Both the Charged Linker Region and ATPase Domain of Hsp90 Are Essential for Rad51-Dependent DNA Repair

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The inhibition of Hsp90 in cancerous cells has been correlated with the reduction in double-strand break (DSB repair) activity. However, the precise effect of Hsp90 on the DSB repair pathway in normal cells has remained enigmatic. Our results show that the Hsp82 chaperone, the ortholog of mammalian Hsp90, is indispensable for homologous-recombination (HR)-mediated DNA repair in the budding yeast Saccharomyces cerevisiae. A considerable reduction in cell viability is observed in an Hsp82-inactivated mutant upon methyl methanesulfonate (MMS) treatment as well as upon UV treatment. The loss of Hsp82 function results in a dramatic decrease in gene-targeting efficiency and a marked decrease in the endogenous levels of the key recombination proteins Rad51 and Rad52 without any notable change in the levels of RAD51 or RAD52 transcripts. Our results establish Rad51 as a client of Hsp82, since they interact physically in vivo, and also show that when Hsp82 is inhibited by 17-AAG, Rad51 undergoes proteasomal degradation. By analyzing a number of point mutants with mutations in different domains of Hsp82, we observe a strong association between the sensitivity of an ATPase mutant of Hsp82 to DNA damage and the decreases in the amounts of Rad51 and Rad52 proteins. The most significant observations include the dramatic abrogation of HR activity and the marked decrease in Rad51 focus formation in the charged linker deletion mutant of Hsp82 upon MMS treatment. The charged linker region of Hsp82 is evolutionarily conserved in all eukaryotes, but until now, no biological significance has been assigned to it. Our findings elucidate the importance of this region in DNA repair for the first time.

When cells are exposed to any DNA double-strand break (DSB)-inducing agent, a plethora of proteins are activated and recruited at the broken junction in order to repair the damage. Failure to repair such DNA lesions leads to cell death, loss of genetic information, and malignancy. In eukaryotes, DSBs can be repaired primarily by two pathways: homologous recombination (HR) and nonhomologous end joining (NHEJ) (1, 2). In higher eukaryotes, NHEJ, which does not rely on any homology, is the predominant break repair mechanism. This process often leads to the deletion of a small portion of the genome. The key proteins associated with this pathway include Mre11, Ku70, Ku80, the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), Artemis, and XRCC4 (3). Most of the lower eukaryotic microbes primarily use HR as the major repair pathway.

HR involves resection of the broken end and thus generation of single-stranded DNA (ssDNA) overhangs, which form the binding site for Rad51p. Rad51p, with its ATPase activity, searches for the homologous templates and invades similar sequences. Rad51, Rad52, Rad54, and replication protein A (RPA) are the key proteins involved in the HR-mediated break repair pathway (4, 5). In Saccharomyces cerevisiae, Rad52p facilitates the formation of a hexameric Rad51p-bound ssDNA complex at the broken junction in the presence of RPA. Rad52p plays a central role in HR, and rad52 mutation abolishes all recombination events in yeast (6).

Hsp90 is an important cellular chaperone that is evolutionarily conserved from bacteria to mammals. It is abundantly present (~1 to 2% of total cellular proteins) in cytosol and is involved in the maturation and stability of a special class of client proteins (7, 8, 9, 10). In vitro experiments have shown that Hsp90 cannot refold completely denatured protein. However, it assists in the folding of client proteins where considerable amounts of secondary structures are retained (11). Hsp90 is not a general chaperone like Hsp70, and how it selectively chooses a special class of clients has remained enigmatic. In the budding yeast S. cerevisiae, Hsp90 exists in two isoforms: Hsc82, which is constitutively expressed in the cell, and a paralog, Hsp82, which is induced under stress conditions (12). Hsc82 and Hsp82 are 97% identical in their amino acid sequences, and at least one of them is essential for yeast viability. In recent years, Hsp90 has shown tremendous potential as an anticancer target (13), and thus it is important to gain a better understanding of Hsp90 functions.

