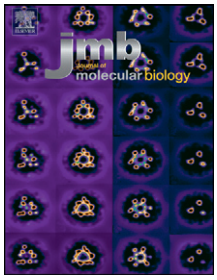

Context-Dependent Remodeling of Rad51–DNA Complexes by Srs2 Is Mediated by a Specific Protein–Protein Interaction

Anna K. Lytle[1], Sofia S. Origanti[1], Yupeng Qiu[2], Jeffrey VonGermeten[1], Sua Myong[2,3] and Edwin Antony[1]

*1 - **Department of Chemistry and Biochemistry,** Utah State University, Logan, UT 84322, USA*
*2 - **Department of Bioengineering,** University of Illinois at Urbana-Champaign, IL 61801, USA*
*3 - **Department of Bioengineering,** Center for Biophysics and Computational Biology, Center of Physics for Living Cells, University of Illinois at Urbana-Champaign, IL 61801, USA*

Correspondence to Edwin Antony: *edwin.antony@usu.edu*
http://dx.doi.org/10.1016/j.jmb.2014.02.014
Edited by S. Kowalczykowski

Abstract

The yeast Srs2 helicase removes Rad51 nucleoprotein filaments from single-stranded DNA (ssDNA), preventing DNA strand invasion and exchange by homologous recombination. This activity requires a physical interaction between Srs2 and Rad51, which stimulates ATP turnover in the Rad51 nucleoprotein filament and causes dissociation of Rad51 from ssDNA. Srs2 also possesses a DNA unwinding activity and here we show that assembly of more than one Srs2 molecule on the 3′ ssDNA overhang is required to initiate DNA unwinding. When Rad51 is bound on the double-stranded DNA, its interaction with Srs2 blocks the helicase (DNA unwinding) activity of Srs2. Thus, in different DNA contexts, the physical interaction of Rad51 with Srs2 can either stimulate or inhibit the remodeling functions of Srs2, providing a means for tailoring DNA strand exchange activities to enhance the fidelity of recombination.

Introduction

Homologous recombination (HR) is an error-free pathway for the repair of DNA double-strand breaks, and inherited deficiencies in HR proteins give rise to gross chromosomal rearrangements that are features of cancer and other diseases [1]. The Rad51 recombinase promotes the pairing and exchange of homologous DNA strands by forming nucleoprotein filaments that catalyze the alignment of a broken DNA end with a homologous sequence that serves as a template for the repair of DNA double-strand breaks and inter-strand crosslinks [2]. Positive and negative regulators of HR activity control the assembly and stability of Rad51 nucleoprotein filaments and are essential for the maintenance of normal genomic structure [1].

The yeast Srs2 protein is an SF-1 (*superfamily-1*) helicase/translocase that suppresses HR activity and is required for the stability of genomic DNA in *Saccharomyces cerevisiae* [3]. In distinction to other prokaryotic SF-1 helicases, Srs2 has a unique C-terminal region of ~400 residues that interacts with several DNA replication and repair proteins [4,5]. Deletion of the C-terminal region from Srs2 eliminates the physical interaction with Rad51, impairing the anti-recombinogenic functions of Srs2 [4–6]. We previously showed that the Srs2–Rad51 protein–protein interaction activates ATP hydrolysis within the Rad51 nucleoprotein filament and promotes Rad51 dissociation from single-stranded DNA (ssDNA), providing a mechanism for the suppression of HR activity [4]. Srs2 has several additional activities including translocation on ssDNA and unwinding of double-stranded DNA (dsDNA) [4,7] that, in principle, could limit HR activity and/or resolve dead-end products of DNA strand exchange [8,9].

Additional biological roles for Srs2's DNA unwinding activity are being discovered [10–12]. In addition to preventing recombination, Srs2 can influence the outcome of HR by promoting synthesis-dependent strand annealing in preference to crossover products [8,11,12]. Srs2 can also unwind triplex DNA structures in concert with DNA polymerase δ and

thereby prevent the expansion of trinucleotide repeats [13–15]. Yeast strains lacking Srs2 show a 40-fold increase in the rate of trinucleotide repeat expansion [13,16]. This activity appears to be independent of Rad51's function in HR but requires ATP hydrolysis by Srs2, and presumably, its translocation on DNA [13]. DNA translocation and unwinding activities of Srs2 may also assist cells in recovering from checkpoint-mediated arrest after the completion of double-strand break repair, perhaps by removing proteins from DNA to signal the completion of repair [17,18].

Given the importance of Srs2's C-terminal region for regulating HR functions such as the disassembly of Rad51 filaments, we investigated whether or not this region also influences the helicase activity of Srs2. Here, we report that the unique C-terminal domain of Srs2 is dispensable for DNA unwinding activity but functions to selectively block unwinding of dsDNA that is coated with Rad51. This surprising result indicates that the interaction of Srs2 with the Rad51 protein facilitates two different anti-recombinogenic functions of Srs2, promoting the disassembly of Rad51–ssDNA filaments and inhibiting the unwinding of branched DNA intermediates formed during HR.

Results

Rad51 protects dsDNA from unwinding by Srs2

In principle, the DNA unwinding activity of Srs2 could down regulate HR activity by dissociating branched DNAs and crossovers formed during recombination [19], in addition to preventing DNA strand exchange *a priori* by disassembling Rad51 filaments [5,20]. Here, we examined the DNA substrate requirements and effects of Rad51 on the DNA unwinding activity of Srs2. Since the non-conserved C-terminal domain of Srs2 is required for efficient disassembly of Rad51 filaments on ssDNA [4], we examined whether this C-terminal domain also contributes to DNA unwinding. Helicase activity was measured using a 25-base-pair (bp) dsDNA substrate with a 3′- to 15-nt ssDNA flanking region to allow loading of Srs2 onto the DNA (see Experimental Procedures). As described previously [4], the full-length Srs2 protein is prone to severe aggregation and hence we examined Srs2$^{C\Delta276}$ (residues 1–898), which has all of the known Rad51-specific functions of Srs2, and a slightly smaller Srs2$^{C\Delta314}$ protein that does not interact with Rad51 and fails to clear Rad51 filaments from ssDNA [4]. Both Srs2$^{C\Delta276}$ and Srs2$^{C\Delta314}$ proteins are efficient helicases *in vitro* (Fig. 1a and d). The Srs2$^{C\Delta314}$ protein fails to rescue the hyper-recombination phenotype of a yeast Srs2 deletion strain, whereas a slightly longer construct (residues 1–875) similar to Srs2$^{C\Delta276}$ can partially complement the loss of Srs2 [6]. These results indicate that the C-terminal region of Srs2 is not required for helicase activity *in vitro*, although it contributes to the regulation of HR functions *in vivo*.

Although the C-terminal region of Srs2 is not required for DNA unwinding, we considered whether its interaction with Rad51 might alter DNA unwinding activity. Purified Rad51 protein was added to the DNA unwinding substrate before the addition of Srs2 (Fig. 1b and e). Rad51 binds to the dsDNA substrate in a saturable manner (K_D = 1.5 ± 0.3 μM; Fig. S1) and interferes with DNA unwinding by Srs2$^{C\Delta276}$ (Fig. 1b and c). However, Rad51 has no effect on the DNA unwinding activity of the shorter Srs2$^{C\Delta314}$ protein (Fig. 1e and f). Dupaigne *et al*. reported that the DNA unwinding activity of full-length Srs2 is stimulated by the addition of Rad51 and the single-strand binding protein RPA [11] instead of the suppression that we observe in the presence of Rad51. Although these authors studied full-length Srs2 protein using a longer DNA substrate (200 bp *versus* the 25-bp dsDNA used here) for their helicase assays, we suspected that the longer incubation time used by Dupaigne *et al*. (15 min *versus* 2 min in our studies) might allow Rad51 to dissociate from DNA and relieve the inhibition of DNA unwinding activity.

Intrinsic dissociation of Rad51 from dsDNA permits unwinding by Srs2

We therefore measured the steady-state rates of DNA unwinding under multiple turnover conditions, in the presence and absence of Rad51. Under steady-state conditions, the truncated Srs2$^{C\Delta314}$ protein unwound the DNA at a rate of 0.041 ± 0.005 min^{-1}, and the addition of Rad51 (45 nM) slowed the rate to 0.027 ± 0.008 min^{-1} (Fig. 2a and b). The longer Srs2$^{C\Delta276}$ protein is completely inhibited by Rad51 during the first 5 min of the reaction (Fig. 2c and d); however, after a significant lag, Srs2$^{C\Delta276}$ unwinds DNA at a rate (0.043 ± 0.004 min^{-1}) comparable to the initial rate of unwinding by Srs2$^{C\Delta314}$ (Fig. 2c). We also measured the kinetics of DNA binding by Rad51, which dissociates rapidly from a short 15-nt ssDNA ($t_{1/2}$ = 0.1 min; Fig. 2e); however, a longer-lived Rad51 complex is formed on the DNA unwinding substrate ($t_{1/2}$ = 13 min; Fig. 2e). A comparable rate of human Rad51 dissociation from dsDNA has been reported ($t_{1/2}$ ~ 5 min) [21]. Thus, the observed lag period of several minutes before DNA unwinding activity is observed may be due to the slow dissociation of Rad51 from the dsDNA. However, the inhibitory effect of Rad51 on the DNA unwinding activity of Srs2$^{C\Delta276}$, but not Srs2$^{C\Delta314}$, suggests that it is due to a specific protein–protein interaction with the Srs2$^{C\Delta276}$ protein and not to nonspecific stabilization of the DNA duplex by Rad51.

