecule is translated from the sample to the reaction side of the flow cell. The results show that it takes on average 15 sec to form bright spots at 11 nM Rad54, whereas at 225 nM protein, lag time decreases 4-fold (Figure 5b). Once the bright spots form, they move at similar rates, consistent with them being oligomers of Rad54. Therefore, we conclude that the bright spots contain a Rad54 oligomer (of unknown size) that translocates dsDNA in an ATP-dependent manner. The formation of oligomers observed here is consistent with previous work showing that Rad54 forms variable-sized, active oligomers on dsDNA^{15; 23; 24}. We note that it is conceivable that the bright spots could contain either a single large oligomer >40 nm in diameter, or several smaller ones.

At elevated protein, Rad54 condenses DNA in the absence of ATP

The ATP-independent reactions observed at >350 nM Rad54 were unexpected (Fig. 5a). Furthermore, the rates were approaching that of the ATP-dependent reactions (1,100 bp/sec), which were on average almost 2-fold slower than those observed in the ATP-dependent portion of the protein titration. Analysis reveals that these reactions are very different from those

observed at ≤ 112 nM protein (Figure 6). First, the position data produces curves that are almost identical to one another (Fig. 6, top panel). Second, the curves are essentially biphasic with an initial rapid phase followed by longer slower phase. Third, the observed “processivity” for these reactions is significantly reduced compared to those shown in Figure 4. Fourth, the curves in the initial phase are linear with no evidence of pausing and/or backtracking, similar to that observed for DNA condensation by protamine (Fig. 6, top panel, inset)⁴¹. In fact, this portion of the curves can be fit to a straight line, with r^2 -values of >0.996 (data not shown). Fifth, during the rapid initial phase, the rate of DNA molecule shortening slowly increases to a maximum velocity of between 5 and 8,000 bp/sec. Thereafter, the rate rapidly decreases within 2 seconds to almost zero for some DNA molecules while for others, it decreases 4-fold and then slowly decreases to 0 (Fig. 6, lower panel, inset). The half-Lorentzian shapes of the curves in this portion of the reaction are consistent with protein binding leading to DNA condensation. The rate initially increases as more protein binds, and then rapidly declines as additional protein binds. The additional binding precludes complete collapse of the DNA molecule. Thus from the above-mentioned, we conclude that at elevated concentrations of protein, binding of Rad54 to DNA results in collapsing or condensing of DNA molecules in an ATP-independent manner.

Translocating Rad54 oligomers are dynamic

During motion, Rad54 oligomers translocate dsDNA into their position. In addition to changing in velocity and position, these protein and DNA complexes could be dynamic as reflected by changes in shape, size and fluorescence intensity during the course of motion along the length of the DNA. To determine if this was occurring, analysis of individual bright spots from the movies presented in Figure 4 was undertaken. This was achieved using the Object tracking function of Image Pro, which was set to simultaneously monitor oligomer position, aspect, area, fluorescence intensity and fluorescence heterogeneity. Aspect is the ratio of the longer dimension to the shorter dimension of the bright spot and was used monitor changes in shape of the translocating complex. The area reports changes in overall size of the complex as DNA is translocated into it. The density is an indication of the increase in fluorescence intensity as additional DNA is translocated into the locus of the oligomer, while fluorescence heterogeneity indicates the fraction of pixels within a bright spot that deviates more than 10% from the average intensity within that bright spot correlating with the position of the translocating Rad54 oligomer.

The results show that as anticipated, translocating complexes are dynamic. The position of the oligomer is shown for comparison in each of the panels of Figure 7 so that a direct comparison can be made to the data presented in Figure 4. For the DNA molecule shown in panels A, C and E of Figure 7 (the upper DNA in Figure 4a), a 2-fold variation in the aspect ratio occurs within the first two seconds of translocation. Thereafter, during the “elongation phase” of translocation, the aspect ratio decreases to 2.9 ± 0.4 (*i.e.*, is elliptical), remaining relatively unchanged until it encounters the bead. During the same time period, a 4-fold variation in aspect ratio for the molecule in panels B, D and F of Figure 7 (the lower DNA in Figure 4a), was observed. Thereafter, the complex stabilizes with an average aspect ratio of 1.7 ± 0.4 (*i.e.*, is almost spherical). This ratio is maintained until 10.4 sec, when it increases abruptly from 1.4 to 4.5, decreases back to 1.4 and then increases again to 3.6 immediately before encountering the bead. The changes in aspect are reflected predominantly in changes in the width of the complex with that length remaining virtually unchanged (data not shown). This suggests that the protein content may remain constant while the DNA character is constantly changing. Therefore, the variations in aspect ratio indicate that during translocation, the Rad54-DNA complex is continuously elongating and condensing, changing overall shape from a spherical to an elliptical structure.

During the course of the reaction for the upper DNA molecule, the density of fluorescence signal increases slowly with in the first 1.8 seconds on the reaction. It then increases rapidly reaching an average maximum density of 212 which is maintained until the complex collides with the bead (Fig. 7C). Simultaneously, the area of this complex increases linearly in size until 6.4 sec, where a 70% increase in the area occurs. In contrast, during the first three seconds on the reaction for the lower DNA molecule the complex is observed to move very rapidly and simultaneously increases very rapidly in fluorescence intensity (Fig. 7D). The density remains constant for an additional two seconds during which time the translocation impasse is reached. At this point the fluorescence signal within the locus of the oligomer decreases as the complex backtracks. In parallel with density, the area of this oligomer increases linearly in size until 3.8 sec and then remains unchanged for an additional 2.1 sec. It then decreases in area over a 1.3 sec period and then resumes increasing in area until it encounters the bead. A similar large fluctuation in area is observed immediately before encountering the bead. The fluctuation most likely reflects interference from the fluorescent dye bound to the beads resulting in inaccurate measurements. The somewhat monotonous increase in area observed for the complex in panel E correlates well with the monotonous translocation trajectory. Similarly, the large fluctuations in area for the oligomer in panel F correlate with the translocation impasse observed between 4 and 6.5 sec. Further, the decrease in area correlates with the period during which the complex is observed to pause and backtrack several hundred base pairs. Since only the DNA is visible in this experiment, the decrease in area that occurs during backtracking is consistent with DNA being released from the oligomer.

