

Role of ATP-binding motifs on DNA-binding activity and biological function of Rhp51, a Rad51 homologue in fission yeast

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Rhp51, a RecA and Rad51 homologue of *Schizosaccharomyces pombe*, plays a pivotal role in homologous recombination and recombinational repair. It has a set of the well-conserved type A and type B ATP-binding motifs, which are highly conserved in all *RecA* homologues. In a previous study [Kim, Lee, Park, Park and Park (2001) *Nucleic Acids Res.* **29**, 1724–1732], we reported that a single mutation of the conserved lysine in A motif [Lys¹⁵⁵ → Ala (K155A)] destroyed the DNA repair ability of Rhp51 and that overexpression of this mutant protein conferred dominant negativity. In the present paper, we investigated DNA-binding properties of recombinant Rhp51 and its mutant proteins. Purified Rhp51 protein showed ATP-dependent double- and single-strand DNA-binding activities. To characterize the role of ATP-binding motifs, we generated Rhp51 K155A and Rhp51

Asp²⁴⁴ → Gln (D244Q), which have a single amino acid substitution in A and B motifs respectively. Interestingly, K155A and D244Q mutations impaired ATP-dependent DNA binding in a different manner. K155A lost the DNA binding itself, whereas D244Q maintained the binding ability but lost the ATP dependency. However, despite the difference in DNA-binding ability, both mutations failed to rescue the methylmethane sulphonate and UV sensitivity of the *rhp51Δ* mutant. Together, these results suggested that not only the DNA binding but also the ATP dependence in DNA binding is required for proper *in vivo* functioning of Rhp51.

Key words: DNA double-strand break repair, homologous recombination, RecA.

INTRODUCTION

Rad51 is a ubiquitous protein that is found in all eukaryotes and plays a key role in homologous recombination and recombinational repair. Rad51 is the structural and functional homologue of *Escherichia coli* RecA in eukaryotes. RecA and Rad51 share very similar overall structure, including a set of highly conserved type A and type B ATP-binding motifs [1] and have similar enzymic properties. Purified *Saccharomyces cerevisiae* and human Rad51 (ScRad51 and HsRad51 respectively) promote homologous pairing and strand-exchange reactions *in vitro*, which is a key step in homologous recombination [2,3]. ScRad51 forms nucleoprotein filaments on single-stranded (ss) DNA in a manner similar to RecA, which may invade homologous duplex DNA [4,5]. However, the strand-exchange activity of Rad51 is much lower than that of RecA, and Rad51 requires additional co-activators such as Rad52, Rad54 and Rad55–57 heterodimers to achieve the maximum level of strand-exchange activity [6–12].

The conserved ATP-binding motifs of Rad51 are crucial for the functioning of Rad51. Purified Sc- and Hs-Rad51 have ATP-hydrolysis activity [2,5]. Mutation in the A motif [ScRad51 Lys¹⁹¹ → Ala (K191A)] completely abolished its ATP-hydrolysis activity and also eliminated its strand-exchange activity and its *in vivo* biological activity to rescue methylmethane sulphonate (MMS) sensitivity of *rad51* deletion mutant [13,14]. Furthermore, overexpression of ScRad51 K191A in wild-type cells conferred dominant-negative effect on MMS [15]. Biochemical studies in the ATP-binding motifs of ScRad51 protein revealed that ATP binding, but not ATP hydrolysis, is important for the DNA-binding and strand-exchange activity [14]. In the presence of the non-hydrolysable ATP analogue adenosine 5'-[γ-thio]-

triphosphate, both Sc- and HsRad51 performed partial strand exchange [3,14] and ScRad51 bound both ss and double-stranded (ds) DNA, although it required elevated levels of proteins [16,17]. In addition, two analogous mutants, ScRad51 K191R and HsRad51 K133R, which bound but did not hydrolyse ATP, not only performed strand-exchange reaction *in vitro* but are also active biologically *in vivo* [14,18]. All these observations imply that ATP binding is sufficient for the proper functioning of Rad51. However, the reason as to why Rad51 hydrolyses ATP is unclear.

