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ORIGINAL PAPER

The in vitro activity of a Rad55 homologue from *Sulfolobus tokodaii*, a candidate mediator in RadA-catalyzed homologous recombination

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Abstract Archaea have recombination proteins similar to those of eukaryote, but many have not been characterized. Here, the characterization of a Rad55 homologue from *Sulfolobus tokodaii* (stRad55A) was reported. StRad55A protein preferred binding to ssDNA and had ssDNA-dependent ATPase activity. In addition, UV light could induce the expression of this protein, which was different from RadB, a RadA paralog found in euryarchaeota. Most importantly, stRad55A could release the suppression of excessive stSSB (single strand DNA binding protein from *S. tokodaii*) on the strand exchange catalyzed by stRadA (RadA homologue from *S. tokodaii*), by interacting directly with both stRadA and stSSB. StRad55A may function as a mediator to accelerate the displacement of stSSB by stRadA.

Keywords Archaea · Recombination · Mediator · StRad55

Introduction

Genome sequencing has revealed that archaea encode homologues to the eukaryotic DNA recombinational repair proteins. The recombinationase RadA from archaea is more similar to the eukaryotic Rad51 protein (40% amino acid identity) than to the bacterial RecA protein (20%). Rad51 and RadA possess a somewhat conserved N-terminal domain, while RecA has a similar domain at its C-terminus (Komori et al. 2000b). In activities, the slower rates of both ATP hydrolysis and DNA strand exchange, suggested the RadA protein behaves more similarly to the eucaryal Rad51 protein than to the bacterial RecA protein (Seitz et al. 1998). In addition, homologues of the eukaryotic recombination repair protein Mre11 and Rad50 are present in all the 37 archaeal genomes whose sequence is available now. The recombination in archaea seems to be more eukaryal than bacterial and study on archaeal DNA recombination proteins may shed insight into the much more complex eukaryotic recombination apparatus (Grogan 2000; Lee et al. 2004). However the details of recombinational repair mechanism have not been fully revealed specially the genes involved in the initiation step still remains a mystery in archaea.

In eukaryote, Rad5l accelerates the homologous strand exchange though interaction with other proteins, such as Rad52, RPA, Rad54, Rad55, Rad57, Rad59 and others (Seitz et al. 1998; Fortin and Symington 2002; Symington 2002). RPA (replication protein A) binds and protects exposed ssDNA ends until Rad51 binds to them to initiate the recombination repair. However, Rad51 by itself cannot bind to RPA-coated ssDNA, and it becomes the critical step in recombination to displace RPA from ssDNA by Rad51. This process is accomplished with the help of so-called mediator proteins Rad52 and the Rad55–Rad57 heterodimer (Symington 2002).

The N-terminus of Rad52 protein is highly conserved in most eukaryotic species, and is responsible for binding DNA and facilitates the homologous ssDNA annealing, whereas the C-terminus confers species-specific interaction

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with Rad51 (Singleton et al. 2002; Symington 2002). Rad52 apparently recruits Rad51 onto ssDNA covered with RPA by binding to both RPA and Rad51 and promotes formation of the Rad51 filament (Miyazaki et al. 2004; Reich et al. 2001).

Rad55 and Rad57 proteins are identified as *Saccharomyces cerevisiae* Rad51 paralogs because of their close sequence homology to Rad51, and they form a heterodimer that facilitates formation of the functional Rad51 complex on RPA-coated ssDNA (Johnson and Symington 1995; Reich et al. 2001; Sung 1997). In contrast to yeast, five Rad51 paralogs (Rad51B, Rad51C, Rad51D, Xrcc2, and Xrcc3) have been identified in mammals (Albala et al. 1997; Cartwright et al. 1998; Dosanjh et al. 1998; Liu et al. 1998). These proteins share 20–30% sequence identity with Rad51 and with each other (Thacker 1999), and are thought to mediate DNA strand exchange events of Rad51 during the process of HR (Takata et al. 2001).

Although there is no homologue of RAD52, there are RadA paralogs in archaea (Grogan 2000; Seitz et al. 1998). In euryarchaeota of archaea, RadB, a RadA paralog, has also been identified in the genome (Akiba et al. 2005; Komori et al. 2000a). RadB retains the major domain of RadA, but lacks the N-terminal domain. This protein has a very weak ATPase activity and a strong DNA binding affinity. RadB directly interacts with RadA or Hjc, and may be involved in HR in archaea (Komori et al. 2000a). Different from euryarchaeota, more than one RadA paralogs are present in the crenarchaeal genomes. Specifically, through sequence analysis, four RadA paralogs were found in *Sulfolobus tokodaii*. They have not the N-terminal domain of RadA either, and phylogenetic analysis based on the entire amino acid sequences of 32 Rad51/RadA and their paralogs from eukaryote and archaea (Appendix Figure in Electronic supplementary material), indicates that they show more homology to Rad55 in yeast than to RadB in euryarchaeota, so we named them *stRad55* genes. However the biochemical characters and the real functions of the Rad55 homologues remain a mystery.

In this study, we investigated the functions of one of the four RadA paralogs in *S. tokodaii*, stRad55A (ST0579), and found that stRad55A could eliminate the inhibition effect of stSSB (SSB from *S. tokodaii*) on the recombination of stRadA by binding to both stRadA and stSSB.

Materials and methods

Strains, plasmids, and chemicals

The pET15b vector and *Escherichia coli* stains TG1 and BL21-CondonPlus (DE3)-RIL were from Invitrogen (CA, USA). Taq DNA polymerase, restriction enzymes, DNA ligation kit, and T4 polynucleotide kinase were purchased from Takara (Takara Biotechnology Dalian Co., Ltd., Dalian, China), and used according to the manufacturer's recommendation. [γ-^{32}P]ATP was obtained from GE Healthcare (Buckinghamshire, UK). The M13mp18 circular ssDNA and replicative form I DNA (about 95% supercoiled) were purchased from Sigma (St Louis, USA). The oligonucleotides were synthesized and gel purified by Invitrogen (CA, USA).

