Sulfolobus tokodaii RadA paralog, stRadC2, is involved in DNA recombination via interaction with RadA and Hjc

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Rad51/RadA paralogs found in eukaryotes and euryarchaea play important roles during recombination and repair, and mutations in one of the human Rad51 paralogs, Rad51C, are associated with breast and ovarian cancers. The hyperthermophilic crenarchaeon *Sulfolobus tokodaii* encodes four putative RadA paralogs and studies on these proteins may assist in understanding the functions of human Rad51 paralogs. Here, we report the biochemical characterization of stRadC2, a *S. tokodaii* RadA paralog. Pull-down assays revealed that the protein was able to interact with the recombinase, RadA, and the Holliday junction endonuclease, Hjc. stRadC2 inhibited the strand exchange activity of RadA and facilitated Hjc-mediated Holliday junction DNA cleavage *in vitro*. RT-PCR analysis revealed that stRadC2 transcription was immediately reduced after UV irradiation, but was restored to normal levels at the late stages of DNA repair. Our results suggest that stRadC2 may act as an anti-recombination factor in DNA recombinational repair in *S. tokodaii*.

Rad51 paralogs are ubiquitous proteins in eukarya. In *Saccharomyces cerevisiae*, Rad55 and Rad57 proteins have been identified as Rad51 paralogs because of their high sequence homology to Rad51, and they form a heterodimer that facilitates formation of the functional Rad51 complex on RPA (replication protein A, RPA)-coated single-stranded (ss) DNA [1,2]. Genetic analysis suggested that Rad55 and Rad57 might play more important roles in the recombination repair (HR) of stalled replication forks than in DSB repair [3]. In contrast to yeast, five Rad51 paralogs (Rad51B, Rad51C, Rad51D, Xrcc2, and Xrcc3) have been identified in mammals. These proteins share 20%–30% sequence identity with Rad51 and with each other, and are

thought to mediate DNA strand exchange events during HR [4–6]. The knockout mutants of each of the five Rad51 paralogs were viable in chicken B-lymphocyte DT40 cell lines, but showed spontaneous chromosomal aberrations, hypersensitivity to DNA-damaging agents, and low levels of HR [7].

It has been reported that archaeal genomes encode recombination repair proteins that are generally more similar to eukaryotic equivalents than to bacterial ones [8]. Accordingly, archaea possess recombinase RadA paralogs [9]. In euryarchaea, *Pyrococcus furious* has one RadA paralog, named RadB. The protein has a strong binding affinity for DNA and a very weak ATPase activity that is not stimulated by DNA [10]. In addition, RadB can interact with RadA directly and repress the recombination activity of RadA [11]. In *Haloferax volcanii*, deletion of *radB* results in growth

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retardation, increased sensitivity to UV light and recombination defects, suggesting a role of RadB as a recombination mediator [9]. The hyperthermophilic crenarchaea, another branch of archaea, has several RadA homologs, such as SSO2452, SSO1861 and SSO0777 in *Sulfolobus solfataricus*, and ST0579, ST0838, ST1830 and ST2522 in *S. tokodaii* [12–14]. These homologs were suggested to be RadA paralogs, because they have homology to RadA and some of them are involved in DNA repair, although their exact relationship with RadA has not been fully determined [12,13]. For convenience, in this study we will refer to these homologs as RadA paralogs. Interestingly, sequence alignment showed that some of these proteins, for instance, SSO2452 and SSO1861 in *S. solfataricus*, and ST0579 and ST1830 in *S. tokodaii* were more similar to the KaiC-like proteins than to any known RadA/Rad51 paralog found throughout archaea and eukarya. Therefore, these proteins were named RadC by Haldenby [9]. However, the real roles of RadC proteins in archaea remain unclear. Based on the latest information from whole-genome sequencing, we designate the four RadA paralogs in *S. tokodaii* as stRadC1 (ST0579), stRadC2 (ST1830), ST0838 and ST2522.

