

Interaction of human recombination proteins Rad51 and Rad54

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ABSTRACT

The cDNA for human protein HsRad54, which is a structural homolog of *Saccharomyces cerevisiae* recombination/repair protein Rad54, was cloned and expressed in *Escherichia coli*. As demonstrated by analysis *in vitro* and *in vivo*, HsRad54 protein interacts with human Rad51 recombinase. The interaction is mediated by the N-terminal domain of HsRad54 protein, which interacts with both free and DNA-bound HsRad51 protein.

INTRODUCTION

Recent studies have led to the discovery of human Rad51 protein, HsRad51, a homolog of bacterial recombination protein RecA and yeast Rad51 protein (1,2). The *Saccharomyces cerevisiae* *RAD51* gene belongs to the *RAD52* epistasis group and, together with other members of the group, is implicated in homologous recombination and repair of DNA double-strand breaks in mitosis and meiosis (reviewed in 3). In addition to a high degree of structural homology (67% identity at the amino acid level), human and yeast Rad51 proteins share significant functional similarity. Both proteins form nucleoprotein filaments on single-stranded (ss) and double-stranded (ds) DNA, possess DNA-dependent ATPase activity and promote DNA strand exchange between ssDNA and homologous dsDNA (4–8). However, while yeast *RAD51* is dispensable for cell growth, mammalian *rad51* is an essential gene (9,10).

The yeast *RAD54* gene is also a member of the *RAD52* epistasis group. Mutants of *RAD54* are highly sensitive to X-rays and methyl methanesulfonate (MMS) and are defective in both spontaneous and induced mitotic recombination. Rad54 protein is a member of the SNF2/SWI2 subfamily of DNA-dependent ATPases, which are involved in chromatin remodeling and transcriptional regulation. The central part of its amino acid sequence contains seven characteristic motifs, including Walker-type nucleotide binding motifs (11–13). However, so far there have been no reports on biochemical activities of Rad54 protein.

Mammalian Rad54 protein, whose gene was recently cloned, can complement MMS sensitivity of a yeast *rad54* mutant (14). In addition, recent studies have shown that homozygous *rad54* mutants in mouse and chicken are highly radiation and MMS sensitive and have reduced levels of homologous recombination

(15,16). All these data point to a high degree of functional conservation between the yeast and the higher eukaryotic homologs of members of the *RAD52* group, particularly Rad51 and Rad54.

It has been shown that several proteins of the yeast *RAD52* epistasis group can interact with each other. Thus, Rad51 protein can associate with Rad52, Rad54 and Rad55 proteins and Rad55 protein interacts with Rad57 protein (9,17–19). These results suggest that the proteins involved in homologous recombination in *S.cerevisiae* function as multiprotein complexes. Similar complexes may also exist in mammalian cells. In particular, it has been shown that human Rad51 protein, like its *S.cerevisiae* counterpart, interacts with human Rad52 protein *in vivo* and *in vitro* (20).

It has been found recently that yeast Rad51 and Rad54 proteins interact *in vivo* and *in vitro* and that the interaction is mediated by the N-terminal part of Rad54 protein (21,22). While the central part of Rad54, which contains the DNA-dependent ATPase motifs, is well conserved between the yeast and human Rad54 proteins, the N-terminal part is considerably less conserved (14). We therefore addressed the question of whether human Rad51 and Rad54 proteins could interact with each other and whether the N-terminal part of HsRad54 would mediate this interaction.

Given the recent appreciation of the roles of DNA repair and recombination in hereditary diseases and cancer, characterization of human enzyme complexes is very important. The data in this study show that human Rad51 protein interacts *in vitro* and *in vivo* with human Rad54 protein and that this interaction is mediated by the N-terminal part of HsRad54. This finding provides additional evidence that the structure of putative multiprotein complexes of homologous recombination may be conserved in yeast and mammals.

