Biochemical Characterization of the Human RAD51 Protein

I. ATP HYDROLYSIS

Received for publication, October 12, 2001, and in revised form, February 7, 2002
Published, JBC Papers in Press, February 11, 2002, DOI 10.1074/jbc.M109915200

Gregory Tombline and Richard Fishel

The prototypical bacterial RecA protein promotes recombination/repair by catalyzing strand exchange between homologous DNAs. While the mechanism of strand exchange remains enigmatic, ATP-induced cooperativity between RecA protomers is critical for its function. A human RecA homolog, human RAD51 protein (hRAD51), facilitates eukaryotic recombination/repair, although its ability to hydrolyze ATP and/or promote strand exchange appears distinct from the bacterial RecA. We have quantitatively examined the hRAD51 ATPase. The catalytic efficiency ($k_{cat}/K_m$) of the hRAD51 ATPase was ∼50-fold lower than the RecA ATPase. Altering the ratio of DNA/hRAD51 and including salts that stimulate DNA strand exchange (ammonium sulfate and spermidine) were found to affect the catalytic efficiency of hRAD51. The average site size of hRAD51 was determined to be ∼3 nt (bp) for both single-stranded and double-stranded DNA. Importantly, hRAD51 lacks the magnitude of ATP-induced cooperativity that is a hallmark of RecA. Together, these results suggest that hRAD51 may be unable to coordinate ATP hydrolysis between neighboring protomers.

The bacterial RecA ATPase and its homologs facilitate DNA recombinaction/repair, although the role of ATP hydrolysis in these processes is not fully understood (1). All members of the RecA family, including the human RAD51 protein (hRAD51), contain classic Walker A/B motifs, which are fundamentally required for ATP hydrolysis (1, 2). These motifs are generally conserved among proteins that bind and hydrolyze NTPs (3). RecA proteins mutated at the Walker A/B motifs lack the ability to bind and/or hydrolyze ATP in vitro (4). While cells expressing these RecA Walker A/B mutants remain viable, they display dramatically reduced levels of recombination and increased radiation sensitivity (5).

Multiple mitotic and meiotic RecA homologs contribute to eukaryotic recombinaction/repair. Each of these homologs is likely to have distinct requirements for ATP binding and/or hydrolysis in recombinaction/repair. For example, four nonessential RecA homologs have been identified in Saccharomyces cerevisiae: RAD51, RAD55, RAD57, and DMC1 (6–9). Mutation of the Walker A/B motifs of RAD51 and RAD55 results in radiation sensitivity as well as meiotic recombinaction deficiency (9, 10). Mutation of the DMC1 Walker A motif results in a dominant meiotic null mutant (11). However, similar mutations of RAD57 do not display radiation sensitivity and are only modestly deficient for meiotic recombinaction (9, 10).

The complexity of the recombinaction/repair system is further amplified in higher eukaryotes, since eight vertebrate RecA homologs have been identified (12). In contrast to RecA or the yeast homologs, the vertebrate RAD51 gene appears to be required for cellular viability, since Rad51−/− mice display early embryo lethality and embryo-derived cell lines could not be established (13, 14). Similarly, chicken DT40 B-cells lacking endogenous RAD51 were not viable (15). Taken together, these results are consistent with the notion that the RecA homologs of higher eukaryotes are not redundant. Interestingly, the chicken DT40 RAD51-deficient cells could be rescued by the overexpression of an hRAD51 Walker A/B mutant protein that was able to bind but not hydrolyze ATP (16). In addition, these rescued cells displayed no increase in radiation sensitivity compared with wild type cells but were less efficient at facilitating recombinaction-dependent gene targeting at several loci (16). This data suggests that the contributions of hRAD51 ATP binding and hydrolysis to viability and recombinaction/repair may be distinct.

Biochemical analysis has proven useful in elucidating the role of ATP hydrolysis in the recombinaction functions of the RecA protein. It has been suggested that cycles of ATP hydrolysis allow protomers within the RecA nucleoprotein filament to alternate between distinct ATP- and ADP-bound conformational states (1, 17). The cycling between conformational states appears to drive directional strand exchange during recombinaction. Historically, these alternating conformations were suspected to facilitate strand exchange by redistributing protomers within the nucleoprotein filament. This assumption was based upon the differential affinities of RecA for DNA in the presence of ATPγS (high affinity) versus in the presence of ADP (low affinity) (1, 17). An alternative model suggested that strand exchange is facilitated by ATP hydrolysis-dependent rotation of the RecA nucleoprotein filament (18). Time lapse electron microscopy of RecA nucleoprotein filaments formed in the presence of ATPγS appeared consistent with this latter proposal (19). These slowly hydrolyzing nucleoprotein filaments remained in an extended yet dynamic state, where the protomers appeared to rotate.

The coupling of ATP binding/hydrolysis to recombinaction by hRAD51 is less certain. In comparison with electron micros-
copy images of RecA, the hRAD51:ssDNA nucleoprotein filaments formed in the presence of ATP·S appeared less extended, suggesting a diminished response to ATP (20). Yet, ATP was required for hRAD51 to extend the helical pitch (DNA-unwinding) of dsDNA (20) as well as to promote strand exchange between homologous DNA substrates (21–24).