Since Srs2 and Rad51 physically interact in the absence of DNA [4–6], we also considered the

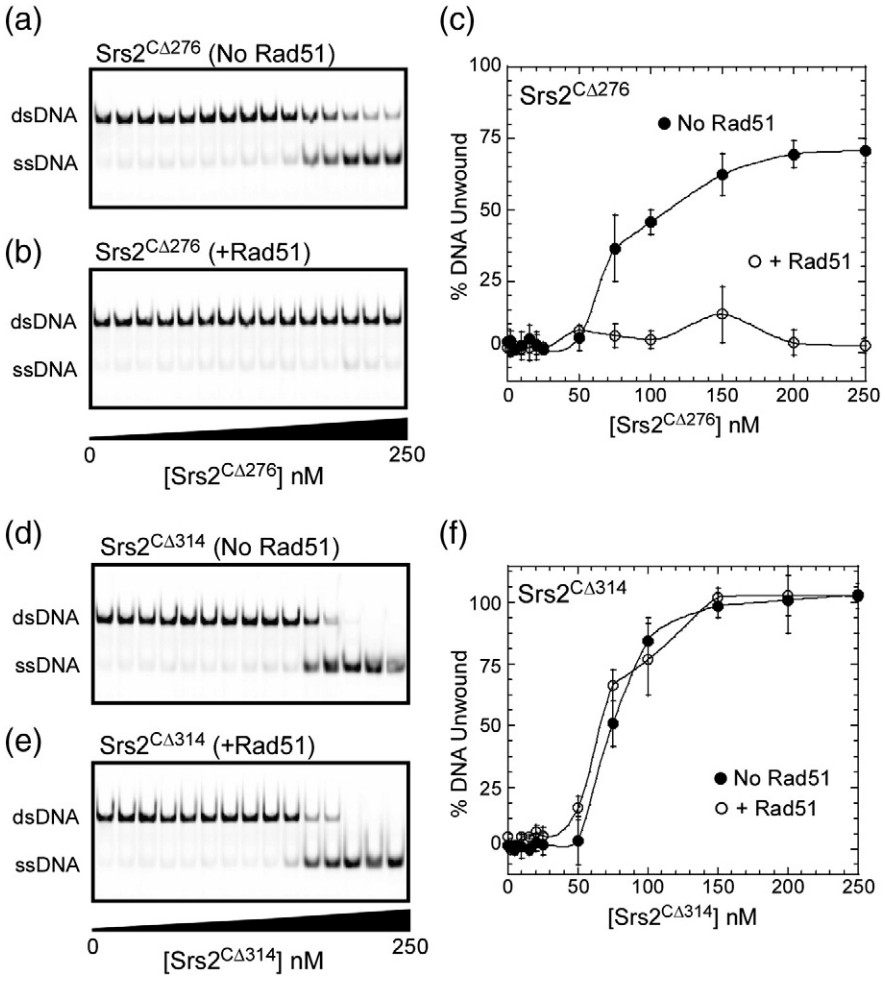

Fig. 1. Rad51 inhibits DNA unwinding by Srs2. (a) DNA unwinding activity of the $Srs2^{C\Delta276}$ protein (b) is inhibited by 45 nM Rad51. Increasing concentrations of $Srs2^{C\Delta276}$ were mixed with the DNA substrate (25 bp dsDNA with 3′ 15 nt ssDNA overhang) then incubated for 2 min before analyzing product formation. (c) Plotting the percent DNA unwound in (a) and (b) as a function of Srs2 concentration shows a threshold for DNA unwinding at about 50 nM Srs2 and marked inhibition of helicase activity by Rad51. (d) An analysis of DNA unwinding by the truncated $Srs2^{C\Delta314}$ protein (e) shows that it is resistant to inhibition by Rad51. (f) A plot of the results from (d) and (e) shows that Rad51 does not inhibit the DNA unwinding activity of $Srs2^{C\Delta314}$. The $Srs2^{C\Delta314}$ construct lacks the Rad51 interaction domain. The experiments were carried out for 2 min.

possibility that Rad51 inhibits DNA unwinding by forming a complex with free Srs2 in solution and preventing it from binding and loading onto the DNA. To address this possibility, we examined the DNA unwinding activity of $Srs2^{C\Delta276}$ in the presence of a mutant rad51-E221D protein that hydrolyzes ATP about 10-fold faster than wild-type Rad51 and consequently binds to DNA with very low affinity [4]. The weak DNA binding activity of rad51-E221D requires the ssDNA tail of the DNA unwinding substrate and does not bind to a blunt-ended duplex (Fig. S1). Although rad51-E221D stably interacts with the Srs2 protein in the absence of DNA [4], it does not inhibit the DNA unwinding activity of $Srs2^{C\Delta276}$ when rad51-E221D is present in large excess over DNA and $Srs2^{C\Delta276}$ (Fig. 2f). These results indicate that the DNA-bound form of Rad51 inhibits DNA unwinding by Srs2, but only in conjunction with the specific protein–protein interaction between Rad51 and the C-terminal region of Srs2.

The binding site size of Rad51 on ssDNA is roughly 3.3 nt. In our unwinding experiment, there are a total of 60 nM Rad51 binding sites including both the 25-bp dsDNA region and the 15-nt 3′ overhang. We measured the Rad51 concentration dependence of DNA unwinding by $Srs2^{C\Delta276}$ and find that a sub-stoichiometric amount of Rad51 is sufficient to inhibit the DNA unwinding activity (Fig. S2). On the contrary, even a 4-fold excess of Rad51 (with respect to number of Rad51 binding sites) is not sufficient to block the DNA unwinding activity of $Srs2^{C\Delta314}$ (Fig. S2). This experiment again shows that the interaction between Srs2 and Rad51 is mandatory for the inhibition of DNA unwinding.