MATERIALS AND METHODS

Cloning of cDNA and protein expression

HsRad54 protein, which consists of 747 amino acid residues, contains a number of arginine residues encoded by AGA and AGG codons that are rarely used by *Escherichia coli*. For that reason we cloned the coding sequence of HsRad54 protein into vector pSBETa, which carries the *argU* gene (23). This gene encodes tRNA^{arg4}, which facilitates translation of AGA and AGG codons (24). The entire coding sequence of HsRad54 protein was inserted between *Nde*I and *Bam*HI sites of the vector.

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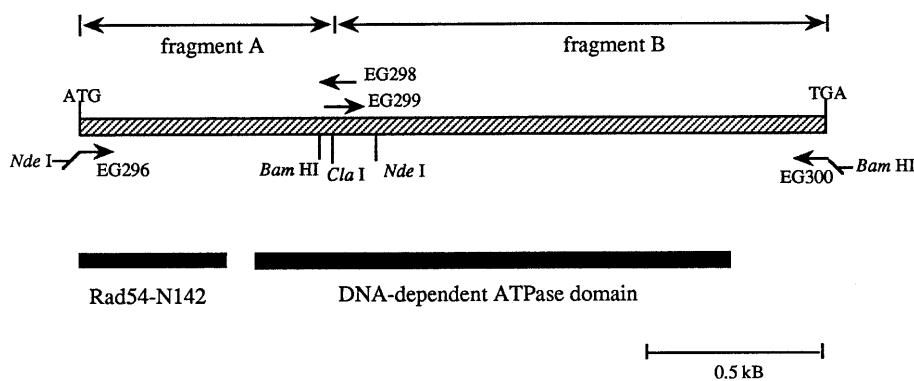


Figure 1. Cloning of HsRad54 cDNA. The HsRad54 cDNA and the primers used for amplification of the gene are indicated. Fragments A and B used for cloning are as specified in Materials and Methods. Also shown are fragments corresponding to amino acids 1–142 of HsRad54 (HsRad54-N142 protein) and the homology-defined putative DNA-dependent ATPase domain (amino acids 178–638, as specified by Kanaar *et al.*; 14). HsRad54 protein consists of 747 amino acid residues, with a calculated molecular weight of 84.3 kDa.

Since Hsrad54 cDNA has internal *NdeI* and *BamHI* sites, we made the Hsrad54 cDNA from two fragments, A and B (Fig. 1). The fragments were obtained by PCR reaction, using the Expand High Fidelity PCR kit (Boehringer Mannheim Corp.). The first cDNA strand made from total RNA of human kidney cell line 293 (ATCC CRL1573) was used as a template. For amplification of fragment A primers EG296 (GGAATTCCATATGCGTGC-CAGCTTGGCTCCCAGCCAGC) and EG298 (GATCCTCC-ATCGATGGCCAG) were used [the underlined sequences indicate homology with the published sequences of the Hsrad54 gene (14)]. Primer EG296 has a *NdeI* restriction site followed by the ATG start codon and two arginine codons, CGT and CGC, which replaced AGG codons at the Arg2 and Arg3 positions. Primer EG298 carries a *ClaI* restriction site. Fragment B was amplified using primers EG299 (CTGGCCATCGATGGAG-GATC) and EG300 (CGCGGATCCTTAGTGATGGTGATGG-TGATGGCGGAGGCCCGCTGTTCTCC). Primer EG299 contains a *ClaI* site. Primer EG300 carries the sequence that codes for six histidine residues, followed by a stop codon and a *BamHI* recognition site. PCR-amplified fragments A and B were digested by *NdeI* and *ClaI*, and *ClaI* and *BamHI*, respectively and were used together for ligation to vector pSBETa, which had been digested by *NdeI* and *BamHI*. The resulting plasmid pEG1042 carries the entire coding region of the HsRad54 protein followed by six histidine residues. Synthesis of the protein is under control of the T7 phage promoter.