Several recent studies suggest that hRAD51 can be induced to resemble RecA, both structurally and functionally. hRAD51 forms an extended nucleoprotein filament with the transition state mimetic ADP-AlF4− that appears analogous to activated RecA (19). Two additional reports indicated that yeast and human RAD51-mediated DNA strand exchange is greatly enhanced by ammonium sulfate and/or spermidine (25, 26). The mechanistic basis for ammonium sulfate and spermidine stimulation of RAD51 function is unknown. It is important to note that the rate of RAD51 strand exchange remains 3–5-fold lower than RecA (25, 26). Furthermore, RAD51 converts a maximum of 30–60% of ssDNA to form II products in 60 min, whereas RecA converts 100% ssDNA to form II in 15 min (25, 26).

Strand exchange by RecA has been shown to be largely independent of ATP hydrolysis (27–30). However, RecA-dependent bypass of heterologous DNA absolutely requires ATP hydrolysis (30–32). ATP hydrolysis-dependent bypass of heterologous DNA has been argued to require the generation of rotary torque and/or recycling of the RecA protomers during the strand exchange reaction (30, 33, 34). RAD51 displays a dramatically reduced capacity to bypass heterologous DNA during strand exchange (26, 35–37). Taken together, these observations suggest that altered ATP processing might account for the disparities between hRAD51 and bacterial RecA activities.

To understand the ATP-dependent recombination functions of hRAD51, we have performed the first quantitative examination of the hRAD51 ATPase. Our results allow a detailed mechanistic basis for ammonium sulfate and spermidine stimulation of RAD51 function is unknown. It is important to note that the rate of RAD51 strand exchange remains 3–5-fold lower than RecA (25, 26). Furthermore, RAD51 converts a maximum of 30–60% of ssDNA to form II products in 60 min, whereas RecA converts 100% ssDNA to form II in 15 min (25, 26).

Strand exchange by RecA has been shown to be largely independent of ATP hydrolysis (27–30). However, RecA-dependent bypass of heterologous DNA absolutely requires ATP hydrolysis (30–32). ATP hydrolysis-dependent bypass of heterologous DNA has been argued to require the generation of rotary torque and/or recycling of the RecA protomers during the strand exchange reaction (30, 33, 34). RAD51 displays a dramatically reduced capacity to bypass heterologous DNA during strand exchange (26, 35–37). Taken together, these observations suggest that altered ATP processing might account for the disparities between hRAD51 and bacterial RecA activities.

To understand the ATP-dependent recombination functions of hRAD51, we have performed the first quantitative examination of the hRAD51 ATPase. Our results allow a detailed comparison with the bacterial RecA protein, the only other homolog in this family in which similar studies have been performed. Of particular importance is our observation that hRAD51 appears to lack the magnitude of ATP-induced cooperativity displayed by RecA. Our results suggest that other regulatory factors or RecA homologs may be required to assemble and control an hRAD51 nucleoprotein filament.

**EXPERIMENTAL PROCEDURES**

**Materials**—Chemicals of the highest grade were obtained from Amresco (Solon, OH) or Sigma. Phosphoric/sulfuric acid-washed charcoal was obtained from Sigma (catalog no. C-5510). ATP was purchased from Amersham Biosciences, dissolved in water, and adjusted for pH. ATP concentration was determined by absorbance at 260 nm with ε = 1.54 × 104 M−1 cm−1. [γ-32P]ATP was purchased from PerkinElmer Life Sciences. Bacteriophage φX174 DNA was purchased from New England Biolabs (Beverly, MA). Linear, blunt-ended dsDNA (RFII) was prepared by treatment of bacteriophage φX174 dsDNA (RFI) with endonuclease Stu1 (New England Biolabs) followed by phenol extraction and ethanol precipitation. The DNA was resuspended in 10 mM Tris-HCl (pH 8), 1 mM EDTA and analyzed by agarose gel electrophoresis. The concentration of DNA is expressed as mol of nt (ssDNA) or bp (dsDNA).

**Purification of hRAD51—**hRAD51 was purified as previously described (38) with several modifications. Briefly, hRAD51 cDNA was subcloned into pET24d (Novagen), and induction was performed in the E. coli strain BL21pLysS. Cells were lysed by three freeze/thaw cycles.
and centrifuged at ~160,000 × g for 1 h. The supernatant was dialyzed overnight against 4 liters of 100 mM Tris acetate (pH 7.5), 5% glycerol, and 7 mM spermidine HCl. The hRAD51 precipitate was collected by centrifugation and resuspended (~10 mg/ml) in P buffer (100 mM potassium phosphate (pH 7.0), 10% glycerol, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol) (fraction I). Fraction I was separated by chromatography through Reactive Blue-4-agarose (Sigma). Protein was eluted with a 750 mM step of NaCl and dialyzed overnight against 4 liters of H buffer (20 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol) (fraction II). Fraction II was loaded onto a heparin-Sepharose CL-6B column and eluted with a 750 mM NaCl step (fraction III). Fraction III was dialyzed overnight against 4 liters of H buffer (fraction IV), loaded onto a Mono-Q column (HR5/5 prepacked from Amersham Biosciences), and eluted with a gradient of 150–750 mM NaCl over 20 ml (fraction V). hRAD51 was dialyzed twice against 2 liters of modified H buffer (containing 0.1 mM EDTA) and was stored on ice. Occasionally, additional purification steps were necessary to remove trace contaminants. In this case, the protein was dialyzed against modified P buffer (P buffer containing 100 mM NaCl) after Mono-Q chromatography on a Mono-S column equilibrated with modified P buffer, and eluted with a 15-mL gradient of 100–750 mM NaCl. This was followed by dialysis against modified H buffer (0.1 mM EDTA). Purified hRAD51 can be stored in modified H buffer at 0 or –80 °C for several months without appreciable loss of ATPase activity. Protein concentration was determined by amino acid analysis (Keck Facility, Yale University). Preparations were found to be nuclease-free by incubation with both single- and double-stranded phage DNA as well as small oligonucleotides.