Hsp90 functions as a dimer, and the protein folding is mediated by the participation of different cochaperones and the ATPase activity of the chaperone (14, 15). Hsp82 is an 82-kDa protein with three distinct domains. The amino-terminal domain (amino acids 1 to 220) has an ATP binding pocket that gives rise to the Bergerat fold. A recent crystal structure of Hsp90 revealed that the function of Hsp82 is dependent on its binding and the hydrolysis of ATP (16, 17, 18). The N-terminal domain is linked to the middle domain (amino acids 273 to 525) through a charged linker...
region, which is an important regulator of Hsp90 function. The charged linker region contains a pentad repeat of the motif (D/E)(D/E)(D/E)KK and is absent from prokaryotic Hsp90. Previously, it was shown that this domain is dispensable for steroid hormone receptor regulation and the pheromone signaling pathway (19). However, a more recent study has demonstrated that deletion of the linker has a distinctive effect on client activation (20).

A study of the crystal structure revealed that the cochaperone Aha1 interacts with the middle and N-terminal domains of Hsp90 and facilitates the binding of a subset of clients (21, 22, 23). The carboxy-terminal domain of Hsp90 has a dimerization domain (24). It has a conserved MEEVD motif at the extreme end, which is the binding site for a special class of cochaperones that contain multiple copies of a tetratricopeptide repeat (TPR) (25). Because the interaction between the chaperone and its clients is very transient, it is very challenging to identify the interactome of Hsp90. Different high-throughput studies aimed at finding out the genomewide interactors of Hsp92 have identified some of the key proteins involved in the DNA repair pathway (26, 27). However, due to the lack of any detailed study, it remains ambiguous whether Hsp90 plays any role in the DNA repair pathway under normal cellular conditions. A potent Hsp90 inhibitor, 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG), has been found to inhibit radiation-induced double-strand break repair in tumor cells by reducing the phosphorylation of DNA-PKcs (28). Also in tumor cells, Hsp90 phosphorylation has been found to be correlated with DNA damage (30), and Rad51 focus formation at the damaged sites has been found to be delayed by NVP-AUY922, an inhibitor of Hsp90 (31). Another study has demonstrated that 17-allylamino-17-demethoxygeldanamycin (17-AAG) inhibits DSB repair in tumor cells by BRCA2 degradation; however, it has no effect in normal cells (32).

So far, no detailed work has been carried out to investigate whether Hsp90 plays any role in the DNA repair pathway in lower eukaryotes. In order to understand whether the DSB repair phenotypes, observed in tumor cell lines upon treatment with Hsp90 inhibitors, stem from direct or an indirect involvement of Hsp90, we have examined the role of yeast Hsp82 in the DSB repair pathway by employing well-characterized *S. cerevisiae* mutant strains. This is the first report which demonstrates that in lower eukaryotes, Hsp82 is indispensable for the homologous-recombination mechanism and that Rad51 is a direct client of Hsp82. Our findings reveal that in an Hsp82-inactivated background (Δhsp82 Δhsc82), the endogenous levels of Rad51p and Rad52p are drastically reduced and HR-mediated DSB repair is severely compromised. Structure-function correlation studies have revealed differential effects of several Hsp82 mutants on gene-targeting efficiency, which is well correlated with endogenous levels of Rad51p and Rad52p. Our study shows that the deletion of a charged linker region causes a pronounced defect in gene targeting and Rad51 focus formation upon treatment with methyl methanesulfonate (MMS).

**MATERIALS AND METHODS**

**Yeast strains.** The strains used in this study are listed in Table 1. The iG170Dhsp82 strain, HH1a-p2HG/Hsp82, and HH1a-p2HG/Hsp82(Δ211-259) were kindly provided by Didier Picard (33, 34). The hsp82 T221, A41V, G81S, T101I, G313S, and iA587T point mutants, and the control strain F82a were provided by Susan Lindquist (35). The yeast expression vector (2μ plasmid) harboring ScRad51 (36) was transformed into HH1a-p2HG/Hsp82(Δ211-259) and the iG170Dhsp82 strain to generate strains TSY1 and SLY69, respectively. The blank vector was transformed into the iG170Dhsp82 strain and HH1a-p2HG/Hsp82(Δ211-259) to generate strains TSY3 and TSY2, respectively.