In a recent study [19], we reported that Rhp51 (Rad51 homologue in *Schizosaccharomyces pombe*) K155A mutation, which is equivalent to ScRad51 K191A, caused severe functional defects: it failed to rescue MMS and UV-light sensitivity of *rhp51* deletion mutant and conferred dominant negativity to wild-type cells when overexpressed. These dominant-negative mutants showed similar morphologies and phenotype with *rhp51* deletion mutants, both in the presence and absence of DNA damage [19]. In the present study, we describe the DNA-binding properties of Rhp51 and its ATP-binding mutants. Since most mutational studies on the ATP-binding motifs of Rad51 were performed in only the A motif, we generated mutation in the conserved residue in the B motif and investigated its cellular and DNA-binding properties.

EXPERIMENTAL

Strains and media

Wild-type *S. pombe* haploid strain JY334 (*h⁺ ade6-M216 leu1-32*) and the *rhp51* deletion mutant (*rhp51Δ*) JAC1/51D (*h⁻ ade6-704*)

Abbreviations used: D244Q, Asp²⁴⁴ → Gln; ds, double-stranded; K155A, Lys¹⁵⁵ → Ala; MMS, methylmethane sulphonate; ss, single-stranded.

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leu1-32 rhp51::ura4⁺) were used as host for complementation assay. *S. pombe* cells were grown and maintained in standard rich medium (YES) or in minimal medium (EMM) supplemented with appropriate nutrients as described by Alfa et al. [20].

Plasmids and site-directed mutagenesis

Site-directed mutagenesis procedure for the generation of *rhp51* Asp²⁴⁴ → Gln (D244Q) mutation and the cloning strategy in Splac551 plasmid are equivalent to those of *rhp51* K155A, as described by Kim et al. [19], except that the primer 5'-TTAG-TTGTCCAAGTTGTACT-3' was used for mutagenesis. For generation of the pET51 plasmid, the open reading frame of the *rhp51*⁺ gene was amplified by PCR using primers 5'-CAAGTTACCATGGCAGATACAGAGGTGG-3' (the first Met codon and a new *Nco*I site are in boldface and underlined respectively) and 5'-CACTAACTCGAGTTAGACAGGTGC-GATAATTTC-3' (the stop codon and a new *Xho*I site are in boldface and underlined respectively) and placed in *Nco*I-*Xho*I sites of pET28b (Novagen, Madison, WI, U.S.A.). Mutant *rhp51* genes were cloned in the same way.

Survival test

Each transformant was grown until the mid-exponential phase ($A_{595} = 0.5-1$) in EMM medium and appropriate dilutions containing 500–1000 cells were plated on to solid media in the presence of MMS or irradiated by UV after spread. The cells were incubated for 4–5 days and the resulting number of colonies was counted. Experiments were performed at least three times and averaged.

Purification of proteins

E. coli BL21(DE3) harbouring pET51 plasmid was induced by 0.1 mM isopropyl β -D-thiogalactoside at 37 °C for 3 h, harvested, resuspended in lysis buffer (50 mM Tris/HCl, pH 8.0, containing 1 mM EDTA/100 mM NaCl/0.5 mM dithiothreitol) and lysed by sonication. The resulting cell lysates were precipitated by 40% saturation of ammonium sulphate, resuspended in potassium buffer [21] and purified by a series of column chromatography using Q-Sepharose, heparin, hydroxyapatite and Mono-Q. The final Mono-Q fraction was dialysed against potassium buffer containing 0.2 M KCl and stored at -70 °C. Rhp51 K155A and Rhp51 D244Q were also produced using the same method. The protein concentration was determined by measuring the absorption at 280 nm using a molar absorption coefficient of 15720 M⁻¹ · cm⁻¹.

DNA-binding assay

*Eco*RI-digested pBluescript SK(+) (Stratagene, La Jolla, CA, U.S.A.) or ϕ X174 viral DNA (NEB, Beverly, MA, U.S.A.) was incubated with Rhp51 protein in 10 μ l reaction mixture containing 20 mM Hepes-KOH (pH 7.9), 1 mM dithiothreitol, 3 mM MgCl₂ and 3 mM ATP for 15 min at 37 °C. The protein-DNA complexes were resolved in 0.7% agarose gel electrophoresis and visualized by ethidium bromide staining. For cross-linking protein-DNA complexes, the reactions were treated with 0.2% (v/v) glutaraldehyde and incubated further for 15 min at 37 °C before gel electrophoresis. The concentration of DNA was expressed as that of the nucleotide.