ATP Hydrolysis—Unless otherwise indicated, the 10-μl reactions contained 1 μM hRAD51 and 6 μM DNA (nt or bp). All reactions were performed in an A buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM dithiothreitol, 2 mM magnesium acetate). In addition to the indicated amounts of ATP, 1 μCi of γ-[32P]ATP was included in each reaction. To achieve consistency in such small reactions, mixes containing either DNA/ATP or hRAD51 were added separately using a repeat pipettor (Eppendorf). Reactions were initiated by adding the hRAD51 mix last and incubated at the indicated amount of ATP. Each reaction was incubated at 37 °C for 30 min and processed with Norit as in Fig. 2. Data points represent the average of at least three replicate experiments. A, ATPase data for either ssDNA (●) or dsDNA (RFI) (●) were fit to the Michaelis-Menten equation. B, in the presence of ssDNA (●), the Hill coefficient (nH) = 0.91, and in the presence of dsDNA (RFI) (●), nH = 0.74.

### RESULTS

#### ATP Dependence of Hydrolysis—Using a modification of a previously published method (38), we purified hRAD51 to near homogeneity (Fig. 1). The ATPase activity of hRAD51 was measured by the Norit method (see “Experimental Procedures”), and the unique conversion of ATP to ADP was confirmed by thin layer chromatography (TLC) (39). We found the Norit method to be superior to TLC or PAGE analysis because of the ease of data acquisition, reduced expense, and the large number of analyses that could be performed in tandem. Past experience has suggested that the separation efficiency of Norit exceeds 90% and can be increased by prolonged incubation of the terminated reactions at 0 °C (39, 40).

While a previous report indicated that ATP hydrolysis by hRAD51 was very inefficient (kcat/dsDNA) = 0.2 min⁻¹, kcat/ssDNA = 0.1 min⁻¹, kcat = 0.03 min⁻¹), these values were based on a single ATP concentration (200 μM) (21). To confirm and extend these studies and to examine the rate-limiting step(s) within the hydrolysis cycle, we performed classic Michaelis-Menten analysis to define the Kₘ and kₐₙ (Vmax/
Cooperativity of the hRAD51 ATPase

We determined these values for the hRAD51 ATPase activity in the presence of ssDNA, supercoiled dsDNA (RFII), linear dsDNA (RFIII), and in the absence of DNA. The $k_{cat}$ values ($k_{cat}$(ssDNA) = 0.21 min$^{-1}$; $k_{cat}$(dsDNA) = 0.07 min$^{-1}$; $k_{cat}$(dsRFII) = 0.07 min$^{-1}$; $k_{cat}$(dsDNA) = 0.07 min$^{-1}$; Fig. 2, A and B, and Table I) agreed with values previously reported (21). These data indicate that the $k_{cat}$(ssDNA) for hRAD51 is ~150-fold lower than bacterial RecA ($k_{cat}$(ssDNA) = 28 min$^{-1}$), and the $k_{cat}$(dsDNA) is ~220-fold lower than bacterial RecA ($k_{cat}$(dsDNA) = 22 min$^{-1}$) in the absence of DNA is ~4-fold higher than the bacterial RecA ($k_{cat}$(dsDNA) = 0.015 min$^{-1}$) (41–43). In addition, hRAD51 displays an equal or higher apparent affinity for ATP ($K_{m}$(ssDNA) = 23 ± 3 μM; $K_{m}$(dsDNA/RFII) = 27 ± 4 μM; $K_{m}$(dsDNA/RFIII) = 26 ± 5 μM; $K_{m}$(dsDNA) = 110 ± 22 μM) compared with bacterial RecA ($S_{0.5}$(ssDNA) ~ 20–60 μM; $S_{0.5}$(dsDNA/RFII) ~ 100 μM at pH 6.2; $S_{0.5}$(dsDNA) ~ 100 μM) (17, 41), the substrate concentration where half-maximal activity occurs is $S_{0.5}$ in a cooperative system and equals $K_{m}$ in a noncooperative system (44). In general, DNA appears to decrease the hRAD51 $K_{m}$ equivalently (Fig. 2, A and B, and Table I). Calculation of the hRAD51 ATPase catalytic efficiency ($k_{cat}/K_{m}$) suggests that ssDNA (150 s$^{-1}$ M$^{-1}$) induces the ATPase no more than 2–3-fold more than dsDNA (~68 s$^{-1}$ M$^{-1}$) and at least 5-fold more than in the absence of DNA (11 s$^{-1}$ M$^{-1}$). The catalytic efficiency of hRAD51 is ~50-fold less than bacterial RecA (8300 s$^{-1}$ M$^{-1}$).