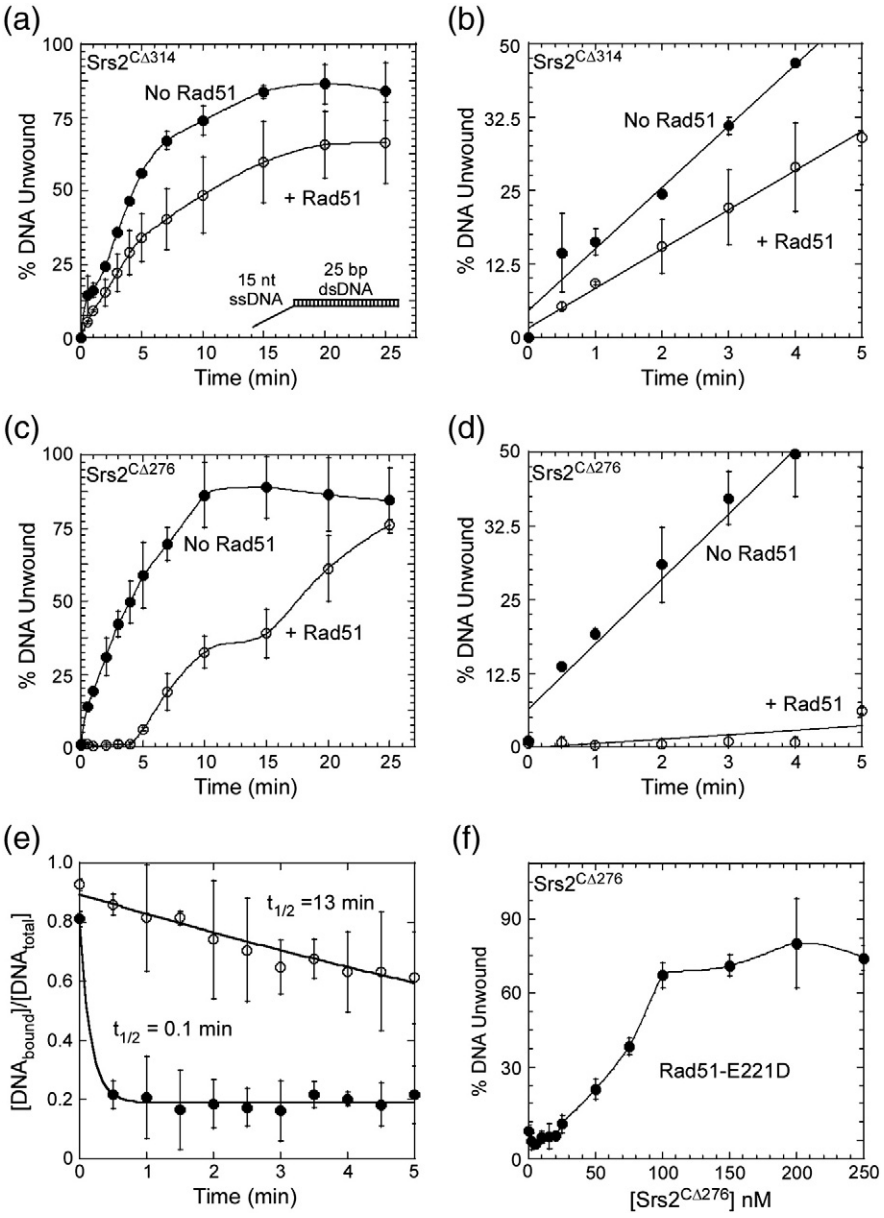

Fig. 2. Rad51 physically blocks DNA unwinding by Srs2. (a) A longer time course of the DNA unwinding catalyzed by 200 nM $Srs2^{C\Delta314}$, which does not physically interact with Rad51 [4], reveals modest inhibition by Rad51. (b) The initial part of the data (up to 6 min) fit to a linear equation. The initial rates of DNA unwinding are 0.041 ± 0.005 min^{-1} and 0.027 ± 0.008 min^{-1} in the absence and presence of Rad51, respectively. (c) The DNA unwinding activity if $Srs2^{C\Delta276}$ is strongly inhibited by Rad51 at early time points, followed by a substantial increase in helicase activity later in the reaction. The initial rates of DNA unwinding are 0.043 ± 0.004 min^{-1} and 0.002 ± 0.001 min^{-1} in the absence and presence of Rad51, respectively. (e) Rad51 dissociation from DNA was measured by a filter binding assay. Rad51 dissociates quickly ($t_{1/2} \approx 0.1$ min) from a 15-nt ssDNA corresponding to the unpaired tail of the DNA substrate for unwinding. The half-life on the tailed duplex DNA substrate for the helicase assays is $t_{1/2} \approx 13$ min. (f) The $rad51_{(E221D)}$ mutant binds weakly to dsDNA (Fig. S1) and fails to inhibit the DNA unwinding activity of the $Srs2^{C\Delta276}$ helicase.

Multiple Srs2 molecules are required for DNA unwinding

Srs2 exhibits a concentration threshold for helicase activity, with no detectable DNA unwinding observed at Srs2 concentrations below 50 nM (Fig. 1a and d). This observation suggests that multiple Srs2 molecules might be required to interact with the DNA substrate before unwinding activity is observed. To test this hypothesis initially, we pre-equilibrated the DNA with a sub-threshold concentration (40 nM) of an enzymatically inactive

form of Srs2, srs2-K41A, in which the conserved Lys in the ATP binding site has been mutated to Ala [5], followed by addition of increasing amounts of $Srs2^{C\Delta276}$. Pre-equilibration of the DNA with srs2-K41A completely blocks the DNA unwinding activity of $Srs2^{C\Delta276}$ that was added subsequently even when added in large molar excess (Fig. 3a). This result shows that multiple active Srs2 molecules must be bound on the DNA in order to unwind.

To further examine the Rad51-mediated inhibition of $Srs2^{C\Delta276}$-catalyzed DNA unwinding, we pre-incubated the DNA substrate with 45 nM Rad51 and 40 nM $Srs2^{C\Delta276}$ protein (that is inhibited by Rad51) (Fig. 1b). Note that Rad51 does not stably bind to the short ssDNA segment in these experiments with a $t_{1/2}$ = 0.1 min (Fig. 2e) and presumably should allow assembly of Srs2. When 40 nM Srs2 is present in the reaction, we observe no DNA unwinding activity (Fig. 1). We then added increasing concentrations of the $Srs2^{C\Delta314}$ protein to the reaction containing DNA and 40 nM $Srs2^{C\Delta276}$, and we tested for DNA unwinding activity (Fig. 3b). When the $Srs2^{C\Delta276}$–Rad51–DNA complex is challenged with the $Srs2^{C\Delta314}$ helicase, no detectable unwinding activity indicating the formation of a stable, inhibited complex of Srs2 and Rad51 bound to the DNA is observed (Fig. 3b). Conversely, we observe DNA unwinding when we first pre-incubate the DNA with 40 nM $Srs2^{C\Delta314}$ (which does not interact with Rad51) followed by addition of increasing concentrations of $Srs2^{C\Delta276}$ (Fig. 3b). These experiments suggest the existence of a high-affinity DNA binding site for Srs2 that is required but not sufficient for DNA unwinding. We conclude that occupancy of a high-affinity site on the DNA by Srs2 is a prerequisite for helicase activity, and the interaction between Rad51 and the Srs2 bound at this site creates a long-lived block of DNA unwinding.

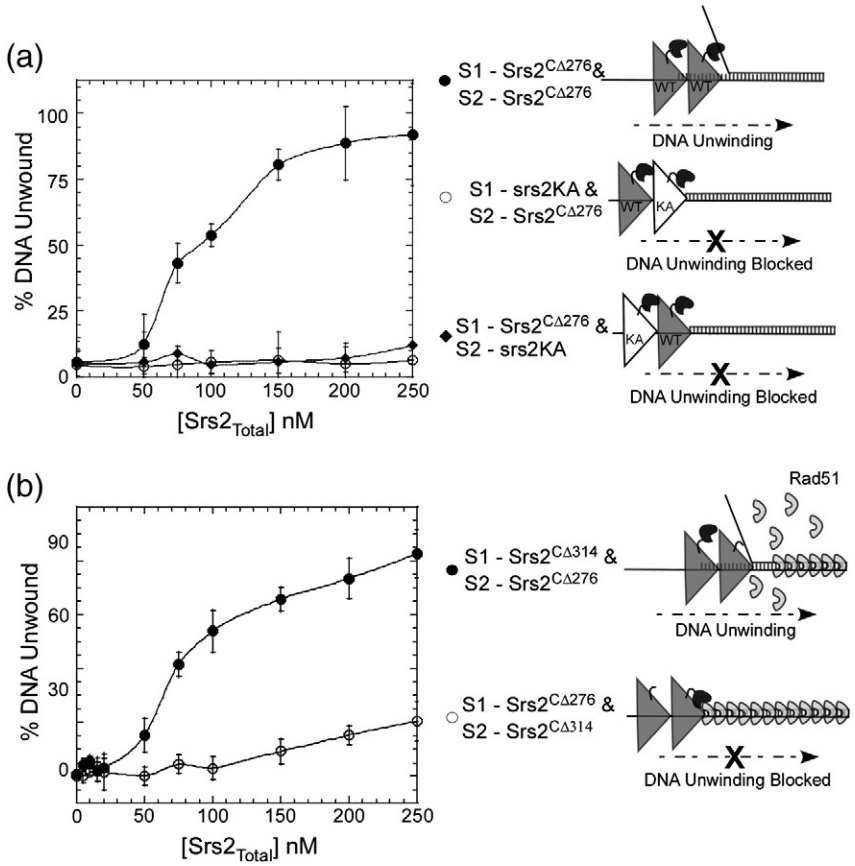

Fig. 3. Rad51 inhibits DNA unwinding of Srs2 through a direct physical interaction. (a) The DNA substrate for unwinding was pre-incubated with a low concentration (40 nM) of Srs2 protein that is insufficient to catalyze DNA unwinding, followed by addition of higher concentrations Srs2 proteins and measurement of the helicase activity after a 2-min incubation. (a) Pre-incubation with 40 nM catalytically inactive $srs2_{(K41A)}$ blocks subsequent DNA unwinding activity by $Srs2^{C\Delta276}$ (○) in comparison to a control reaction that was pre-incubated with 40 nM $Srs2^{C\Delta276}$ before addition of more $Srs2^{C\Delta276}$ (●). (b) In the presence of Rad51 (45 nM), pre-incubation of DNA with $Srs2^{C\Delta276}$ substantially inhibits helicase activity of $Srs2^{C\Delta314}$ even when added in vast molar excess (○). On the contrary, pre-incubation with $Srs2^{C\Delta314}$, which does not physically interact with Rad51 [4], preserves the helicase activity of $Srs2^{C\Delta276}$ in the presence of Rad51 (●).

To directly visualize whether multiple Srs2 molecules are bound on the DNA, we next examined DNA binding to Srs2 by monitoring the formation of Srs2–DNA complexes using electrophoretic mobility shift analysis (EMSA). Increasing amounts of $Srs2^{C\Delta276}$ were added to 5 nM DNA [25 bp dsDNA with a 3′ $(dT)_{15}$ overhang] in the presence of the non-hydrolyzable ATP analog AMP-PNP to prevent unwinding activity (Fig. 4). There are two complexes observed in the band shift experiment. The first complex migrating faster is observed across all Srs2 concentrations (labeled complex 1; Fig. 4). An additional slower-migrating band is observed only in lanes containing 75–250 nM Srs2 in the reaction (labeled complex 2; Fig. 4). A third much weaker complex is also observed closer to the well suggesting that at higher Srs2 concentrations more than two Srs2 molecules can bind to this DNA substrate. Similar results were also obtained for the shorter $Srs2^{C\Delta314}$ protein (data not shown). Occupancy of multiple UvrD molecules (the bacterial homolog of Srs2) has been observed on a similar DNA substrate [22–24]. Multiple UvrD molecules have also been observed during DNA unwinding in single-molecule analysis [25].

