Rad51 Protein from the Thermotolerant Yeast *Pichia angusta* as a Typical but Thermodependent Member of the Rad51 Family

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The Rad51 protein from the methylotrophic yeast *Pichia angusta* (Rad51$_{pa}$) of the taxonomic complex *Hansenula polymorpha* is a homolog of the RecA-RadA-Rad51 protein superfamily, which promotes homologous recombination and recombination repair in prokaryotes and eukaryotes. We cloned the RAD51 gene from the cDNA library of the thermotolerant *P. angusta* strain BKM Y1397. Induction of this gene in a rad51-deficient *Saccharomyces cerevisiae* strain partially complemented the survival rate after ionizing radiation. Purified Rad51$_{pa}$ protein exhibited properties typical of the superfamily, including the stoichiometry of binding to single-stranded DNA (ssDNA) (one protomer of Rad51$_{pa}$ per 3 nucleotides) and DNA specificity for ssDNA-dependent ATP hydrolysis [poly(dC) > poly(dT) > dX174 ssDNA > poly(dA) > double-stranded M13 DNA]. An inefficient ATPase and very low cooperativity for ATP interaction position Rad51$_{pa}$ closer to Rad51 than to RecA. Judging by thermoinactivation, Rad51$_{pa}$ alone was 20-fold more thermostable at 37°C than its *S. cerevisiae* homolog (Rad51$_{sc}$). Moreover, it maintained ssDNA-dependent ATPase and DNA transferase activities up to 52 to 54°C, whereas Rad51$_{sc}$ was completely inactive at 47°C. A quick nucleation and an efficient final-product formation in the strand exchange reaction promoted by Rad51$_{pa}$ occurred only at temperatures above 42°C. These reaction characteristics suggest that Rad51$_{pa}$ is dependent on high temperatures for activity.

*Pichia angusta*, one of the species of the taxonomic complex *Hansenula polymorpha* (25), is a methylotrophic yeast which has attracted considerable attention as a promising host for the production of recombinant proteins because of its powerful promoter elements, its ability to grow at high density on inexpensive substrates, and the unusual properties of its homologous and nonhomologous recombination systems promoting a multiple tandem integration of a nonlinearized plasmid in the host chromosome (for reviews, see references 11, 28, and 31). One additional characteristic that might have a biotechnological advantage is its thermotolerance, an ability to grow at temperatures (42°C and higher) which are not acceptable for other yeast strains.

Among 2,500 *P. angusta* novel protein-encoding genes now identified, 6% have no homologs in *Saccharomyces cerevisiae* (3). Little is known about the recombination or DNA repair proteins in *P. angusta*, such as the homologous DNA transferase Rad51 and its paralogs. This recombinase is a RecA-RadA-like protein, found in all three domains of life (4), which forms filaments on single-stranded DNA (ssDNA) in the presence of ATP (and thus possesses ATPase activity) and promotes homologous pairing and strand exchange, which are the two main steps in the initiation of homologous recombination and recombination repair, as well (6, 16, 27). All members of the RecA-RadA-Rad51 recombinase superfamly form nucleoprotein filaments of similar structures and stoichiometries, and they display similar preferences in DNA substrates for DNA-dependent ATP hydrolysis (26, 30, 32). Although similar in ATP-dependent recombination functions, these members are divided into two subfamilies: RecA-like and RadA-Rad51-like proteins. The latter group displays weak ATP hydrolysis activity and lacks the magnitude of the ATP-induced cooperativity displayed by RecA (8, 19, 35).

Here, we characterize the basic recombination activities of Rad51$_{pa}$, a new member of the Rad51 family from *P. angusta*. Some properties were compared with those of Rad51 from *S. cerevisiae* (Rad51$_{sc}$), mainly in the context of protein thermostability. In two principle recombination activities analyzed here, a quick nucleation and an efficient final-product (FP) formation in a dX174 DNA strand exchange reaction, Rad51$_{pa}$ demonstrated thermodependence rather than thermostolerance.

