Physical Interaction between Replication Protein A and Rad51 Promotes Exchange on Single-stranded DNA*

Received for publication, January 5, 2004, and in revised form, March 10, 2004
Published, JBC Papers in Press, March 31, 2004, DOI 10.1074/jbc.M400029200

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Replication protein A (RPA) is displaced from single-stranded DNA (ssDNA) by Rad51 during the initiation of homologous recombination. Interactions between these proteins have been reported, but the functional significance of the direct RPA-Rad51 interaction has yet to be elucidated. We have identified and characterized the interaction between DNA-binding domain A of RPA (RPA70A) and the N-terminal domain of Rad51 (Rad51N). NMR chemical shift mapping showed that Rad51N binds to the ssDNA-binding site of RPA70A, suggesting a competitive mechanism for the displacement of RPA from ssDNA by Rad51. A structure of the RPA70A-Rad51N complex was generated by experimentally guided modeling and then used to design mutations that disrupt the binding interface. Functional ATP hydrolysis assays were performed for wild-type Rad51 and a mutant defective in binding RPA. Rates of RPA displacement for the mutant were significantly below those of wild-type Rad51, suggesting that a direct RPA-Rad51 interaction is involved in displacing RPA in the initiation stage of genetic recombination.

Homologous recombination (HR) is a fundamental means for processing DNA in eukaryotes and is used for repairing double-strand DNA breaks, segregating genes during meiosis, and resolving stalled replication forks. HR proceeds by multiple pathways whose common features include invasion of a homologous duplex by single-stranded DNA (ssDNA), formation of a joint molecule, DNA strand exchange, and resolution of one or more Holliday junctions (1). Dynamic protein interactions are fundamental to progression through the ordered steps of DNA-processing pathways (2), and HR is no exception (3, 4). Each step of HR, including presynapsis, strand exchange, and postsynapsis, is catalyzed by a network of interacting proteins comprising Rad51, Rad52, and many other recombination mediator proteins (5, 6).

The “catalytic triad” of presynapsis is Rad51, Rad52, and replication protein A (RPA). When double-strand breaks occur in DNA, RPA binds and protects exposed ssDNA ends until they can be coated by Rad51 (7). It also removes secondary structures that prevent formation of a continuous Rad51 filament (8). Rad51 forms a helical filament along the exposed ssDNA, hydrolyzing ATP in the process (9). It then catalyzes the homologous pairing and strand exchange steps of HR (10). Rad52 facilitates the displacement of RPA by Rad51, a step necessary for efficient nucleoprotein filament formation, via direct interactions with both RPA and Rad51 (11). In strand exchange reactions, Rad52 catalyzes HR by helping Rad51 overcome the inhibitory effects of RPA-coated ssDNA (12, 13).

The structural interactions of the presynaptic proteins have been studied extensively. NMR and x-ray crystal structures are available for each domain of RPA (14–17) and Rad51 (18–20) and for the DNA-binding/oligomerization domain of Rad52 (21). Interactions between several of the domains have been characterized (16, 22–26), but there are no published structures of any complexes. Indeed, the structural basis for the catalytic effect of Rad52 is still not fully understood.

One of the key unanswered questions is whether or not Rad52 facilitates a direct interaction between Rad51 and RPA. Evidence supporting this idea comes from studies of the yeast protein network that have shown that Rad52 cannot by itself displace RPA from ssDNA; Rad52 needs Rad51 (11). Also, a direct interaction between human RPA and Rad51 has been reported by Golub et al. (23). Evidence against a direct RPA-Rad51 interaction is based on studies showing that Escherichia coli single-stranded DNA-binding protein can substitute for RPA in several steps of the strand exchange reaction (8) and that the fact that a ternary RPA-Rad51-DNA complex has never been isolated (11). However, the latter findings pertain to yeast proteins, and species specificity may be a determining factor in how different systems perform HR. For instance, a RAD52-null mutation renders Saccharomyces cerevisiae cells nearly incapable of double-strand break repair, whereas the same mutation in mouse cells has only a modest effect on HR levels and no effect at all on DNA repair or meiosis (27). Likewise, Rad51 protein is essential for viability of mammalian organisms, whereas in yeast cells it is dispensable (28). Although functional redundancy may explain some or most of these phenomena, species-specific protein interactions likely also play a part. Indeed, Shen et al. (22) have pointed out that the Rad51-interacting region of human Rad52 has no sequence similarity with the same region of yeast Rad52, implying that the interaction is species-specific. Subsequent data from Kurumizaka et al. (24) established that human Rad52 binds to a completely
different domain of Rad51 than the yeast protein.