hRAD51 Lacks ATP-induced Cooperativity—A commonly used method for determining ATP-induced cooperativity is by calculating the Hill coefficient (44). The Hill coefficient was originally developed for the analysis of cooperative fractional saturation for a ligand binding to multiple interdependent sites (45). If one assumes that the rate of an enzymatic reaction is proportional to the fractional saturation of the enzyme, then a slope (Hill coefficient) greater than one derived from a plot of the fractional rate vs. substrate concentration ($[S]/V_{max}$) shows positive cooperativity (44). In general, the largest value for cooperativity occurs at half-maximal fractional saturation. As the fractional saturation approaches unity, the Hill coefficient also approaches 1. This appears to be the case for the RecA ATPase (41, 46). The Hill coefficient ($n_{H}$) of RecA varies from $n_{H} = 3$ to $n_{H} = 11$ at ATP concentrations below or slightly above the $S_{0.5}$. At ATP concentrations above the $S_{0.5}$ (>100 μM), ATP-induced cooperativity becomes less apparent, since the Hill coefficient equals 1.

Three methods of analysis of hRAD51 ATPase data indicate that hRAD51 lacks ATP-induced cooperativity. First, in the absence or presence of DNA, the rate of ATP hydrolysis was easily fit to a simple hyperbolic curve (the Michaelis-Menten equation; Fig. 2A). Second, the slope of the same ATPase data plotted by the double-reciprocal method is linear (not concave upward; Fig. 2B). Third, the slope of hRAD51 ATPase data plotted as a fractional rate vs. ATP concentration (Hill coefficient) was ~1 in all conditions (ssDNA $n_{H} = 0.79$; dsDNA (RFI) $n_{H} = 0.76$; dsDNA (RFIII) $n_{H} = 0.74$; absence of DNA $n_{H} = 0.70$; Fig. 2C and Table I). hRAD51 also failed to display ATP-induced cooperativity in the range of ATP concentrations.
utilize dsDNA as a cofactor for hydrolysis at pH 6.2. However, the amount of ATP (supplemented with $[\gamma^{32}P]ATP$) in ATPase reactions were varied by mixing the indicated amount of hRAD51 with 6 μM DNA (nt or bp) or the indicated amount of DNA with 3 μM hRAD51. Each reaction contained 250 μM ATP and was incubated for 30 min at 37 °C. Each reaction was processed by the Norit method as in Fig. 2. Each point represents the average of three replicate experiments. ●: reactions where ssDNA was added; ■: reactions where dsDNA (RFII) was added; ○: reactions performed in the absence of DNA.

### DNA Dependence of the hRAD51 ATPase

$K_m$ and $V_{\text{max}}$ were determined by Michaelis-Menten analysis (see also Fig. 6).

**TABLE II**

| nt ssDNA | $K_m$ (μM) | $V_{\text{max}}$ (μM·min⁻¹) |
|----------|-----------|-----------------------------|
| 0.5      | 11.4 ± 4.8| 0.073 ± 0.006               |
| 1.0      | 35 ± 5.0  | 0.122 ± 0.006               |
| 1.5      | 23 ± 9.9  | 0.148 ± 0.018               |
| 3.0      | 13.2 ± 7.4| 0.164 ± 0.018               |
| 4.5      | 10.9 ± 3.0| 0.165 ± 0.008               |
| 6.0      | 8.1 ± 4.9 | 0.129 ± 0.012               |
| 15.0     | 3.6 ± 5.0 | 0.087 ± 0.012               |

| bp dsDNA (RFII) | $K_m$ (μM) | $V_{\text{max}}$ (μM·min⁻¹) |
|-----------------|-----------|-----------------------------|
| 0.5             | 8.5 ± 4.7 | 0.072 ± 0.001               |
| 1.0             | 13 ± 11.5 | 0.083 ± 0.015               |
| 1.5             | 9.7 ± 6.2 | 0.113 ± 0.015               |
| 3.0             | 20.3 ± 6.6| 0.126 ± 0.010               |
| 4.5             | 8.8 ± 5.6 | 0.064 ± 0.009               |
| 6.0             | 21.4 ± 7.0| 0.130 ± 0.012               |
| 15.0            | 6.54 ± 5.0| 0.079 ± 0.005               |

Below the $K_m$ (Fig. 3). These data contrast with the cooperativity shown by RecA.

The Effect of pH on hRAD51 ATPase—RecA can efficiently utilize dsDNA as a cofactor for hydrolysis at pH 6.2. However, at pH 8.0, the dsDNA-dependent ATPase can only be measured after a lag of several hours (1, 17, 42). This lag at higher pH is absent when the carboxyl-terminal domain of bacterial RecA is deleted or when purified unwound dsDNA is used as a cofactor (43, 47, 48). We examined the pH dependence of the hRAD51 ATPase between 6.2 and 8.2 and found no difference in ATPase activity for either ssDNA or dsDNA (RFIII) (Fig. 4, A and B, respectively). It is interesting to note that a comparison of the hRAD51 and RecA sequences suggests that the carboxyl-terminal domain is missing in hRAD51 (1, 2, 49).