**Construction of epitope-tagged RAD52 in various strain backgrounds.** In order to tag the MYC epitope at the C-terminal end of RAD52, we used plasmid pF6a-13MyckanMX6 (37) as a template and primers OSB68 (5′ AAG ACC AAA GAT CAA TCC CTA GTC AGG CAA GCC TAC TGC GAT CCC CGG GTT ATT AAT TAA 3′) and OSB69 (5′ ATA ATG ATG CAA ATT TTT TAT TTG CTT CAG CCA AGG GGA ATT CCA GCT GTT TAA AAC 3′), with upstream and downstream sequences of RAD52. The 2.3-kb PCR product was used to target the RAD52 loci of W303 and the iG170Dhsp82 and T101I mutants by homologous recombination to generate SLY47, SLY49, and MVS36, respectively. In order to tag the RAD52 locus of strain HH1a-p2HG/Hsp82(Δ211-259) with Myc, we first isolated the plasmid from this mutant strain. The hsp82(Δ211-259) gene was PCR amplified and was cloned into the pRS313 yeast expression vector (CEN plasmid). The recombinant plasmid with the HIS marker was transformed into the RAD52 MYC-tagged T101I strain (MYS36), and the transformants were selected on a SC-His (synthetic medium without histidine) plate. Thus, we generated strain SLY65 by plasmid swapping to create the RAD52 MYC-tagged hsp82(Δ211-259) strain.

**MMS and UV sensitivity assays.** In order to perform the return-to-growth assay in the presence of the DNA-damaging agent methyl methanesulfonate (MMS), fresh yeast cells were grown in yeast extract-peptone-dextrose (YPD) at 25°C to an optical density (OD) of 0.5. The cells were then divided into two parts with equal volumes. One part of the cells was exposed to 0.03% (vol/vol) MMS (Sigma-Aldrich) for 2 h, followed by plating on YPD medium at the required temperature (either 25°C or 37°C) after a 10-fold serial dilution. The other batch was grown in the absence of MMS for 2 h; its growth was then normalized to that of the MMS-treated cells, and it was subsequently plated at the required temperature. The plates were incubated for 36 h, and the growth of the two batches was compared.

In order to determine the difference in MMS sensitivity more quantitatively, we did a viability assay. Briefly, about 1,000 cells (as estimated from optical density) from MMS-treated or mock-treated cultures were plated and allowed to grow. The plates were incubated for 36 h, and the growth of the two batches was compared.

For the UV sensitivity assay, cells of each strain were grown in YPD medium to an OD of 0.5. Then they were diluted, and 1,000 cells were spread on YPD medium and were exposed to the doses of UV radiation indicated in Fig. 1 and 4. UV irradiation was carried out using Stratagene Stratalinker 1800. The plates were then incubated for 4 days; the number of viable colonies for each dosage of UV radiation was counted; and the percentage of survivability was calculated by considering the growth of each strain without UV irradiation to be 100%.

**Gene-targeting assay.** The ADE2 gene was knocked out at the ADH4 loci of F82a, the hsp82 T221, A41V, G81S, T101I, G313S, G170D, and iA587T point mutants, and the hsp82(Δ211-259) strain. The targeting cassette (36) contained the homologous stretches of upstream and downstream sequences of ADH4 flanking the ADE2 gene and a KANMX6 selectable marker upstream of the ADH4 sequence. Two micrograms of the DNA was transformed into each strain. Transformants were first grown on an SC-Ade plate and were subsequently replica plated on a G418 sulfate-containing plate. In each case, the Ade+ G418 colonies were counted. Gene-targeting efficiency was normalized by transforming an equal amount of uncut replicating plasmid into the respective strains to nullify any variation arising from the difference in competence for DNA uptake between strains. The gene-targeting efficiency, expressed as a percentage, was calculated as (number of Ade+ G418 colonies in each mutant)/(number of Ade+ G418 colonies in F82a) × 100.