MATERIALS AND METHODS

Strains, plasmids, and media. Thermotolerant *P. angusta* strain BKM Y1397 was kindly supplied by the Russian Collection of Microorganisms. The strain was grown in yeast extract-peptone-dextrose (YPD) medium (1% peptone, 0.5% yeast extract, 2% glucose) at 30, 37, 40, 46, and 49°C for 3 days after an initial inoculation of not more than 1,000 cells per ml. The growth curves (data not shown) revealed that 37, 40, and even 42°C appeared to be optimal growth temperatures, whereas 50 and 46°C markedly inhibited cell growth, though the cells not only maintained survival but even grew slightly at 49°C. *S. cerevisiae* diploid strain D4004 (MATa/MATa ade2-248/ade2-248 leu2-3,112/leu2-3,112 ura3-160,288/ura3-160,288 trpl1/trpl1 RAD51/RAD51) and its *rad51*-1 derivative D3164 (MATa/MATa ade2-248/ade2-248 leu2-3,112/leu2-3,112 ura3-160,288/ura3-160,288 trpl1/trpl1 rad51-1/1) were used to study complementation of the ionizing radiation (IR) sensitivity of the *S. cerevisiae* rad51-1 diploid mutant with the cloned RAD51$_{pa}$ gene.

cDNA library construction and RAD51$_{pa}$ cloning procedure. The *P. angusta* cDNA library was constructed in a Uni-ZAP XR vector (Stratagene) with poly(A) RNA purified from BKM Y1397 cells. A fragment of the RAD51$_{pa}$ gene

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amplified by PCR using degenerate primers designed from conserved re-
gions of the Rad51 protein family. The resulting PCR product was used as a
probe for subsequent library screening. A positive clone containing Rad51
was recloned into a PET21b vector (Novagen). The pYES2::Rad51 plasmid
was constructed by recloning the Rad51 gene into the EcoRI-XhoI sites of
the pYES2 plasmid under a GAL1 promoter. Both pYES2 and pYES2::Rad51
were used in comparative analysis of IR sensitivity.

**DNA manipulation.** Standard procedures described previously (29) were
used for DNA manipulation. Linear dX174 double-stranded DNA (dsDNA) was
prepared from its circular form by cleavage with the PstI endonuclease, followed
by extraction with phenol-chloroform, precipitation with ethanol, and resuspen-
sion in Tris-EDTA (pH 8.0) buffer. The concentrations of DNA substrates are
expressed as nucleotide equivalents. The concentrations of poly(dT), poly( dC),
poly(dA), ssDNA, and dsDNA were determined using the following extinction
coefficients at 280 nm: 1.565 \( \times \) 10\(^4\) M\(^{-1}\)cm\(^{-1}\); 2.600, 7.400 \( \times \) 10\(^4\) M\(^{-1}\)cm\(^{-1}\);
8.600 \( \times \) 10\(^4\) M\(^{-1}\)cm\(^{-1}\); and 6.500, 6.500 \( \times \) 10\(^4\) M\(^{-1}\)cm\(^{-1}\), respectively.

**Protein purification.** The Rad51 protein was produced in Escherichia coli
strain BL21(DE3) (F\(ompT\) hsdS\(d+\) rK\(+\) mK\(+\) dcm gal urea\(300\)) carrying the
pET21b::Rad51 plasmid. RNA\(^\text{IV}\) and RNA\(^\text{V}\) were coexpressed to facilitate the translation of the RAD51 minor codons (1). The primary procedures of
Rad51 protein purification, including the selective extraction with polyvinyl P
followed by phosphocellulose chromatography, were similar to those described
earlier (7). The protein fraction containing Rad51 was further purified by
separation on Bio-Gel hydroxyapatite, Cyabron Blue (Hi-Trap Blue), and
MonoQ (Hi-Trap Q) columns. Finally, Rad51 eluted from the MonoQ column
at 400 mM KCl was stored at \( -70^\circ\)C at a concentration of 1.2 mg/ml. Purity of the
protein fraction was more than 95%, as determined by sodium dodecyl sulfate-
polyacrylamide gel electrophoresis analysis followed by Coomassie blue staining.
The final Rad51 protein fraction contained no detectable exonuclease or en-
donuclease activities. S. cerevisiae Rad51 protein (Rad51) was purified from insect cells as
described previously (21). Yeast replication protein A (RPA) was expressed in
E. coli by use of plasmid pM126 (provided by S. Brill and B. Stillman) and was
purified as described previously (5).

The concentrations of proteins were determined using the following extinction
coefficients at 280 nm: 1.565 \( \times \) 10\(^4\) M\(^{-1}\)cm\(^{-1}\) for Rad51, 2.60 \( \times \) 10\(^4\) M\(^{-1}\)cm\(^{-1}\), and
8.600 \( \times \) 10\(^4\) M\(^{-1}\)cm\(^{-1}\) for RPA.