In our previous study, we demonstrated that stRadC1 and ST0838 (corresponding to the old designations of stRad55A and stRad55B, respectively) might be involved in the recombination catalyzed by RadA [12]. Different from stRadC1 and ST0838, stRadC2 did not show obvious induction following DNA damage. Here, we identified the interacting proteins of stRadC2 by pull-down assays and found that the protein can interact with both RadA and Hjc. Further investigations revealed that stRadC2 inhibited RadA-mediated recombination and facilitated the resolution of Holliday junction DNA via Hjc, the Holliday junction endonuclease. These results suggest that stRadC2 might work at the late stages of archaeal HR.

1 Materials and methods

1.1 Strains, plasmids and chemicals

The vector, pET-15b, and *Escherichia coli* stains, TG1 and BL21-CondonPlus (DE3)-RIL, were from Invitrogen (California, USA). *S. tokodaii* strain 7 was purchased from JCM (Japan Collections of Microbes, Cat. No. 10545), and was cultured at 80°C as described previously [15]. Taq DNA polymerase, restriction enzymes, DNA ligation kit, and T4 polynucleotide kinase were purchased from Takara (Dalian, China) and used according to the manufacturer's recommendations. $(\gamma$-^{32}P)ATP was obtained from GE Healthcare (Buckinghamshire, UK). Single-stranded M13mp18 DNA was bought from Sigma (Saint Louis, MI, USA). Oligonucleotides were synthesized and gel-purified by Sangon (Shanghai, China).

For UV-irradiation of cells, cell suspensions in 10 mmol

L^{-1} phosphate buffer (pH 7.0) were irradiated at room temperature with 200 J m^{-2}. Then, cells were re-suspended in fresh medium and incubated at 80°C with shaking. After incubation, cells were harvested by centrifugation, washed twice with 10 mmol L^{-1} phosphate buffer, and stored at –80°C before sample preparation.

S. tokodaii proteins, RadA (recombinase, ST0297), Hjc (Holliday junction endonuclease, ST1444), SSB (ssDNA binding protein, ST0503), pyruvate kinase (ST1617), and other DNA repair proteins used in this paper were the corresponding homologues of *S. solfataricus* and came from our laboratory stocks.

1.2 DNA manipulations

Plasmid and chromosomal DNAs were isolated from *E. coli* using the alkaline lysis method and from S*ulfolobus* using the lysozyme lysis method [16]. The gene encoding *S. tokodaii* stRadC2 (ST1830) was amplified by PCR using the up-stream primer 5′-GG<u>CATATG</u>GAGGGGTATACTGT-GATT-3′, and the down-stream primer 5′-TA<u>GGATCC</u>A-TAGCTACGCCCTCGATAAG-3′ (*Nde* I and *Bam*H I sites are underlined). The amplified fragment was digested with *Nde* I and *Bam*H I and then ligated into the expression vector pET15b to produce the recombinant vector pET15b/stRadC2.

RNA from UV- or mock-treated cells was prepared using an extraction kit from Qiagen (Hilden, Germany) according to the procedure described in the manual. The RNA was quantified spectrophotometrically and then reverse-transcribed with random primers using a First-Strand cDNA Synthesis Kit (Shennergy Biocolor Bioscience & Technology Company, China). The subsequent PCR was carried out with the above stRadC2 primers, according to standard protocols [16].

1.3 Biochemical assays

1.3.1 DNA substrates

The 84 mer ssDNA, 84 bp double stranded (ds) DNA, and four 72 mer oligonucleotides, which could form Holliday junction DNA by annealing, were consistent with the DNA substrates used in our previous publication [17]. The DNA substrates used here were γ-^{32}P-labeled at their 5′ ends as described previously [18], and stored in TE buffer (10 mmol L^{-1} Tris-HCl, pH 7.0, and 0.5 mmol L^{-1} EDTA).