3′ ssDNA overhang length dependence of DNA unwinding and ATP hydrolysis suggests oligomerization of Srs2 on DNA

ATP hydrolysis fuels the DNA unwinding activity of Srs2 [7]. In addition to directly monitoring DNA unwinding, we analyzed ATP hydrolyzed by Srs2 during DNA unwinding (Fig. 5a). A biphasic ATP hydrolysis profile is observed with little to no ATPase activity below 50 nM Srs2 in the reaction and a hyperbolic increase in ATPase activity at higher Srs2 concentrations (Fig. 5a). These results mimic the results observed for the DNA unwinding activity (Fig. 1c). This lends additional support to our conclusion that multiple Srs2 molecules need to assemble on the DNA to initiate DNA unwinding. To test whether the assembly of the Srs2 molecules occurs on the 3′ ss (*s*ingle-*s*tranded) tail of the DNA substrate, we performed the ATPase experiment on DNA substrates with increasing lengths of the 3′ ssDNA overhang. The dsDNA was fixed at 25 bp and the length of the 3′ ss tail was varied from 0 to 30 nt. A very sharp dependence of 3′ ss tail length dependence of ATP hydrolysis is observed. Little to no ATPase activity is observed on substrates with tail lengths 0–10 nt (Fig. 5b). On longer tailed substrates (12–30 nt), ATPase activity is observed (Fig. 5b). Similar results were obtained for the $Srs2^{C\Delta314}$ protein as well (data not shown).

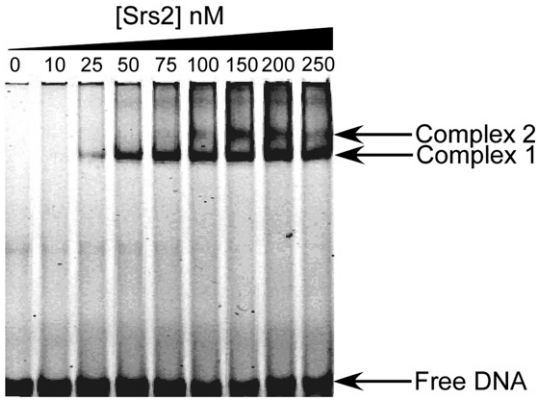

Fig. 4. Multiple Srs2 molecules bind to DNA during the unwinding reaction. An EMSA experiment showing formation of $Srs2^{C\Delta276}$–DNA complexes. Increasing concentrations of $Srs2^{C\Delta276}$ were incubated with DNA (25 bp dsDNA with a 3′ 15-nt overhang). A slower-migrating band is observed in the presence of 10–250 nM protein (Complex 1). A second slower-migrating band is observed only at Srs2 concentrations 75 nM or higher (Complex 2). The non-hydrolyzable ATP analog AMP-PNP (2 mM) was included to prevent DNA unwinding by Srs2.

The 3′ ss tail length dependence is also observed in DNA unwinding experiments (Fig. 5c). We analyzed the DNA unwinding activity of $Srs2^{C\Delta276}$ on DNA substrates with a 25-bp double-stranded region and increasing lengths of the 3′ ss tail (0–30 nt). Similar to the ATPase results (Fig. 5b), there is no DNA unwinding activity on substrates with 3′ ss tail lengths less than 12 nt. However, robust unwinding is observed on longer 3′ ss tail substrates (Fig. 5c). The crystal structure of SF-1 helicases such as UvrD, PcrA and Rep all show a minimum of 7 nt of 3′ ss tail occluded in the helicase core [26–28]. Based on these structures, we suggest that one Srs2 molecule can bind to ~7–10 nt of the 3′ ss tail but is not sufficient to unwind DNA. When the length of this tail is increased, more than one Srs2 molecule assembles leading to DNA unwinding activity.

In a previous study, using the full-length version of Srs2 and longer DNA substrates (200 bp dsDNA with a 409-nt 3′ ssDNA tail), Dupaigne *et al.* showed that Rad51 is able to stimulate the DNA unwinding activity of Srs2 [11]. Since our findings are in contrast to their report, we repeated our DNA unwinding experiments on the longer DNA substrate and find that $Srs2^{C\Delta276}$ is able to unwind this DNA just as efficiently as the shorter DNA unwinding substrates (Fig. 6a). Moreover, when we incubated the long DNA substrate with Rad51, followed by addition of $Srs2^{C\Delta276}$, a time-dependent inhibition of Srs2 unwinding was observed (Fig. 6b). When we performed this experiment with the $Srs2^{C\Delta314}$ protein, we did not observe any DNA unwinding activity as this protein cannot remove the Rad51 filament from the long 409-nt ssDNA 3′ tail (data not shown). The reason as to why we observe robust DNA unwinding with our Srs2 preparation *versus* their protein is not immediately obvious, and as a consequence, we have investigated the unwinding

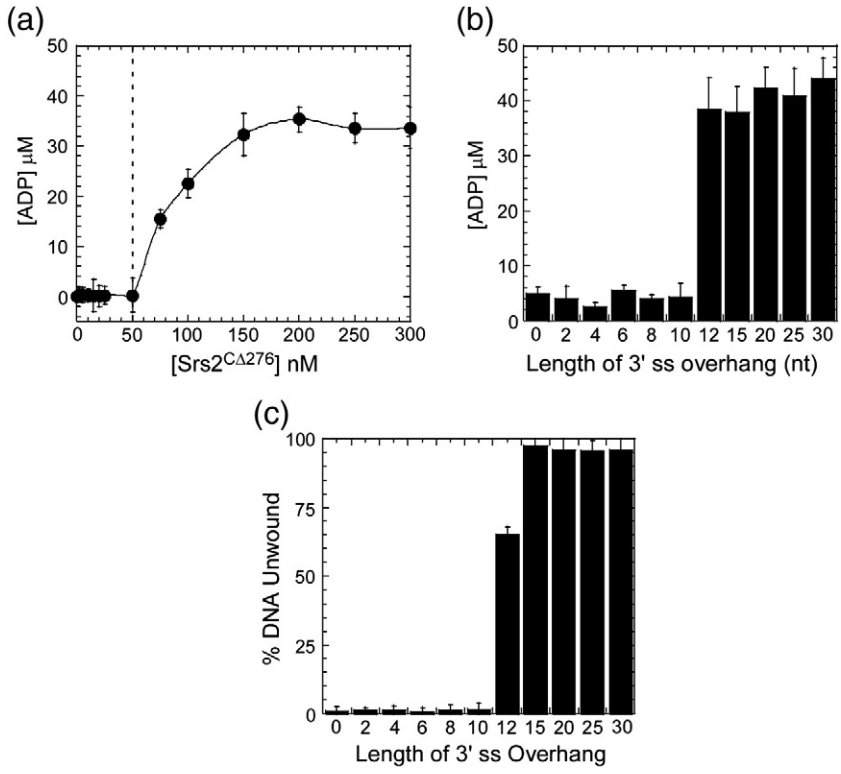

Fig. 5. 3′ ssDNA tail dependence of Srs2 ATPase and DNA unwinding activity (a) The extent of ATP hydrolysis during DNA unwinding by Srs2 recapitulates the biphasic behavior observed for DNA unwinding with a similar concentration threshold of 50 nM Srs2 (dotted line). (b) Stimulation of ATP hydrolysis by Srs2 is observed only on DNA substrates with 3′ ssDNA overhangs of 12 or more nucleotides. (c) The 3′ ssDNA length dependence of Srs2 DNA unwinding activity shows that a minimum of 12 nt is required to observe DNA unwinding, possibly corresponding to multiple Srs2 molecules binding to the DNA.

activity of the $Srs2^{FL}$ protein as well (described below). One discrepancy between the two studies may be attributable to the poor solubility of the full-length Srs2 protein and its tendency to aggregate in solution, which we encountered and reported in our earlier study [4]. The truncated Srs2 protein alleviates