**DNA-dependent ATP hydrolysis.** ATP hydrolysis was measured as described
previously (17). Oxidation of NADH was measured as the decrease in absorb-
ance at 340 nm by using an extinction coefficient of 6.22 \( \times \) 10\(^3\) M\(^{-1}\)cm\(^{-1}\). Unless
otherwise noted, the TMD reaction buffer used in all experiments contained 25
mM Tris-HCl (pH 7.5), 10 mM Mg\(_2\)Cl, and 1 mM diethiothreitol (DTT), and
the incubation temperature was 37°C. Rad51 and a DNA cofactor [poly(dT),
poly(dC), poly(dA), or dsDNA] were added to a reaction mixture containing 1
mM ATP, 3 mM phosphoenolpyruvate, 30 U of pyruvate kinase ml\(^{-1}\),
12.6 mM NADH, and 30 U of lactate dehydrogenase ml\(^{-1}\) in TMD buffer.

The hydrolysis of ATP by the Rad51 protein was analyzed by using the Hill
equation (9) that expresses the rate constant per monomeric Rad51 protein
as:

\[
\frac{V}{V_{\text{max}}} = \frac{[ATP]}{K_{\text{m}} + [ATP]},
\]

where \( V_{\text{max}} \) is the maximal velocity at the infinite ATP concentration, and
\( [ATP] \) is the ATP substrate concentration. The velocity of the ATPase reaction
at [ATP], \( V_{\text{max}} \), is the Hill coefficient. All kinetic parameters were determined by fitting data to this
equation at various concentrations of ATP. Microsoft Excel was used in the
calculation of the parameters.

The thermodynamic parameters for the rate-determining step, changes in
enthalpy (\( \Delta H \)), entropy (\( \Delta S \)), and activation-free energy (\( \Delta G \)), were obtained from the Arrhenius plot for Rad51 ATPase activity with the formula
\( \ln(k_{a}) =\)

\[
A - E_{a} - RT \ln(RT) - RT \ln(R) + \ln(A/\beta_{h}h_{k}),
\]

where \( E_{a} \) is the activation energy, \( A \) is the intercept of the Arrhenius plot, \( R \) is the
the gas constant, \( T \) is the absolute temperature, \( h_{k} \) is the Boltzman constant, \( \beta_{h} \) is the
turnover number, and \( h \) is the Planck constant (18).

**DNA strand exchange.** DNA strand exchange was performed using the aro-
garose gel assay described previously (22). In the reaction, a 60 mM concentration
of circular dX174 dsDNA was preincubated with 20 \( \mu \)M Rad51 in the presence of
TMD buffer and 2.0 mM ATP with its regenerating system for 5 min at 37°C.
Then, E. coli ssDNA-binding protein and yeast RPA were added to RPA con-
centration of 0.6 and 1 \( \mu \)M, respectively. After 7 min, the reaction was started by
the addition of linear dX174 dsDNA to achieve a concentration of 60 \( \mu \)M. The
reaction was terminated after 120 min by the addition of EDTA, sodium dodecyl
sulfate, and protease K to final concentrations of 5 mM, 0.5%, and 0.1 mg/ml,
respectively, and then deproteinized by incubation at 37°C for 30 min. The
products of the reaction were analyzed by gel electrophoresis on 1% agarose gel
in Tris-acetate-EDTA buffer and visualized by staining with ethidium bromide.

The same reaction conditions were used for DNA strand exchange promoted
by Rad51 except that TMD buffer was replaced with MMD buffer (40 mM
K-MES [K-morpholineethanesulfonic acid] [pH 6.5], 2.5 mM MgCl\(_2\), 1 mM
DTT) and 5 mM spermidine was added to the reaction mixture as described
previously (34). After visualization, the products of DNA strand exchange were
quantified, and the amounts of FPs and joint molecules (JM) were expressed as
percentages of the original amounts of dsDNA converted into these products.

**Nucleotide sequence accession number.** The sequence data have been sub-
mitted to the DDBJ, EMBL, and GenBank databases under accession number
AAP74362.