1.3.2 Pull down assays

S. tokodaii cells were suspended in protein binding buffer (20 mmol L^{-1} Tris-HCl pH 7.0, 2% glycerol, 1 mmol L^{-1} EDTA, 100 mmol L^{-1} NaCl, 1 mmol L^{-1} DTT, and 0.6 mmol L^{-1} PMSF) and disrupted on ice with an ultrasonicator at 600 W output for a total of 2 min and the debris was removed by centrifugation (16000×*g*, 20 min). The total

proteins were treated with DNase, and then subjected to ammonium sulfate precipitation at 80% saturation to eliminate DNA fragments. The precipitate was collected by centrifugation at 25000×*g* for 20 min. The resulting pellet was dissolved in binding buffer, and passed through a Ni-column. His-tagged stRadC2 was added to the flow-through fractions and incubated at 70°C for 20 min. Proteins in association with stRadC2 would bind the nickel column, and were eluted with 0.8–1 mol L^{-1} NaCl after washing the column with binding buffer. Eluted proteins were then separated on polyacrylamide gels and identified using MALDI-TOF mass spectrometry.

His-tagged stRadC2 was also used to analyze its interaction with purified recombination proteins based on a previously described protocol [16]. First, His-tagged protein was incubated with non His-tagged protein in binding buffer at 70°C for 20 min, then the reaction mixture was loaded onto a Ni-NTA sepharose column (Qiagen). After washing with three column volumes of washing buffer (20 mmol L^{-1} Tris-HCl pH 7.0, 2% glycerol, 200 mmol L^{-1} NaCl, 1 mmol L^{-1} DTT, 40 mmol L^{-1} imidazole, and 0.6 mmol L^{-1} PMSF), the proteins were eluted with 200 mmol L^{-1} imidazole and assayed by SDS-PAGE.

1.3.3 Three-strand-exchange assay

Reaction mixture (20 μL) containing SEB reaction buffer (20 mmol L^{-1} Tris–HCl, pH 7.0, 5 mmol L^{-1} MgCl$_2$, 2% glycerol, 1 mmol L^{-1} ATP, and ATP renew system that included 5 mmol L^{-1} phosphoenolpyruvate and 1 μg pyruvate kinase), 15 μmol L^{-1} circular single-stranded M13 virion DNA, and RadA protein (5 μmol L^{-1}) was first incubated at 65°C for 5 min. Then linear M13 dsDNA was added at a concentration of 15 μmol L^{-1}. After a second incubation at 65°C for 30 min, SDS (a final concentration of 1%) and proteinase K (a final concentration of 1 μg μL^{-1}, Takara) were added to the reaction solution. A further incubation at 37°C for 40 min was required to fully degrade the protein in the reaction. Samples were 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 3 V cm^{-1}.

1.3.4 Holliday junction DNA cleavage by Hjc

Assays were carried out in reaction buffer (20 mmol L^{-1} Tris-HCl, pH 7.5, 0.5 mmol L^{-1} DTT, 5 mmol L^{-1} MgCl$_2$, and 50 mmol L^{-1} NaCl) containing 10 nmol L^{-1} γ-^{32}P labeled four-way DNA junction with initial equilibrium at 60°C for 5 min. The reactions were initiated by the addition of 5 nmol L^{-1} Hjc in a final reaction volume of 20 μL. After the indicated times, reactions were stopped by the addition of EDTA and SDS to final concentrations of 50 mmol L^{-1} and 1%, respectively. Samples were then analyzed by denaturing gel electrophoresis and phosphor imaging as described [16].

2 Results

2.1 Analysis of the amino acid sequence and radiation inductivity of stRadC2

The protein stRadC2 (ST1830) is composed of 283 amino acids. Domain alignment of stRadC2 with the homologous proteins, RadA and stRadC1 from *S. tokodaii*, Rad55 and Rad51 from *S. cerevisiae*, and RadB from *Thermococcus kodakarensis*, is shown in Figure 1A. Compared with RadA/Rad51, stRadC2 possesses the conserved ATPase core domain but lacks the N-terminal domain of RadA/Rad51, similarly to Rad55, RadB and stRadC1. In particular, the amino acid residue numbers of RadB and stRadC1 are similar to that of stRadC2. Alignment of protein sequences of RadB, stRadC1, and stRadC2 is shown in Figure 1B, and their secondary-structure elements were marked above the sequences. stRadC2 possesses low amino acid identities with stRadC1 (about 34%) and RadB (about 18%). Though their secondary structures are generally similar, the C-terminus of stRadC2 has two helixes, both of which are absent in RadB and one of which is missing in stRadC1. Based on the alignment, we think that stRadC2 is probably a paralog of RadA and may have particular function in *S. tokodaii*.