To make the protein containing only the N-terminal 142 amino acid sequence of HsRad54 protein, we used primers EG296 (see above) and EG297 (CGCGGATCCTTAGTGATGGTGATGGT-GATGGACAGGGAGTTTCTCC). The latter carries a sequence coding for six consecutive histidine residues, followed by a stop codon and a *BamHI* recognition site. The amplified fragment was digested by *NdeI* and *BamHI* and inserted between *NdeI* and *BamHI* sites of the pSBETa vector. The resulting plasmid pEG1041 codes for the 142 N-terminal residues of HsRad54 protein (HsRad54-N142 protein) followed by six histidine residues.

Expression of HsRad54 and HsRad54-N142 proteins from plasmids pEG1042 and pEG1041 respectively was carried out in *E. coli* strain NovaBlue (DE3) (Novagen Inc.), which carries a gene for T7 phage RNA polymerase inducible by isopropyl β -D-thiogalactopyranoside (IPTG). Transformed bacteria, grown to an

OD₅₉₅ of 0.6 in LB medium with 30 μ g kanamycin/ml at 37°C, were induced by addition of 2 mM IPTG and harvested after 2 h.

Fragment 4 of BRCA-1 protein, which contains amino acid residues 758–1064 (BRCA-1*; 25), was amplified by PCR using primers EG294 (CGCGGATCCAAACTGAAAGATCTGTA-GAGAG) and EG295 (TGCGGGTCGACTGGAACCTATTT-CATTAATAC). Regions of homology in these primers are identical to those used by Scully *et al.* (25). Primers EG294 and EG295 contain *BamHI* and *SalI* restriction sites respectively. The amplified fragment of BRCA-1 was inserted into expression vector pQE-32 (Qiagen Inc.) as an N-terminal fusion with six histidine residues, producing plasmid pEG1038. The plasmid was introduced into *E. coli* M15 (pREP4) (Qiagen Inc.) and protein was induced by IPTG in the same way as described above.

Isolation of 6 \times His-tagged HsRad52 protein has been described elsewhere (26). The same protocol was used for isolation of the HsRad51-N142 and BRCA-1* proteins. Purification of human Rad51 protein was described earlier (8).

Analysis of Rad51–Rad54 interaction using 6 \times His tag binding beads

Analysis by Western blotting. The purified 6 \times His-tagged N-terminal fragment of HsRad54 protein (0.7 μ M) was incubated with 0.4 μ M HsRad51 protein in 100 μ l buffer B [50 mM sodium phosphate buffer, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 2 mM β -mercaptoethanol, 100 μ g bovine serum albumin (BSA)/ml] for 90 min at room temperature. Then, 20 μ l of a 50% slurry of Ni-NTA-agarose resin (Qiagen Inc.), pre-equilibrated in buffer B, were added. After 90 min incubation at room temperature, the beads were washed three times with 400 μ l buffer B and twice with 400 μ l buffer B with 50 mM imidazole. After an additional wash with 100 μ l buffer B with 50 mM imidazole, proteins bound to the beads were released by adding 100 μ l buffer B with 350 mM imidazole. The presence of Rad51 in different fractions was analyzed by Western blotting using polyclonal rabbit anti-HsRad51 antibodies and anti-rabbit IgG conjugated to alkaline phosphatase.

Analysis by a 'DNA pull-down' assay. For analysis of the interaction of HsRad54 protein with HsRad51 protein bound to DNA, 1 μ M purified HsRad51 protein was preincubated for 3 min at 37°C with