RESULTS

Rhp51 is an ATP-dependent DNA-binding protein

Rad51 from budding yeast and human has ATP-hydrolysis activity, DNA-binding activity and homologous-pairing/strand-exchange activity *in vitro* [2,3]. To characterize enzymic activities of Rhp51 protein, we expressed and purified Rhp51 from *E. coli* to near homogeneity by a series of column chromatography experiments (Figure 1). The purified protein, however, displayed neither ATP-hydrolysis activity nor homologous-pairing/strand-exchange activities (results not shown).

On the other hand, ssDNA- and dsDNA-binding activities of Rhp51 were observed by gel-mobility-shift assay. With increasing amounts of protein, ssDNA-protein complexes displayed progressively decreased mobility in the presence of ATP (Figure 2A, left panel, lanes 2–6), implying the formation of a Rad51-like nucleoprotein filament [4]. Such an ssDNA binding of Rhp51 was observed more evidently after glutaraldehyde treatment, which cross-linked DNA-protein complex formation (Figure

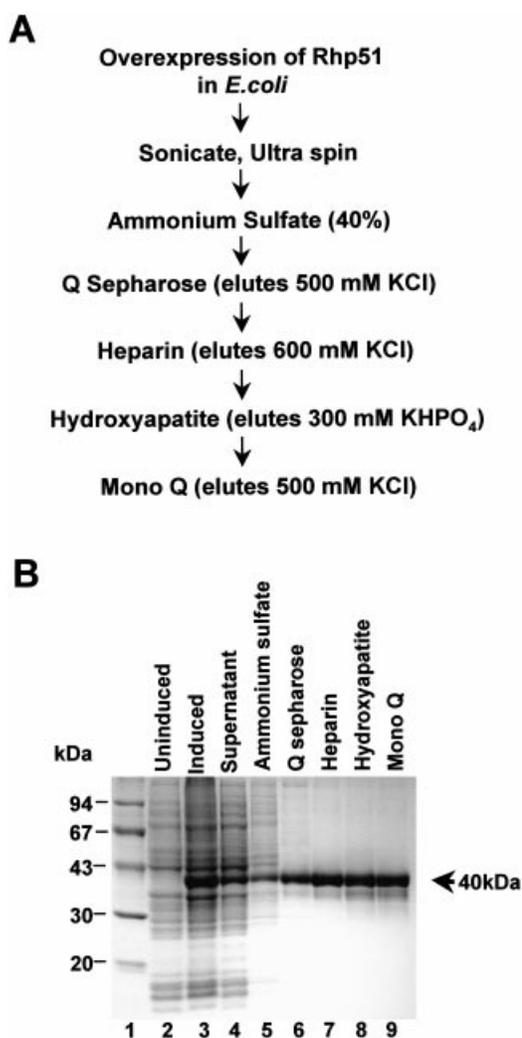


Figure 1 Purification of Rhp51 protein

(A) Purification scheme. (B) Each fraction of column chromatography was analysed by SDS/PAGE (12% gel) and visualized by staining with Coomassie Blue. Arrow indicates the purified protein.