Finally, and in addition to changes in shape, size and accumulation of fluorescence signal, the fluorescence intensity within the translocating complexes also varies during the course of the translocation reaction as indicated by the level of heterogeneity (Fig. 7E). As for area, the level of heterogeneity correlates with the translocation stage, varying as much as 5-fold within the first 2 seconds of translocation and then stabilizing for the remainder of the trajectory, i.e., possibly indicating a steady-state for translocation (Fig. 7F). The fluctuations in fluorescence intensity result from increases in intensity as dsDNA is translocated into the locus of the oligomer, concomitant with the loss of YOYO-1 as DNA strands are partially separated (evidence provided in a subsequent section). Thus, this analysis shows that the translocating Rad54 oligomers are dynamic, varying in size and shape during the course of a translocation reaction.

Translocation requires ATP hydrolysis

The data in Figures 4, and 5a and b, support the conclusion that the dynamic bright spots contain Rad54 oligomers and that their formation and subsequent motion is ATP-dependent. If these are actively translocating Rad54 oligomers and not DNA-condensing complexes, then motion should require ATP hydrolysis. Therefore, single DNA molecule translocation experiments were done to determine whether competitive inhibitors of the ATPase activity of Rad54, namely ADP, AMP-PNP or ATP γ S, could inhibit motion (Table I). As magnesium ions displace YOYO-1 from the DNA (data not shown and ³⁷), it is necessary to maintain the ratios of nucleoside to metal ions in the experiment at approximately 1. Therefore these reactions contained 2 mM MgOAc, 2 mM ATP and 200 μ M of each competitive inhibitor. In the presence of ATP γ S, no change in the DNA was observed, identical to that observed in the absence of ATP (Table I). In the presence of either ADP or AMPPNP, bright spots formed less frequently than in the presence of ATP (41 and 21%, respectively) and of these, only a very small number translocated (0 for ADP and 3% for AMPPNP). Because formation of Rad54 oligomers on dsDNA does not require a nucleoside triphosphate cofactor^{14; 24}, the bright spots observed here are not simply the result of the protein binding to the DNA leading to DNA condensation. Instead, they are the result of ATP hydrolysis-dependent, motor action.

Previous work showed that Rad54 hydrolyzes ATP efficiently in the presence of Mn^{2+} or Mg^{2+} ions and that the rate was affected by magnesium concentration²⁰. Consistent with this work, we demonstrate that Rad54 oligomers translocate dsDNA at comparable rates in the presence of either metal cation. Further, the rate of translocation decreased 2.8-fold when magnesium concentration was lowered from 1 to 0.5 mM (Table I).

As DNA translocation requires ATP hydrolysis, the rate of motion should be dependent on the concentration of ATP and the apparent affinity for ATP in single molecule translocation experiments should be comparable to the K_m^{ATP} observed in bulk-phase assays in the presence of linear dsDNA. To address this issue, bulk-phase rates of ATP hydrolysis on linear dsDNA were measured and compared to single molecule translocation rates (Figure 8a). Both data sets were well approximated by the Michaelis-Menten equation consistent with previous results^{19; 34}. Further, the apparent K_m^{ATP} in single molecule translocation experiments and the bulk-phase K_m^{ATP} is, within experimental error, the same (Figure 8a). Taken together, these data demonstrate that the bright spots are actively translocating Rad54 oligomers which require ATP hydrolysis for motion.

Translocating Rad54 oligomers induce topological changes in dsDNA

Previous work has shown that Rad54 induces positive supercoils ahead of protein movement and negative supercoils trailing it^{15; 21; 23}. These supercoils are sensitive to cleavage by topoisomerases. Further, the negative supercoils produce transient strand separation and DNA within these regions is sensitive to cleavage by the single-strand specific nuclease P1²¹. To determine the topological state of the DNA at the locus of Rad54 oligomers observed here, single DNA molecule translocation experiments were performed in the presence of Wheat Germ topoisomerase (WGT) or P1 nuclease. WGT was used as it cleaves both positively and negatively supercoiled DNA and P1 was used to determine whether the DNA contained transient, single-stranded regions.

Control reactions with either WGT or P1 alone, produced no discernible changes in optically trapped DNA (data not shown). In the presence of either WGT or P1, Rad54 oligomers formed on 100% of the DNA molecules visualized, but their resulting behavior was significantly different from the control reactions performed with Rad54 alone (Table I; 50 nM Rad54). In the presence of WGT, 94% of the bright spots disintegrated less than 0.5 sec after being detected, with the remainder translocating <5kB and stopping permanently (Table I). Similarly, in the presence of P1, 80% of bright spots disintegrated shortly after being detected, with the remaining 20% either not moving at all, or translocating with reduced processivity (*i.e.*, ≤ 10 kB) before disintegrating. As the DNA preparations used in this study are ~20% nicked (data not shown), the abolishment of translocation is not simply the presence of a discontinuity in one strand of the DNA causing translocating oligomers to derail when a nick is encountered. Instead, strand cleavage of DNA within loops at the location of the Rad54 oligomer by WGT or P1 abolishes translocation. Therefore, the DNA within bright spots or dynamic Rad54 oligomers is both topologically constrained and partially strand separated, consistent with previous bulk-phase results^{15; 21}.

Rad54 is a robust ATPase^{13; 20} and it is reasonable therefore to think that some fraction of the observed bulk-phase ATPase activity may be associated with topological changes in the DNA that arise during translocation. To test this, we added P1 nuclease to ongoing Rad54 ATPase assays on linear dsDNA. This resulted in an 11-fold reduction in k_{cat} (Figure 8b), suggesting that a significant fraction of the ATPase activity associated with Rad54 results from oligomers inducing conformational changes in the DNA during translocation. The reduction caused by P1 nuclease is specific to topological changes induced in the DNA by Rad54, as addition of P1 to ATPase assays containing *E. coli* RecG (which does not induce topological changes in dsDNA during translocation) does not affect k_{cat} (Fig. 8b and⁴²). Since the

relaxation of Rad54-induced supercoils by WGT or cleavage of the resulting, transient ssDNA regions by P1 nuclease abolishes translocation, we conclude that DNA supercoil formation is intrinsic to efficient translocation by Rad54 oligomers.