This article reports the biochemical and structural character-
ization of a specific interaction between human RPA and Rad51.
The initial work was carried out using the known minimal structural domains, and then the findings were tested in the context of the full-length proteins. Our results provide the first evidence that the physical interaction between RPA and Rad51 plays a role in initiating HR events in humans. They also support a general functional mechanism of regulat-
ing DNA processing by altering the ability of RPA to bind ssDNA.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—The Rad51 expression plasmid was obtained from Dr. Jeffrey Holt of Vanderbilt University. Full-length Rad51 protein was purified by the method of Kurumizaka et al. (24). The N-terminal domain (Rad51N, residues 1–93) was subcloned into pET15b (Novagen) using PCR amplification followed by ligation into the digested vector. RPA constructs (RPAT70AB, residues 181–422; RPAT70A, residues 181–291; and full-length RPA heterotrimer) were expressed kindly provided by Dr. Alexey Bochkarev of the University of Oklahoma Health Sciences Center. For all constructs, protein expression was achieved by growing overnight cultures of Rosetta pLysS cells (Nova-
gen) at 37 °C in LB. These were used to inoculate 1-liter batches of LB, which were grown at 37 °C until A600 ~ 0.7. Isopropyl-1-thio-
galactopyranoside was added to 1 mM, and the cultures were incubated for 3 h longer. Cells were harvested by centrifugation and frozen at −80 °C until lysis. To obtain isotopically labeled proteins, cultures were grown in M9 minimal medium containing 15N,NCl as a nitrogen source.

For RPAT70AB, RPAT70A, and Rad51N, cell pellets were resuspended in an appropriate volume (~5 ml/g) of 50 mM Tris, pH 8.0, 0.5 mM NaCl, 10 mM imidazole, 5 mM BME, 10% glycerol. Cells were lysed by sonication in the presence of lysozyme, and the soluble fraction was isolated by centrifugation. The cleared supernatant was loaded onto a 25-ml Ni2+-affinity column in lysis buffer. The His-tagged protein was eluted with a 25-ml gradient of 10–500 mM imidazole. RPAT70AB was further purified by passage over a MonoQ column, followed by elution in a gradient of 10–500 mM KCl in 10 mM Tris, pH 7.0, 1 mM BME.

His tags were removed from RPAT70A and Rad51N as follows. The fractions from the Ni2+-column were exchanged by dialysis or centrifug-
ugal concentration into 10 mM Tris or Heps, pH 7.0, 20 mM KCl, 1 mM BME. Thrombin was added at ~1 unit/mg of protein, and aliquots were taken at various time points to monitor cleavage of the His tag by SDS-PAGE. At completion, the sample was concentrated and loaded onto a 16/60 Superdex-75 sizing column in the same buffer. The desired protein was efficiently separated from both high molecular weight contain-
tants and the His tag by this step, and protein yields were typically 10–15 mg/liter. Full-length RPA heterotrimer was purified by essen-
tially the same protocol as described for RPAT70A, excluding the throm-
bin cleavage step.

Proteolysis Reactions—20 ng of proteinase K were added to ~20 μg of Rad51N or RPAT70AB or the combination thereof in a reaction volume of 85 μl. 10-μl aliquots were removed at the specified times for SDS-PAGE analysis. Reactions were run in 20 mM Tris, pH 8.0, 1 mM BME at room temperature. Protease stock solution was made fresh on the day of the experiment.

NMR Data Acquisition—NMR samples of 15N-labeled Rad51N, RPAT70A, and RPAT70AB were prepared at 0.3–0.5 mM in 10 mM Tris, pH 7.0, 20 mM KCl, 1 mM BME. 1H-15N HSQC experiments were performed at 300 K on a Bruker Avance 800-MHz spectrometer using an inverse detection probe head equipped with triple-axis gradients. Unlabeled protein additions were made directly from a concentrated stock with concentration and pH adjustments made to the NMR sample as needed. Spectra were processed and visualized with XEASY software (Accelrys, Inc.). Precisions for both RPAT70A and Rad51N spectra were obtained by transfer from previously collected and published data (18, 29). For the NMR displacement experiment, a single-stranded oligonucleotide (d-CCTCA) was added from a concentrated stock into a sample of the prefomed RPAT70A-Rad51N complex. Resonances were observed for the 15N-la-
beled Rad51N protein domain over the three sequential additions resulting in a slight excess of oligonucleotide.

Chemical shift mapping was performed by recording the chemical shift of each peak before the titration and again at saturation. The normalized change in chemical shift was calculated by the formula

\[ \text{obs} = \frac{1}{\sqrt{\Delta H^2 + \Delta N^2} + \text{HH}} \]

where ΔH and ΔN are the linear change along the 1H and 15N axes, respectively, and n is the ratio of the chemical shift dispersion in 15N and 1H. Chemical shift perturbations were deter-
mined to be significant if they were ≥0.03 ppm for RPAT70A and ≥0.04 ppm for Rad51N.