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Western blotting. For the estimation of levels of Rad51 and 13-Myc-tagged Rad52 proteins, we grew SLY47, the iG170Hsp82 strain, SLY49, MVS36, SLY65, SLY69, TSY1, TSY2, and TSY3 overnight at 25°C. Next morning, the overnight cultures were diluted at a 1:100 ratio and were grown again for 3 to 4 h at 25°C until the OD at 600 nm (OD600) reached 0.5. For the temperature-sensitive strains, the cells were divided into two groups; one part was incubated at 25°C for 4 h, and another part was incubated at 37°C overnight. After that, equal numbers of cells were taken and precipitated, and protein was isolated from them by the trichloroacetic acid (TCA) method, followed by Western blotting (38). A rabbit anti-Rad51 antibody (Promega), a mouse anti-Hsp82 antibody (Calbiochem), and a mouse anti-Act1 antibody (Abcam) were used at a 1:5,000 dilution. A rabbit anti-Myc antibody (Abcam) was used at a 1:8,000 dilution. A horseradish peroxidase-conjugated anti-rabbit secondary antibody (Promega) and a horseradish peroxidase-conjugated anti-mouse secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) were used at a 1:10,000 dilution. The Western blots were developed using a chemiluminescent detection system (Pierce). The bands on the gel were quantified using GeneTools (Syngene), and the relative densities thus obtained were plotted using GraphPad Prism software. The mean values from four independent experiments were plotted with standard deviations (SD). All blots were normalized against actin.

Real-time RT-PCR. Total RNA was isolated from the iG170Hsp82 strain after growth at 25°C and at 37°C for 4 h. In another assay, wild-type strain HH1a-p2HG/Hsp82 and the charged linker deletion mutant, as well as P82a and the hsp82 T101I point mutant, were either left untreated or exposed to 0.03% MMS for 2 h, and by using the acid phenol method as described previously (38), we isolated RNA from these strains. We synthesized cDNA exactly as described in reference 38. We used OSB16 (5′ TGA CCA AACT CTAT TTA CAC CTC C 3′) and OSB14 (5′ TTA GAA ACA CTT GGG GGG CAA G 3′) and OSB45 (5′ CTA CTC GTC TTC TTC GCT G 3′), and to amplify 208 bp of the 3′ end of RAD51, we used primers OSB44 (5′ GTG GTG ATC CAA CCG CAA G 3′) and OB45 (5′ CTA CTC GTC TTC TTC GCT G 3′), and to amplify 307 bp at the 3′ end of RAD52, we used primers OSB133 (5′ TGG GAA TCA AGT ACC GCG TG 3′) and OSB134 (5′ TGG GAA TCA AGT ACC GCG TG 3′) = 2μTATAcRad51