Translocation initiates in the flexible regions of the DNA

Rad54 is a dsDNA motor that is expected to bind nonspecifically at any position along the length of a DNA molecule as observed previously¹⁹. Unexpectedly, translocation observed here initiates on the DNA distal to the bead in the flow and progresses from that position towards the bead and against the direction of buffer flow. This site of visualization of the initiation of translocation arises due to flow-induced extension of the DNA resulting in the tension on the DNA being maximal closest to, and minimal furthest from the bead^{43; 44}. Therefore, the DNA is rigid proximal to, and flexible distal to the bead in the flow. As a result, Rad54 initiates efficient translocation in the flexible region of the stretched DNA molecule (*i.e.*, at the position distal to the bead in the flow). This may arise due to Rad54 binding internally and subsequently translocating without visibly altering the DNA¹⁹. Once in the vicinity of the distal end, the Rad54 oligomer progresses towards the bead, inducing a visible alteration in the DNA that we require to observe motion. The flexible portion of the DNA moves with the Rad54 oligomer, permitting continued movement toward the bead and is concomitant with displacement of some fraction of the dye and shortening of the DNA molecule. The presence of YOYO-1 does not alter the location of binding of Rad54 since the position of oligomer formation and the subsequent translocation rates was unaffected when the concentration YOYO-1 was lowered by a factor of two (data not shown and Table I).

It is critical to note that oligomer formation is not DNA end-dependent. In less than 1% of optically trapped DNA-bead complexes, the free end of the DNA loops back and attaches nonspecifically to the bead so that no free end exists. Under these conditions, Rad54 oligomer formation occurs at the position distal to the bead in the flow, positioned in the approximate center of the looped DNA (data not shown). Once assembled, translocation occurs in similar fashion towards the bead, with both arms of the loop decreasing in length at similar rates. In addition, in approximately 5% of the movies analyzed, a small segment (less than a few hundred base pairs) of the fluorescent DNA is observed extending out the back of the translocating complex as it moves towards the bead (data not shown). In these cases, it is clear that the assembled oligomers cannot be exactly at the free end of the DNA. Experiments done under conditions of reduced flow (where the entire DNA molecule is considerably more flexible) reveal formation of Rad54-dependent, mobile bright spots at internal positions on the DNA (data not shown). Furthermore, experiments were done using GST-Rad54 bound to either fluorescent anti-GST antibodies or to Quantum Dots coated with anti-GST antibodies. Under these conditions, single optically trapped, fluorescent-DNA molecules were translated into a channel containing 112 nM tagged Rad54 (which is observed as a white channel due to the tag fluorescence). The DNA was held in this channel for a period of 5 seconds and then pulled back into the first channel. To our surprise, we did not observe clusters of enhanced fluorescence bound at internal sites. Instead, we observed enhancement only at a position distal to the bead in the flow (data not shown). Therefore, we conclude that Rad54 oligomer formation is not DNA end-dependent and does not occur appreciably at internal sites under standard conditions. Instead, visualization of the action of Rad54 occurs in the most flexible region of the stretched polymer, where it can establish efficient translocation.

Rad54 oligomers cross-bridge dsDNA molecules in proximity

The presence of Rad54 in the coaggregate assay leads to an increase in the formation of DNA networks (Fig. 2b and 3b). It is not immediately obvious how only the translocation activity of Rad54 could produce the 15-fold stimulation that we observe. Therefore, Rad54 must possess additional properties responsible for this stimulation. In the movie shown in Fig. 3b,

DNA molecules from neighbouring traps were held together in a Rad54-dependent complex when they were in close proximity (arrow 2). We termed this property cross-bridging and resulted from Rad54 oligomers binding multiple DNA molecules simultaneously. Cross-bridging could occur because DNA binding induces oligomerization of Rad54^{15; 23}, and by definition, these oligomers must possess more than one DNA binding site. Therefore, we surmised that linear arrays of Rad54 oligomers bound in juxtaposition on DNA may be able to bind to neighboring DNA molecules simultaneously, contributing to the formation of DNA networks. This positioning of oligomers on the DNA may be comparable to those observed assembling at the end of Rad51 nucleoprotein filaments²⁴.

Before proceeding to the cross bridging assay (presented schematically in Figure 10a and described below), it is necessary to demonstrate the presence of Rad54 oligomers bound to the DNA at internal sites under translocation conditions. To demonstrate their presence, conditions sub-optimal for translocation were used to maximize binding of Rad54 oligomers in juxtaposition along the length of a DNA molecule. These conditions included using elevated levels of Rad54 (>350 nM protein) and increased sucrose concentration, which would further stretch the dsDNA as observed previously⁴³. The results show that under these conditions in single DNA molecule translocation assays, the bright spot formed on the DNA distal to the bead in the flow as before (Figure 4a), but its progress was severely impeded (Figure 9a). To demonstrate that the bound oligomers are not terminal complexes, experiments were repeated in 70 mM NaCl. We surmised that under these conditions, the internally bound complexes may not be tightly associated with the DNA and could be displaced by the active, translocating oligomer. As expected, the bright spot formed on the DNA as before, but its subsequent translocation was rapid (Figure 9b).

It is conceivable that rapid translocation observed in the presence of 70 mM NaCl occurs since internal binding of Rad54 oligomers was inhibited by the NaCl and never happened in the first place as we had expected. Therefore experiments were performed in a 3-channel flow cell in reaction buffer containing 40% sucrose (Figure 9c). Here, the first channel contained DNA-bead complexes as before; the second channel contained 377 nM Rad54 and ATP while the third channel contained ATP, 70 mM NaCl and no free Rad54. Once optically trapped DNA was translated into the Rad54 channel, binding occurred, as evidenced by the formation of the bright spot on the DNA. Thereafter, the complex was translated into the third channel containing the NaCl to destabilize the binding of the internally bound oligomers while allowing the active oligomer to translocate. The analysis shows that bright spot formation occurred in channel 2, but its subsequent translocation was inhibited as expected (Figure 9d). Upon entering the third channel, rapid translocation resumed as the NaCl destabilized the binding of internal oligomers allowing the active complex to displace them during translocation (Figure 9d). Therefore, we conclude that at these elevated concentrations of Rad54, protein binding occurs along the length of the DNA, resulting in inhibition of the complex bound in the region of DNA distal to the bead in the flow.