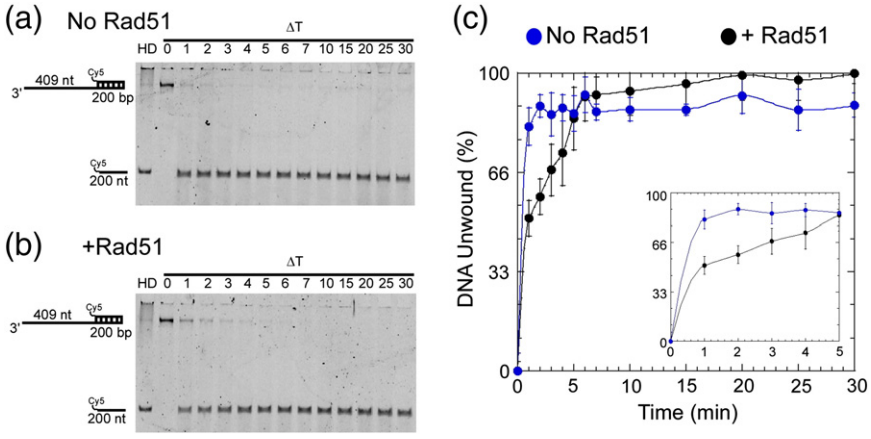

Fig. 6. DNA unwinding activity of $Srs2^{CΔ276}$ on long DNA substrates. (a) DNA unwinding activity of $Srs2^{CΔ276}$ was monitored on a 200-bp dsDNA substrate with a 409-nt 3′ ssDNA overhang. The short 200-bp annealed strand had a Cy5 probe that was used to observe the unwound strand in EMSA. (a) $Srs2^{CΔ276}$ unwinds DNA rapidly in the absence of Rad51. (b) When the DNA is incubated with Rad51, it retards the DNA unwinding activity of $Srs2^{CΔ276}$. (c) Quantification of the percent DNA unwound as a function of time and showing that the unwinding activity is slowed down when Rad51 is bound to the DNA substrate.

this problem and is also able to unwind long DNA substrates, which appears not to be the case with the full-length Srs2 protein [11].

Rad51 inhibits the DNA unwinding activity of full-length Srs2

As mentioned above, our findings are in contrast to the observation that Rad51 stimulates the DNA unwinding activity of Srs2 [11]. Since a key difference between the studies are the versions of Srs2 used in the experiments, we performed our unwinding experiments with purified full-length Srs2 protein ($Srs2^{FL}$). $Srs2^{FL}$ tends to aggregate in solution and is sensitive to the buffer conditions for *in vitro* studies. Purifying the protein in buffer containing 20 mM potassium phosphate (pH 8.0) and 20% glycerol alleviated some of the aggregation issues (data not shown), and we were able to observe DNA unwinding activity (Fig. 7a). $Srs2^{FL}$ unwinds DNA but requires an ~2.5-fold excess of

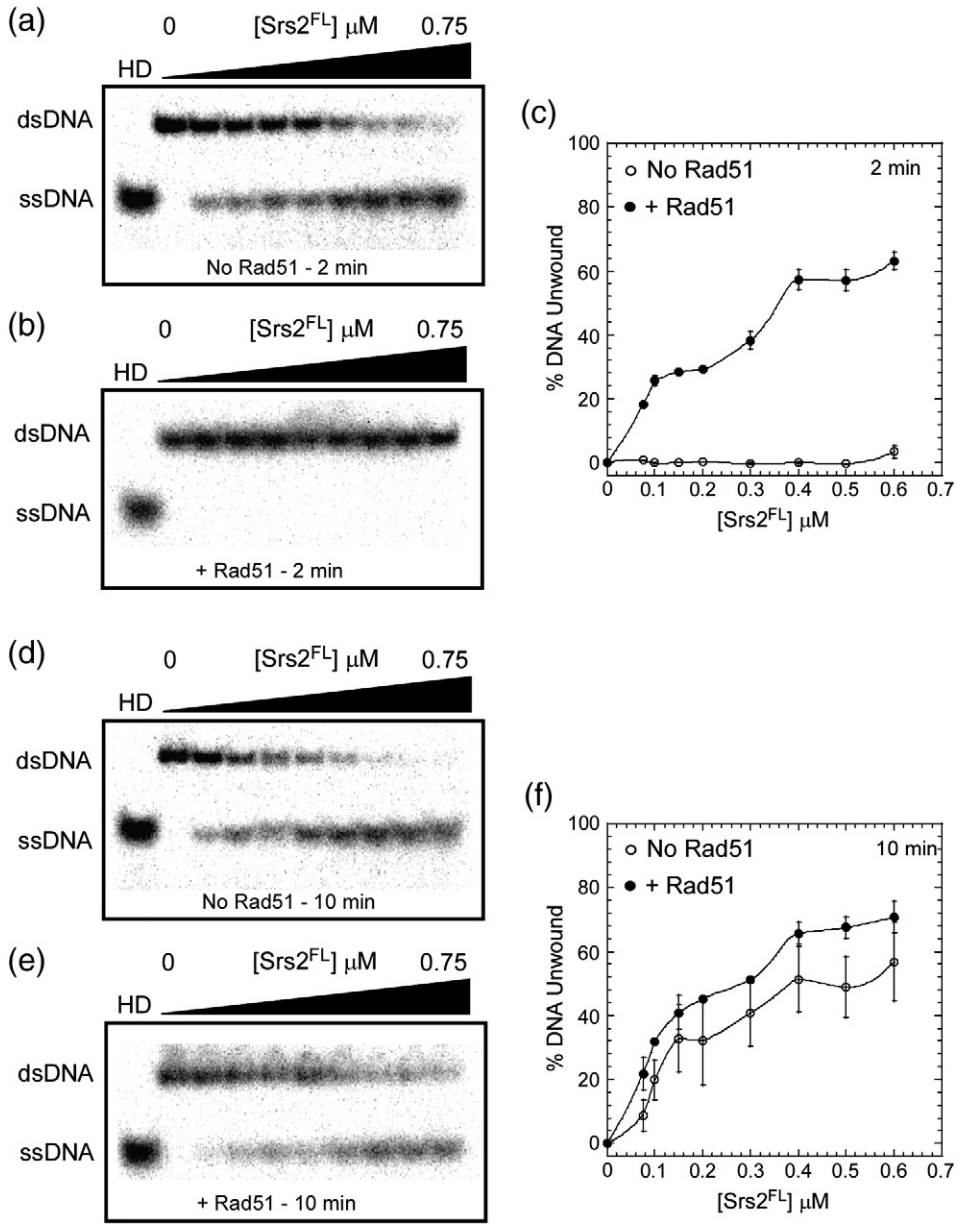

Fig. 7. Rad51 inhibits the DNA unwinding activity of full-length Srs2. DNA unwinding by Srs2 was measured for 2 min in (a) the absence or (b) presence of Rad51. (c) Quantification of results from (a) and (b) and showing that Rad51 inhibits DNA unwinding over a 2-min period. Similar experiments performed over 10 min in (d) the absence or (e) presence of Rad51 and (f) their respective quantifications show that the inhibition of DNA unwinding by Rad51 is significantly reduced.

protein to reach maximum DNA unwound (400 nM $Srs2^{FL}$ for 60% unwinding; Fig. 7a) compared to the truncated version (150 nM $Srs2^{C\Delta276}$ for 60% unwinding; Fig. 1a and c). By titrating a broader range of $Srs2^{FL}$ concentrations in the experiments, we were able to capture DNA unwinding and assess the effect of Rad51. $Srs2^{FL}$ unwinds a 25-bp dsDNA substrate with a 3′- to 15-nt overhang (Fig. 7a). Pre-incubation of the DNA substrate with Rad51 inhibits DNA unwinding when the unwinding reaction is incubated for 2 min (Fig. 7b). As observed for $Srs2^{C\Delta276}$ truncation, allowing the unwinding reaction to proceed for 10 min removes the Rad51-dependent inhibition and unwinding is observed (Fig. 7d–f). These results show that the Rad51-mediated inhibition of DNA unwinding by Srs2 is observed for both the $Srs2^{FL}$ (Fig. 7c) and the $Srs2^{C\Delta276}$ truncation and is lost only in the version of Srs2 lacking the ability to interact with Rad51 ($Srs2^{C\Delta314}$; Fig. 1f).