**High Salt Activation of the hRAD51 ATPase**—In the absence of DNA, high salt concentrations increased the $k_{\text{cat}}$ of bacterial RecA ATPase to a value that is equivalent to the $k_{\text{cat(ssDNA)}}$ observed at low salt concentrations. However, the high salt-dependent RecA ATPase reaction displays a $K_m$ that approaches 1 mM and fails to display ATP-induced cooperativity (50). ATPase activity under these conditions has been generally attributed to salt-induced structural transitions within the RecA nucleoprotein filament (51). High salt (1.5 M NaCl) also increases the hRAD51 ATPase activity ($k_{\text{cat}} = 0.40$ min⁻¹; Fig. 5A and Table I) such that it approaches the ATPase activity observed with low salt in the presence of ssDNA (150 mM NaCl; $k_{\text{cat(ssDNA)}} = 0.21$ min⁻¹; Fig. 2A and Table I). In addition, hRAD51 fails to display ATP-induced cooperativity in high salt ($pH = 8.0$; Fig. 5B and Table I). In contrast to RecA, the $K_m$ of the hRAD51 ATPase in high salt ($K_m = 17$ μM) resembles the $K_m$ at low salt when DNA is present ($K_m \sim 20$ μM). While the $K_m$ represents a number of variables (44), these results are consistent with the notion that hRAD51 is incapable of forming an actively hydrolyzing aggregate (see Ref. 50).

**DNA Dependence of the hRAD51 ATPase**—Michaelis-Menten...
TABLE III
ATPase data as a function of DNA concentration to determine site size (N)

| nt ssDNA | DNA/RAD51 v cat | v/[DNA] | N |
|----------|----------------|---------|---|
| µM | nt/monomer | µM · min⁻¹ | min⁻¹ | |
| 0.5 | 0.16 | 0.32 ± 0.03 | 0.64 ± 0.05 | 3.76 |
| 1 | 0.33 | 0.58 ± 0.08 | 0.55 ± 0.07 | 3.40 |
| 1.5 | 0.50 | 0.73 ± 0.06 | 0.49 ± 0.04 | 2.85 |
| 2 | 0.66 | 0.79 ± 0.10 | 0.40 ± 0.04 | 2.35 |
| 2.5 | 0.83 | 0.79 ± 0.06 | 0.32 ± 0.02 | 1.88 |
| 5 | 1.66 | 0.89 ± 0.02 | 0.15 ± 0.005 | NA |
| 10 | 3.33 | 1.35 ± 0.07 | 0.14 ± 0.007 | NA |
| 15 | 5.0 | 1.49 ± 0.07 | 0.10 ± 0.005 | NA |
| 20 | 6.66 | 1.53 ± 0.07 | 0.08 ± 0.003 | NA |
| 25 | 8.33 | 1.54 ± 0.20 | 0.06 ± 0.008 | NA |

bp dsDNA (RFI)  DNA/RAD51 v cat | v/[DNA] | N |
| µM | bp/monomer | µM · min⁻¹ | min⁻¹ | |
| 0.5 | 0.16 | 0.53 ± 0.05 | 1.06 ± 0.112 | 9.14 |
| 1 | 0.33 | 0.53 ± 0.01 | 0.53 ± 0.011 | 4.57 |
| 1.5 | 0.50 | 0.57 ± 0.04 | 0.35 ± 0.025 | 2.28 |
| 2 | 0.66 | 0.55 ± 0.10 | 0.28 ± 0.051 | 2.41 |
| 2.5 | 0.83 | 0.61 ± 0.10 | 0.24 ± 0.044 | 2.07 |
| 5 | 1.66 | 0.70 ± 0.05 | 0.14 ± 0.010 | NA |
| 10 | 3.33 | 0.92 ± 0.10 | 0.09 ± 0.010 | NA |
| 15 | 5.0 | 1.24 ± 0.13 | 0.08 ± 0.008 | NA |
| 20 | 6.66 | 1.30 ± 0.05 | 0.06 ± 0.002 | NA |
| 25 | 8.33 | 1.26 ± 0.12 | 0.05 ± 0.005 | NA |

TABLE IV
ATPase data as a function of hRAD51 concentration to determine site size (N)

| ssDNA/51 | v cat | Kcat  | N |
|----------|-------|-------|---|
| µM | nt/monomer | µM · min⁻¹ | min⁻¹ | |
| 0.1 | 60:1 | 0.022 ± 0.010 | 0.218 ± 0.103 | 2.23 |
| 0.3 | 20:1 | 0.056 ± 0.013 | 0.318 ± 0.043 | 2.23 |
| 0.6 | 10:1 | 0.104 ± 0.024 | 0.173 ± 0.041 | 2.81 |
| 1.0 | 6:1 | 0.172 ± 0.032 | 0.172 ± 0.032 | 2.81 |
| 2.0 | 3:1 | 0.272 ± 0.035 | 0.136 ± 0.017 | 3.57 |
| 3.0 | 2:1 | 0.449 ± 0.057 | 0.150 ± 0.019 | 3.24 |

| dsDNA/51 | v cat | Kcat  | N |
|----------|-------|-------|---|
| µM | bp/monomer | µM · min⁻¹ | min⁻¹ | |
| 0.1 | 60:1 | 0.034 ± 0.007 | 0.259 ± 0.067 | 1.38 |
| 0.3 | 20:1 | 0.046 ± 0.003 | 0.156 ± 0.009 | 2.29 |
| 0.6 | 10:1 | 0.062 ± 0.014 | 0.114 ± 0.023 | 3.14 |
| 1.0 | 6:1 | 0.118 ± 0.009 | 0.109 ± 0.009 | 3.28 |
| 2.0 | 3:1 | 0.212 ± 0.019 | 0.097 ± 0.010 | 3.69 |
| 3.0 | 2:1 | 0.288 ± 0.034 | 0.095 ± 0.011 | 3.76 |