To investigate the role of stRadC2 in *S. tokodaii*, an RT-PCR assay of stRadC2 induction was performed after UV irradiation (Figure 1C). For 2–8 h after UV irradiation, fragmented genomic DNA was observed. Post-irradiation, RadA was induced, and pyruvate kinase (ST1617, an important enzyme in glycolysis) was apparently inhibited. Notably, the transcription of stRadC2 was inhibited between 2–6 h post-irradiation, which is different from that of stRadC1 and ST0838 [12]. However, the expression of stRadC2 was restored 8 hr post-irradiation when RadA was induced and pyruvate kinase was inhibited.

2.2 Pull-down analysis of stRadC2-interacting proteins from the total protein extract of *S. tokodaii*

To analyze the function of stRadC2, the protein was expressed in *E. coli* strain BL21 (DE3) and isolated by heat treatment and Ni^{2+}-NTA affinity column purification. His-tagged stRadC2 was used to isolate interacting proteins from the total protein extract of *S. tokodaii*. Seven proteins that bound to the Ni-NTA agarose beads through interaction with His-tagged stRadC2 were isolated and identified as CarB (carbamoyl phosphate synthase large subunit, ST1504), FBP (fructose-bisphosphatase, ST0318), hypothetical protein (ST1922), EF-2 (elongation factor 2, ST0437), RadA (ST0297), Hjc (Holliday junction endonuclease, ST1444), and a fragment of stRadC2 (Figure 2A). CarB is an important enzyme in the biosynthesis of arginine and the pyrimidine nucleotides, FBP is a key enzyme in gluconeogenesis, and EF-2 mediates translocation in protein synthesis
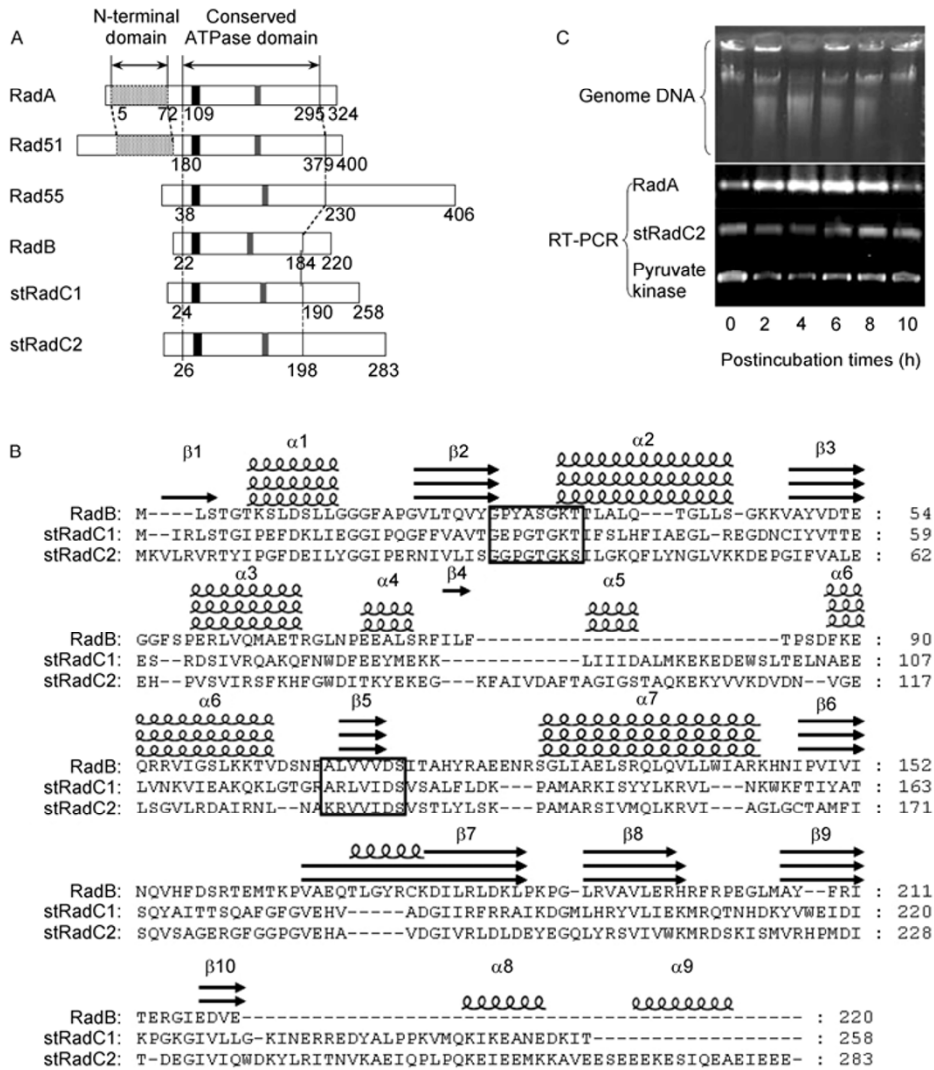
Figure 1 Domain and sequence analysis of stRadC2 from *S. tokodaii*. A, The domain structures of stRadC1, stRadC2 and RadA from *S. tokodaii*, Rad55 and Rad51 from *S. cerevisiae*, and RadB from *T. kodakarensis*. The rectangles indicate Walker A and B motifs, respectively. B, Sequence alignment of