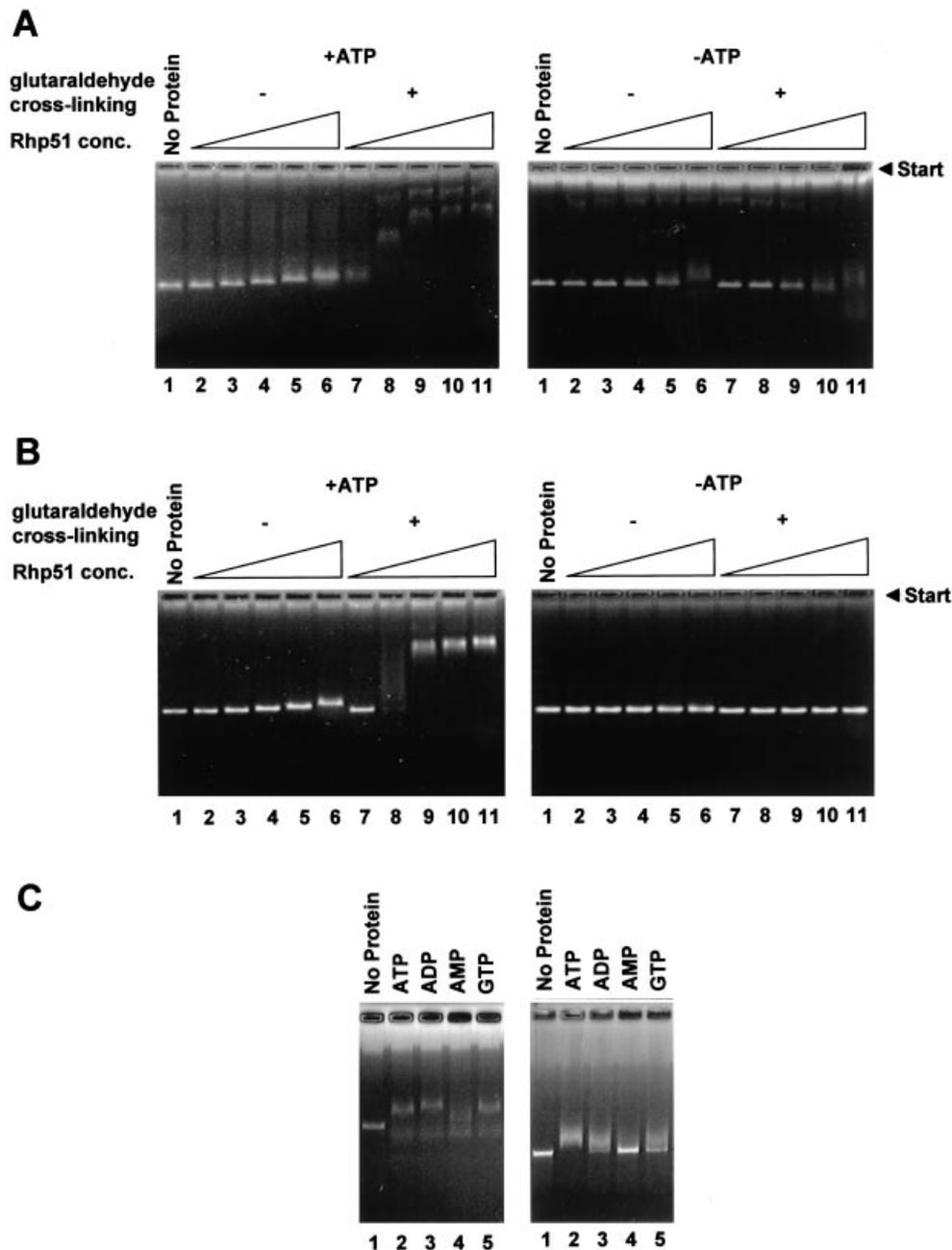


Figure 2 ATP-dependent ssDNA and dsDNA binding of Rhp51

Increased concentrations of Rhp51 (1.8, 3.6, 7.3, 14.6 and 29.2 μM) were incubated with 15.4 μM of ϕX174 viral DNA (**A**) or 15.7 μM *Eco*RI-linearized pBluescript SK(+) (**B**) for 15 min in the presence (left panel) or absence (right panel) of ATP. Protein–DNA complexes, either cross-linked by glutaraldehyde (lanes 2–6 of each gel) or not (lanes 7–11 of each gel), were analysed by 0.7% agarose gel electrophoresis and visualized by staining with ethidium bromide. (**C**) Nucleotide cofactor requirements for DNA binding of Rhp51 were analysed with various nucleotide cofactors. Each reaction contains 13.8 μM protein with 9.2 μM *Eco*RI-linearized pBluescript SK(+) (left panel) or 9.4 μM ϕX174 viral DNA (right panel).

2A, left panel, lanes 7–11). Omission of ATP, however, decreased the mobility shift of ssDNA (Figure 2A, right panel). Rhp51 also bound to linear duplex DNA in the presence of ATP (Figure 2B, left panel). Rhp51 bound to the dsDNA in a more ATP-dependent manner, since omission of ATP caused almost no dsDNA binding of Rhp51 (Figure 2B, right panel). Glutaraldehyde fixation also revealed that for a nucleotide/protein ratio of approx. 2, both ssDNA (Figure 2A, left panel, lane 9) and

dsDNA (Figure 2B, left panel, lane 9) binding of Rhp51 was saturated, suggesting that Rhp51 bound to every two nucleotides. We examined whether other nucleotides can replace ATP. Substitution of ATP by ADP, AMP or GTP showed that Rhp51 utilized not only ATP but also ADP and GTP for dsDNA binding, whereas Rhp51 bound to ssDNA weakly in the presence of AMP (Figure 2C). These results indicated that ATP is required for the binding of Rhp51 to both ssDNA and dsDNA.