The Site Size of the hRAD51 ATPase—The hRAD51 ATPase appeared to increase linearly with increasing hRAD51 protein (Fig. 7A). This linear increase was evident in the absence of DNA as well as in the presence of ssDNA and dsDNA. A clear saturation of the hRAD51 ATPase (v) was observed at a molar ratio of 3–4 nt/1 hRAD51 (Fig. 7B; see also Fig. 6A). Interestingly, there appeared to be an initial saturation of ATPase activity that occurred at approximately a 1 nt/1 hRAD51 monomer molar ratio (Fig. 7B). These data suggest that the
hRAD51 ATPase may possess two modes of DNA stimulation. The bacterial RecA site size (N) appeared to vary between 3 and 6 nucleotides per monomer and was dependent upon whether ATP hydrolysis or DNA binding was measured (17). This paradox was resolved by dividing the v_{ATPase}/[DNA] (at constant excess protein) by the v_{ATPase}/[protein] (at constant excess DNA) and revealed that N was ~3 (53). Using this methodology, we examined the site size of hRAD51 for both ssDNA and dsDNA (Table III and Table IV). We found that the v_{ATPase}/[DNA] for RecA remained constant with increased DNA concentrations, while the v_{ATPase}/[DNA] of hRAD51 decreased significantly (Table III). These results produced a range of site sizes that depended upon calculations using the average v_{ATPase}/[protein] and individual v_{ATPase}/[DNA] values (Table III) or the average v_{ATPase}/[DNA] and individual v_{ATPase}/[protein] values (Table IV). While averages of these calculations yielded a site size of ~3 nt (bp), these values appeared to be too variable to provide a precise determination.

The Effect of Ammonium Sulfate and Spermidine on the hRad51 ATPase—Two recent reports demonstrate that extensive DNA strand exchange promoted by the yeast and human RAD51 proteins could be induced with ammonium sulfate and/or spermidine (25, 26). Under these modified conditions, hRAD51 displays a reduced affinity for dsDNA (25), and the transition from intermediates to products during DNA strand exchange was enhanced (26). Although ATPase activity was not examined, these studies suggested that ammonium sulfate and/or spermidine provided a condition for efficient DNA strand exchange in the absence of significant ATP hydrolysis. To address the effects of ammonium sulfate and/or spermidine, we performed a detailed analysis of the hRAD51 ATPase under similar conditions. We found that ammonium sulfate increased the K_m ~3–4-fold in the presence of ssDNA (Fig. 8A) or dsDNA (Fig. 8B) cofactors (Table V). In contrast, spermidine did not significantly affect the hRAD51 ATPase (Fig. 8; Table V). Ammonium sulfate also elicited a modest decrease in the rate of hydrolysis (V_max) in the presence of dsDNA (Fig. 8B; Table V). However, the rate of hydrolysis was modestly enhanced in the presence of ssDNA (Fig. 8A; Table V). It is worth noting that under both conditions (ammonium sulfate and/or spermidine) the Hill coefficient remained ~1 (data not shown), and the data were easily fit to the Michaelis-Menten equation (Fig. 8).

**DISCUSSION**

ATP binding and hydrolysis are coupled to the recombinational strand exchange function(s) of bacterial RecA (1, 4, 17). This is exemplified by the observation that the free energy of strand exchange and ATP hydrolysis appear equivalent in temperature-dependent studies (54). In addition, coordinated ATP hydrolysis appears exceedingly efficient between protomers within the RecA nucleoprotein filament (Hill coefficient of >11). The ATP hydrolysis activity of other RecA family members has not been well characterized. Initial studies suggested that hRAD51 was inefficient at promoting extensive recombinational strand exchange and displayed a weak ATPase activity compared with RecA (21–23). More recent studies have demonstrated enhanced RAD51 strand exchange activity in the presence of ammonium sulfate and spermidine (25, 26). Our studies were initiated to detail the mechanistic differences between RecA and hRAD51.

The hRAD51 ATPase appears fundamentally distinct from

![Fig. 8. The effect of ammonium sulfate and spermidine on the hRAD51 ATPase. ATPase assays were performed by incubating 0.5 μM hRAD51 and, if present, 6 μM ssDNA (nt) (A) or dsDNA (bp) (B) with the indicated amount of ATP (which was supplemented with |γ-32P|ATP). Each reaction was incubated at 37 °C for 1 h and then terminated by the addition of 10% activated charcoal (Norit) in 10 mM EDTA. The samples were centrifuged, and the supernatant containing free phosphate was counted by the Cerenkov method. Data points represent the average of at least three replicate experiments. ATPase data were fit to the Michaelis-Menten equation. ATPase reactions were performed under standard buffer conditions (●) in the presence of 100 mM (NH_4)_2SO_4 (○), in the presence of 100 mM (NH_4)_2SO_4 and 4 mM spermidine (▲), or in the presence of 100 mM (NH_4)_2SO_4 and 4 mM spermidine (●). The insets clearly indicate the effect of (NH_4)_2SO_4 on K_m values. These data are summarized in Table V.]