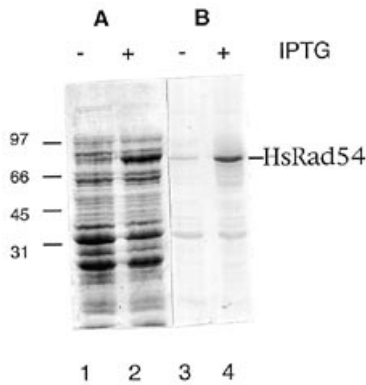


Figure 2. HsRad51 protein binds to HsRad54 protein. (A) Coomassie Blue stained gel. (B) Far-Western blotting. About 1×10^7 NovaBlue (DE3)/pEG1042 cells, in which expression of the *HsRad54* gene was induced by IPTG (lanes 2 and 4) or the same amount of non-induced bacteria (lanes 1 and 3) were boiled with loading buffer and proteins were separated on a 12% SDS-polyacrylamide gel and transferred to a membrane. The membrane was then incubated with HsRad51 protein and washed extensively. HsRad51 protein retained by the membrane was detected using anti-HsRad51 antibodies. Numbers at left indicate protein molecular weight standards in kDa.

2.5 μ M (nucleotide concentration) 32 P-labeled 83mer oligonucleotide W16(-) (8) in 20 μ l buffer C (20 mM Tris-HCl, pH 7.2, 25 mM NaCl, 2 mM $MgCl_2$, 1 mM ATP- γ -S, 50 μ g BSA/ml). Then, 6 \times His-tagged HsRad54-N142 protein was added at 1.5 μ M and incubation continued for 50 min at room temperature, followed by addition of 5 μ l Ni-NTA-agarose slurry and another 15 min incubation. The beads were spun down and washed four times with 200 μ l buffer C containing 20 mM imidazole. Radioactivity in the supernatant obtained after centrifugation of the beads and on the washed beads was measured by Cherenkov counting.

Interaction of HsRad51 protein with proteins bound to nitrocellulose membrane

A protocol for far-Western blotting was described earlier (21,27). Briefly, proteins were electrophoresed through a 12% SDS-polyacrylamide gel and transferred to Immobilon-P membrane (Millipore Corp.). The membrane was immersed in 8 M urea and 1% mercaptoethanol in FW buffer (20 mM Tris-HCl, pH 7.5, 60 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 5% glycerol, 0.02% NP-40). Proteins adsorbed to the membrane were renatured by incubation in 10 sequential 2-fold dilutions of urea in FW buffer. The membrane was then blocked for 1 h in 5% non-fat dry milk in FW buffer and incubated further for 1 h with 5.4 μ M HsRad51 in FW buffer containing 2% BSA. The bands which retained HsRad51 protein were detected using anti-HsRad51 antibodies.

A modification of the far-Western assay was devised to measure interactions of purified proteins on membranes. Purified test proteins, diluted in 10 mM Tris-HCl, pH 7.5, 50 μ g BSA/ml were spotted onto a Biotrace NT nitrocellulose membrane (Gelman Sciences). The membrane was air dried for 30 min at room temperature and blocked for 1 h in FW buffer containing 5% non-fat dry milk. After washing in FW buffer, the membrane was incubated for 1 h with 5.4 μ M HsRad51 in FW buffer containing 2% BSA. The membrane was then washed extensively in FW buffer and any HsRad51 protein retained on the membrane was detected using anti-HsRad51 antibodies.