stRadC1, stRadC2 and RadB. Secondary structure elements of the three proteins are shown above the alignment. Spiral lines represent α-helical segments and horizontal arrows represent β-sheets. C, RT-PCR assay of stRadC2 transcription after UV radiation. The irradiated cells were incubated after irradiation at 80°C for different times (0, 2, 4, 6, 8, 10 h), and total RNAs were then extracted for RT-PCR assay. The transcription of RadA and pyruvate kinase was assayed as controls. The genomic DNA from irradiated cells was also isolated, digested with *Hin*d III and *Eco*R I, and separated by agarose gel electrophoresis.
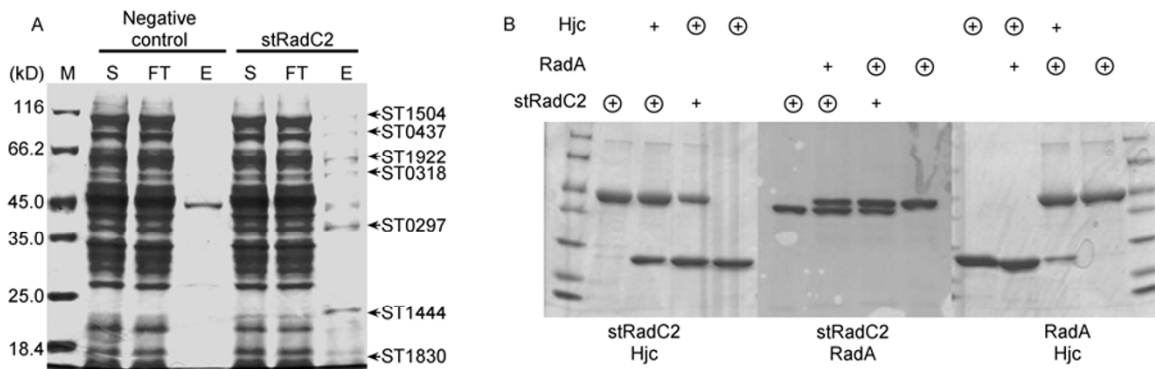
Figure 2 Interaction assays among stRadC2, RadA and Hjc proteins. A, Pull-down of stRadC2 interacting proteins from the total extract of *S. tokodaii*. Lane S, total protein from *S. tokodaii*; lane FT, proteins in flow through; lane E, proteins eluted with NaCl. Ni-NTA agarose was used as a control to exclude resin-binding proteins. B, Direct binding of purified stRadC2 protein with RadA and Hjc. His-tagged (circled+symbol) or non His-tagged (+symbol) proteins were used to investigate reciprocal interactions.

and facilitates the events of translational elongation. All three of these proteins are needed for normal cellular metabolism [19]. RadA and Hjc are DNA damage repair proteins, which are involved in DNA recombination and Holliday-junction cleavage, respectively. The result that stRadC2 could pull down these proteins suggests that stRadC2 might be involved in both metabolism and DNA repair. These proteins may also bind the column non-specifically. Surprisingly, only a fragment but not the full length stRadC2 was found in the pull-down sample, a phenomenon that needs further investigation. Here, our focus was primarily on the known repair proteins, RadA and Hjc, to study the role of stRadC2 in archaeal DNA repair.