**RESULTS**

**Rad51 amino acid sequence.** The predicted Rad51 amino acid sequence is presented in Fig. 1 as the sequence alignment of Rad51-related proteins from three yeast species and one Rad51 protein from a human. The choice of Rad51 proteins used for the alignment represents the obvious differ-
ence in recombination DNA repair systems operating in eu-
karyotes. Lower eukaryotes, such as S. cerevisiae and Schizo-
saccharomyces pombe, display strong DNA repair, whereas
recombinational repair in higher eukaryotes, such as Homo sapiens, is significantly less efficient. This sequence alignment
displays equal amino acid identities and similarities among
Rad51, and Rad51 homologs. In a comparison of the se-
quencies of those protein regions where comparison is possible
for all four of the Rad51 proteins analyzed (between residues
24 and 317 of Rad51) (Fig. 1), Rad51 displays about 71, 75,
and 69% identity as well as 84, 87, and 82% homology to
Rad51c and Rhp51 from S. pombe and Rad51 from H. sapiens,
respectively. Thus, the Rad51 protein is equally similar to
lower and higher eukaryotic Rad51 proteins.

**Rad51 protein partially complements the Rad51 defi-
ciency in S. cerevisiae.** The Rad51 gene was inserted into the
pYES2 expression vector under the galactose-inducible pro-
motor PGAL1. Both pYES2 and pYES2::RAD51 plasmids were transformed into
S. cerevisiae strain D3164, and the IR sensitivities of the transformed strains were compared as de-
scribed in Materials and Methods (Fig. 2). At an IR dose of 25
kilorads, the yeast strain (D3164/pYES2::RAD51) with in-
duced Rad51 was about 10-fold more resistant than the same
strain without induction (Fig. 2). However, the D4004 strain
with a normal RAD51 gene was >1,000-fold more resistant to
IR under the same conditions than the strain with Rad51
deficiency (D3164). Thus, Rad51 induced by galactose only
partially complemented the survival of the DNA repair-defi-
cient strain D3164. These data indicate that, even though
Rad51c and Rad51 proteins are homologous, Rad51c only
partially complements in vivo functions of Rad51c in yeast.
This partial complementation indicates the complexity of the
DNA repair mechanism in yeast, in which the interaction of
Rad51 with other proteins plays a significant role.

**Characteristics of ssDNA-dependent ATP hydrolysis cata-
lized by Rad51.** The Rad51 protein was purified to near
homogeneity, and its ability to hydrolyze ATP was determined.
Since ATP is an obligatory component of the presynaptic com-
plex Rad51c::ATP: ssDNA, ssDNA-dependent ATP hydrol-
sis is commonly used to monitor nucleoprotein complex for-
mation. Figure 3 shows the Rad51:ssDNA-binding isotherm
as monitored by poly(dT)-dependent ATP hydrolysis.
olation of linear portions of this isotherm demonstrates how an estima-
tion of Rad51 Pa-ssDNA stoichiometry was done. The stoici-
ometry was 3.0 nucleotides per one protomer of Rad51Pa filament, which is in accordance with values found for other members of the RecA-RadA-Rad51 protein family (15, 20, 35).

The levels of ability of different DNA cofactors to stimulate ATP hydrolysis promoted by Rad51Pa protein were determined by the comparison of the $k_{cat}$ turnover numbers (calculated as $V_{max}/[Rad51Pa]_0$) for different DNA substrates (Table 1). Rad51Pa hydrolyzed ATP only when both DNA and Mg$^{2+}$ were present. The order of the levels of ability to stimulate ATP hydrolysis starts with poly(dC) as the most efficient and then poly(dT), X174 ssDNA, poly(dA), and M13 dsDNA.

FIG. 1. Sequence alignments of three yeast Rad51 proteins (from P. angusta, S. cerevisiae, and S. pombe, designated PaRad51, ScRad51, and SpRhp51, respectively) and an H. sapiens Rad51 protein (HsRad51). A dash indicates a gap introduced into the sequences to optimize the alignment. A period represents a residue identical to that from Rad51Pa. Bold letters show similarity between amino acid residues in given positions of the alignment. Asterisks indicate boundaries of the alignment chosen for quantitative comparison of sequences (residues 24 to 317).

FIG. 2. Compensation of the Rad51Sc protein deficiency with Rad51 Pa as measured by the resistance to $\gamma$-irradiation of strain D3164/pYES2::RAD51Pa, in which the expression of the Rad51Pa protein was induced by galactose (0.2%). Controls were D4004 (Rad51Sc phenotype) (filled diamonds), D3164/pYES2 without galactose in the growth medium after irradiation (Rad51 Sc phenotype) (filled triangles), D3164/pYES2 with galactose after irradiation (Rad51 Sc phenotype) (open triangles), and D3164/pYES2::RAD51Pa without galactose after irradiation (Rad51 Pa phenotype) (open squares).