E. coli cells were grown at 37°C in Luria–Bertani (LB) broth or on LB plates solidified with 1.5% agar. *S. tokodaii* strain 7 was cultured at 80°C in complete medium as described previously (Brock et al. 1972).

Genetic assays

Radiation resistance

S. tokodaii cells were grown to early stationary phase and used for determining the cell survival rate. For UV irradiation, the cell suspension (15 ml) in 130 mm glass Petri dishes was stirred gently with a magnetic rod and irradiated with a germicide UV lamp (254 nm, 30 W) at room temperature for several different doses (from 0 to 400 J/m^2), which were adjusted by changing the time exposed to UV-ray source of the samples. After the treatments, the cells were diluted in fresh medium and incubated at 80°C for 5 days. The density of the cells was measured every 12 h to generate the growth curves.

RT-PCR assays

The RNA from the cells treated with UV light or mock was prepared by RNA extract kit according to the manual. After the RNA was quantified by spectrophotometer, reverse transcription was carried out with random primers using the First-Strand cDNA Synthesis Kit (Shennergy Biocolor Bioscience & Technology Company), and the succedent PCR with the primers for stRad55A (F 5′-GCTGAGGGA CTTAGGGAAGGAGATA-3′; R 5′-CCTCTCTTCTCTCG TTTAT TTTACC-3′) and stRadA (F 5′-CAGGAATACCA CTAACTACAGCCC-3′; R 5′-ATAGAGTATGACCACC TACAGCCAC-3′), were run according to the standard protocol (Ausubel et al. 1995).

Western blotting assay

The *S. tokodaii* cells were disrupted with an ultrasonicator at 600 W output for a total of 2 min on ice and the debris was removed by centrifugation (15,000×*g*, 20 min). The proteins in the supernatant were separated on SDS-PAGE

gel and the target proteins were detected with corresponding antibodies (rabbit IgG, laboratory stock).

Cloning, expression and purification of the protein

The gene for stRad55A (ST0579) from *S. tokodaii* was amplified using PCR with the following primers, the upper-stream primer: 5′-GGGCATATGATTAGACTGTCAACTGGTA-3′, and the down-stream primer: 5′-CCGGATCCATAGAAGCTTGCAAGAAATAG-3′ (the underlined indicate the *Nde*I site and *Bam*HI site, respectively). The amplified fragment was digested with *Nde*I and *Bam*HI, and ligated into an expression vector pET15b to produce a recombinant vector named pET15/Rad55A.

The constructed expression vector was transformed into *E. coli* strain BL21-CodonPlus (DE3)-RIL, and the expression and purification of the proteins were according to the manual of the Ni^{2+}–NTA agarose column (Qiagen). The concentration of the purified stRad55A protein was determined by UV absorbance at 280 nm.

Biochemical assays

DNA substrates

The replicative form I DNA was linearized with *Eco*RI. The 84-mer oligonucleotide and the double-stranded fragment complement with the oligonucleotide, of which the sequences were taken from M13mp18, were γ-^{32}P-labelled at the 5′ end as described before (Ausubel et al. 1995). DNA substrates were stored in TE buffer (10 mM Tris–HCl, pH 7.2, 0.5 mM EDTA).

DNA binding assay

Various concentrations of stRad55A protein were incubated with 1 μM γ-^{32}P-labeled single-stranded or a double-stranded 84-mer oligonucleotide (as nucleotide concentration) in SEB buffer (20 mM Tris–HCl, pH 7.0, 5 mM $MgCl_2$, 2% glycerol, and 2.5 mM ATP) at 60°C for 10 min. The reaction products were then analyzed by 6% native polyacrylamide gel in 1× TBE buffer and the gel was exposed to the PhosphorImager screen and scanned with Typhoon.

ATPase assay

The ATPase activity assay was performed in a 20 μl reaction mixture containing 50 mM HEPES buffer, pH 7.0, 1 mM $MgCl_2$, 10 μM ATP, 0.2 μl [γ-^{32}P] ATP (10 mCi/ml) and 100 nM stRad55A in the absence or presence of single-stranded DNA or double-stranded DNA (40 fmol) at 60°C. After the reaction, the samples were cooled on ice and stopped by adding 2 μl of 100 mM EDTA to the reaction. Two microlitres of each aliquot was spotted onto a polyethyleneimine–cellulose plate (Merck, Germany). ATP and Pi were separated by chromatography in 1 M formic acid/0.5 M LiCl. After being dried, the plate was exposed to a PhosphorImager screen and scanned. The spontaneous hydrolysis was corrected in the calculation of the ATPase activity.

In vitro binding assays of stRad55A, stRadA and stSSB

The binding buffer contained 20 mM Tris–HCl (pH 7.0), 5% glycerol, 1 mM EDTA, 100 mM NaCl, 1 mM DTT, and 0.6 mM PMSF. StRad55A, stRadA and stSSB proteins with or without his-tags come from the store of our lab and their purification are more than 95%. Purified protein without $(His)_6$-tag was first loaded onto the Ni–NTA agarose column to make sure no binding to the column by itself. Then the protein was incubated with another his-tagged purified protein on ice for 30 min. The mixture was then loaded onto a Ni–NTA column that was equilibrated with ten volumes of binding buffer. After extensive washing, the binding proteins were eluted with 300 mM imidazole and analyzed by SDS-PAGE (Ausubel et al. 1995; Sheng et al. 2005).