Having demonstrated the presence of Rad54 bound to internal sites on the DNA, a novel assay involving dual-optical traps was used (Figure 10a). For these experiments, the mobile trap is used to bring two DNA molecules into proximity to facilitate intermolecular interactions. If Rad54 oligomers are bound in juxtaposition along the length of the DNA then we expected that oligomers on one DNA might bind to the neighbouring DNA molecule held in the other optical trap. Since two homologous λ DNA molecules were used as DNA substrates, Rad51 was not included. This was done to eliminate complications arising from the pairing and exchange of strands of DNA and from the potential binding of multiple DNA molecules by a helical Rad51 nucleoprotein filament. Under these conditions, we expected that Rad54-dependent bright spots would form on the DNA distal to the bead as before, but their subsequent

motion would be inhibited due to binding of Rad54 along the length of the DNA, impeding the progress of the active complex.

Using the above-mentioned reaction conditions, DNA-bead complexes trapped side-by-side in separate optical traps were translated into a channel containing Rad54 and ATP. Once binding occurred (as indicated by bright spot formation distal to the bead), the mobile trap was moved to bring the DNA molecules into proximity to facilitate intermolecular interactions such as those that might be found within DNA networks (Figure 10b). After ~3 sec, traps were separated resulting in the DNA molecules unzipping. The unzipping effect results from DNA being dislodged from the juxtaposed oligomers (*i.e.*, the teeth of the zipper) in sequential fashion as lateral force is applied when the mobile trap is separated from the fixed trap. By measuring the locations of the zipper teeth or positions at which the DNA becomes dislodged in sequential movie frames, it is possible to determine the spacing of the oligomers on the optically trapped DNA molecule. The results show that under the conditions used, oligomers are spaced an average of 104 ± 185 bp apart (Figure 10c). This spacing reflects the positions at which disruption of the cross-bridge can be detected as traps are separated. As the spacing is below the resolution limit of the system, it is at best an approximation of oligomer spacing. Control reactions done in the absence of Rad54 show that as the mobile trap moves a trapped DNA molecule past a DNA molecule held in the fixed trap, DNA molecules simply slide past one another without adhering (Figure 10d). Therefore, we conclude that Rad54 oligomers cross-bridge or bind DNA molecules positioned in proximity. DNA cross-bridging is the second activity of Rad54 that contributes to the formation of DNA networks.

The GST-tag negatively affects the cross-bridge activity of Rad54

The widely used GST tag negatively impacts Rad54 as evidenced by the effects on the K_m^{ATP} and k_{cat} for ATP hydrolysis and the ability to nonspecifically aggregate DNA. Previous work demonstrated that GST-tagged Rad54 protein can stimulate the formation of DNA coaggregation. The stimulation observed in Rad51-Rad54 reactions relative to Rad54 alone was only modest (1.4-fold higher). In sharp contrast, we demonstrate that the presence of untagged Rad54 results in at the least a 15-fold stimulation of DNA network formation in this formation is observed in both in bulk phase and at the level of single DNA molecules.

To determine whether the GST-tag influences the ability of Rad54 protein to stimulate cross-bridge formation, we performed the cross-bridge assay using GST tagged Rad54 protein. Here, DNA-bead complexes were trapped side-by-side as before, and translated into a channel containing GST-Rad54. Once binding occurred (as indicated by bright spot formation distal to the bead), the mobile trap was moved to bring the DNA molecules into proximity to facilitate cross-bridging (Figure 11). After ~10 s, traps were separated to reveal the position of the first cross-bridge. In sharp contrast to cross-bridge formation facilitated by untagged Rad54, these cross-bridges are stable complexes that cannot be disrupted by the forces resulting from optical trap separation. This is clear as continued motion of the mobile trap distorts the DNA in the fixed trap instead of becoming unzipped from it. Once the mobile trap is shuttered, the bead in the mobile trap is released, and the cross-bridges hold the DNA molecules together in a stable complex that can withstand greater than 50 pN of force resulting from the flow of the buffer within the channel (the force was calculated using the Stokes drag equation for the drag experience by the bead as described previously⁴¹). Therefore, and in addition to the effects on ATP binding and hydrolysis, the GST tag negatively influences the ability of Rad54 protein in the stimulation of DNA networks and subsequently may compromise its role in DNA strand exchange possibly leading to erroneous results.

Rad54 cross-bridging is not simply DNA aggregation

As Rad54 can form large complexes in the presence of DNA²⁴, it is important to demonstrate that cross-bridging is not simply aggregation of DNA by these large complexes. Instead it should be an interaction that occurs between two neighboring DNA molecules positioned in relatively close proximity. Therefore, cross-bridging experiments using untagged Rad54 protein were performed with one trap holding a bead with a single DNA attached, while the mobile trap holds two beads each with one DNA attached. Following cross-bridging, separation of the traps reveals that only 1 DNA from the mobile trap participates in cross-bridging with the single DNA in the fixed trap (Figure 12). Identical results were obtained using DNA-beads with more than one DNA attached per bead (data not shown). Thus cross-bridge formation occurs between Rad54 oligomers bound to DNA molecules positioned in close proximity (*i.e.*, less than 1 μm , the diameter of the bead), and is not the result of large aggregates of protein bound to the DNA.

Conclusion

The findings presented herein are without precedent for a translocating DNA motor and provide insight into the role of Rad54 in DNA strand exchange. We demonstrated that the presence of catalytic amounts of Rad54 results in enhanced formation of three-dimensional DNA networks or coaggregates which are key intermediates on the reaction pathway (Figure 2b). This stimulation is intrinsic to Rad54 and results from the combination of the dsDNA translocase and the previously undiscovered cross-bridging activity of Rad54 oligomers.