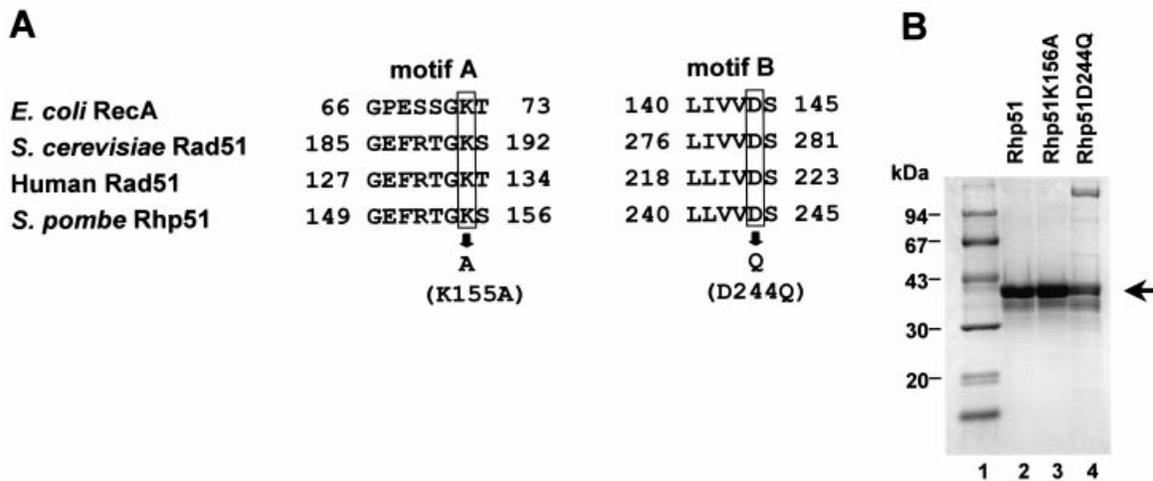


Figure 3 Purification of Rhp51 K155A and D244Q

(A) Comparison of amino acid sequences of conserved ATP-binding motif A and B from *E. coli* RecA, *S. cerevisiae* Rad51, human Rad51 and *S. pombe* Rhp51. (B) Rhp51 K155A and Rhp51 D244Q were purified with the same purification procedure with Rhp51 and 10 μ l of final fractions of each proteins were analysed by SDS/PAGE (12% gel).

Mutation in ATP-binding motifs of Rhp51 alters DNA-binding properties

To characterize the effect of conserved ATP-binding motifs on the DNA-binding activity of Rhp51, we introduced single-point mutation at each of the A (K155A) or B motifs (D244Q) and purified the resulting proteins (Figure 3). The ssDNA- and dsDNA-binding activities of Rhp51 K155A and Rhp51 D244Q were compared with those of Rhp51 in the presence of ATP and Mg^{2+} . The Rhp51 K155A lacked dsDNA binding completely but showed reduced ssDNA binding (Figures 4A and 4B, lanes 5–7 respectively), whereas Rhp51 D244Q showed slightly enhanced DNA-binding activity (Figures 4A and 4B, lanes 8–10). In the absence of ATP or Mg^{2+} , Rhp51 K155A did not bind to either ssDNA or dsDNA, suggesting that Rhp51 K155A is deficient in DNA-binding activity, regardless of the presence of ATP or Mg^{2+} (Figures 4C and 4D, lanes 5–7). However, unlike the wild-type protein, both ssDNA- and dsDNA-binding activities of Rhp51 D244Q were not affected by the presence or absence of ATP or Mg^{2+} (Figures 4C and 4D, lanes 8–10), indicating that Rhp51 D244Q lost the ATP and Mg^{2+} dependence for DNA binding but maintained the DNA-binding ability itself. Taken together, the K155A and D244Q mutations changed the ATP-dependent ssDNA- and dsDNA-binding activities of Rhp51 differently.