Fluorescence Data Acquisition—Titrations and displacement assays were carried out at 25 °C on a Fluoromax-3 fluorometer from Jobin Yvon. Trypsin fluorescent was monitored by excitation at 295 nm and detection of emission at 345 nm. Slit widths were 1 mm. Contributions of buffer and Rad51N to the signal were subtracted from each measurement. RPAT70A has two tryptophans, one at the DNA-protein-
binding site and one at a remote location. For protein-DNA interaction titrations, RPAT70A concentrations were ~20 μM, and Rad51N was added up to a 3+ molar excess. Single stranded DNA (d-CCTCA) was added in 1–2 μM increments from a concentrated stock.

Calculation of Dissociation Constants—The NMR and fluorescence data were plotted as cumulative fluorescence or chemical shift change versus concentration of the added protein or DNA molecule. The result-
ing curves were fit to the following equation

\[ \frac{\Delta F_{\text{max}}}{\Delta F_{\text{max}}} = \frac{K_0 + N_0 + R_0 - (K_0 + N_0 + R_0)_{\text{obs}}}{2K_0} \]

which was derived from the basic formulas

\[ K_0 = \frac{(R_0 - RN)}{RN} \]

\[ RN = R_0 \frac{(\Delta F_{\text{max}})}{\Delta F_{\text{max}}} \]

where \( F_{\text{max}} \) is the observed fluorescence change at each titration point, \( F_{\text{max}} \) is the maximum observed fluorescence change during the titra-
tion, \( R_0 = \text{total RPA concentration, } N_0 = \text{total Rad51N concentration, } R_0 = \text{complex concentration. In each formula, } F \) can be substituted with \( \delta \) for the NMR chemical shift change; both types of data are fit in the same way. DNA concentration was substituted for protein where appropriate. The program ZAP (31) was used to perform the least squares fitting procedure, and the values for \( K_0, F_{\text{max}}, \) and the constant mole-
cule concentration were allowed to float.

Docking Calculations—The program HADDOCK (30) was used to dock RPAT70A and Rad51N using ambiguous restraints. Protein Data Bank files were obtained for Rad51N (code 1B22) and RPAT70A (code 1FGU). Only the coordinates for the A domain of RPAT70A were used. Active and passive residues were selected according to the specifications of Dominguez et al. (30). The HADDOCK protocol defines active residues as those that undergo a significant chemical shift perturbation and have a high solvent accessibility in the free protein structure. Passive residues are those that either undergo a less significant chemical shift perturbation or are surface neighbors to active residues and that have a high solvent accessibility. Interface segments were defined as resi-
dues 16–22, 54–60, 66–70, and 70–78 for Rad51N and residues 208–220 and 258–277 for RPAT70A. Chemical shift perturbations were the only experimental restraint type incorporated into the calculation. De-
fault settings were used for the topology and parameter files, for struc-
ture sorting, and for all simulated annealing stages. In the final run reported here, 1000 structures were docked, and 200 of those were refined and analyzed according to the HADDOCK algorithm. The best 100 structures were selected for water refinement in the last stage. Minimum cluster size was set to 4 with a maximum pairwise backbone root mean square deviation of 1.5 A. Structures were visualized in InsightII version 2000.1 (Accelrys Inc.) and visualized with R�.

Rad51N Mutants—Point mutations were incorporated into the Rad51N or full-length Rad51 expression plasmids using the QuikChange site-directed mutagenesis kit from Stratagene. Briefly, PCR was performed using complementary primers that incorporated the desired mutation. The entire plasmid was amplified, and the meth-
ylated parental strand was digested by DpnI enzyme. All mutations were confirmed by DNA sequencing using the Vanderbilt DNA-se-
quecing facility. Mutant proteins were expressed and purified as was done for the wild type, and their behavior in solution was indistinguish-
able from the wild type.

ATP Hydrolysis Assays—The ssDNA-dependent ATP hydrolysis ac-
ivity of Rad51 was measured following the protocol of Sagiya and Kowalczykowski (11) with a few modifications. Experiments were per-
formed in a buffer of 30 mM Tris, pH 7.5, 50 mM KCl, 5 mM MgCl2, 1 mM dithiothreitol, and 100 mM (NH4)2SO4. A standard experiment con-
tained ~1 μM Rad51 protein, 0.3 mM phosphoenolpyruvate, 8 units/ml

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pyruvate kinase, 13 units/ml lactate dehydrogenase, 2.5 mM ATP, and 250 μM NADH. For the Rad51-first conditions, the sample was equilibrated at 37 °C for 20 min, followed by addition of φX-174 viral ssDNA (New England Biolabs, 2.7 μM final concentration). The absorbance at 340 nm was monitored for the next 40–60 min, after which RPA was added to 0.5 μM.