We note that although isolated Rad54 can cross-bridge and translocate dsDNA efficiently, it does not act globally within the nucleus, a condition detrimental to the cell. Instead, Rad54 acts at the site of a DNA double-strand break by virtue of its multiple interactions with Rad51 nucleoprotein filaments⁴⁵. Here, it may bind along the length of the filament¹⁷ or to filament ends²⁴, producing a protein nanomachine with multiple dsDNA binding sites (Figure 1, left inset). In this context, Rad54 oligomers can bind and hold multiple dsDNA molecules in proximity to Rad51 *via* their cross-bridging activity. This allows Rad51 to sample multiple DNA molecules simultaneously for a homologous target. Concurrently, Rad54 oligomers may also translocate dsDNA rapidly across the Rad51 filament surface thereby contributing to a more rapid homology search. In the process, DNA loops leading to transient strand separation are produced at the site of the Rad54 oligomer. As suggested previously, these loops may play a critical role during DNA strand exchange^{15; 21}. In support, DNA loop cleavage results in disruption of efficient ATP-hydrolysis dependent translocation by Rad54 on single molecules of DNA and in complete disruption of bulk-phase ATP hydrolysis. The requirement for ATP-hydrolysis is further supported by previous results showing that non-hydrolyzable ATP analogues and mutations that disrupt the ATPase activity of Rad54 have disastrous effects on the ability to induce topological changes in DNA and on the ability to stimulate DNA strand exchange^{13; 15}.

We have shown that Rad54 translocates rapidly and processively on dsDNA. The rates observed here are 8.8-fold higher than those observed previously, where translocation was visualized by fluorescent antibody-tagging of GST-Rad54¹⁹. These slower translocation rates may result from a combination of factors. In the previous study, the antibody was bound to Rad54 at a ratio 10:1 (antibody:GST) to visualize Rad54. As Rad54 forms variable sized oligomers on DNA and an antibody MW is ~150kDa, the resulting antibody-Rad54 complexes being studied may be as large as 1 Mda in size. The increase in diameter of the resulting complex relative to untagged Rad54 will produce increased drag leading to a reduction in the translocation rate. In addition, the presence of the antibody reduces bulk-phase ATPase activity by as much as 70% by an unknown mechanism¹⁹. If the hydrolysis of ATP is coupled to translocation as observed for other dsDNA translocases⁴⁶, then the reduced rates of ATP

hydrolysis for the antibody-tagged protein indicates that one would expect a reduced rate of translocation relative to the unmodified protein. In fact, the observed concentration rates are reduced by 70% compared to what we observe in the study. In addition, we provide evidence that a significant fraction of the ATPase activity observed in bulk phase is related to the ability of the enzyme to form loops in the DNA. All translocation events in the previous study were limited to internal sites on the DNA; that is, where the stretched polymer is more rigid⁴⁴ and DNA loop formation was not observed. Thus under these conditions, a more slowly moving Rad54 complex results and which does not induce DNA loop formation. In contrast, using untagged Rad54 we demonstrate rapid, ATP hydrolysis-dependent translocation mediated by dynamic Rad54 oligomers. In the process, Rad54 induces topological changes in the DNA, changes which we demonstrate are intrinsic to efficient motor function. The rapid rates observed herein for Rad54 oligomers ($2,629 \pm 912$ bp/sec; Fig. 4c) are comparable to those observed for FtsK⁴⁷. This DNA motor functions during cell division to transport chromosomes and assembles into even larger oligomers than Rad54 and which translocate at speeds in excess of 5,000 bp/sec⁴⁸. The rapid rates we observed are for Rad54 oligomers bound in the flexible region of the optically trapped DNA distal to the bead and the flow (but not at the end). Similar rapid rates are observed for the *E. coli* RecBCD enzyme which binds exclusively to the free ends of dsDNA; these ends in single DNA molecule experiments are positioned within the flexible region of the DNA. That is, distal to the DNA in the flow. The translocation rates observed for single molecules of RecBCD are identical to those observed in bulk-phase⁴⁹. Thus the rates reported here for untagged Rad54 protein accurately reflect the translocation velocity of human Rad54 protein.

In conclusion, the dsDNA translocase and cross-bridging activities are critical to the role of Rad54 in stimulating the formation of DNA networks. Disruption of these activities results in a failure to form these networks producing an inefficient DNA strand exchange reaction, which is observed when Rad51 functions in the absence of Rad54.

Materials and Methods

Chemicals

Phosphoenol pyruvate (PEP), nicotinamide adenine dinucleotide (NADH), heparin, pyruvate kinase, lactate dehydrogenase and the single stranded DNA (ssDNA) cellulose resin were obtained from Sigma Chemical Company. ATP, DEAE sepharose Fast Flow resin and the 16/10 heparin FF column were obtained from Amersham Pharmacia Biotech. The hydroxyapatite Bio-Gel[®] resin was from Bio-Rad Laboratories. Dithiothreitol (DTT) was purchased from Acros Organics. Wheat Germ Topoisomerase 1 (WGT) was from Promega.

All solutions were prepared using Barnstead Nanopure water. Stock solutions of Phosphoenol pyruvate (PEP) were prepared in 0.5 M Tris-acetate (pH 7.5). ATP was dissolved as a concentrated stock in 0.5 M Tris-HCl (pH 7.5), with the concentration determined spectrophotometrically using an extinction coefficient of $1.54 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$. NADH was dissolved in 10 mM Tris-acetate (pH 7.5), concentration determined using an extinction coefficient of $6,250 \text{ M}^{-1} \text{ cm}^{-1}$, and stored in small aliquots at -80°C . Dithiothreitol (DTT) was dissolved as a 1M stock in nanopure water and stored at -80°C .

Proteins

Human Rad54⁴⁵ was supplied by Patrick Sung, Yale University. Rad51 was purified as described¹¹. Human GST-Rad54 was provided by Alex Mazin, Drexel University. Wheat germ topoisomerase (WGT) was from Promega; P1 nuclease was from USBiological. RecG was purified as described⁴². BSA was purchased from New England Biolabs.

Optical trapping and video, fluorescence microscopy

Reactions were performed in either two- or three-channel, micro-machined flow cells with inlet ports connected to Hamilton Gas Tight syringes controlled by a syringe pump (PHD 2000, Harvard Apparatus, MA, USA). Flow cells were held in place on a Märzhäuser stage in a Leica DMIRE2 fluorescence microscope. The DMIRE2 was modified to mate with a Laser Manipulator LM-02 (Solar TII, Belarus) containing galvano-scanning mirrors (VM500, GSI Lumonics, MA). Optical traps were formed by passing an Nd: YVO₄ infrared laser (wavelength=1064 nm, 5W, Spectra Physics) through the LM-02 which split the beam into S- and P-components to form fixed and mobile optical traps in the focal plane of the microscope. The position and motion of the mobile trap was controlled by the scanning-mirrors. The resulting, independent laser beams were focused through an oil-immersion objective lens (Plan Apo 100x, 1.4 NA, Leica) to a position 20 μ m above the lower surface of the flow cell. Fluorescent DNA-bead complexes were excited using an XCite120 (XFO) using a GFP-endow fluorescence cube (Chroma Technology, VT). Fluorescence images were captured at video rate by an EB-CCD camera (Hamamatsu) and recorded on digital videotape (DV184) using a digital VCR (Sony DSR-11).