Rad51 retards the DNA unwinding of Srs2 6-fold in single-molecule experiments

Using single-molecule FRET (*f*luorescence *r*esonance *e*nergy *t*ransfer) (smFRET) experiments, we recently showed that Rad51 inhibits the unwinding activity of the truncated Srs2 protein ($Srs2^{C\Delta276}$) [29]. Since an ~3-fold excess of $Srs2^{FL}$ protein is required to observe ample DNA unwinding in our bulk experiments (Fig. 7a), we used smFRET analysis to measure unwinding in the presence and absence of Rad51 (Fig. 8). We used a Cy3–Cy5 FRET pair positioned at the DNA junction to monitor unwinding (Fig. 8a). One strand is immobilized using biotin on the surface and the unwound strand dissociates leading to a drop in FRET signal (Fig. 8a and b). The time window of the FRET signal (δT) change from its highest point to its lowest point upon addition of Srs2 is quantified as the "unwinding time" (Fig. 8c). Three examples of such unwinding times depicted as a drop in FRET signal are shown in Fig. 8c. Similar unwinding times are measured in the presence of Rad51 by first pre-incubating the DNA substrate with Rad51 followed by the addition of Srs2 to the reaction (Fig. 8d). The presence of Rad51 significantly elongates these unwinding times (Fig. 8d). These unwinding times are measured for multiple single molecules and the average unwinding time is calculated as previously described [29]. In the absence of Rad51, the average time to unwind the DNA for $Srs2^{FL}$ *versus* $Srs2^{C\Delta276}$ was 16.8 ± 0.98 s (Fig. 8e) and 5.98 ± 0.04 s (Fig. 8g), respectively. Intrinsically, the unwinding rate of $Srs2^{FL}$ is ~3-fold slower compared to the truncated $Srs2^{C\Delta276}$ protein and, at the moment, we do not clearly understand the basis for this difference. However, when Rad51 is pre-bound to the DNA, the average unwinding times are 108.49 ± 3.41 s (Fig. 8f) and 33.96 ± 0.1 s (Fig. 8h) for the $Srs2^{FL}$ and $Srs2^{C\Delta276}$ proteins, respectively. Hence for the $Srs2^{FL}$ protein, the presence of Rad51 increases the average unwinding time by ~6.5-fold *versus* ~5.7-fold for the $Srs2^{C\Delta276}$ protein. Therefore, the Rad51-dependent inhibition of the unwinding activity of Srs2 is not attributable to the differences between the $Srs2^{FL}$ and the $Srs2^{C\Delta276}$ proteins. These results again support our model that Rad51 inhibits the DNA unwinding activity of Srs2. Rad51 dissociation from dsDNA allows Srs2 to proceed with DNA unwinding. This inhibition is observable only when Srs2 physically interacts with Rad51.

Discussion

The SF-1 helicase/translocase Srs2 interacts with the Rad51 recombinase in two different contexts, but with differing results. This interaction is required for Srs2 to disassemble a Rad51–ssDNA nucleoprotein filament [4,5,20] but inhibits dsDNA unwinding by Srs2 (Figs. 1 and 2). Both of these Rad51-dependent activities are mediated through a protein–protein interaction with the C-terminal region of Srs2. The $Srs2^{C\Delta314}$ mutant lacking this C-terminal segment fails to disassemble Rad51–ssDNA filaments [4], and its wild-type helicase activity is not inhibited by Rad51 (Figs. 1 and 2). Srs2 catalyzes Rad51 dissociation from ssDNA by stimulating Rad51 to hydrolyze ATP, in a filament clearing reaction that also requires the ATP-dependent translocation of Srs2 along the ssDNA [4]. On dsDNA, the interaction of Srs2 with Rad51 prevents DNA unwinding by Srs2. In this context, a stable Srs2–DNA complex that is resistant to challenge with high concentrations of $Srs2^{C\Delta314}$ is formed (Fig. 3b), a protein that is normally immune to inhibition by Rad51 (Figs. 1 and 2). Whether the inhibition of DNA unwinding by Srs2 is coupled to the ATPase activity of Rad51 remains to be explored.

Changes in the oligomeric nature of several SF-1 helicases are known to regulate their functions [30]. While some SF-1 helicases have been shown to unwind DNA as monomers (Dda) [31], others need to multimerize in order to display helicase activity [30,32–35]. In the case of *Escherichia coli* UvrD [32,33,36,37], the monomeric enzymes are rapid and processive ssDNA translocases but display minimal helicase activity and must form at least a dimer in order to activate helicase activity [25]. This is also the case for the *E. coli* Rep helicase. In the case of Rep, it has been shown that a sub-domain (2B) is auto-inhibitory for Rep monomer helicase activity and dimerization relieves this inhibition [34,35,38]. Although the current report has not examined the mechanism by which Srs2 unwinds duplex DNA, our results indicate that multiple Srs2 molecules are required to bind the DNA substrate in order to activate DNA unwinding activity. Qualitatively, we observe two concentration-dependent phases of Srs2 binding to the DNA substrate used

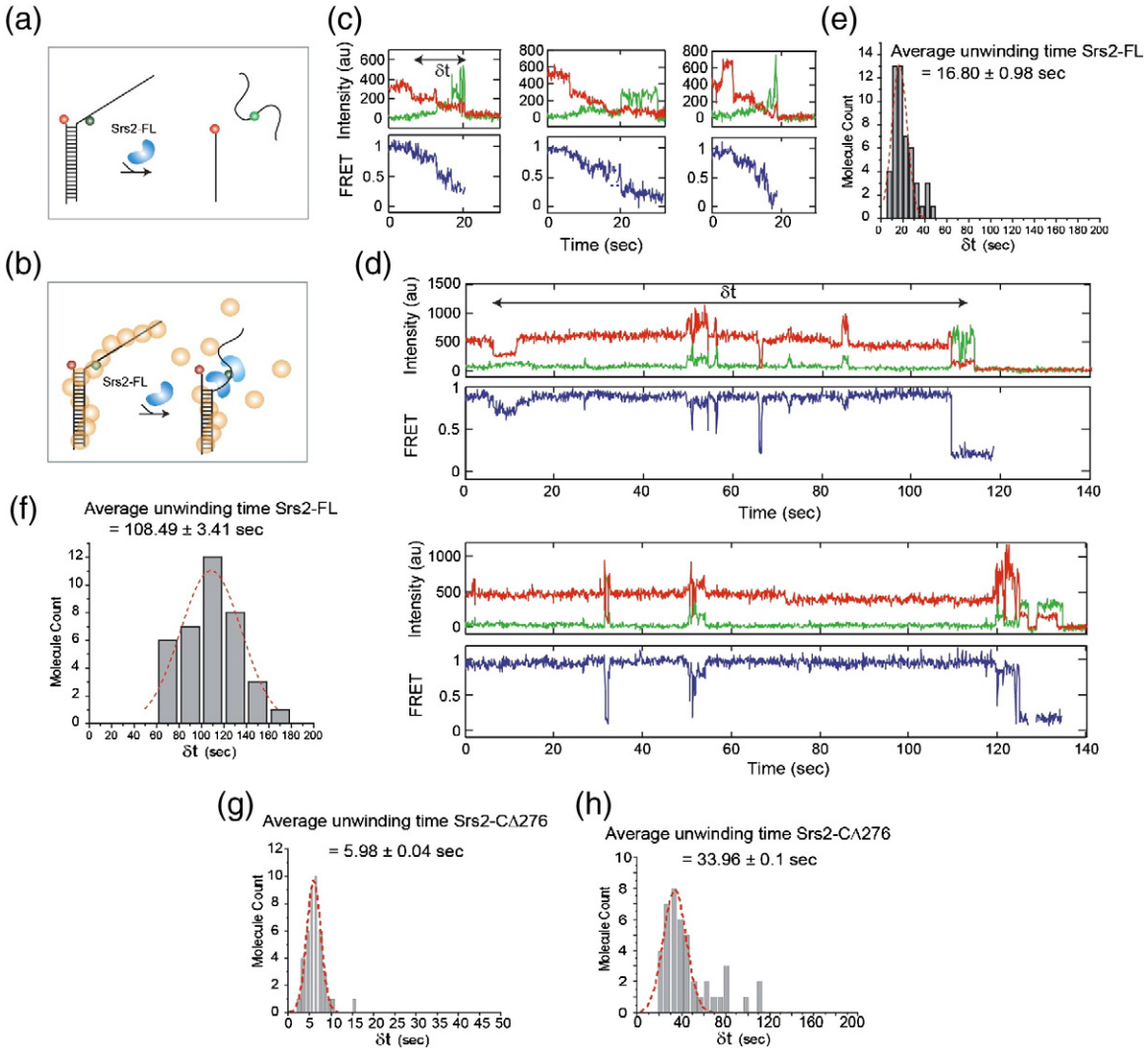

Fig. 8. Rad51-dependent inhibition of DNA unwinding is observed for both $Srs2^{FL}$ and $Srs2^{C\Delta276}$ proteins in smFRET analysis. (a and b) Schematic of unwinding DNA constructs used in this study showing an 18-bp dsDNA substrate with a 20-nt 3′ overhang with Cy3 and Cy5 probes located near duplex junction. Upon DNA unwinding, a decrease in the FRET signal is observed. (c and d) $Srs2^{FL}$ unwinding in the presence of Rad51 is delayed as indicated by slower FRET decrease than unwinding without Rad51. (e and f) Histograms and Gaussian distribution fits show that the $Srs2^{FL}$ unwinding duration is about 6× longer in the presence of Rad51. (g and h) Histograms and Gaussian distribution fits show that the $Srs2^{C\Delta276}$ unwinding duration is about 5–6× longer in the presence of Rad51. Errors in fit results are in SEM (*s*tandard *e*rror of the *m*ean). Rad51 retards the DNA unwinding time by ~5.67-fold and ~6.45-fold for $Srs2^{C\Delta276}$ and $Srs2^{FL}$ proteins, respectively.

for unwinding assays. The high-affinity interaction of Srs2 with the DNA appears to have a pivotal role in both the helicase activity of Srs2 and its inhibition by Rad51. Pre-incubation of the DNA with a sub-threshold 40-nM concentration of a catalytically inactive Srs2 mutant prevents subsequent DNA unwinding by an excess of catalytically active Srs2 protein (Fig. 3). Occupancy of this high-affinity DNA binding sites may interfere with DNA loading or translocation of the active helicase. The high-affinity DNA site also participates in the Rad51-dependent inhibition of the $Srs2^{C\Delta276}$ helicase, and remarkably, pre-incubation with a low concentration of $Srs2^{C\Delta314}$ protects against Rad51 inhibition of DNA unwinding initiated by a high concentration of $Srs2^{C\Delta276}$ (Fig. 3b). Pre-incubation with srs2-K41A causes a sustained block of DNA unwinding (Fig. 3a), and we infer that the $Srs2^{C\Delta314}$ protein also remains bound to the DNA and may function with $Srs2^{C\Delta276}$ in the DNA unwinding reaction (Fig. 3b). Crystal structures of related SF-1 helicases UvrD [26] and PcrA [27] in complex with DNA show a binding site on ssDNA

that spans approximately 7 nt. By analogy, two molecules of Srs2 could bind to the 15-nt ssDNA tail of the preferred substrate for DNA unwinding (Fig. 5c).