For RPA-first conditions, 0.3 μM RPA was equilibrated at 37 °C in the reaction buffer and assay components with 2.7 μM ssDNA. After 20–60 min, Rad51 protein was added at various concentrations as shown in Fig. 4A. A_{340} was recorded for 60 min thereafter. The rate of hydrolysis was determined from the difference in A_{340} at 45 and 55 min after the addition of Rad51 protein ([ΔA_{340}/Δt × 9880] = rate of ATP hydrolysis in μM/min). Average background values (the ATP hydrolysis rates measured just prior to addition of Rad51 protein) were determined to be 0.21 and 0.22 for the wild-type and mutant data sets, respectively.

RESULTS

The goal of these studies was to demonstrate that the exchange of RPA for Rad51 on ssDNA requires physical interaction between the proteins. Our strategy was to identify the exact points of contact and then use functional assays to test the relevance of the interaction. The domain mapping of the binding sites was greatly facilitated by the knowledge of the domain structures of both RPA and Rad51. This allowed a rapid determination of the contact points and the design of mutations to probe the accuracy of the structural model. The corresponding mutations in intact Rad51 were then used in an RPA displacement assay to demonstrate the functional relevance of physical interaction between the two proteins.

Defining the Binding Site—The RPA heterotrimer consists of 70-, 32-, and 14-kDa subunits. The 70-kDa subunit (RPA70) possesses four structured domains: N, A, B, and C. The N domain is involved primarily in protein interactions, whereas domain C binds only to ssDNA. The bifunctional region comprising domains A and B (RPA70AB) contains both high affinity ssDNA-binding sites and protein association capability. Golub et al. (23) have previously localized a Rad51-binding site to residues 181–326 of RPA70, which includes the A domain and about one-fourth of the B domain. Since fractional portions of a structured domain are not likely to be functionally relevant, we began this investigation by determining precisely where Rad51 interacts within the tandem RPA70AB construct (residues 181–422).

Since the C-terminal domain of Rad51 is associated with catalytic activity, we surmised that the N-terminal domain (residues 1–93) might be involved in interactions with RPA. Indeed, limited proteolysis experiments on RPA70AB revealed that addition of Rad51N results in a reduction of the rate of digestion by proteinase K (Fig. 1A). Previous studies (18, 29) had shown that both RPA70AB and Rad51N are amenable to digestion by proteinase K (Fig. 1). Peaks of Rad51N. Peaks are labeled according to their residue-specific assignments, and arrows designate which peaks belong to domains A and B, SC, side chain amide.

Fig. 1. Localization of the Rad51-binding site. A. 4–12% SDS-PAGE analysis of proteolysis rates for Rad51N (left), RPA70AB (right), and the combination thereof (N + AB, center). Lane labels correspond to the starting sample (St), 5 or 30 min (5’ or 30’), or 1 h (1hr). B, overlay of the NMR spectra for RPA70AB in the absence (gray) and presence (charcoal) of Rad51N. Peaks are labeled according to their residue-specific assignments, and arrows designate which peaks belong to domains A and B, SC, side chain amide.

Fig. 2C depicts the interacting surface of each protein based on NMR chemical shift mapping. The data reveal that Rad51N has separate binding sites for DNA and RPA, whereas RPA70A binds to both ssDNA and Rad51N at the same site. This result is suggestive of competition at the RPA70A binding site.

Competition for the Binding Site in RPA70A—Modulation of the ssDNA binding affinity of RPA by protein interaction has its precedent in the SV40 viral replication system. Studies in our laboratory on the interaction of the origin-binding domain of T-antigen with RPA70AB reveal a clear allosteric effect on the binding of ssDNA by RPA70AB.2 This prompted us to test whether or not Rad51 operates by the same mechanism. Thus, the effect of the Rad51N-RPA70A interaction on the affinity of RPA70A for ssDNA was examined. Fluorescence titrations observing the addition of ssDNA to RPA70A were carried out in the presence and absence of saturating concentrations of

2 A. I. Arunkumar and W. J. Chazin, unpublished results.
Rad51N (Fig. 3A). The difference between the calculated dissociation constants in the absence and presence of Rad51N was well within the error of the measurement. Thus, the interaction between Rad51N and RPA70A has no significant effect on the ssDNA binding capacity of RPA70A. To confirm this conclusion, the NMR chemical shift perturbations of RPA70A upon addition of Rad51N were fit to the standard binding equation. The average dissociation constant of $10^{8}/H_{1006}$ estimated in this manner is about $100^{8}/H_{1003}$ weaker than the average affinity for ssDNA (31).