Three-strand-exchange assay

The reaction solution (20 μl) containing reaction buffer SEB (20 mM Tris–HCl, pH 7.0, 5 mM $MgCl_2$, 2% glycerol, stSSB, and 2.5 mM ATP), 15 μM circular single-stranded M13mp18 virion DNA, and the stSSB protein (0.5 or 10 μM) were first incubated in the reaction buffer at 60°C for 5 min. Then RadA was added to a concentration of 5 μM. After a second incubation at 60°C for 5 min, the M13 dsDNA and ATP were added to the finally concentration of 15 μM and 5 mM, respectively. After a third incubation at 60°C for 50 min, SDS (a final concentration of 0.5%) and proteinase K (a final concentration of 1 μg/μl, Takara) were added to the reaction solution. A further incubation at 37°C for 30 min was required to fully degrade the protein in the reaction. Sample was mixed with 2 μl of loading buffer composed of 50% glycerol and 0.1% bromophenol blue and resolved on a 1% TAE agarose gel at 2 V/cm.

Binding of recombination proteins to M13mp18 ssDNA immobilized on agarose beads

Immobilized M13mp18 ssDNA on CNBr-activated Sepharose 4B (GE) was prepared as described previously

(Ausubel et al. 1995; Wolner et al. 2003). Ten microlitres of the agarose beads containing 200 ng M13 (+) strand were mixed with the indicated amounts of each protein in 20 µl of SEB buffer for 5 min at 60°C by constant tapping. The beads were captured by centrifugation at low speed. After removing the supernatant, the bead-bound stRadA, stRad55A, and stSSB were eluted with 20 µl of 5% SDS, and were analyzed by SDS-PAGE.

Results

Analysis of the amino acid sequence of stRad55A

StRad55A (ST0579) is composed of 258 amino acids. Domain alignment of stRad55A with its homologous proteins, RadA from *S. tokodaii*, Rad55 and Rad51 from *S. cerevisiae*, and RadB from *Thermococcus kodakarensis* is shown in Fig. 1a. RadA and Rad51 are composed of two domains: the highly conserved ATPase domain, which makes up the core oligomeric structure, and the N-terminal domain, which is not conserved in bacterial RecA and involves in binding to double strand DNA (Lovett 1994). Compared with RadA/Rad51, stRad55A has the conserved ATPase core domain but not the N-terminal domain, which is similar to the Rad55 proteins in eukaryote, or RadB in euryarchaeota. The ATPase domain of stRad55A shares more sequence identity with Rad55 than RadB. Compared with yeast Rad55, stRad55A has a small and lysine-rich amino acid tail in the C-terminus, which endows the protein a basic isoelectric point (about 8.4). However, the C-terminal domain of stRad55A is bigger than that of RadB besides little sequence identity between them. The alignment of the secondary structure elements of stRad55A and RadB by computational analysis is shown in Fig. 1b, which presents further structural comparability in the ATPase domain and difference in the C-terminal tail (Akiba et al. 2005).

StRad55A and stRadA could be induced by UV light

To find suitable conditions for UV irradiation of *S. tokodaii* cultures, we analyzed the effect of different UV doses on the

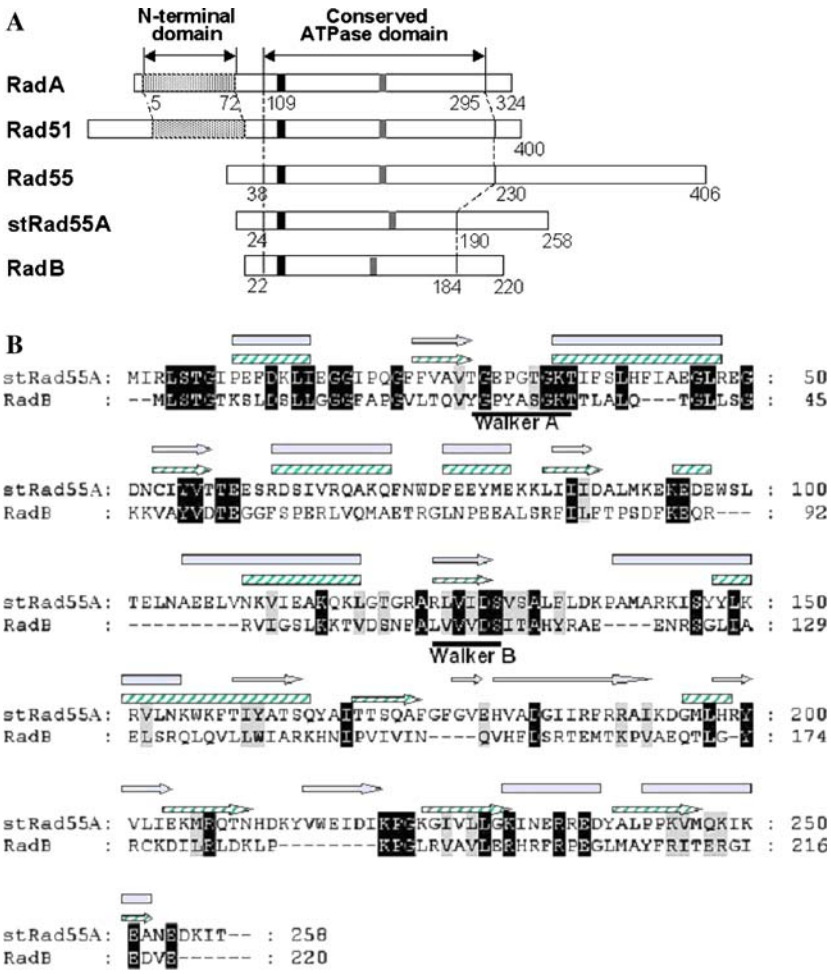


Fig. 1 Domain and sequence analysis of stRad55A from *S. tokodaii*. **a** The domain structures of stRadA and stRad55A from *S. tokodaii*, Rad55 and Rad51 from *S. cerevisiae*, and RadB from *T. kodakarensis*. The *filled* and *shaded boxes* indicate the Walker A and B motifs, respectively. **b** Sequence alignment of stRad55A with RadB. The amino acid coordinates are shown to the *right of the lines*. The secondary structure elements of stRad55A (*gray*) and RadB from *T. kodakarensis* (*grained*) are shown *above the alignment* with the same notation. *Strips* represent α-helical segments and *horizontal arrows* represent *β*-sheets

cell growth. As shown in Fig. 2a, the cells had the ability to survive at the dose below 400 J/m^2. It has been reported that *Sulfolobus solfataricus* could survive the treatment at the dose of 3,300 J/m^2 and resume normal growth only after approximately seven-generation time (Salerno et al. 2003). The UV tolerance of *S. tokodaii* was clearly weaker than that of *S. solfataricus*, which may be due to the difference between two strains and the radiation processes. The recovery exhibited a short delay at 50 J/m^2 and was prolonged as the doses increased. When the dose was at 400 J/m^2, 7 days was needed to restore the cell growth. So, we chose 100 and 200 J/m^2 as the irradiation doses for in vivo transcription and translation analysis of the proteins.