DNA-bead preparation and single DNA molecule reactions

DNA-bead complexes were prepared as described previously³⁷. Sample syringes contained 30 mM Tris-acetate (pH 7.5), 100 mM DTT, 20% sucrose, 9.9×10^7 DNA-bead complexes and 0.2 μ M YOYO-1. Reaction syringes contained 30 mM Tris-acetate (pH 7.5), 100 mM DTT, 20% sucrose, 112 nM Rad54, 2 mM Mg(OAc)₂, 2 mM ATP and additional proteins as indicated. Reactions were done at 23°C at a linear flow velocity of 74 μ m/sec. Cross-bridging reactions contained 377 nM Rad54 and 40% sucrose.

Data analysis

Images were captured on an IBM compatible computer interfaced with the DSR-11 *via* a PIXCI SV5 frame-grabber controlled by XCAP software (EPIX Inc.). Captured videos were converted into individual, sequential frames to make measurements using Image-Pro Plus v6.1 (Media Cybernetics). Calibration of the microscope optics was achieved using a Stage Micrometer (Leica), resulting in 8.54 pixels per micron. Under the assay conditions used, the average corrected length of individual, stretched, fluorescent λ DNA molecules was 18 μ m (n=700). DNA lengths were corrected for slight bead blooming (*i.e.*, beads appeared 1.2x larger than anticipated). Thus for all calculations, as λ DNA is 48,502 bp in length, the average number of bp/ μ m is 2,695. Under flow conditions, the fluctuation of a stationary fluorescent spot (*i.e.* a permanently arrested Rad54 oligomer complex) was measured to determine the noise level in analysis of translocation trajectories. Fluctuations in both X and Y position were ascertained for 11 spots over an average of 109 frames per analysis and found to be 174 ± 31 bp parallel to the flow (X; 65 ± 12 nm) and 152 ± 47 bp perpendicular to the flow (Y; 57 ± 17 nm). The rate of DNA translocation was calculated by measuring the observed length of DNA in each frame of a time course, or using the object tracking function of Image Pro to track the position of bright spots as a function of time. Data were exported from Image Pro and analyzed using GraphPad Prism (v 5.0). To ascertain the time-dependent position of each oligomer, the data from individual trajectories were smoothed with a 2nd order Savitzky-Golay filter with a time constant of either 1 or 2.5 sec, as described previously⁴⁰. Then, the instantaneous velocity for each Rad54 oligomer was determined by using a differential average over the length of the movie from the point at which oligomers were observed until they either ceased moving entirely or encountered the bead. The instantaneous velocities were averaged to obtain the translocation rate. These rates were corrected for Rad54-independent dye loss that occurs when DNA molecules are translated into channels containing magnesium³⁷. Processivity (N) is defined

as the distance a bright spot moves from the time it is first detected to the point at which motion ceases, frequently when complexes encounter the bead.

Coaggregate assay

Reactions were done as described ⁷. Reactions (30 μ l; 37°C) contained 20 mM Tris-OAc (pH 7.5), 0.1 mg/ml BSA, 1 mM DTT, 20 % sucrose (where indicated), 20 U/ml of pyruvate kinase, 15 mM PEP, 1mM Mg(OAc)₂, 1 mM CaCl₂, 10 μ M nts ϕ X174 ssDNA (NEB), 3 μ M Rad51, 0.27 μ M Rad54 and 116 μ M nts linear, heterologous pPB67. Reactions were initiated by addition of Rad51 to the ssDNA and incubating for 15 min, followed by Rad54 (5 min) and dsDNA (10 min). Reaction processing was performed as described ⁷.

Optical tweezers pairing assay

A 5kB PCR fragment (3 μ M nts) homologous to the end of λ DNA distal to the bead was incubated with 1 μ M Rad51 followed by 0.25 μ M Rad54, as described for the coaggregate assay. These nucleoprotein filaments were introduced into the second channel of a 2 channel flow cell. The reaction buffer contained 20 U/ml of pyruvate kinase and 15 mM PEP to maintain a constant concentration of ATP.

ATPase assays

ATP hydrolysis was monitored using either a coupled-spectrophotometric ⁵⁰, or a thin layer chromatography assay ⁴⁶. Reaction buffer and temperature were the same as that used for single molecule experiments, except that sucrose was not present and for the spectrophotometric assay, the components of the ATP-regenerating system were present.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Cuiling Xu, Jackson Buss, Margaret Hollingsworth of The University at Buffalo and Tomohiko Sugiyama and Noriko Kantake of Ohio University for their critical reading of the manuscript. This work was supported by a Susan G. Komen Breast Cancer Foundation Grant # BCTR0601350 and NIH Grant GM66831 to PRB.

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Figure 1. Schematic of the DNA strand exchange reaction

The three, kinetically distinguishable stages of a three-strand reaction are shown, with ssDNA in red and homologous, linear dsDNA in green. These stages apply to reactions performed by either RecA or Rad51 as discussed in the text ⁶. Lower panels are schematics showing the potential disposition of DNA, Rad51, and Rad54 proteins within presynaptic filaments (left) and DNA networks (right). Presynaptic filament I is adapted from ²⁵ and filament II is from ²⁴. For simplicity, a Rad54 oligomer is shown bound only to the 5'-end of Rad51 filament II, although a similar situation could exist with the oligomer bound to the 3'-end or embedded within a discontinuous Rad51 filament.

Figure 2. Rad54 stimulates coaggregate formation

(a) Schematic of the bulk-phase coaggregate assay⁷. Single-stranded circular DNA was ϕ X174 ssDNA (jagged circle) and the heterologous, linear dsDNA was 5'-end labeled, pPB67. * = ^{32}P . (b). Analysis of bulk-phase coaggregate assays which were done twice.