In complementary NMR experiments, ssDNA was added to a sample of 15N-enriched Rad51N preloaded with RPA70A. Fig. 3B shows that when increasing amounts of ssDNA are added, the NMR signals of Rad51N revert from their positions in the protein complex back to locations characteristic of free Rad51N. This result confirms the location of the binding site indicated by chemical shift mapping and also shows direct competition between ssDNA and Rad51N for the binding site on RPA70A.

We note that the inability of Rad51N to displace RPA70A from a pentadeoxynucleotide runs counter to its known biochemical function in vivo. To understand this, it is important to consider that recombination is initiated by full-length Rad51 and RPA on long tracts of DNA. Clearly, there are additional factors beyond what is found in this study of domains extracted from their normal biological context. It is therefore imperative to test the functional relevance of the Rad51N-RPA70A interaction. Consequently, mutagenesis experiments and biochemical assays using intact proteins have been performed as described in the following sections.

**Point Mutations at the Binding Site**—To determine the functional relevance of the interaction of RPA70A and Rad51N, mutations were designed to interfere with the interaction. To provide a basis for choosing mutations at the binding site, chemical shift mapping data were used to generate an ensemble of structural models by docking the known structures of RPA70A and Rad51N. HADDOCK version 1.0 (30) produced a cluster of 58 structures in which the C-terminal helix of Rad51N (residues 69–80) was oriented orthogonally to the axis of the RPA70A ssDNA-binding site. A minor cluster of 10 structures predicted the same general interface but had Rad51N reoriented by essentially a 180° rotation. The key common feature of all structures was the insertion of the acid-rich N-terminal segment of Rad51N into the ssDNA-binding cleft of RPA70A (Fig. 4A).

All residues selected for site-directed mutagenesis were located in the interface between RPA70A and Rad51N in the model. The most evident feature of the complex was a high degree of electrostatic complementarity at the binding interface. Electrostatic interactions are long range and dynamic, and a number of specific Rad51N residues were involved in salt bridges with one or more RPA70A residues in the majority of the structural models. Three of these Rad51N residues (Asp-72, Lys-80, and Glu-17) were selected for mutagenesis. Asp-72 and Lys-80 are in the C-terminal helix of Rad51N and were mutated to arginine and glutamate, respectively. Glu-17 is positioned in the middle of a run of glutamates (residues 16–18) at the N-terminal end of Rad51N, and this residue was mutated to a lysine. Fig. 4B shows the spectra of RPA70A before and after the addition of wild-type Rad51N and the three mutants.
D72R and K80E cause the same chemical shift changes as the wild-type protein. In contrast, the lack of chemical shift perturbations upon addition of E17K shows that this mutation greatly diminishes the interaction between Rad51N and RPA70A. Charge reversal at the binding site likely alters the binding equilibrium, resulting in the observation of less molecular interaction at the concentrations used.

**Functional Effects of Altering the Binding Site**—The functional effects of disrupting the RPA70A-Rad51N interaction were tested in the context of the full-length proteins using an RPA displacement assay. Under specific conditions, Rad51

![FIG. 3. Affinity of RPA70A for ssDNA.](image)

**A**, fluorescence titrations of RPA70A or RPA70A-Rad51N with ssDNA. **B**, displacement of RPA70A from Rad51N by addition of ssDNA. Equivalent regions of the $^{15}$N-$^1$H HSQC spectrum of Rad51N show how the peaks move in response to (upper panel) the addition of RPA70A and (lower panel) the subsequent addition of ssDNA. The upper spectrum overlay has the original Rad51N spectrum shown in black, an intermediate titration point in charcoal, and the RPA70A-saturated state in gray. The lower overlay has the same gray spectrum, with an intermediate addition of ssDNA shown in charcoal, and the ssDNA saturation point is shown in black.

![FIG. 4.](image)

**A**, a representative model of the RPA70A-Rad51N interaction. The structure shown is the lowest energy structure from the major cluster of the HADDOCK calculation. For clarity (and because the N-terminal tail of Rad51 is disordered up to residue 16), only residues 16–20 from Rad51N are shown. The backbone of RPA70A is rendered as a grey ribbon, and the Rad51N segment is shown as a stick model colored white, with the side chain of Glu-17 displayed and colored black. **B**, NMR assay of Rad51N mutants. The panels compare equivalent regions of the $^{15}$N-$^1$H HSQC spectra of RPA70A in the absence (black) and presence (gray) of saturating Rad51N. Spectra are shown for the wild-type Rad51N (WT) and for three mutants. Peaks are labeled according to their residue-specific assignments. The wild-type spectra have a different spectral width, so they are scaled slightly differently.
alone can displace RPA from ssDNA, albeit much more slowly than in the presence of Rad52. The displacement can be monitored directly by observing the increase in fluorescence of RPA or indirectly by measuring the ATP hydrolysis rate of Rad51 as it polymerizes along the DNA. Both methods have been demonstrated by Sugiyama and Kowalczykowski (11) as an accurate way to monitor the same event, nucleation of Rad51 on ssDNA that has been coated with RPA. The time course of Rad51 binding measured by ATP hydrolysis exactly matches that of RPA displacement measured by fluorescence, and both are dependent on the order in which the proteins are added to the reaction. Furthermore, data from both methods are subject to the catalytic effects of Rad52, indicating that the same events are being observed in each type of experiment (11). We chose the ATP hydrolysis assay for its ability to measure both ATPase activity and RPA displacement since preliminary characterizations of the ATPase activity of the Rad51 proteins were required. This allowed us to use the same set-up for all of the experiments. After producing the E17K mutation in the full-length Rad51 protein, ATP hydrolysis assays were carried out on both the wild-type and mutant proteins.