The interaction among purified stRadC2, RadA, and Hjc was examined *in vitro*. As shown in Figure 2B, stRadC2 could bind with RadA and Hjc, and only a minor interaction was found between RadA and Hjc. This result suggests that the three proteins may interact and form a complex, although other methods such as gel filtration and yeast two hybrid are needed to verify the protein interactions and formation of the complex.

2.3 StRadC2 is a negative regulator of RadA recombination

To further understand the interaction between stRadC2 and RadA (or Hjc), biochemical assays of stRadC2 on the activities of RadA and Hjc were carried out. As shown in Figure 3A, RadA protein could enhance the homologous strand exchange reaction (lane 2), while stRadC2 protein could not (lane 1). With the addition of a gradient of stRadC2 protein (from 1 to 7 μmol L^{-1}) to the strand exchange reaction, the amount of reaction product clearly decreased (lanes 3–6).

Thus, purified stRadC2 could inhibit the recombination activity of RadA.

To understand the inhibition mechanism, the effect of the order in which proteins were added on the DNA strand exchange activity was analyzed. When RadA was incubated with DNA before stRadC2, the level of recombination product was not significantly reduced (lane 3 in Figure 3B). However, when stRadC2 was incubated with DNA before RadA, the level of product was clearly reduced (lane 4 in Figure 3B), suggesting that stRadC2 blocked the binding of RadA to ssDNA. When the two proteins were mixed with each other before incubation with DNA, the recombination was inhibited dramatically (lane 5 in Figure 3B). Thus, the interaction of stRadC2 and RadA repressed their binding to ssDNA.

To further confirm that the stRadC2 inhibition of RadA recombination is related to their binding to DNA, gel shift assays with γ-^{32}P labelled ssDNA were performed to test the interactions between RadA and stRadC2 (Figure 3C). When RadA was pre-incubated with ssDNA before the addition of stRadC2, little free ssDNA was released (lane 3). When tRadC2 was added first or the two proteins were mixed before adding to ssDNA, a greater amount of free ssDNA was released (lanes 4 and 5). Thus, the stRadC2 inhibition of RadA recombination activity is likely to be mainly caused by interference of the binding of RadA to ssDNA.