TABLE 1  Yeast strains used in this study

| Strain      | Genotype                                      | Source                      |
|-------------|----------------------------------------------|----------------------------|
| SLY20       | MATα leu2-3,112 trp1 ura3-1 ade2-1 his3-3,115 VIII::ADE2 | Laskar et al. (38)          |
| SLY47       | MATα leu2-3,112 trp1 ura3-1 ade2-1 his3-3,115 VIII::ADE2 Rad52-13MYC-KANMX6 | This study                  |
| SLY4        | MATα leu2-3,112 trp1 ura3-1 ade2-1 his3-3,115 VIII::ADE2 Hsp82::HIS3 | Laskar et al. (38)          |
| SLY5        | MATα leu2-3,112 trp1 ura3-1 ade2-1 his3-3,115 VIII::ADE2 Hsp82::KAN6 | Laskar et al. (38)          |
| iG170Hsp82 mutant | MATα can1-100 ade2-1 his3-3,115 leu2-3,112 trp1-1 ura3-1 hsp82::LEU2 hsc82::LEU2 | D. Picard                   |
| HH1a-p2HG/Hsp82 | MATα hsp82::LEU2 hsc82::LEU2 ade2 his3 leu2 trp1 ura3 HSP82-2μ-HIS (p2HG/Hsp82) | D. Picard (34)              |
| HH1a-p2HG/Hsp82 (Δ211-259) | MATα hsp82::LEU2 hsc82::LEU2 ade2 his3 leu2 trp1 ura3 HSP82 (Δ211-259)-2μ-HIS (p2HG/Hsp82) | D. Picard (34)              |
| DP533       | Δhsc82::kanMX4 Δhsp82::kanMX4/2μ-HSC82-URA3 [YEpLac195] Δpdr::loxP-Leu2::loxP trp1-289 leu-2,112 his3-Δ200 URA3-52 ade2-101Δcys2-801 | D. Picard (34)              |
| SLY49       | MATα can1-100 ade2-1 his3-3,115 leu2-3,112 trp1 ura3-1 hsp82::LEU2 hsc82::LEU2 HIS3::HSP82G170D Rad52-13MYC-KANMX6 | This study                  |
| MVS36       | MATα can1-100 ade2-1 his3-3,115 leu2-3,112 trp1 ura3-1 hsp82::LEU2 hsc82::LEU2 CEN pTGPD/T3-138 Rad52-13MYC-KANMX6 | This study                  |
| SLY65       | MATα can1-100 ade2-1 his3-3,115 leu2-3,112 trp1 ura3-1 hsp82::LEU2 hsc82::LEU2 pRS313hsp82(Δ211-259) Rad52-13MYC-KANMX6 | This study                  |
| SLY69       | MATα can1-100 ade2-1 his3-3,115 leu2-3,112 trp1 ura3-1 hsp82::LEU2 hsc82::LEU2 HIS3::HSP82G170D 2μpTAScRad51 | This study                  |
| TSY1        | MATα hsp82::LEU2 hsc82::LEU2 ade2 his3 leu2 trp1 ura3 HSP82(Δ211-259)-2μ-HIS [p2HG/Hsp82(Δ211-259)] 2μ (pTAScRad51) | This study                  |
| TSY2        | MATα hsp82::LEU2 hsc82::LEU2 ade2 his3 leu2 trp1 ura3 HSP82(Δ211-259)-2μ-HIS [p2HG/Hsp82 (Δ211-259)] 2μ-pTA | This study                  |
| TSY3        | MATα can1-100 ade2-1 his3-3,115 leu2-3,112 trp1 ura3-1 hsp82::LEU2 hsc82::LEU2 HIS3::HSP82G170D 2μ-pTA | This study                  |
| P82a (control) | MATα can1-100 ade2-1 his3-3,115 leu2-3,112 trp1 ura3-1 hsp82::LEU2 hsc82::LEU2 CEN pTGPD/P82 | S. Lindquist (35)           |
| T22I mutant | MATα can1-100 ade2-1 his3-3,115 leu2-3,112 trp1 ura3-1 hsp82::LEU2 hsc82::LEU2 CEN pTGPD/T3-142 | S. Lindquist (35)           |
| A41Y mutant | MATα can1-100 ade2-1 his3-3,115 leu2-3,112 trp1 ura3-1 hsp82::LEU2 hsc82::LEU2 CEN pTGPD/T1-40 | S. Lindquist (35)           |
| G815 mutant | MATα can1-100 ade2-1 his3-3,115 leu2-3,112 trp1 ura3-1 hsp82::LEU2 hsc82::LEU2 CEN pTGPD/T1-15 | S. Lindquist (35)           |
| T101I mutant | MATα can1-100 ade2-1 his3-3,115 leu2-3,112 trp1 ura3-1 hsp82::LEU2 hsc82::LEU2 CEN pTGPD/T3-138 | S. Lindquist (35)           |
| G313S mutant | MATα can1-100 ade2-1 his3-3,115 leu2-3,112 trp1 ura3-1 hsp82::LEU2 hsc82::LEU2 CEN pTGPD/T4-47 | S. Lindquist (35)           |
| ia587T mutant | MATα can1-100 ade2-1 his3-3,115 leu2-3,112 trp1 ura3-1 hsp82::LEU2 hsc82::LEU2 CEN pTGPD/P82 A587T::HIS3 | S. Lindquist (35)           |