UV light can induce RadA protein in thermophiles archaea (Reich et al. 2001; Ries et al. 2000). Our previous studies had also proved that *S. tokodaii* RadA could be induced after treatment with UV light (results in publication). Here, the induction of stRad55A was analyzed as shown in Fig. 2b, c. The results showed that the protein was induced at 200 J/m^2 (lane 3), and the transcription and translation levels were approximately twofolds as those in the mock. However, the transcription and translation levels of stRad55A did not increase at the dose of 100 J/m^2. On the contrary, there was some decrease in both assays (lane 2). The lesions caused by the dose at 100 J/m^2 might be insufficient to induce transcription and translation of stRad55A. When the dose increased up to 200 J/m^2, more lesions appeared, and recombinational repair played more important role in the repair, in which stRad55A was needed. The result may be coincident with the fact that Rad55 is a recombinational repair protein in *S. cerevisiae*.

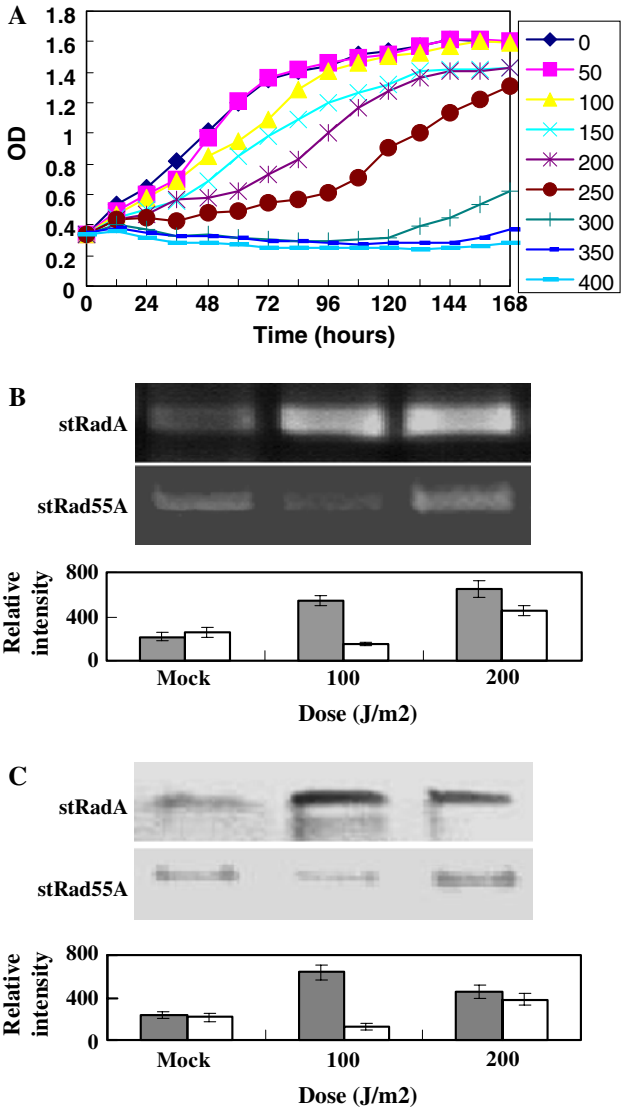


Fig. 2 Induction of stRad55A by UV light. **a** The growth curves of *S. tokodaii* after UV treatment at different doses (in J/m^2). **b** RT-PCR assay. *Lane 1* mock without UV light; *lanes 2* and *3*, treatment with 100 and 200 J/m^2 UV light, respectively. **c** Western blotting. Forty micrograms of crude protein extracts from *S. tokodaii* that was irradiated (*lanes 2* and *3*) or as mock (*lane 1*) were loaded on each lane of a SDS-PAGE gel. Anti-stRad55A antibody was used to detect the amount of the protein in the *upper panel*. Anti-stRadA antibody was used to show the induction of stRadA as a control. Quantification of the intensity of stRadA (*gray bar*) and stRad55A (*open bar*) bands by Gel Imager System (Bio-Rad Laboratories, Hercules, CA), was shown in the *lower panels* in **b** and **c**, respectively. The *values* represent the mean values and standard deviations of three independent experiments, respectively ($P < 0.05$)

Purification of recombinant stRad55A protein

To further inspect the activity of stRad55A in *S. tokodaii*, we expressed the protein in *E. coli*. The profile of the recombinant stRad55A protein purified by a Ni^{2+}–NTA affinity column was shown in Fig. 3. The final protein was obtained by dialysis against a storage buffer (10 mM Tris–acetate, pH 7.0, 50 mM NaCl, 0.1 mM DTT, and 20% glycerol) and was store at –20°C for further analysis in vitro.

The DNA binding activity and ATPase activity of purified stRad55A protein

In order to characterize the DNA binding activity of stRad55A, we performed band-shift assay with γ-^{32}P labeled DNA (Fig. 4a). With increasing amount of stRad55A in the reaction, both ssDNA and dsDNA appeared as the lag bands on gels. About 1.6 μM proteins could retard ssDNA completely, but only about 30% for dsDNA. StRad55A prefered to bind ssDNA, which was accordant with the DNA binding ability of RadB, Rad55, and Rad51/RadA proteins (Johnson and Symington 1995; Komori et al. 2000a; Lovett 1994; Seitz et al. 1998).