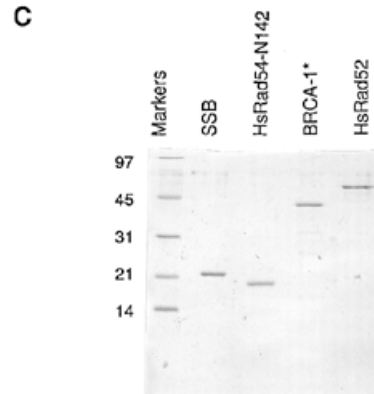
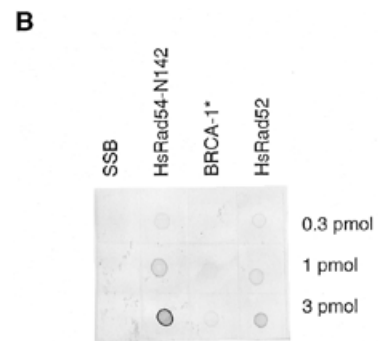
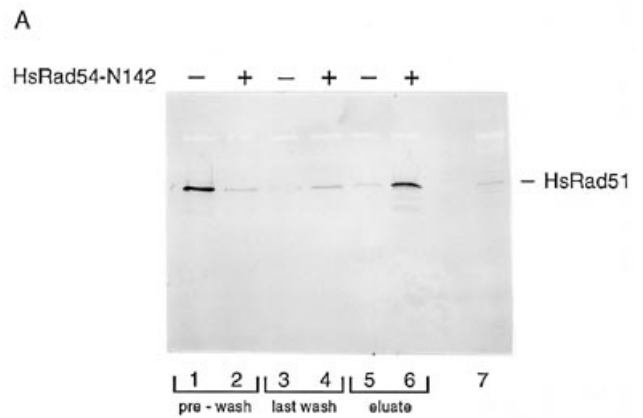


Figure 3. The N-terminal fragment of HsRad54 protein binds to HsRad51 protein. (A) HsRad51 protein was incubated with 6 \times His-tagged HsRad54-N142 protein (lanes 2, 4 and 6, marked +) or with an equal amount of BSA (lanes 1, 3 and 5, marked -). Ni-NTA-agarose beads were added to both samples. The beads were washed six times and proteins bound to the beads were released by incubation with 350 mM imidazole. Aliquots of supernatants from centrifugations preceding the washes (lanes 1 and 2) and after the last wash (lanes 3 and 4) and eluates (lanes 5 and 6) were subjected to Western blotting. Lane 7, protein extract of rat cell line TGR-1 (30), used as a Rad51 protein marker. HsRad51 protein was detected using polyclonal rabbit antibodies. (B) Far-Western in dot-blot format. Equimolar amounts of the protein shown were spotted on nitrocellulose membrane. The membrane was incubated with HsRad51 protein, whose retention on the membrane was detected using anti-HsRad51 antibodies. Numbers at right indicate the amount of blotted protein. (C) Coomassie Blue stained gel of the protein preparations used in the experiment shown in (B). Numbers at left indicate protein molecular weight standards in kDa.

Table 1. Interaction of HsRad54-N142 protein with HsRad51 protein bound to ssDNA (DNA pull-down assay)

Proteins in reaction	Radioactivity in supernatant (c.p.m.)	Radioactivity on Ni-NTA-agarose beads (c.p.m.)
None	4.8×10^3 (91%)	5.0×10^1 (1%)
Rad51	4.3×10^3 (81%)	8.2×10^1 (2%)
Rad54-N142	4.1×10^3 (77%)	1.2×10^2 (2%)
Rad51+Rad54-N142	3.2×10^3 (60%)	2.0×10^3 (38%)

Numbers in parentheses indicate the percentage relative to the amount of input radioactivity, which was $\sim 5.3 \times 10^3$ c.p.m.

Table 2. Two-hybrid analysis of HsRad54-N142 protein interactions

Protein fused to Gal4 DNA binding domain	Protein fused to Gal4 activation domain	β -Galactosidase activity (Miller units)
HsRad54-N142	HsRad51	1.8×10^2
HsRad51	HsRad54-N142	<0.5
HsRad54-N142	ScRad51	<0.5
ScRad51	HsRad54-N142	<0.5
HsRad54-N142	HsDmc1	<0.5
HsDmc1	HsRad54-N142	<0.5
HsRad54-N142	HsRad54-N142	<0.5
HsRad51	HsRad51	1.2×10^1

β -Galactosidase activity was measured in liquid assay using ONPG as a substrate. The Miller units of β -galactosidase are the average values from experiments with at least three colonies.