FIG. 3. Rad51 Pa-poly(dT)-binding isotherm monitored by ATP hydrolysis. Each point on the curves represents a result with an individual sample. The spectrophotometric assay was performed at 37°C in TMD buffer containing 4 mM Rad51Pa, 1 mM ATP with its regenerating system, the NADH-lactate dehydrogenase system, and poly(dT) at the concentration indicated. Each point in the curve (solid line) was averaged from the results of two or three repeat experiments. Broken line, extrapolation of the linear portions of the Rad51Pa-ssDNA-binding isotherm.
Cooperativity is determined by calculating the Hill constant $n_H$. Photometric ATPase assays were performed by incubating 1.0 $\mu$M of ATP. The inset represents the original kinetic data. The spectroscopically labeled ssDNA was 28 min$^{-1}$. Without DNA, $K_M$ was 0.02 ± 0.02 M. Without MgCl$_2$, $K_M$ was 0.18 ± 0.03 M. Without DNA, $k_{cat}$ was 0.01 ± 0.01 M$^{-1}$s$^{-1}$. (the least efficient). Similar orders for different DNAs were shown previously for other RecA-like proteins (32, 36). Additionally, ssDNA-dependent ATP hydrolysis catalyzed by Rad51$_{Pa}$ is 25-fold less efficient than that catalyzed by E. coli RecA under similar conditions (for RecA from E. coli, $K_M$ for ssDNA was 28 min$^{-1}$). These results suggest that Rad51$_{Pa}$ belongs to the RadA-Rad51 subfamily of inefficient ATPases.

The last conclusion is further supported by the finding that Rad51$_{Pa}$ showed very low, if any, ATP-induced cooperativity. Cooperativity is determined by calculating the Hill constant $n_H$ (a measure of cooperativity) as a slope derived from a plot of fractional rate $v/[V_{max} - v]$ versus the substrate concentration ([ATP]), presented in logarithmic coordinates (10). A Hill plot based on kinetic measurements of poly(dT)-dependent ATP hydrolysis at 37 and 47°C is shown in Fig. 4 (inset). The steady-state kinetic parameters ($K_M$, $k_{cat}$, and $n_H$), summarized in Table 2, were determined by fitting data to the Hill equation as described in Materials and Methods. The data indicate that Rad51$_{Pa}$ displays a lower apparent affinity for ATP ($K_M$) than does E. coli RecA [$S_0.5$ is 60 $\mu$M for poly(dT) at 37°C and pH 7.5; in a cooperative system, the substrate concentration where half-maximal activity occurs is $S_0.5$ (14, 27). Furthermore, the Rad51$_{Pa}$ protein possesses weak cooperativity ($n_H$ is close to 1), and its catalytic efficiency ($k_{cat}/K_M$) is approximately 35-fold less than that of E. coli RecA. Interestingly, the Rad51$_{Pa}$ protein is still thermoresistant at 47°C, and its catalytic efficiency is practically equal to that observed at 37°C.

Thermoinactivation of Rad51$_{Pa}$ compared with that of Rad51$_{Sc}$. Since P. angusta is thermotolerant, it is reasonable to propose that Rad51$_{Pa}$ should be more thermoresistant than its homolog from bakery yeast. First, we determined the thermostability of the Rad51$_{Pa}$ and Rad51$_{Sc}$ proteins by preincubating them for 10 min at different temperatures. Levels of protein inactivation were determined by measuring residual ssDNA-dependent ATP hydrolysis activity at 37°C. Figure 5A summarizes the inactivation profiles for both proteins. Rad51$_{Sc}$ is completely inactivated after 10 min of incubation at 37°C. By contrast, Rad51$_{Pa}$ remains completely active, and preincubation at the significantly higher temperature of 54°C was required to inactivate it completely. It has been shown that the stability of Rad51 is significantly increased when protein is in the nucleoprotein complex with ssDNA (2, 12, 24). The semi-inactivation period for Rad51$_{Pa}$ at 47°C was 10 min, whereas the stability of Rad51$_{Sc}$ in the complex with poly(dT) at the same temperature was increased to 1 h (data not shown). Additionally, the inactivation kinetics of the Rad51$_{Pa}$ and Rad51$_{Sc}$ proteins at different temperatures were performed (Fig. 5B and C). Rad51$_{Pa}$ was more resistant to inactivation across all tested temperatures. Note that it was 20 times more thermostable at 37°C than Rad51$_{Sc}$ was. Moreover, Rad51$_{Pa}$ retained 10% of the ssDNA-dependent ATPase activity even after 30 min of preincubation at 47°C, whereas Rad51$_{Sc}$ was completely inactive after 5 min. These results show that Rad51$_{Pa}$ is more resistant to temperature inactivation than the Rad51$_{Sc}$ protein.