Then the ATPase activity of stRad55A was analyzed. First, the ATPase activity of the protein was examined over

a wide range of temperatures to determine the optimal reaction conditions. StRad55A was found to exhibit the highest level of ATP hydrolysis at temperature of 80°C, whereas the ATPase activity was dramatically reduced at temperatures lower than 60°C (Fig. 4b), indicating that the ATPase activity of stRad55A was thermal dependent.

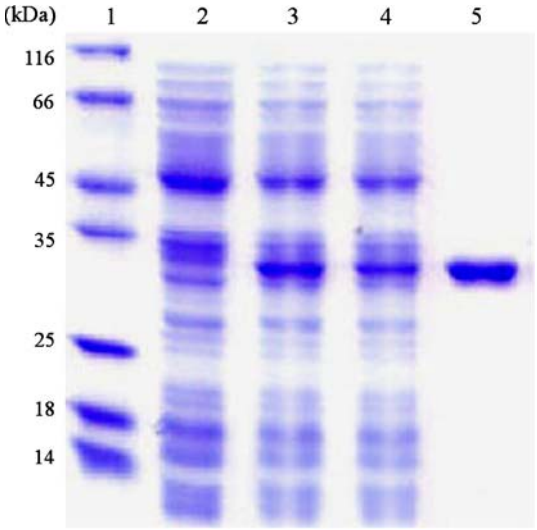


Fig. 3 SDS-PAGE analysis of proteins in different purification steps. *Lane 1* protein molecular weight marker; *lane 2* expression cell without IPTG induction; *lane 3* total cell lysate after 4-h IPTG induction; *lane 4* soluble fractions of the induced cell; *lane 5* purified recombinant stRad55A protein by Ni^{2+}–NTA agarose column

Considering ATP hydrolyzes spontaneously at higher temperature, 60°C was selected as the reaction temperature in the following ATPase assay.

Next, we inspected the ATPase activity in the presence of ssDNA and dsDNA (Fig. 4c). It was shown that the ATP hydrolysis activity of stRad55A was weak in the absence of DNA, and drastically stimulated by ssDNA, but not dsDNA. StRad55A exhibited an ssDNA-dependent ATPase activity. The ATPase activity is different from that of RadB, which had a very weak ATPase activity that is not stimulated by DNA (Komori et al. 2000a), but was in accordance with the ATPase activity of RadA in archaea (Lee et al. 2004; Seitz et al. 1998), or Rad51 and Rad55 in eukaryote (Johnson and Symington 1995; Lovett 1994).

StRad55A could block the DNA strand-exchange promoted by stRadA, but release the inhibition of stSSB on the activity

The effect of stRad55A on the DNA strand-exchange activity in the presence of superfluous stSSB was tested according to the method described in "Materials and methods".

StRad55A alone could not catalyze the strand exchange between circle ssDNA and linear dsDNA (data not shown), but exhibited an inhibition to the activity of stRadA (Fig. 5a). The strand exchange product promoted by

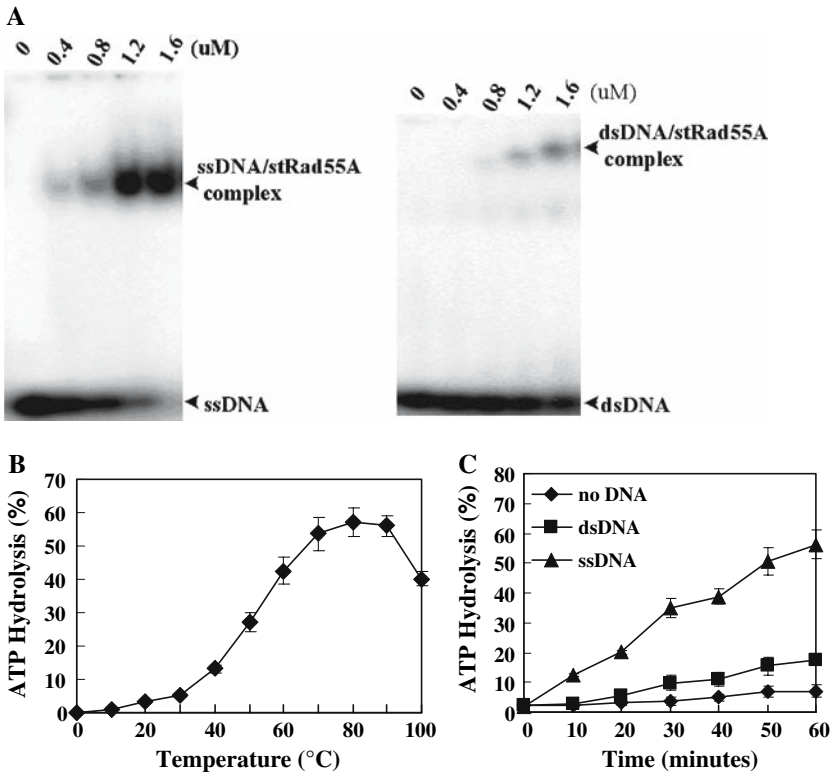
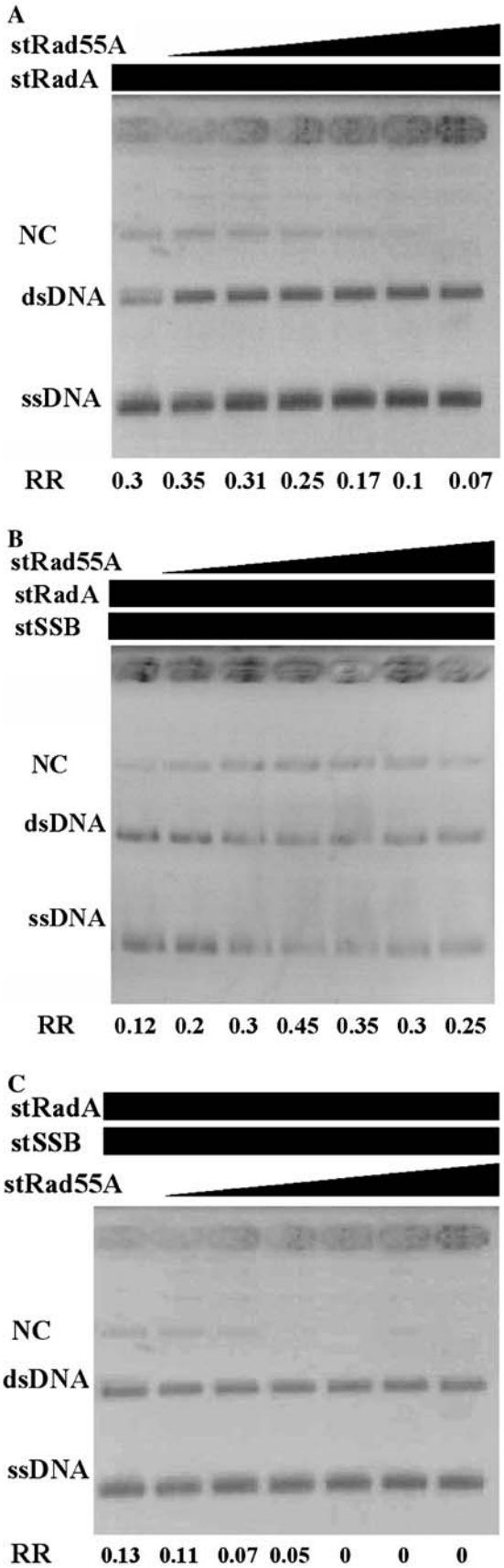