| Table V Summary of the effect of ammonium sulfate and spermidine on the hRAD51 ATPase |
|----------------|----------------|----------------|----------------|
|                | K_m [μM] | V_{max} [μM·min^{-1}·10^{-2}] | k_{cat} [min^{-1}] | k_{cat}/K_m [s^{-1}·μM^{-1}] |
| ssDNA          |          |                              |                  |                             |
| No addition    | 9.5 ± 1.6| 5.5 ± 0.2                    | 0.11             | 193                         |
| with (NH_4)_2SO_4 | 39.8 ± 4.4| 7.3 ± 0.3                   | 0.15             | 63                          |
| with spermidine| 12.5 ± 2.9| 5.4 ± 0.3                   | 0.11             | 146                         |
| with (NH_4)_2SO_4 and spermidine | 37.5 ± 5.2 | 6.1 ± 0.3 | 0.12 | 53 |
| dsDNA (RFI)    |          |                              |                  |                             |
| No addition    | 13.3 ± 2.5| 7.7 ± 0.4                   | 0.15             | 194                         |
| with (NH_4)_2SO_4 | 29.0 ± 7.0| 5.4 ± 0.5                   | 0.11             | 62                          |
| with spermidine| 11.8 ± 2.3| 6.3 ± 0.3                   | 0.13             | 178                         |
| with (NH_4)_2SO_4 and spermidine | 43.6 ± 6.2 | 6.6 ± 0.3 | 0.13 | 51 |
RecA. As has been previously reported (21, 22), the \( k_{cat} \) for hRAD51 is \(-150\)–\(-200\)-fold lower than the \( k_{cat} \) for the bacterial RecA. Combined with the 2–3-fold lower \( K_m \) of hRAD51 reported here, these observations translate to an \(-50\)-fold difference in the catalytic efficiency (\( k_{cat}/K_m \)) of hRAD51 compared with the bacterial RecA. The catalytic efficiency of hRAD51 is \(-7\) orders of magnitude below a diffusion-limiting process (55).

The majority of sequence homology between hRAD51 and RecA is within a central domain containing classic Walker A/B adenine nucleotide binding motifs (1, 2, 49). hRAD51 contains an N-terminal domain that is absent in RecA and is missing a C-terminal domain that is present in RecA (1, 2, 49).

Based upon NMR and mutagenesis data, it has been suggested that the N terminus of hRAD51 may functionally substitute for missing a C-terminal domain that is present in RecA (1, 2, 49). It appears that the N-terminal domain of hRAD51 may functionally substitute for the C-terminal domain of RecA (RecA5327). The \( k_{cat} \) of the RecA5327 mutant was stimulated by both ssDNA and dsDNA equally and without a lag (48). Likewise, the \( k_{cat} \) of hRAD51 in the presence of ssDNA or dsDNA appeared only modestly different. However, acidic pH allowed the RecA to utilize dsDNA as a co-factor for ATP hydrolysis without a lag, an effect that has been generally ascribed to charge neutralization of the C-terminal domain (1). Therefore, a range of pH had no effect on the hRAD51 ATPase. These data suggest that the hRAD51 N terminus may not fully substitute for the RecA C terminus.

We determined the DNA site size of the hRAD51 ATPase to be \(-3\) nt (bp) for ssDNA or dsDNA. Similar methodologies were used to determine that the bacterial RecA has a site size of 3 nt for ssDNA (53). This correlative site size (3 nt or bp) for hRAD51 appears to indicate that most of the protein in the purified fractions was active. While we have estimated the minimal site size of hRAD51 to be 3 nt, 6–8 nt of ssDNA per hRAD51 monomer provoked the optimal catalytic efficiency of the hRAD51 ATPase. This observation may indicate that hRAD51 binds an additional ssDNA molecule, similar to RecA. Saturation of a second RecA ssDNA binding site reduced the \( S_{0.5} \) from 60 to \(-20\) \( \mu \)M. However, the \( V_{max} \) and ATP-induced cooperativity of RecA remained unaffected by excess ssDNA (52, 53). In contrast, excess ssDNA lowered the \( V_{max} \) of the hRAD51 ATPase, and the \( K_m \) approached the \( K_p \) for ATP binding (57). While the effect of excess ssDNA on the \( S_{0.5} \) of RecA appeared similar to hRAD51, the RecA ATPase retained an \(-10\)-fold difference between the \( S_{0.5} \) and \( K_m \) for ATP binding. The persistent difference between the \( S_{0.5} \) and \( K_p \) of RecA is thought to correlate with a threshold of nucleoprotein filament ATP saturation that must precede efficient/cooperative ATP hydrolysis (17). It may be that the hRAD51 ATPase is not subject to regulation by a threshold of ATP saturation. The reduced \( V_{max} \) displayed by the hRAD51 ATPase in the presence of excess ssDNA also suggests a change in the rate-limiting step (see Ref. 57).