ATPase activity was confirmed for both wild-type and mutant Rad51 by adding ssDNA (40X-174 virion, 5.3 kb) to them in the presence of ATP (Table I). The normalized ATP hydrolysis rates were equivalent within the error of the measurement, and both proteins exhibited a ~3.2-fold increase in hydrolysis rate upon addition of RPA. This immediate jump in ATP hydrolysis in response to addition of RPA has also been reported by Sugiyama and Kowalczykowski (11) and arises from the RPA-induced relaxation of secondary structures in the ssDNA that cause discontinuous Rad51 polymerization. These results confirmed that the mutation does not affect ATP hydrolysis activity, as was expected, since Glu-17 is not in the ATP-binding site or at the self-association interface for Rad51. According to our data, it only affects the interaction of Rad51N with RPA70A.

Having established the ATPase activity of the wild-type and mutant Rad51 proteins, each Rad51 protein was added to a preformed RPA-ssDNA complex, and ATP hydrolysis was observed for 60 min. As described above, this allowed us to monitor both the loading of Rad51 on the ssDNA and the simultaneous displacement of RPA. Fig. 5A shows the results of repeating this assay at various concentrations of Rad51 proteins. For both wild-type and mutant Rad51, the ATP hydrolysis rate increased as a function of protein concentration. This relationship is also seen for yeast Rad51 and confirms that nucleation of the Rad51 protein is the rate-limiting step in nucleoprotein filament formation (11). Comparison of the wild-type values with values reported for yeast Rad51 agrees with the published 3-fold difference in ATP hydrolysis activity for the two proteins (11, 38). The ATP hydrolysis rate increased more rapidly as a function of concentration for wild-type Rad51 than for the E17K mutant, indicating that the loss of protein association in the mutant results in decreased ability to displace RPA. Our data suggest that Rad51 nucleation is mediated, at least in part, by the interaction between Rad51N and RPA70A, and therefore ATP hydrolysis decreases in the absence of this interaction.

**DISCUSSION**

The fact that the Rad51 E17K mutant has any level of ATP hydrolysis above the background implies that this isolated protein-protein interaction surface is not the only factor mediating the binding of Rad51 to RPA or stimulating displacement of RPA from ssDNA. We have characterized a single contact

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**TABLE I**

|                     | Rate   |
|---------------------|--------|
|                     | Before RPA | After RPA |
| Wild type           | 0.209 μ/min | 0.683 μ/min |
| E17K mutant         | 0.261 μ/min | 0.838 μ/min |

* Calculated ATP hydrolysis rates were normalized to equivalent Rad51 concentrations. The estimated error for each measurement was <0.15 μ/min.
point between RPA and Rad51, but multiple interactions with RPA are anticipated, as has been shown for both Rad52 (26) and xeroderma pigmentosum group A protein (XPA (32, 33)). XPA modulates the function of RPA during nucleotide excision repair by inhibiting its strand separation activity (34) and increasing its selectivity for undamaged ssDNA (35, 36). XPA binds to both the 70- and the 32-kDa subunits of RPA, and the total affinity of the full-length proteins is significantly higher (at least 5-fold) than that of the individual domains (33). The same is likely true for RPA and Rad51. Indeed, we have obtained preliminary evidence for an additional interaction between Rad51 and the 32-kDa subunit of RPA (23).

The in vivo network of interactions also includes multiple associations of both RPA and Rad51 with additional recombination mediator proteins. Rad52, for example, interacts with both RPA and Rad51 to catalyze the initiation of HR (13). Within this network, the weakness of individual domain interactions is viewed as a positive contributor to dynamic progression from initiation to the strand exchange phase of recombination. Specifically, the weak interaction between Rad51 and RPA would allow them to dissociate easily when Rad51 filament formation begins. While future experiments will focus on the role of the Rad51-RPA interaction within the context of the ternary Rad51-Rad52-RPA network, we note again that Rad52 is expendable in mice for Rad51-dependent strand exchange. Within this network, the weakness of individual domain interactions is important, at least in mammals. Furthermore, analysis of isolated systems is necessary to deconvolute the contribution of individual interactions to the overall reaction of strand exchange.