The dependence of inactivation coefficient $k_{inact}$ $[V_{max}/[Rad51]]$ on temperature was constructed with coordinates of an Arrhenius plot (Fig. 5D), which permitted the identification of the thermodynamic parameters for the rate-determining step of ATP hydrolysis as described in Materials and Methods.

![FIG. 4. Hill plot of Rad51$_{Pa}$-mediated ATP hydrolysis as a function of ATP. The inset represents the original kinetic data. The spectrophotometric ATPase assays were performed by incubating 1.0 $\mu$M Rad51$_{Pa}$ and 20 $\mu$M poly(dT) with the indicated amount of ATP (with its regenerating system) in TMD buffer (pH 7.5) at the indicated temperatures for 20 min. Lg, log.](https://example.com/figure4.png)

| DNA cofactor | $k_{cat}$ (min$^{-1}$) | $K_M$ (M) |
|-------------|-----------------|---------|
| Poly(dT)    | 1.20 ± 0.45     | 0.31    |
| Poly(dC)    | 1.31 ± 0.36     | 0.31    |
| ssDNA ϕX174 | 1.04 ± 0.31     | 0.06    |
| Poly(da)    | 0.09 ± 0.28     | 0.06    |
| dsDNA M13   | 0.63 ± 0.18     | 0.03    |
| Without MgCl$_2$ | 0.03 ± 0.03 | 0.08 ± 0.03 |

TABLE 1. Comparison of the abilities of different DNA cofactors to stimulate ATP hydrolysis catalyzed by Rad51$_{Pa}$, as assessed by the steady-state kinetic parameter $k_{cat}$.

| DNA cofactor | Avg $K_m$ (μM) | $k_{cat}$ (min$^{-1}$) | $n_H$ | $k_{cat}/K_m$ (s$^{-1}$·M$^{-1}$) |
|-------------|---------------|----------------------|-------|-------------------------------|
| 37          | 86 ± 23       | 1.20 ± 0.45          | 1.23  | 240                           |
| 47          | 141 ± 35      | 2.23 ± 0.37          | 0.97  | 264                           |

At both temperatures, the DNA cofactor was poly(dT). See also Fig. 4.

TABLE 2. Kinetic parameters of poly(dT)-dependent ATP hydrolysis catalyzed by Rad51$_{Pa}$

| Protein | $E_f$ (kcal·mol$^{-1}$) | $|\Delta H|$ (kcal·mol$^{-1}$) | $|\Delta S|$ (kcal·mol$^{-1}$·K$^{-1}$) | $\Delta G$ (kcal·mol$^{-1}$) |
|---------|-----------------|-----------------|-----------------|-----------------|
| Rad51$_{Pa}$ | 71.1 ± 1.6      | 70.4 ± 2.0      | 0.15 ± 0.03     | 24.7 ± 1.6      |
| Rad51$_{Sc}$ | 61.6 ± 1.5      | 61.0 ± 1.7      | 0.13 ± 0.02     | 21.4 ± 1.6      |

*Values are means ± standard deviations. See also Fig. 5D. $E_f$, inactivation energy.
(Table 3). Based on all three parameters (changes in enthalpy \([\Delta H]\), entropy of inactivation \([\Delta S]\), and inactivation-free energy \([\Delta G]\)), we conclude that the higher thermostability of Rad51 Pa than that of Rad51 Sc is attributed to the better structural stability of the Rad51 Pa protein in the ATPase active site.