The DNA unwinding activity of Srs2 may contribute to the down regulation of HR activity by dissociating branched DNA intermediates of recombination pathways [19]. We have shown that the Rad51 recombinase in complex with dsDNA suppresses the helicase activity of Srs2 through a protein–protein interaction that, in another context, enables Srs2 to disassemble Rad51 filaments on ssDNA (Fig. 9) [4]. This DNA context-sensitive regulation of Srs2 by Rad51 would allow Srs2 to efficiently disassemble the Rad51–ssDNA filament without unwinding adjacent regions of dsDNA. Srs2 is subject to phosphorylation by the Cdk kinase at several residues along the C-terminal region [17,39]. One hypothesis would be that phosphorylation controls binding of Srs2 to Rad51 thereby regulating whether it functions to remove the Rad51 filament or unwind the DNA after Rad51 has been displaced [39,40]. Moreover, the unwinding activity can also be accomplished after Rad51 is displaced from the dsDNA by the Rad54 protein. Such a model could account for the lethality of the *srs2 rad54* strains that are suppressed by the loss of Rad51 [41].

Srs2 allosterically modulates ATP turnover by Rad51 [4], and it will be of great interest to determine if there is a reciprocal effect on ATP hydrolysis by Srs2 that is sensitive to the DNA binding context of the Rad51 recombinase. Moreover, the functional complex of Srs2–Rad51–dsDNA might serve a signaling role, for example, to promote resolution of recombination intermediates into non-crossover products (Fig. 9). Our findings have more general significance in demonstrating that the DNA remodeling activity of an ATP-dependent motor protein can be tailored by protein–protein interactions to produce different outcomes.

Experimental Procedures

Oligonucleotides

Unlabeled and fluorescein-isothiocyanate-labeled DNA oligonucleotides used for the DNA unwinding and binding experiments were synthesized on an ABI 394 DNA synthesizer or purchased from Integrated DNA Technologies (Coralville, IA) and were gel purified before annealing. The dsDNA was

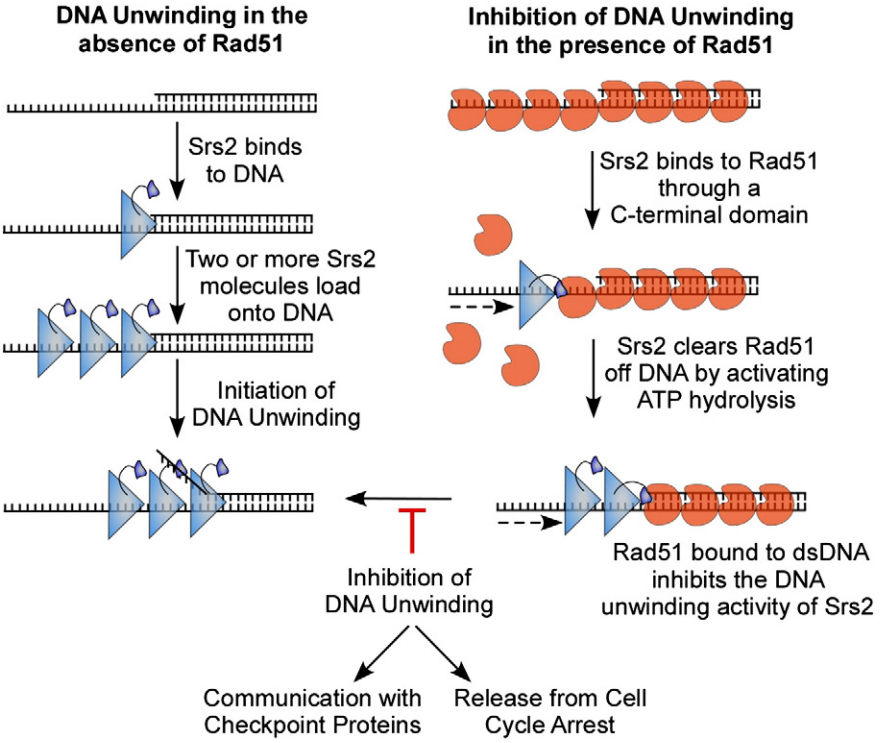

Fig. 9. Context-dependent regulation of Srs2 activity by Rad51. Schematic representation depicts that two or more Srs2 molecules are required to unwind DNA. When Rad51 coats the dsDNA, its specific physical interaction with Srs2 inhibits the DNA unwinding activity. The same physical interaction is required to remove Rad51 molecules from the ssDNA. This context-dependent regulation of Srs2's unwinding activity by Rad51 possibly regulates their activities in HR.

formed by annealing a labeled top strand (EA-T: 5′-CGATCGTCC-TCTAGACAGCTTACGC-3′) and a bottom strand {EA-B: 5′-GCGTAAGCTGTCTA-GAGGAC-GATCG[T]$_{(n)}$, where n = 0–30 nt} to yield a 25-bp dsDNA with 3′ ssDNA overhangs of defined lengths (n). For the smFRET experiments, Cy5 or internally positioned aminomodifier-dT-labeled oligonucleotides were purchased from Integrated DNA Technologies. The following two oligonucleotides were used to form the 18-bp dsDNA substrate with a 17-nt 3′ overhang: pdT3 + 17: 5′-(Cy5)-GCCTCGCTG CCGTCGCCA-(biotin)-3′ + 5′-TGGCGACGGCAGC-GAGGC-(dT)$_3$-(C6dT)-(dT)$_{17}$-3′. The C6 dT was labeled with Cy3 using a Cy3 monofunctional NHS ester (GE Healthcare, Princeton, NJ) as previously described [29].

Proteins

Srs2$^{C\Delta276}$, Srs2$^{C\Delta314}$, Srs2$^{C\Delta276}$-K41A, Rad51 and Rad51-E221D proteins were overexpressed in *E. coli* and purified as described previously [4]. To purify the full-length Srs2 protein, we generated a 9× His-tagged version of Srs2^{FL} with an intervening Precission protease site to facilitate removal of the His tag. Srs2^{FL} protein was overexpressed similar to the truncated versions of Srs2 and the protocol for purification was modified as follows: The biomass was resuspended in Lysis Buffer [50 mM $KHPO_4$ (pH 8.0), 10% sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM 2-mercaptoethanol, 1 M urea, protease inhibitor cocktail and 0.5 M NaCl] and lyzed using lysozyme (0.3 mg/ml) and sonication. Srs2^{FL} was precipitated from the clarified supernatant using 0.26 g/ml ammonium sulfate. The subsequent pellet was resuspended in NTA Buffer [50 mM $KHPO_4$ (pH 8.0), 20% glycerol, 0.5 mM EDTA, 5 mM 2-mercaptoethanol, protease inhibitor cocktail and 0.5 M NaCl] and loaded onto a Ni^{2+}-NTA column. After loading and removal of nonspecific bound proteins, we subjected resin-bound Srs2 to overnight on-column proteolytic cleavage with 1:1000 ratio of Precission protease in Cleavage Buffer [50 mM $KHPO_4$ (pH 8.0), 20% glycerol, 5 mM 2-mercaptoethanol and 0.2 M NaCl]. Cleaved Srs2 was collected and dialyzed into Heparin Buffer [50 mM $KHPO_4$ (pH 8.0), 20% glycerol, 0.5 mM EDTA, 5 mM 2-mercaptoethanol and 0.3 M NaCl] and further subjected to fractionation on a heparin column. Srs2 was loaded through line A on the FPLC and as 50% B onto the heparin column with Heparin Buffer containing no NaCl in line B. This ensured that Srs2 was in high salt buffer until just before entering the column. This key step prevented precipitation of Srs2. Srs2 was eluted with a linear gradient of 0.15–1 M NaCl and fractions containing Srs2 were pooled and very gently concentrated to a final concentration of ~0.5 mg/ml. Srs2 was dialyzed against storage buffer [50 mM $KHPO_4$ (pH 8.0), 20% glycerol, 0.5 mM EDTA, 10 mM 2-mercaptoethanol and 0.3 M NaCl] and small aliquots were flash frozen in liquid nitrogen and stored at −80 °C.