Cellular effects of mutation in B motif of Rhp51

In a previous study [19], we described a deleterious effect of K155A mutation of Rhp51 on its function. Rhp51 K155A was unable to rescue MMS and UV sensitivity of the *rhp51* Δ strain and also conferred dominant negativity when overexpressed. We then examined if Rhp51 D244Q also lost its biological activities as K155A, by measuring their complementary activities of MMS and UV sensitivity of *rhp51* Δ strain. As shown in Figures 5(A) and 5(B), both Rhp51 K155A and D244Q provide no resistance to *rhp51* Δ against MMS and UV. The complementary activity of both K155A and D244Q were almost indistinguishable with *rhp51* Δ itself, indicating that both mutations fully impaired the biological activity of Rhp51. Moreover, overexpression of

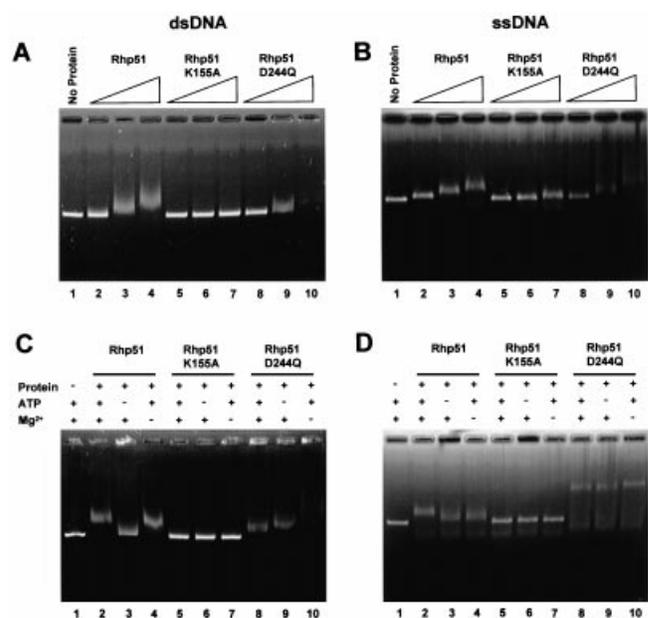


Figure 4 DNA-binding properties of Rhp51 K155A and Rhp51 D244Q

Increasing concentrations (4.6, 13.8 and 23 μ M) each of Rhp51, Rhp51 K155A and Rhp51 D244Q were incubated with 15.4 μ M of ϕ X174 viral DNA (A) or 15.7 μ M *Eco*RI-linearized pBluescript SK(+) (B) for 15 min in the presence of ATP. ssDNA (C) or dsDNA (D) binding of each Rhp51, Rhp51 K155A and Rhp51 D244Q was assessed in the absence of ATP (lanes 3, 6 and 9) or Mg^{2+} (lanes 4, 7 and 10). Each reaction contains 13.8 μ M protein and 9.4 μ M ϕ X174 viral DNA (C) or 9.2 μ M *Eco*RI-linearized pBluescript SK(+) (D).

Rhp51 D244Q in wild-type cells also caused dominant-negative effect over MMS and UV damage to a similar extent with Rhp51 K155A (results not shown). Taken together, these results suggest that, although K155A and D244Q mutations have different effects on the DNA-binding activity of Rhp51, their biological effects in cells were similar.

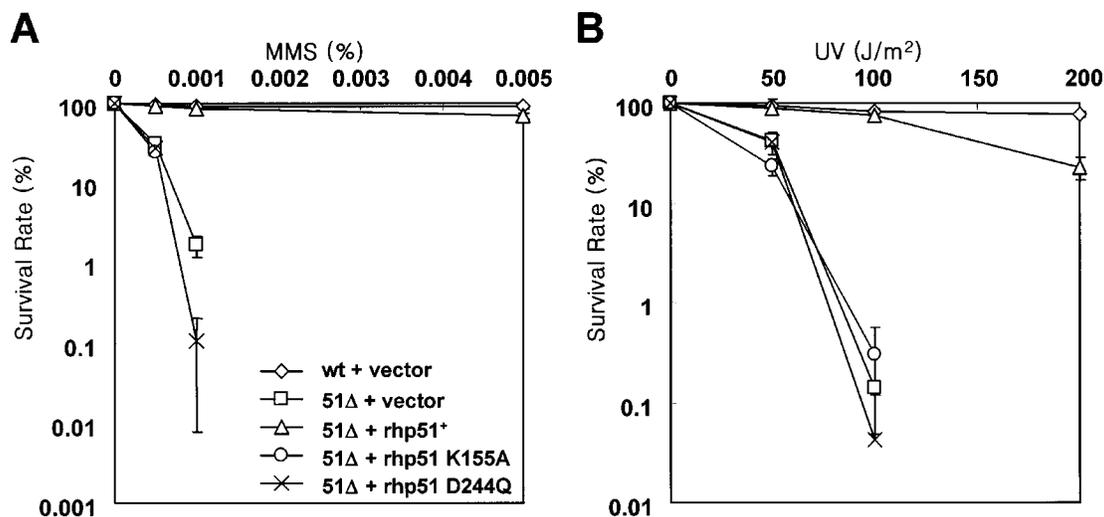


Figure 5 Rescue of MMS and UV sensitivity of *rhp51Δ* cells by Rhp51 K155A or D244Q