Like Rad51N, XPA binds to the ssDNA-binding site of RPA70A (33). This suggests a competitive mechanism whereby XPA regulates the function of RPA during nucleotide excision repair. Our results also suggest a scenario in which Rad51 displaces RPA from ssDNA by competing for its primary ssDNA-binding site. Together, the data support competition for overlapping binding sites as a potential mechanism for regulation of the ssDNA binding activity of RPA. In addition, the preference of Rad51 for RPA70A (versus DNA-binding domain D) maximizes the influence of a very weak protein interaction. Since RPA70A has the highest affinity, it is the first of the DNA-binding domains to load onto ssDNA (31), and competition with this domain has the largest possible effect on the ssDNA binding affinity of RPA.

Structural modeling indicates that the N-terminal tail of Rad51 inserts into the ssDNA-binding cleft of RPA70A. If this is true, it is likely that the trio of glutamates (residues 16–18) in the N-terminal tail mimics the phosphate backbone of ssDNA. The RPA70A-binding site appears to be designed to accommodate a fixed number of negatively charged moieties (14). Although the site can adjust dynamically to DNA sequence diversity (37), it cannot tolerate the replacement of a negative charge with a positive charge. Such a charge reversal is shown here to disrupt the interaction of RPA70A with Rad51N. In contrast, charge reversal at Asp-72 and Lys-80 failed to affect the protein interaction significantly. This is most likely because the C-terminal helix of Rad51N is at the periphery of the interaction site and thus is not a major contributor to the total interaction energy. This conclusion is consistent with the fact that residues in this helix are not perturbed during chemical shift-mapping experiments. Both methods indicate that a highly localized region of Rad51N interacts with RPA70A.

In summary, our results support the generally accepted functional coupling between Rad51 and RPA, and we have characterized a specific contact point between Rad51N and RPA70A that influences this coupling. Whereas Rad51N has separate sites for DNA and protein interactions, RPA70A utilizes a multifunctional binding site for interactions with Rad51N. A single-site mutation rendering Rad51N incapable of binding to RPA70A led to a decrease in the ability of Rad51 to displace RPA from ssDNA. These results suggest that the RPA70A-Rad51N interaction plays a fundamental role in filament formation by Rad51, facilitating subsequent initiation of recombination. Our data support the view that direct competition for overlapping binding sites on RPA is a general mechanism for influencing the choice of, or progression through, DNA-processing pathways. Our findings fit well with a simple model for how the RPA-Rad51 interaction mediates initiation of HR (Fig. 5B).

This model serves as a useful starting point, motivating further investigations of interactions between Rad51 and RPA as well as future studies incorporating an increasing number of recombination mediators that facilitate homologous recombination events.

Acknowledgments—We thank Jaison Jacob for assistance in setting up the NMR experiments, Dr. Steve Korban for invaluable advice on the ATP hydrolysis assays, Drs. Alexandre Bonvin and Jarrod Smith for help with running HADDOCK, and Craig Vander Kooi and Dr. Shibani Bhattacharya for critical reading of the manuscript.