A. **B.** **Figure 3. Rad54 stimulates DNA networks in real-time**

(a). Schematic of the single molecule approach used. Upper panel, the arrangement of a two-channel flow cell and syringe pump as viewed from the top (not to scale). The sample syringes contain fluorescent, λ DNA-bead complexes and reaction syringes contain either nucleoprotein filaments and ATP (coaggregate assay; left inset) or proteins and/or ATP (translocation assay; right inset). The positions of the trapped DNA molecules within the flow cell are highlighted by the grey ellipse in the centre of the flow cell and the blue X indicates the positions of the optical traps, typically positioned within 5 μm of one another. The arrow above the flow cell indicates the direction of buffer flow and the direction in which the optically trapped, fluorescent-labeled, DNA molecules are stretched. The arrow to the left of the flow cell indicates the direction of stage movement which occurs once DNA-bead complexes have been trapped in each of the two optical traps. The result of this motion is to relocate optically-trapped DNA molecules (green strands, insets) from the sample to the reaction side of the flow cell where reactions occur. Left inset, real-time, coaggregate assay using DNA-beads with 3–7 DNA molecules attached. Right inset, single DNA molecule, Rad54 translocation assay. The black arrow in the insets indicates the direction of movement of the trapped DNA-bead complexes from the sample to the reaction side of the flow cell. F = fixed optical trap; M=

mobile trap. (b). Sequential frames from a movie showing Rad54-dependent, DNA network formation in real-time. Arrow 1, a nucleoprotein filament invades the lower trapped complex resulting in bending and twisting of the trapped DNA resulting in a cross-bridge being formed with the second trapped DNA complex (arrow 2). Arrows 3 and 4, additional invasion events followed by translocation and cross-bridging by Rad54 to produce a coaggregate or DNA network (n=4; n = the # of movies analyzed per reaction condition).

A. **B.**

C.

Figure 4. Rad54 oligomers translocate dsDNA

(a). Selected, sequential frames from a video recording of dsDNA translocation by Rad54. The direction of translocation is from right to left. Numbers at the lower right of each frame indicate real time. Arrows indicate formation of a bright spot correlating with Rad54 oligomer position; for the two DNA molecules shown, this occurs at different times. The inset shows a schematic of the protein-DNA structure within the bright spot. These contain a Rad54 oligomer and topologically constrained DNA whose strands are partially separated. (b). Record of position (upper) and velocity (lower) for Rad54 oligomers. Blue trace, upper DNA molecule and red trace, lower DNA molecule from the movie shown in (a). The black lines indicate the smoothed data used to determine position and translocation velocity. The large apparent error results from the large differences in instantaneous velocity for each oligomer. (c) Analysis of eleven trajectories. Upper panel, oligomer position and lower panel, velocity. Only the region from 10 to 35 sec is shown and only the smoothed traces of oligomer position are presented for

clarity. $\text{Rate}^{\text{corr}}$ is the average translocation velocity corrected for dye loss in the absence of Rad54. (d). Record of position (upper) and velocity (lower) for Rad54 oligomers formed under different concentrations of protein. The coloured traces indicate raw data while the black lines indicated smoothed data. The traces are from movies representative of reactions observed at each protein concentration.

Figure 5. Translocation of Rad54 oligomers is ATP-dependent

(a) Analysis of translocation rates of Rad54 oligomers from a protein titration done in the presence (■, n=76) or absence (□, n=27) of ATP. Reactions using DNA stained with 0.1 μ M YOYO-1 are shown for comparison (○, n=11). The shaded region indicates the ATP-dependent region of the titration. (b). Analysis of the kinetics of lag time for oligomer formation and translocation velocity of the resulting complexes. Lag time is defined as the time required for bright spot detection once a DNA molecule is translated from the sample to the reaction side of the flow cell. Data for lag time were fit to a single exponential producing an R^2 value of 0.496 while data for translocation rate were approximated to a straight line yielding a slope of -1.1 and an R^2 value of 0.002.

Figure 6. At elevated concentrations of protein, Rad54 mediates ATP-independent, condensation or collapsing of DNA

Seven condensation trajectories are shown from reactions done at 377 nM Rad54 in the absence of ATP; reaction conditions are otherwise identical to those of Figures 4 and 5. Upper panel, record of position and lower panel, record of instantaneous condensation rate. Insets highlight the early portions of trajectories where rapid condensation occurs. Rates within this region (grey box, lower inset, $4,690 \pm 825$ bp/sec), while the rates of DNA condensation for the remainder of each trajectory are 295 ± 97 bp/sec. This produces combined average rate of DNA shortening of 987 ± 450 bp/sec.

Figure 7. Translocating Rad54 oligomers are dynamic

(A-F). Analysis of oligomers in the movie frames shown in Figure 4. Reactions contained 112 nM Rad54, 2 mM ATP and 2 mM $\text{Mg}(\text{OAc})_2$. As a reference the positions of the oligomers as a function of time are shown in each panel (black traces). Bright spots formed distal to the optically trapped beads in the flow were analyzed using the Object Tracking Function of Image Pro as described in the text. Panels A, C and E are the top DNA molecule in Figure 4a; panels B, D and F correspond to the lower DNA molecule. A, B, The aspect ratio of each bright spot; C and D, the fluorescence intensity of each complex; E and F, the area (blue) and fluorescence heterogeneity (red) for each complex. The time in each panel is indicated as relative time, normalized to the start point or the point at which the analysis of the bright spot was initiated in the time course.

Figure 8. Translocation requires ATP hydrolysis

(a). Superposition of single DNA-molecule translocation (●) and bulk-phase ATP hydrolysis (○) rates. Data were fit to the Michaelis-Menten equation to yield the parameters shown. The resulting R^2 values are 0.978 for ATPase data and 0.394 for all translocation data or 0.8433 when the average rate at each concentration of ATP is used in the curve fitting instead. A minimum of 4 DNA molecules were analyzed at each ATP concentration in single molecule experiments; $n = 50$. Data points for the single molecule data represent the mean rate and error bars indicate the standard error of the mean. The bulk-phase ATPase data are from a single TLC assay employing ^{32}P -ATP. (b). P1 nuclease inhibits Rad54 ATPase activity on linear dsDNA. Coupled spectrophotometric ATPase assays were used and contained 100 nM Rad54 or 100 nM RecG. Once a steady-state rate was achieved, 10 units of P1 nuclease were added and 3 minutes later, the steady-state rate was re-evaluated. Data shown are from a single experiment for each protein.