Analysis of interactions of HsRad54 in the yeast two-hybrid system

The nucleotide sequence corresponding to the first 142 amino acids of HsRad54 protein was amplified by PCR from cDNA prepared from human 293 cells. The primers used were EG296 (see above) and EG301 (GGTGAATTCAATGGACAGGGAGTTTCTCC). The resulting fragment was cloned into two-hybrid vectors pGBT9 and pGAD GH (Clontech Laboratories Inc.) as fusions with the Gal4 DNA binding and activation domains, respectively. The same vectors were used to produce fusions of yeast Rad51 and human Rad51 and Dmc1 proteins. Interactions were analyzed in yeast reporter strain SFY526 (28) by measuring the levels of β -galactosidase produced in a liquid assay, using *O*-nitrophenyl- β -D-galactopyranoside (ONPG) as substrate (29). Yeast transformants were selected at 30°C on plates with synthetic complete (SC) medium lacking leucine and tryptophan. For the β -galactosidase assay, cells from individual colonies were grown to saturation in the same medium. At least three colonies were assayed in each case and the average values are presented in Table 1.

RESULTS

Human Rad51 and Rad54 proteins interact *in vitro*

We used a far-Western protocol to study the interaction of HsRad51 and HsRad54 proteins. To this end, we expressed 6 \times His-tagged full-length HsRad54 protein in *E. coli*. The expressed protein (molecular weight 85.2 kDa) was mostly insoluble, but could be purified on Ni-NTA-agarose resin, which binds the 6 \times His tag under denaturing conditions (data not shown). For the binding study, total proteins from an *E. coli* strain overproducing full-length HsRad54 protein were separated on an SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. To renature the

transferred proteins, the membrane was first incubated with 8 M urea and then with solutions of decreasing concentrations of urea. After final washing without urea and blocking, the membrane was incubated with a solution of HsRad51 protein. Following extensive washing, bound Rad51 protein was detected using anti-HsRad51 antibody. HsRad51 protein bound to full-length HsRad54 protein in this assay (Fig. 2).

The interaction with HsRad51 protein is mediated by the N-terminal part of HsRad54 protein

We overproduced in *E. coli* and isolated a 142 residue N-terminal fragment of HsRad54 protein (HsRad54-N142) as a fusion with six histidine residues on its C-terminus (Fig. 3C). Using Ni-NTA-agarose beads, we found that HsRad51 protein binds to HsRad54-N142 protein, which results in its co-precipitation with the HsRad54-N142-Ni-NTA-agarose complex (Fig. 3A, compare lanes 1 and 2). HsRad51 protein was retained by the beads in the presence of HsRad54-N142 and can be removed from the beads by 350 mM imidazole (Fig. 3A, lanes 3–6).

We also tested the interaction of HsRad54 protein with HsRad51 protein which has formed a nucleoprotein filament on DNA. A complex was formed between HsRad51 and a 32 P-radio-labeled single-stranded 83mer oligonucleotide and incubated with 6 \times His-tagged HsRad54-N142 protein at a 1.5:1 Rad54/Rad51 molar ratio. We then looked at the ability of Ni-NTA beads to pull down the radiolabeled DNA from the reaction in the presence and in the absence of Rad54. Indeed, when HsRad54-N142 protein was present there was an $\sim 35\%$ drop in the radioactive count in the supernatant after beads were removed by centrifugation (Table 1). Furthermore, the radio-labeled DNA remained quantitatively associated with the sedimented beads after several washes. In contrast, when Rad51, Rad54 or both proteins were omitted from the reaction there was very little binding of DNA to the beads (Table 1). This experiment demonstrates that human Rad54 protein can bind human Rad51 which is associated with DNA.