Fig. 4 DNA binding and ATPase activity assays. **a** Assay of the binding activities of stRad55A to ssDNA (*left panel*) and dsDNA (*right panel*). The DNA substrates and the amount of the protein are shown on the *top of the photos*. The reactions were performed at 60°C for 15 min. After reactions, the samples were separated on 6% native polyacrylamide gels and analyzed using PhosphorImager. **b** The ATPase activity of stRad55A measured at a range of temperatures. **c** The ATPase activity of stRad55A in the absence or presence of ssDNA or dsDNA. ATPase assays were carried out as described in "Materials and methods". Values are the mean ± standard deviations obtained from three different experiments



◄ **Fig. 5** DNA strand exchange assay. The reactions were carried out as described in "Materials and methods", and the reaction mixtures contained 15 μM M13 circular ssDNA, 15 μM M13 linear dsDNA, 5 μM stRadA protein, and gradient stRad55A or stSSB protein. The product (nicked circular dsDNA) and the recombination ratio (RR = product/dsDNA) are denoted as *NC* and *RR*, respectively. *Lane 1* no stRad55A; *lanes 2–7* 1, 2, 3, 4, 5, and 6 μM stRad55A, respectively. **a** The effect of stRad55A on the DNA strand exchange activity of stRadA. **b** StRad55A could release the inhibition of stSSB on stRadA-promoted strand exchange when superfluous stSSB pre-binding with ssDNA. **c** StRad55A could not promote the recombination when stRad55A pre-binding with ssDNA before stSSB protein

stRadA decreased strikingly with the addition of the gradient stRad55A to the reaction solutions (lanes 2–7 in Fig. 5a).

We had proved that joint molecule formation catalyzed by stRadA was repressed when ssDNA was pre-saturated with stSSB (data in publication). Here, ssDNA was pre-saturated with superfluous stSSB and the recombination activity of stRadA was inhibited (lane 1 in Fig. 5b). With gradient stRad55A was added to the reaction solution, the inhibition caused by stSSB was weakened clearly (lanes 2–6), though high concentration stRad55A displayed inhibition (lane 7).

However, when stRad55A was incubated with ssDNA before adding stSSB, the recombination did not improved, and with the increase of stRad55A protein, the recombination product decreased clearly (Fig. 5c). The function of stRad55A in the recombination was related to the protein adding order.

StRad55A assists stRadA to the saturated stSSB–ssDNA

To understand the process of stRadA recombination in the presence of stRad55A and stSSB, we examined the proteins bound to ssDNA that were fixed on agarose beads and normalized their amount by SDS-PAGE. We used 200 ng M13 ssDNA fixed to agarose beads in the reaction to analyze the interactions among ssDNA, stSSB, stRadA and stRad55A. To saturate ssDNA with stSSB, abundant stSSB in binding buffer flew through the beads several times to bind to ssDNA at 50°C, then washed away the unbinding protein by binding buffer. As RecA and Rad51 proteins bind two or three nucleotides per protein monomer (Seitz et al. 1998), 200 ng M13 ssDNA (0.6 nmol nucleotides) could bind 0.2–0.3 nmol stRadA. We used 0.15 nmol stRadA in our reaction that was not superfluous.

First, we tested the effect of gradient stRad55A (from 0.03 to 0.18 nmol) on the formation of RadA–ssDNA without stSSB. ssDNA was incubated with stRadA for