Figure 9. Rad54 oligomers bind to DNA at internal sites in juxtaposition and impede translocation of the active Rad54 oligomer positioned on the DNA distal to the bead in the flow

(a). Selected, sequential frames from a movie demonstrating inhibition of translocation of the active Rad54 oligomer. Rate = 224 ± 6 bp/sec; N = 7,990 bp. (b). Selected, sequential frames from a movie demonstrating destabilization of internal oligomers in 70 mM NaCl and their subsequent displacement by the translocating Rad54 oligomer. Rate = $6,385 \pm 159$ bp and N = 34,165 bp. (c) Schematic showing the arrangement of the syringe pump and 3-channel flow cell (not to scale). The arrangement the flow cell and syringe pump is identical to that shown in Figure 3 except that a third channel is used. DNA-bead complexes are introduced into the sample channel where they are trapped. Trapped complexes are translated into channel two (designated Reaction 1) containing 40% sucrose, 377 nM Rad54, and ATP. Once the bright spot has formed and its motion slowed, the Rad54-DNA-bead complex is translated into the third channel (designated reaction 2) containing 40% sucrose and only ATP and 70 mM NaCl (and no free Rad54). (d) Analysis of a representative DNA molecule translated from channels 1 through 3 as described in panel (c). Time zero indicates the time at which the DNA molecule was trapped; entry into the second and third channels is indicated by the arrows. Analysis for each of the movies in this figure was performed by monitoring DNA length.

A. **B.** **C.**

D. **Figure 10. Rad54 oligomers cross-bridge dsDNA**

(a) Schematic of the cross-bridge assay presented as sequential movie frames. Two DNA-bead complexes are optically trapped and translated into the Rad54 channel to permit protein binding and oligomer formation (red or green spheres at the DNA ends). The mobile trap (M, red) is moved relative to the fixed trap (F, green) to bring DNA molecules into close proximity to permit cross-bridging (frame 1). After a few seconds, traps are separated by moving the mobile trap in the direction of the black arrow to reveal cross-bridges (frames 2 – 6). When mobile trap is moved in the direction of the arrow, the lateral force disrupts cross-bridges resulting in an “unzipping” effect as DNA is sequentially dislodged from Rad54 oligomers (pentagons).

(b) Sequential movie frames demonstrating Rad54-mediated cross-bridge formation followed by unzipping ($n=10$). From $t=8.65 - 9.47$ sec, traps are brought together to permit cross-bridge formation (curved arrow). At $t=12.77$ sec, trap separation reveals the position of the first cross-bridge (arrow at $t=13.60$ sec). Continued motion of the mobile trap results in unzipping of Rad54 cross-bridges ($t=14.42 - 16.9$ sec).

(c) Analysis of cross-bridge of Rad54 oligomer position from the movie shown in panel b. Cross-bridge or Rad54 oligomer position is determined from the sequential positions from which the DNA is dislodged as traps are separated. A value of zero indicates no change detected between successive frames and is not a spacing on zero.

(d). Sequential movie frames from a control reaction done in the absence of Rad54. ($n = 15$).

Figure 11. GST-Rad54 oligomers cross-bridge dsDNA producing terminal complexes

Sequential frames from a movie showing stable cross-bridge formation by GST-Rad54. At $t=0$, DNA molecules have been bound by Rad54 as evidenced by bright spots distal to the beads in the flow. From $t=8.71 - 13.07$ s, trap-M is moved to position DNA molecules in proximity to facilitate cross-bridge formation. At $t=26.14$ s, separation of traps reveals the position of the first cross-bridge which for GST-Ra54 is a terminal complex (arrow at $t=30.49$ s). Continued motion of trap-M, followed by shuttering of this trap, releases the bead and reveals cross-bridge stability. ($n=24$).

Figure 12. Rad54 mediated cross-bridges are not large aggregates of protein and DNA

Selected, sequential frames from a movie demonstrating cross-bridge formation by untagged Rad54 as in Figure 8, except two beads are present in trap-M. Reaction conditions are exactly the same as in those used in Figure 8. The cross-bridge is indicated by arrow 1 and the DNA molecule that does not participate in the cross-bridge is indicated by arrow 2. (n=6).

Table I

Factors affecting the assembly and translocation of Rad54 oligomers

Reaction Condition	# DNA molecules Analyzed	Fraction forming oligomers (%)	Observed effect; Rate (bp/sec)
112 nM Rad54 ^a	32	100	translocation; $2,961 \pm 2,075$
0.1 μ M YOYO-1 ^b	11	100	translocation; $2,679 \pm 1,640$
50 nM Rad54 ^c	11	100	translocation; $3,193 \pm 1,927$
^d 1 mM Mg ²⁺	16	100	translocation; $4,117 \pm 2,709$
^e 0.5 mM Mg ²⁺	14	100	translocation; $1,448 \pm 1,346$
^f Mg ²⁺ , + Mn ²⁺ (2 mM)	12	100	translocation; $1,743 \pm 1,071$
no ATP ^g	30	0	no change in the DNA
ATP, ADP ^h	32	41	no motion detected
ATP, ATP γ S ^h	33	0	no change in the DNA
ATP, AMP-PNP ^h	38	21	translocation for 3% of these
P1 ⁱ	28	100	oligomers disintegrate ~0.5 sec
WGT ⁱ	28	100	oligomers disintegrate ~0.5 sec

^aComplete reaction with 2 mM Mg(OAc)₂, 2 mM ATP.^{b-g}Same as (a) with only the indicated component altered.^hTo minimize dye displacement by Mg(OAc)₂, mixtures of ATP (2mM) and the analogues were used (0.2 mM). Competitive inhibition of the ATPase activity by each analogue was established in bulk-phase ATPase assays (data not shown).ⁱP1 (10U) or WGT (10U) were present in the reaction channel together with Rad54 (50 nM). Disintegration is defined as cleavage of one strand of dsDNA in the proximity of the Rad54 oligomer (*i.e.*, the bright spot), resulting in disassembly and release of topologically constrained dsDNA. In some cases (<5%), two proximal cleavage events produces dsDNA breaks (data not shown).