2.4 stRadC2 could promote the Holliday junction cleavage activity of Hjc

S. tokodaii Hjc is a homologue of *S. solfataricus* and *P. furiosus* Hjc proteins that have Holliday junction DNA cleavage activity [18,20]. Here, a standard *in vitro* cleavage
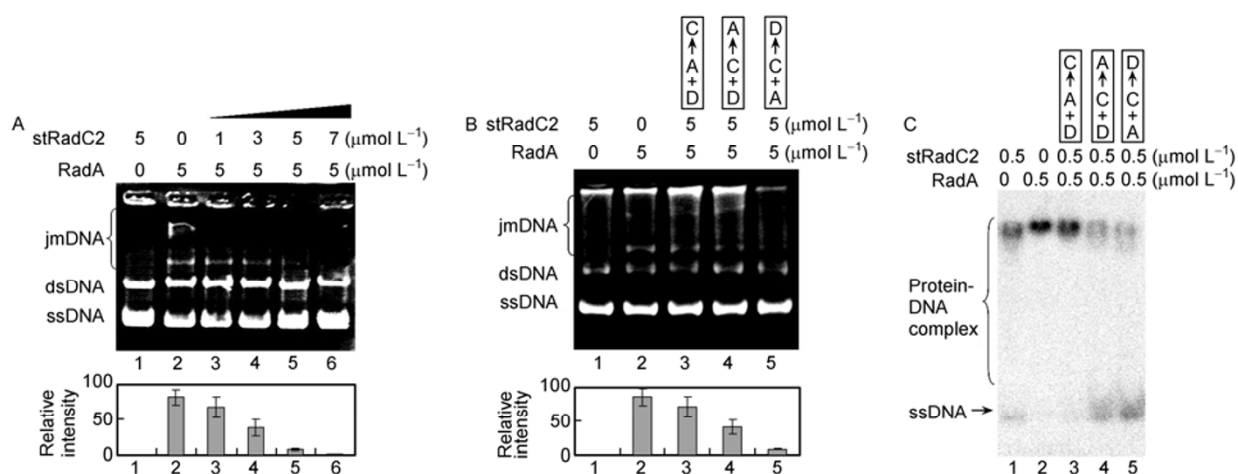
Figure 3 The effect of stRadC2 on the DNA strand exchange activity of RadA. The reaction was carried out as described in Materials and methods, and the product (molecular junction DNA), single strand DNA, and double strand DNA are denoted as jmDNA, ssDNA and dsDNA, respectively. A, A concentration gradient of stRadC2 inhibited the DNA strand exchange catalyzed by RadA. Lane 1, stRadC2; lane 2, RadA; lanes 3–6, RadA and the stRadC2 gradient (from 1 to 7 μmol L^{-1}). B, The effect of the order in which stRadC2 was added to the strand exchange reaction. C, Gel shift assay of the binding of the two proteins to γ-^{32}P labeled ssDNA. Lane 1, stRadC2 protein alone; lane 2, RadA protein alone; l lane 3, RadA pre-incubated with ssDNA; lane 4, stRadC2 pre-incubated with ssDNA; lane 5, the two proteins were pre-incubated before adding DNA. The adding order of stRadC2 (C), RadA (A) and ssDNA (D) is also marked above the figure. The relative intensities of the product bands were measured using a Gel Imager System, and the values are the averages ($\pm$SD) from three independent experiments.

assay was used with a synthetic four-way junction as a substrate, and the cleavage efficiency of Hjc was examined in the presence of a concentration gradient of stRadC2 (Figure 4A). The results showed that stRadC2 promoted the junction DNA cleavage activity of Hjc. As the concentration of stRadC2 increased, so did the cleavage rate. At 8 nmol L^{-1} stRadC2, about 50% of Holliday junction DNA was resolved.

Because stRadC2 could bind to DNA, it is necessary to examine whether stRadC2 facilitated Hjc activity by way of its DNA binding ability. The effect of the order in which proteins were added on the cleavage reaction was analyzed with 5 nmol L^{-1} Hjc and 8 nmol L^{-1} stRadC2. The results showed that the cleavage rates did not show any significant changes with respect to the order in which proteins were added (Figure 4B). The effect of stRadC2 on Hjc binding to Holliday junction DNA labelled with γ-^{32}P was then investigated, and the result showed that the release of junction DNA increased as the concentration of stRadC2 increased (Figure 4C). The activation of stRadC2 on Hjc did not result from its facilitation of binding Hjc to DNA, and the real

mechanism needs further study.