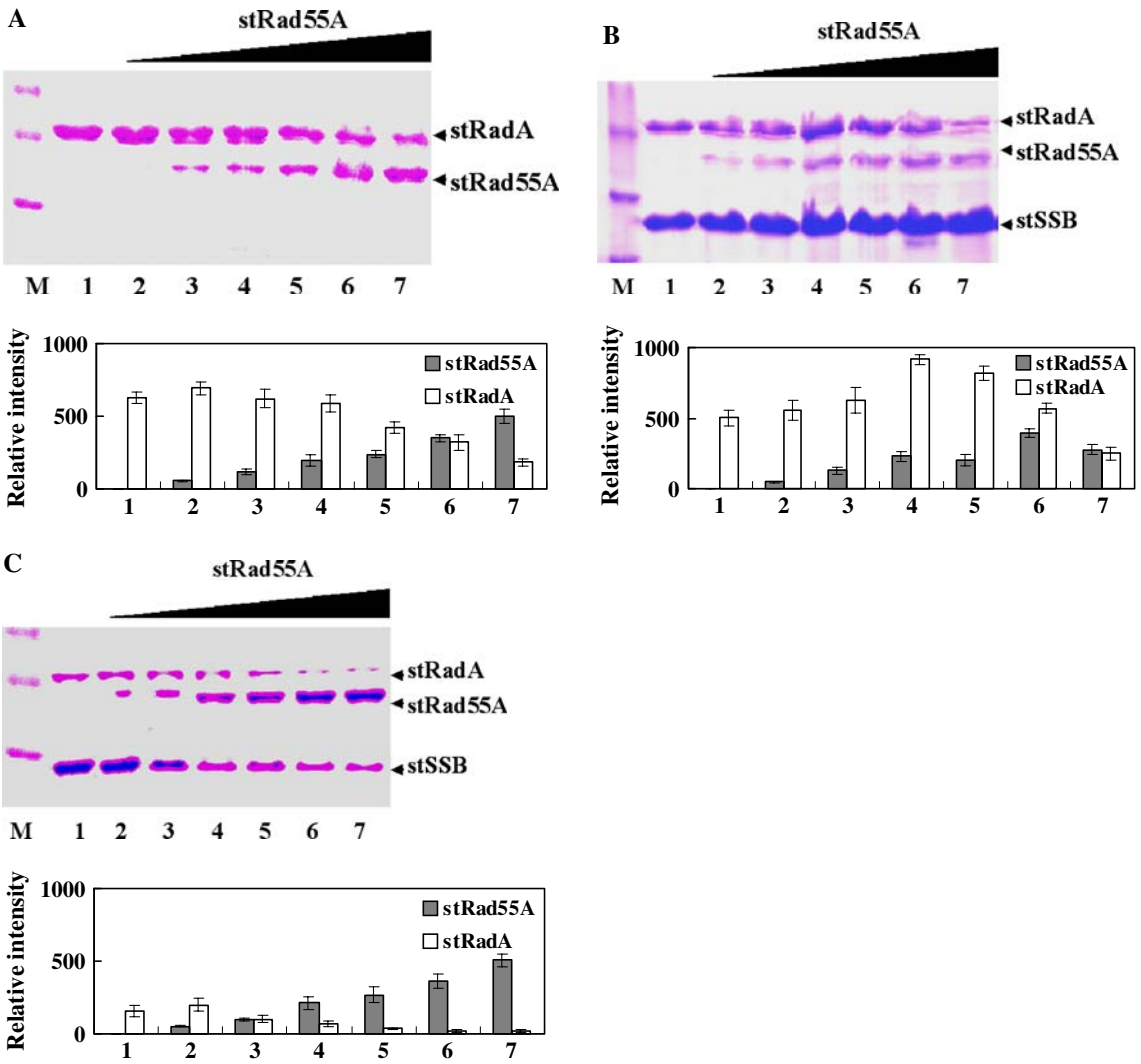


Fig. 6 Quantification of protein complexes binding to ssDNA-Sepharose by SDS-PAGE. **a** The effect of purified stRad55A on the formation of stRadA–ssDNA without stSSB. **b** StRad55A facilitated stRadA binding to stSSB pre-saturated ssDNA. **c** StRad55A enhanced the inhibition of stSSB on stRadA binding to ssDNA when pre-incubated stRad55A with ssDNA. About 0.15 nmol stRadA and gradient stRad55A (from 0.03 to 0.18 nmol) were added to 200 ng M13 ssDNA (0.6 nM nucleotides) without stSSB or saturated with stSSB. After incubation at 60°C for 5 min, the proteins bound to ssDNA were analyzed by SDS-PAGE. *Lane 1* no stRad55A; *lanes 2–7* 0.03, 0.06, 0.09, 0.12, 0.15, and 0.18 nmol stRad55A, respectively. The relative intensities of stRadA (*empty bars*) and stRad55A (*filled bars*) bands were quantified with Gel Imager System (Bio-Rad Laboratories, Hercules, CA), and showed under the map, respectively. The *values* represent the mean values and standard deviations of three independent experiments, respectively ($P < 0.05$)

2 min, then stRad55A was added (Fig. 6a). At low stRad55A concentration, stRadA binding to M13 ssDNA showed a little increase (lane 2, Fig. 6a), which was perhaps due to the disruption of DNA secondary structure by stRad55A. As stRad55A increased, the amount of stRadA bound to ssDNA decreased due to their competition with stRad55A for ssDNA (Fig. 6a, lanes 3–7).

Next, ssDNA was pre-saturated with stSSB, and then 0.15 nmol stRadA and stRad55A in gradient were added to the solution (Fig. 6b). StRadA protein was able to displace stSSB partially and bind to ssDNA (lane 1). When stRad55A was added to the mixture, more stRadA was bound to ssDNA (lanes 2–5). However, stSSB protein was not synchronously released from ssDNA with the increase of stRadA and stRad55A. According to the previous report, Rad51 binds both single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) in an ATP- and Mg^{2+}-dependent manner, and RadA and Rad51 share a common ATP-dependent filamentous quaternary structure for DNA binding and facilitating strand exchange (Akiba et al. 2005; Lovett 1994). There is no ATP added in our reaction. So, only a part of stRadA had chance to bind to ssDNA. With