**Thermodependence of Rad51Pa protein nucleation.** Polymerization of Rad51 Sc on ssDNA in the presence of ATP results from a short lag period of nucleation or presynaptic complex formation which is ready to initiate homologous pairing from the 3' or 5' end (13, 21, 23, 33). The short lag period can be monitored by the ternary-complex ATPase activity. Figure 6 shows that Rad51 Pa nucleation time on ssDNA was reduced from 7 min to about 1 min by increasing the temperature from 37 to 47°C. This result indicates the thermodependence in the presynaptic complex formation of homologous recombination promoted by the Rad51 Pa protein.

**Thermodependence of the DNA strand exchange reaction promoted by the Rad51Pa and Rad51Sc proteins.** Rad51 Pa and Rad51 Sc were compared for their ability to promote DNA strand exchange between circular ssDNA and homologous linear duplex DNA at different temperatures (Fig. 7A). Rad51 Sc promoted DNA strand exchange equally well in the temperature range between 32 and 42°C, but no products were detected at temperatures above 47°C (Fig. 7B and C). About 50% of linear DNA was converted into JM and nicked circular dsDNA (FP) at optimal temperatures, and formation of the latter was maximal at 42°C. In contrast, Rad51 Pa promoted strand exchange most efficiently at temperatures above 42°C. JM were major products of the reaction after incubation at

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**FIG. 5.** Comparative thermoinactivation of the Rad51 Pa (PaRad51) and Rad51 Sc (ScRad51) proteins. (A) Each protein alone was incubated in KMD buffer (20 mM potassium phosphate [pH 7.5], 4 mM MgCl₂, and 1 mM DTT) with 5% glycerol for 10 min at the temperatures indicated. The residual poly(dT)-dependent ATPase activity was measured by spectrophotometric assay in the reaction mixture containing an 8 \(\mu\)M concentration of either Rad51 Pa, or Rad51 Sc, 80 to 160 \(\mu\)M poly(dT), and 1 mM ATP with its regenerating system. Each point of the curves was averaged from the results of three separate experiments performed with different excesses of poly(dT) in order to determine a residual \(V_{\text{max}}\) at the given temperature under conditions at which DNA binding sites of the protein are saturated with poly(dT). One hundred percent signifies the \(V_{\text{max}}\) for the protein without a preliminary temperature treatment. (B and C) Time courses of the thermoinactivation of the Rad51 Pa and Rad51 Sc proteins. The residual poly(dT)-dependent ATPase was measured as described above. (D) Arrhenius plot. A reconstruction of the data from panels B and C is shown. T, temperature. \(K_{\text{ic}}\), inactivation coefficient.
42°C, and incubation at 52°C was required for the accumulation of nicked circular dsDNA. These data indicate that thermodependence is as critical an in vitro property of Rad51 Pa as the ability to exchange extended DNA molecules.

**DISCUSSION**

The RAD51 gene from thermotolerant yeast \textit{P. angusta} was cloned, and its nucleotide sequence was determined. The \textit{RAD51} gene encodes a 40-kDa protein which shows about 70% identity and more than 80% similarity with core regions of Rad51 proteins of both lower and higher eukaryotes (Fig. 1). In vivo, the protein was able to complement, though only partially, the DNA repair deficiency of \textit{S. cerevisiae} \textit{rad51} cells (Fig. 2). The Rad51 Pa protein was overproduced in \textit{E. coli} and purified to homogeneity. Judging by the thermoresistant potential of Rad51 Pa, it seems reasonable to conclude that the thermostability of Rad51 Pa is more than enough to support the growth of its host at 42°C.

Two additional conclusions can be derived from our results. First, Rad51 Pa is a typical representative of the Rad51 subfamily. Indeed, according to such general characteristics as the stoichiometry (Fig. 2) and DNA specificity (Table 1) of Rad51 Pa-ssDNA interactions, the protein is referred to as a member of the RecA-RadA-Rad51 superfamily. However, two particular properties, the inefficient ATPase (Table 2) and very low cooperativity (Fig. 4) in Rad51 Pa, are typical only for RadA-Rad51 proteins (32, 35).

Second, Rad51 Pa is a thermodependent protein rather than a thermostable protein. Indeed, the temperature of 42°C is critical for at least two protein characteristics which are thought to be important for recombination. These characteristics are a sharp enhancement of nucleation in presynaptic filamentation (Fig. 6) and the ability to form extended JM and thus \( \phi X174 \) FPs in the strand exchange reaction (Fig. 7). Interestingly, this critical temperature was very close to the optimal growth temperature interval (37 to 42°C) found for \textit{P. angusta} strain BKM Y1397.

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