DNA unwinding assays

DNA unwinding by Srs2 was measured by the monitoring the displacement of the labeled EA-T oligonucleotide prepared by labeling with γ-^{32}P-ATP (Perkin Elmer) and polynucleotide kinase (NEB). Labeled DNA strands were annealed with a slight excess of an unlabeled complementary strand, and 5 nM annealed DNA was incubated with increasing concentrations of Srs2$^{C\Delta276/C\Delta314}$ (0–0.25 μM) and ATP (2 mM) in DNA unwinding buffer [50 mM Tris–Cl (pH 8.0), 120 mM NaCl, 10 mM $MgCl_2$, 100 μg/ml bovine serum albumin and 0.2 mM β-mercaptoethanol] at 30 °C for the denoted amount of time before the reaction was quenched with stop buffer [100 mM EDTA (pH 8.0), 20% v/v glycerol and 0.4% SDS]. For unwinding experiments with the Srs2^{FL} protein, 5 nM labeled DNA was incubated with increasing concentrations of Srs2^{FL} (0–0.8 μM) in a modified DNA unwinding buffer [20 mM $KHPO_4$ (pH 8.0), 120 mM NaCl, 10 mM $MgCl_2$, 100 μg/ml bovine serum albumin and 0.2 mM β-mercaptoethanol] at 30 ºC for the denoted time and quenched as described above. The dsDNA substrate and dissociated ssDNA product strand were resolved by EMSA in a 10% TBE (*T*ris–*b*orate–*E*DTA) acrylamide gel (100 V at 25 °C in 1× TBE), dried and quantified using a phosphor imager. For the sequential-incubation DNA unwinding experiments, 5 nM ^{32}P-labeled DNA was first incubated with 40 nM either Srs2-WT or Srs2-mutant at 30 °C for 2 min in DNA unwinding buffer with 2 mM ATP followed by addition of increasing concentrations of either Srs2-mutant or Srs2-WT (0–250 nM), incubated for an additional 2 min before quenching and resolving the products as described above.

To measure the effect of Rad51 on DNA unwinding by Srs2, fluorescein isothiocyanate or ^{32}P-labeled DNA was first incubated with 45 nM Rad51 in DNA unwinding buffer and 2 mM ATP at 30 °C for 2 min followed by addition of increasing concentrations of Srs2$^{C\Delta276/C\Delta314}$ (0–0.25 μM) or Srs2^{FL} (0–0.8 μM). For the experiment where [Rad51] was varied, 0–0.25 μM Rad51 (as noted) was first incubated with the DNA substrate at 30 °C for 2 min followed by Srs2 addition. Unwinding was monitored after 2 min of incubation, quenched, resolved and quantified as described above. The steady-state rate of DNA unwinding was measured similarly in a 100-μl reaction containing 5 nM labeled DNA, 100 nM Srs2 (in the presence or absence of 45 nM Rad51) and 2 mM ATP at 30 °C. Reaction aliquots (10 μl) were removed at the indicated times and quenched with 10 μl of stop buffer, vortexed immediately and stored on ice until the EMSA

analysis was performed as described above. DNA unwinding by $Srs2^{C\Delta276}$ on the long DNA substrates was performed as previously described [11]. Briefly, a 200-bp DNA substrate was generated with a 409-nt 3′ ssDNA overhang as previously described [11] with a Cy5 label on the shorter strand. $Srs2^{C\Delta276}$ (0.5 μM) was incubated with 50 nM labeled DNA for the indicated amount of time in the presence of 0.25 μM RPA and in the presence or absence of 3 μM Rad51. Reactions were quenched with an equal volume of quench buffer (25 mM EDTA, 1% SDS and 0.5 mg/ml proteinase K) and analyzed using EMSA (6.5% TBE acrylamide gel). The gel was scanned using a Typhoon Image set at the Cy5 wavelength.

DNA binding EMSA assay

The DNA binding activity of Srs2 was measured using a ^{32}P-labeled DNA substrate (5 nM) with a 25-nt dsDNA region and a 3′ 15-nt ssDNA overhang. Binding was measured by incubating increasing concentrations of $Srs2^{C\Delta76/C\Delta314}$ (0–0.25 μM) in DNA unwinding buffer in the presence of 2 mM AMP-PNP (to prevent Srs2 from unwinding the DNA). Reactions were incubated at 25 °C for 10 min and the formation of the Srs2–DNA complexes were measured using EMSA on an 8% PAGE gel and resolved in 1 × TBE buffer at 100 V at 25 °C.

Rad51 dissociation

Dissociation of Rad51 was measured by monitoring the release of free radiolabeled DNA from preformed Rad51 filaments using nitrocellulose filter binding technique [42]. Rad51–DNA complexes were preformed using 0.2 μM ^{32}P-labeled DNA, 1.8 μM Rad51 and 5 mM ATP in DNA unwinding buffer (100 μl reaction volume). The reaction was incubated at 25 °C for 2 min to allow filament formation followed by addition of 100 μM unlabeled 79-nt dT ssDNA oligonucleotide as a trap to capture the dissociating Rad51 molecules. At the depicted time points, 10 μl of the reaction was removed and passed through a pre-equilibrated filter and washed with 150 μl of the reaction buffer. The filters were dried and bound DNA was quantified using a phosphor imager. The data were fit to a single-exponential equation [$X = A(1 - \exp^{(-kt)})$] to obtain the rate constant, k, for dissociation of rad51 from DNA, where A is the amplitude.

ATPase assays

ATP hydrolysis during DNA unwinding by Srs2 was measured by mixing increasing concentrations of Srs2 (0–0.25 μM) with 5 nM DNA and 2 mM α^{32}P-ATP (Perkin Elmer) for 2 min at 30 °C in DNA unwinding buffer (10 μl reaction volume). The reaction was quenched by adding 10 μl of 0.5 M EDTA and ADP formation was monitored by spotting 1 μl of the reaction on a TLC plate, dried and resolved with 0.6 M phosphate buffer (pH 3.4). ADP formed was quantified using a phosphor imager.

smFRET experiments

smFRET experiments were carried out in Rad51 reaction buffer containing 50 mM Tris–HCl (pH 7.5), 50 mM NaCl and 10 mM $MgCl_2$, with an oxygen scavenging system (0.8% v/v dextrose, 1 mg/ml glucose oxidase, 0.03 mg/ml catalase and 1% v/v 2-mercaptoethanol). All these reagents were purchased from Sigma Aldrich (St. Louis, MO). The measurements were performed at room temperature (21 ± 1 °C) and 1 mM ATP was used in all the experiments unless otherwise specified. We mixed 1 μM Rad51 with Rad51 reaction buffer and ATP and added it to the immobilized DNA as described previously [29]. Srs2 (200 nM) was mixed with Rad51 reaction buffer and ATP and added to a flow chamber that had 100 pM DNA specifically immobilized on a polyethylene-glycol-coated quartz surface through biotin-neutravidin linkage. smFRET measurements were made using a wide-field total internal reflection fluorescence microscope. Cy3 (donor) on DNA was excited by an Nd:YAG laser (532 nM, 75 mW, Coherent CUBE) via total internal reflection. The fluorescence signals from Cy3 and Cy5 were collected through an objective (Olympus Uplan S-Apo; X100 numerical aperture; 1.4 oil immersion) and detected at 100 ms time resolution using an EMCCD (*e*lectron *m*ultiplying *c*harge-*c*oupled *d*evice) camera (iXon DU-897ECS0-#BV; Andor Technology). The camera was controlled using a custom C++ program. Single-molecule traces were extracted from the recorded video file by IDL software (Exelis VIS, Boulder, CO).

Acknowledgements

We thank Dr. Tom Ellenberger and Dr. Tim Lohman (Washington University School of Medicine in St. Louis, MO) for supporting preliminary data collection in their laboratories. We also thank Dr. Peter Burgers (Washington University School of Medicine in St. Louis, MO) for providing us with yeast RPA protein. This work was supported through start-up funds from Utah State University to E.A. S.M and Y.Q. were supported by a an NIH Director's New Innovator Award (1DP2GM105453) and a Research Scholar Grant from the American Cancer Society (RSG-12-066-01-DMC). J.V. was supported by an undergraduate research and creative opportunities grant from Utah State University.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jmb.2014.02.014.

Received 3 August 2013;
Received in revised form 10 February 2014;
Accepted 16 February 2014
Available online 24 February 2014

Keywords:
Srs2;
DNA unwinding;
Rad51;
homologous recombination;
helicase

Abbreviations used:
ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; HR, homologous recombination; EMSA, electrophoretic mobility shift analysis; smFRET, single-molecule FRET; EDTA, ethylenediaminetetraacetic acid.

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