3 Discussion

This study is part of an effort to understand the functions of RadA and its paralogs in the hyperthermophilic archaeon, *S. tokodaii*. Similar to RecA in bacteria, Rad51 in eukaryotes, and RadA in halophilic or methanogenic archaea, *Sulfolobus* RadA is also a DNA damage-inducible recombination repair enzyme [21]. To face the challenges from their hyperthermic environment, *Sulfolobus* express RadA at high basal levels, which is beneficial to the preservation of their genetic integrity. However, like a two-edged sword, excessive recombinase will likely result in inappropriate chromosomal crossing over and endanger genomic stability [22]. In bacteria, RecX and DinI were found as anti-recombinases to limit excessive recombination activity by binding to RecA protein directly [23,24]. In eukaryotic cells, several proteins, including Rad51 paralogs that interact with the human Rad51 protein, inhibit strand exchange and replication fork
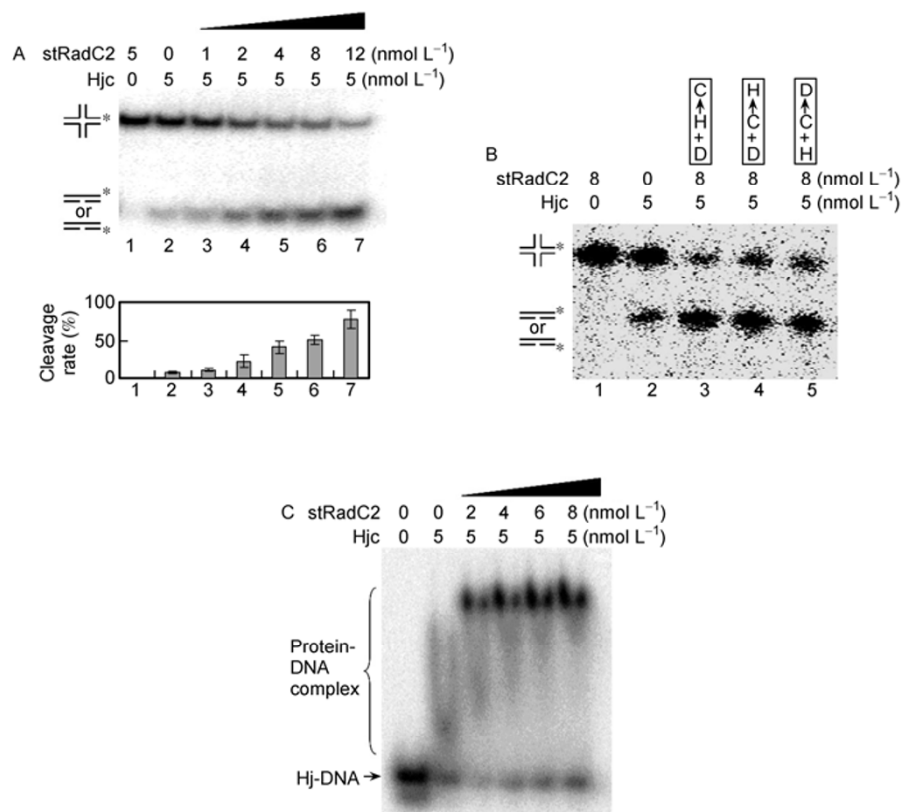
Figure 4   Effect of stRadC2 on junction DNA cleavage and binding activity of Hjc. Holliday junction DNA cleavage was carried out in reaction buffer (20 mmol L^{-1} Tris-HCl, pH 7.5, 0.5 mmol L^{-1} DTT, 5 mmol L^{-1} MgCl$_2$, and 50 mmol L^{-1} NaCl) with 10 nmol L^{-1} γ-^{32}P labeled four-way junction DNA. The binding assay was carried out in the Holliday junction DNA cleavage buffer without MgCl$_2$. A, A gradient of stRadC2 facilitates the cleavage activity of Hjc. B, The effect of the order in which protein was added on junction DNA cleavage by Hjc. Lane 1, stRadC2 alone; lane 2, Hjc alone; lane 3, Hjc pre-incubated with DNA; lane 4, stRadC2 pre-incubated with DNA; lane 5, the two proteins added at the same time. C, Gel shift assay of the effect of a concentration gradient of stRadC2 on Hjc protein binding to junction DNA. The bands were scanned and the cleavage rate (product DNA/total DNA) was calculated. Values are the average (±SD) from six independent experiments.

regression promoted by human Rad51 [25]. Euryarchaeal RadB and crenarchaeal stRadC2 are known to inhibit RadA recombination activity, which has enriched our knowledge on crenarchaeal DNA recombination. In addition, stRadC2 interacts with Hjc and facilitates the resolution of Holliday DNA junctions, which is more similar to its eukaryotic equivalents [3–6] than to its euryarchaeal ones [9,10].

At the late stages of DNA recombination, the cell ends DNA repair and starts normal metabolism. Checkpoint proteins are involved in the switch between DNA damage response and normal metabolism [26]. In Figure 2A, stRadC2 could pull down both DNA repair proteins and normal metabolism-related proteins, suggesting that stRadC2 might be involved in both metabolism and DNA repair. stRadC2 is not induced by DNA damage, negatively regulates DNA recombination, and is up-regulated 8 hr post-irradiation, which indicates that stRadC2 might function at the late stages of DNA repair.

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