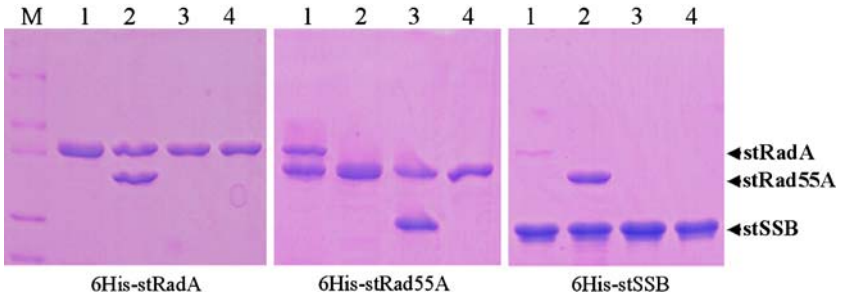


Fig. 7 Pull down assay of the interaction among stRad55A, stRadA and stSSB in vitro. His-tagged stRadA (stRad55A or stSSB) protein was first loaded onto the nickel column followed by adding non-his-tagged stRadA (*lane 1*), stRad55A (*lane 2*), stSSB (*lane 3*) or no protein as control (*lane 4*), respectively. After washing of the protein complex with 10 mM imidazole in binding buffer, the binding complex was eluted by 300 mM imidazole and analyzed by SDS-gel

the increase of stRad55A, ATP may be hydrolyzed quickly and much less stRadA could bind to ssDNA (lanes 6–7). To test this hypothesis, we performed the experiment as above with elevated ATP concention from 2.5 to 10 mM. More stRadA was bound to ssDNA and stSSB was shown to be replaced (data not shown).

Similary, we inspected the proteins binding to the ssDNA when stRad55A added to the reaction before stSSB and stRadA (Fig. 6c). With the stRad55A increasing (from 0.03 to 0.18 nmol) in the reaction, both stRadA and stSSB bound to ssDNA decreased, but stRad55A on ssDNA increased clearly.

So, we summarized that stRad55A is involved in the recombination by blocking the assembly of stRadA to ssDNA and mediating the recombinase on stSSB-coated ssDNA. Physical interaction may occur between stSSB and stRad55A in this process.

Purified stRad55A protein interacts with both stRadA protein and stSSB in vitro

To further investigate the details of stRad55A involving in HR, in vitro pull-down assay using combinations of stRad55A, stRadA, and stSSB was carried out (Fig. 7). It was clearly shown that stRadA failed to form stable complex with stSSB alone, but stRad55A formed a complex with either stSSB or stRadA, which maybe helpful in the displacement of stSSB by stRadA protein.

Discussion

In this paper, our results showed that *S. tokodaii* stRad55A had ssDNA-dependent ATPase activity, preferred binding to ssDNA, and could be induced by UV light, which was different from RadB protein in euryarchaea (Komori et al. 2000a). Most importantly, stRad55A could facilitate stRadA to bind to SSB-coated ssDNA, serving as a mediator in the stRadA-catalyzed DNA strand exchange reaction in this archaeaon.

StRad55A had similar domains and ATPase activity with Rad55 from *S. cerevisiae*, and facilitate the stRadA-mediated DNA strand exchange between stSSB coated ssDNA and dsDNA. However, the promotion of stRad55A on recombination is to release the suppression of excessive stSSB on the strand exchange (Fig. 5b) by binding both stRadA and stSSB, which exhibited some similar function pattern to Rad52. According to the mode proposed by Tomohiko, Rad52 forms a heptameric ring-like structure and this multimerization is required for its function to facilitate RPA release from ssDNA (Stasiak et al. 2000; Sugiyama and Stephen 2002). Here, the purified stRad55A protein could formed an oligomer that has a molecular mass of approximately 180 kDa (Fig. 8), perhaps being hexamer, the same as UvsY that is a functional homologue of Rad52 protein in bacteriophage T4 (Hashimoto and Yonesaki 1991; Sweezy and Morrical 1999).

However, there is also much difference between the activities of stRad55A and Rad52. RAD52 protein also plays a key role in promoting the annealing of complementary single-stranded DNA (New et al. 1998; Stasiak et al. 2000; Sugiyama and Stephen 2002). But, we had not found the protein could promote the annealing of complementary single-stranded DNA (data not shown). Besides, stRad55A protein exhibited an inhibition on stRadA's recombination without stSSB (Fig. 5a) or when stSSB was added followed stRad55A in the mixture (Fig. 5c), which are different from previous biochemical analysis on the activity of Rad52 or Rad55–Rad57 in yeast (Shinohara and Ogawa 1998). Accordingly, the stRad55A exhibited a partial functional homologue of Rad52 in the recombination. Though we could not prove the in vivo activity of stRad55A for the moment, our results suggest that *S. tokodaii* has a different combinational mediator comparing with the known protein in eukaryote and prokaryote.

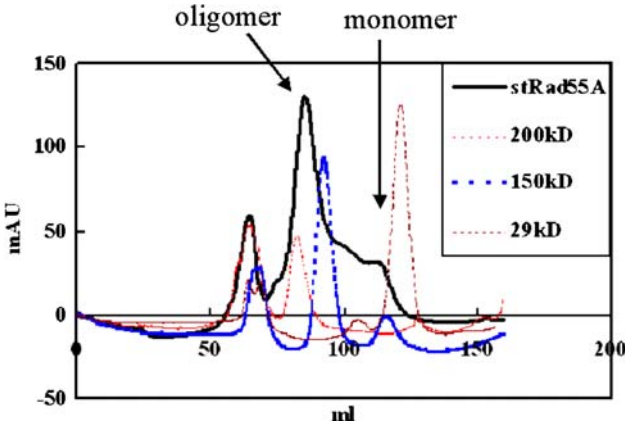


Fig. 8 Elution profiles of stRad55A proteins in gel filtration using Superdex 200 column. StRad55A purified with ammonium sulfate precipitation, heat treatment, and anion exchange chromatography was loaded onto a Superdex 200 column. To assess the molecular size of the protein, protein markers, β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), and carbonic anhydrase (29 kDa) were also loaded for analysis. The *peaks* of monomer and polymer were indicated

Besides stRad55A (ST0579) and RadA, there are three other paralogs of RadA in *S. tokodaii* (ST0838, ST1830 and ST2522). They have the conservative ATPase core domains and the different C-terminal tails. This situation is similar to that in vertebrates that have five Rad51 paralogs. The study of the stRad55A proteins in *S. tokodaii* may be helpful for deciphering Rad51 mediated-recombination mechanism in vertebrates.

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