The specificity of the interaction between HsRad51 and HsRad54N-142 proteins *in vitro* was confirmed by a modification of the far-Western method. In this experiment, purified proteins (Fig. 3C) were spotted on a nitrocellulose membrane which was subsequently incubated with HsRad51 protein. Retention of HsRad51 protein on the membrane was analyzed using anti-Rad51 antibodies. As a negative control for this experiment, we used *E. coli* ssDNA binding protein (SSB). Human Rad52 protein, which binds to HsRad51 protein (20), was used as a positive control. As expected, HsRad51 protein binds to HsRad54-N142 and HsRad52 proteins, but not to SSB protein (Fig. 3B). However, BRCA-1*, which has been reported to interact with human Rad51 protein (25), showed little if any binding to HsRad51 in our assay (Fig. 3B).

HsRad51 and HsRad54 proteins interact *in vivo*

Interaction between HsRad51 and HsRad54-N142 was tested *in vivo*, using the yeast two-hybrid system. As shown in Table 2, there is a strong interaction between the two proteins, as measured by β -galactosidase level in a yeast reporter strain. In contrast, there was no interaction of HsRad54-N142 protein with yeast Rad51 or with human Dmc1 protein, a meiosis-specific RecA/Rad51 homolog which has 52% amino acid identity to HsRad51. The interaction of HsRad51 with HsRad54-N142 was detected only when HsRad51 protein was fused to the activation domain of Gal4. This asymmetry has been earlier observed in the interaction of yeast Rad51 and Rad54 proteins (21).

DISCUSSION

In this study we have demonstrated that human recombination protein Rad51 binds to human Rad54 protein. This interaction parallels the interaction between the yeast Rad51 and Rad54 proteins discovered recently (21,22). As several members of the yeast *RAD52* epistasis group are thought to function as multi-protein complexes, our data, together with the data of others, suggest that similar complexes may also exist in human cells.

In our analysis we first overexpressed full-length HsRad54 protein in *E.coli*. Although the protein produced was mostly insoluble, it was possible to analyze its interaction with HsRad51 following SDS-PAGE and renaturation on a membrane by a far-Western assay. This analysis clearly indicates that full-length HsRad54 protein binds HsRad51 protein. Since it was reported that interaction between yeast Rad51 and Rad54 proteins is mediated by the N-terminal part of Rad54 (21), we expressed and purified a 142 amino acid N-terminal fragment of HsRad54 protein, HsRad54-N142, and analyzed its ability to bind to HsRad51 protein. Using two assays that utilize histidine tag binding beads, we have shown here that this fragment indeed binds HsRad51 protein in solution. In addition, this interaction was confirmed by far-Western analysis using immobilized native Rad54-N142. Importantly, both free Rad51 protein and Rad51 protein which was bound to a single-stranded oligonucleotide could interact with the N-terminal part of HsRad54 protein. This indicates that the binding site for Rad54 protein on Rad51 protein is likely to be distinct from its DNA binding site. Our preliminary analysis showed that Rad54-N142 protein had no significant effect on HsRad51-mediated strand exchange reactions and its ATPase activity (data not shown).

The HsRad51/HsRad54-N142 interaction was also shown *in vivo*, using the yeast two-hybrid system. It is interesting that although there is relatively little identity between human and yeast Rad54 over the N-terminal part of the protein, the species-specific Rad51/Rad54 interaction appears to be conserved. Although expression of the *HsRad54* gene could partially complement MMS sensitivity of a yeast *rad54* Δ mutant (14), no interaction was detected between HsRad54-N142 and yeast Rad51 in our two-hybrid experiments.

Since by structural criteria Rad54 protein belongs to a group of DNA-dependent ATPases, some of which are helicases, it is expected to bind DNA. Possibly, through its interaction with Rad51, Rad54 may be recruited to the sites on DNA where homologous recombination takes place. It may act as a helicase in Rad51-mediated strand exchange reactions. Several precedents for a helicase functioning in strand exchange are known,

including the recombination systems of *E.coli* (31), *S.cerevisiae* (32) and T4 phage (33). Further biochemical analysis of Rad54 protein is needed to test this hypothesis.

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