REFERENCES

1. Crowley, G. A., Connelly, J. C., and Leach, D. R. (2001) Mol. Cell 8, 1163–1174
2. Kowalczykowski, S. C. (2000) Nat. Struct. Biol. 7, 1087–1089
3. Essers, J., Houts muller, A. B., van Veelen, L., Paulusma, C., Nigg, A. L., Pastink, A., Vermeulen, W., Hoeijmakers, J. H., and Kanaar, R. (2002) EMBO J. 21, 2003–2013
4. West, S. C. (2003) Nat. Rev. Mol. Cell Biol. 4, 435–445
5. Bianco, P. R., Tracy, R. B., and Kowalczykowski, S. C. (1998) Front. Biosci. 3, D570–D605
6. Pauques, F., and Haber, J. E. (1999) Microb. Mol. Biol. Rev. 63, 349–404
7. Gastor, S. L., Olivares, H., Ear, U., Hari, D. M., Weichselbaum, R., and Bishop, D. K. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 8411–8418
8. Sugiyama, T., Zaitseva, E. M., and Kowalczykowski, S. C. (1997) J. Biol. Chem. 272, 7940–7945
9. Yu, Y., Jacobs, S. A., West, S. C., Ogawa, T., and Egelman, E. H. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 8419–8424
10. Baumann, P., Benson, F. E., and West, S. C. (1996) Cell 87, 757–766
11. Sugiyama, T., and Kowalczykowski, S. C. (2002) J. Biol. Chem. 277, 31663–31672
12. Sung, P. (1997) J. Biol. Chem. 272, 28194–28197
13. New, J. H., Sugiyama, T., Zaitseva, E., and Kowalczykowski, S. C. (1998) Nature 391, 470–473
14. Bochkarev, A., Pfuetzner, R. A., Edwards, A. M., and Frappier, L. (1997) Nature 385, 176–181
15. Jacobs, D. M., Lipton, A. S., Isern, N. G., Daughdrill, G. W., Lowry, D. F., Gomes, X., and Wold, M. S. (1999) J. Biomol. NMR 14, 321–331
16. Mer, G., Bochkarev, A., Gupta, R., Bochkareva, E., Frappier, L., Ingles, C. J., Edwards, A. M., and Chazin, W. J. (2000) Cell 103, 449–454
17. Bochkareva, E., Kordovez, S., Lee-Miller, S. P., and Bochkarev, A. (2002) EMBO J. 21, 1855–1863
18. Aihara, H., Ita, Y., Kurumizaka, H., Yokoyama, S., and Shibata, T. (1999) J. Mol. Biol. 290, 495–504
19. Pellegrini, L., Yu, D. S., Lo, T., Anand, S., Lee, M., Blundell, T. L., and Venkitaraman, A. R. (2002) Nature 420, 287–293
20. Shin, D. S., Pellegrini, L., Daniels, D. S., Yeletst, B., Craig, L., Bates, D., Yu, D. S., Shivji, M. K., Hitomi, C., Arvai, A. S., Volkmann, N., Tsuruta, H., Blundell, T. L., Venkitaraman, A. R., and Tainer, J. A. (2003) EMBO J. 22, 4566–4576
21. Kagawa, W., Kurumizaka, H., Ishii, R., Fukui, S., Nureka, O., Shibata, T., and Yokoyama, S. (2002) Mol. Cell 10, 359–371
22. Shen, Z., Cloud, K. G., Chen, D. J., and Park, M. S. (1996) J. Biol. Chem. 271, 148–152
23. Golub, E. I., Gupta, R. C., Haaf, T., Wold, M. S., and Radding, C. M. (1998) Nucleic Acids Res. 26, 5388–5393
24. Kurumizaka, H., Aihara, H., Kagawa, W., Shibata, T., and Yokoyama, S. (1999) J. Mol. Biol. 291, 537–548
25. Kreji, L., Damborsky, J., Thomsen, B., Duno, M., and Bendixon, C. (2001) Mol. Cell 8, 886–896
26. Jackson, D., Dhar, K., Wahl, J. K., Wold, M. S., and Borysthal, G. E. (2002) J. Mol. Biol. 321, 133–148
27. Rippke, T., Van Den Ouweland, J., Morillo, R., Bolink, A. G., Baarends, W. M., Van Sloum, P. P., Lohman, P. H., and Pastink, A. (1998) Mol. Cell Biol. 18, 6423–6429
28. Tsuzuki, T., Fujii, Y., Sakumi, K., Tominaga, Y., Nakao, K., Sekiguchi, M., Matsushiro, A., Yoshimura, Y., and Morita, T. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6236–6240
29. Bhattacharya, S., Arunkumar, A. I., Sullivan, S. L., Botuyan, M.-V., Arrow-
30. Dominguez, C., Boelens, R., and Bonvin, A. M. (2003) *J. Am. Chem. Soc.* **125**, 1731–1737
31. Arunkumar, A. I., Stauffer, M. E., Bochkareva, E., Bochkarev, A., and Chazin, W. J. (2003) *J. Biol. Chem.* **278**, 41077–41082
32. Stigger, E., Drissi, R., and Lee, S. H. (1998) *J. Biol. Chem.* **273**, 9337–9343
33. Daughdrill, G. W., Buchko, G. W., Botuyan, M. V., Arrowsmith, C., Wold, M. S., Kennedy, M. A., and Lowry, D. F. (2003) *Nucleic Acids Res.* **31**, 4176–4183
34. Patrick, S. M., and Turchi, J. J. (2002) *J. Biol. Chem.* **277**, 16096–16101
35. Hermanson-Miller, I. L., and Turchi, J. J. (2002) *Biochemistry* **41**, 2492–2498
36. Lee, J. H., Park, C. J., Arunkumar, A. I., Chazin, W. J., and Choi, B. S. (2003) *Nucleic Acids Res.* **31**, 4747–4754
37. Bhattacharya, S., Botuyan, M. V., Hsu, F., Shan, X., Arunkumar, A. I., Arrowsmith, C. H., Edwards, A. M., and Chazin, W. J. (2002) *Protein Sci.* **11**, 2316–2325
38. Baumann, P., and West, S. C. (1998) *Trends Biochem. Sci.* **23**, 247–252