Cells *rhp51Δ* containing multicopy plasmids carrying *rhp51* K155A or *rhp51* D244Q were subjected to a survival test whether these mutant genes were able to rescue their hypersensitivity to MMS (A) and UV (B). ◆, wild-type cells with Splac551 vector; □, *rhp51Δ* with Splac551; △, *rhp51Δ* with p51.551; ○, *rhp51Δ* with p51.551 K155A; ×, *rhp51Δ* with p51.551 D244Q.

DISCUSSION

In the present study, we reported that Rhp51 is an ATP-dependent ssDNA- and dsDNA-binding protein and the mutations in the A or B motif of Rhp51 disrupted its biological activity and changed its DNA-binding activity in a different manner.

We found that Rhp51 protein produced in *E. coli* had an ATP-dependent ssDNA- and dsDNA-binding activity, which was similar but distinct from ScRad51 [13,16,17]. Unlike ScRad51, the Mg^{2+} dependency of dsDNA binding was diminished in Rhp51. In addition, it was also different from ScRad51 in that Rhp51 could utilize both ADP and GTP instead of ATP for ssDNA and dsDNA binding. Therefore the two proteins may differ in their detailed requirements for nucleotide cofactors and/or bivalent cations.

Although we failed to detect ATP hydrolysis and strand-exchange activity of Rhp51, it is unlikely that Rhp51 is devoid of these activities. Similar to our observation, a previous study [13] has reported that ScRad51 produced in *E. coli* also did not show ATP-hydrolysis and strand-exchange activities, but had ATP-dependent DNA-binding activity. In addition, active ScRad51 purified from *S. cerevisiae* showed very weak intrinsic strand-exchange and ATP-hydrolysis activities with very narrow optimal conditions and requirements of single-strand-binding protein [2,14,22]. Therefore it is not clear whether the absence of all the activities of our recombinant Rhp51 was due to the mis-folding or omission of some important post-translational modifications or non-optimal conditions used for the reactions.

ATP dependence of the DNA binding of Rhp51 was changed in a different manner by the K155A and D244Q mutations. Lys¹⁵⁵ in the A motif and Asp²⁴⁴ in the B motif are highly conserved, and their corresponding residues in RecA have been known to interact with β,γ -phosphate of ADP-ATP and with Mg^{2+} to water respectively [23]. The K155A mutation completely eliminated the DNA repair activity of Rhp51 as well as the DNA-binding activity. An equivalent mutant, ScRad51 K191A, lacking ATP-hydrolysis and strand-exchange activity, also showed deficiency in DNA repair [13,14]. On the other hand,

Rhp51 D244Q protein had totally different DNA-binding properties compared with Rhp51 K155A. Rhp51 D244Q maintained the DNA-binding activity but lacks the ATP dependency. Furthermore, it appeared to have an improved affinity to DNA compared with the wild-type protein. Therefore it is intriguing that both K155A and D244Q mutations showed virtually the same cellular defects such as complete deficiency in DNA repair and dominant-negative effect on DNA damage. Rhp51 D244Q mutation was very unique, since it was also different from the ScRad51 K191R mutation that was partially active. It is probable that the ATP-dependent DNA binding is more significant than DNA binding itself for the cellular function of Rhp51. Until now, the reason why RecA and Rad51 hydrolyse ATP has remained questionable, although extensive studies have been done [24]. One proposed function is that ATP hydrolysis is required for the disassembly of RecA monomer from RecA nucleoprotein filament [25], suggesting that the enhanced affinity of Rhp51 D244Q to DNA could be explained by its failure to dissociate from DNA. Therefore whether Rhp51 D244Q bound and hydrolysed ATP should be examined for delineation of the relationship between ATP hydrolysis and the biological function of Rhp51 in the future.

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