It is notable that the hRAD51 ATPase fails to display the magnitude of ATP-induced cooperativity found with the RecA ATPase. Whether the lack of ATP-induced cooperativity is responsible for the reduced catalytic efficiency of the hRAD51 ATPase and/or the distinct recombinational strand exchange is uncertain. It is suspected that ATP-induced cooperativity augments the catalytic efficiency of the RecA ATPase at two potential rate-limiting junctures, 1) achieving the active/extended nucleoprotein filament by saturation of a threshold number of RecA protomers with ATP (1, 17, 58) and 2) maintaining the active/extended nucleoprotein filament during ATP hydrolysis by provoking rapid ADP release (thereby preventing reversibility) (59, 60). In the case of RecA, the cooperative state of the nucleoprotein filament appears critical for recombinational strand exchange as well as bypass of heterologous DNA during strand exchange (1, 17, 30–32).

Specialized cellular or biochemical conditions may induce hRAD51 to achieve and/or maintain an active nucleoprotein filament that is necessary for efficient recombination functions. Such conditions could enhance the rate and cooperativity of the hRAD51 ATPase. Alternatively, such conditions may allow hRAD51 to more effectively utilize ATP but not dramatically affect ATP hydrolysis. In the presence of ammonium sulfate, we observed a modest decrease in the rate of hRAD51-mediated ATP hydrolysis when ssDNA was used as the cofactor. These data appear to be consistent with the interpretation of Sigurdsson et al. (25), which suggested that ammonium sulfate enhanced the activity of hRAD51 to discriminate between ssDNA and dsDNA. The most significant effect of ammonium sulfate is to increase the \( K_m \) for ATP 3–4 fold. The molecular effect of ammonium sulfate on the hRAD51 protein is unknown. However, recent studies with the F 1 ATPase suggest that SO 4 \(-2\) is capable of occupying an intermediate position near the region of the \( \gamma \)-phosphate that, with ADP, mimics an ATP hydrolysis intermediate (61). We have found that ADP release appears rate-limiting for the hRAD51 ATPase (57). Perhaps ammonium sulfate provokes transient ternary hRAD51-ADP-SO 4 \(-2\) complexes that maintain and mimic a more active ATP-bound nucleoprotein filament. Such complexes would be distinct from fully ADP-bound inactive nucleoprotein filaments and may promote efficient DNA strand exchange without dramatically enhancing the rate of hydrolysis. It is important to note that even in the presence of ammonium sulfate and spermidine, RAD51 does not appear to be capable of bypassing heterologous DNA during strand exchange (25, 26). Our studies appear to suggest that it is the continued inability of RAD51 to coordinate ATP hydrolysis between protomers that makes bypassing heterologous DNA during strand exchange largely refractory.

The present studies are consistent with at least two possibilities. One possibility is that the role of ATP binding and hydrolysis by hRAD51 in recombination/repair is different from RecA. Alternatively, other factors may be required to mimic the enhancing effect(s) of ammonium sulfate and/or improve the efficiency of hRAD51.

Acknowledgments—We thank Hans-Jürg Alder and the Kimmel Nuclear Acids Facility for oligonucleotide synthesis and sequencing; Pete Von Hippel, Charles Brenner, Jan Hoek, Albert Wong, Christoph Schmutte, Kang-Sup Shim, Chris Heinen, Samir Acharya, Scott Gradia, and Teresa Wilson for helpful discussions; and Kristine Yoder for helping revise the manuscript.

REFERENCES
1. Roca, A. I., and Cox, M. M. (1997) Prog. Nucleic Acid Res. Mol. Biol. 56, 129–223
2. Brocchieri, L., and Karlin, S. (1998) J. Mol. Biol. 276, 249–264
3. Walker, J. E., Saraste, M., Runswick, M. J., and, Gay, N. J. (1992) EMBO J. 1, 945–951
4. Kowalczykowski, S. C. (1991) Biochimie (Paris) 73, 289–304
5. Konoda, J. T., Logan, K. M., and Knight, K. L. (1994) J. Mol. Biol. 237, 20–34
6. Lovett, S. T., and Mortimer, R. K. (1987) Nature 325, 191–193
7. Kana, J. S., and Mortimer, R. K. (1991) Gene (Amst.) 65, 139–140
8. Bishop, D. K., Park, D. X., Wu, L., and Kleckner, N. (1991) Cell 68, 439–456
9. Shinozawa, A., Ogawa, H., and Ogawa, T. (1992) Cell 69, 457–470
10. Johnson, R. D., and Simington, L. S. (1995) Mol. Cell. Biol. 15, 4843–4850
11. Dresser, M. E., Ewing, D. J., Conrad, M. N., Dominguez, A. M., Barstead, R., Jiang, H., and Kodadek, T. (1997) Genes Dev. 11, 533–544
12. Thacker, J. (1999) Trends Genet. 15, 166–168
13. Lim, D. S., and Hasty, P. (1996) Mol. Cell. Biol. 16, 7133–7143
14. Tsuzuki, T., Fujii, Y., Sakumi, K., Tominaga, Y., Nakao, K., Sekiguchi, M., Matsushiro, A., Yoshimura, Y., and Morita, T. (1996) EMBO J. 15, 598–608
15. Sonoda, E., Sawaki, M. S., Buerstedde, J. M., Beznoubova, O., Shirahata, A., Ogawa, H., Takata, M., Yamaguchi-Iwai, Y., and Takeda, S. (1998) EMBO J. 17, 689–698
16. Morrison, C., Shirahata, A., Sonoda, E., Yamaguchi-Iwai, Y., Takata, M., Weichselbaum, R. R., and Takeda, S. (1999) Mol. Cell. Biol. 19, 6891–6897