Indirect immunofluorescence. The wild-type strain HH1a-p2HG/Hsp82 and the charged linker deletion mutant HH1a-p2HG/Hsp82(Δ211-259) were grown at 30°C to an OD of 0.5 and were then divided into two parts. One part was allowed to grow under the same conditions, and the other part was incubated at 37°C.
other part was treated with 0.03% MMS for 2 h, followed by an indirect immunofluorescence assay. Cells were briefly fixed with 4% paraformaldehyde for 2 h, washed with 0.1 M potassium phosphate buffer and 1 mM MgCl₂, and permeabilized with yeast Lyticase enzyme. Spheroplasts were fixed on a coverslip with poly-l-lysine (Sigma). Samples were blocked with 1% dry milk in phosphate-buffered saline (PBS) for 30 min, incubated with anti-Rad51 (Promega) at a 1:200 dilution for 1 h at room temperature, and washed with PBS 10 to 12 times. Incubation with an Alexa Fluor 488-conjugated anti-goat antibody (Molecular Probes; Life Technologies) at a 1:200 dilution and 4′,6-diamidino-2-phenylindole (DAPI) at a 1:2,000 dilution was carried out for 30 min in the dark. The samples were then washed 10 to 12 times with PBS. Slides were then covered with a mounting solution and were used for confocal microscopy with a Zeiss LSM 510 Meta confocal microscope.

**RESULTS**

**Loss of Hsp82 function affects DSB repair activity.** Previous findings revealed that 17-AAG, a potent inhibitor of Hsp90, causes a drastic reduction in radiation-induced DSB repair in tumor cell lines (DU145 and SQ-5). However, no such effect on DSB repair activity was observed in normal cell lines in the presence of an Hsp90 inhibitor (32). In order to investigate the role of Hsp82 in the DNA repair pathway, we used *S. cerevisiae* as a model system. To this end we have used Δhsp82 or Δhsc82 single mutants as well as a temperature-sensitive mutant (ig170Dhsp82) in which the activities of both Hsp82 and Hsc82 are abrogated. We investigated the sensitivities of these mutants to DNA-damaging agents, such as MMS and UV radiation. These agents cause DNA damage via distinct mechanisms. Both the wild-type strain (SLY20) and the single mutants were exposed to 0.03% MMS for 2 h and were then returned to growth without MMS. Serial dilutions of cells were spotted onto the plates for comparison of the growth of different strains after DNA damage. Since failure to repair damaged DNA leads to the death of the cells, the survival of cells is directly proportional to the efficiency of DNA repair. We observed that the single mutants SLY5 (Δhsp82) and SLY4 (Δhsc82) did not show significantly more sensitivity to MMS in the plate assay than wild-type cells (Fig. 1A). The fractions of MMS-treated cells that survived in the return-to-growth experiment were measured, and the growth of single mutants was found to be about 85% that of the wild type (Fig. 1B). The sensitivities of single mutants to UV radiation were also determined by scoring their abilities to survive on different doses of UV radiation. Determination of percentages of viability showed no significant difference in the tolerance of UV radiation between the single mutants and the wild-type cells (Fig. 1C). These results suggest that the two paralogs HSP82 and HSC82 are redundant to each other. The temperature-sensitive ig170Dhsp82 strain (35) was used to investigate the effect of the abrogation of both Hsp82 and Hsc82 functions on DNA damage sensitivity. We incubated the ig170Dhsp82 strain at 25°C (permissive temperature) as well as at 37°C (restrictive temperature) for 2 h and 4 h and treated the cells with MMS. In the course of our studies, we noted that the ig170Dhsp82 strain, when preincubated at 37°C for a short interval (2 h), became significantly more sensitive to MMS than the strain grown at the permissive temperature (25°C). Prolonged incubation at 37°C (for 4 h) rendered the cells even more hypersensitive to MMS. However, the MMS sensitivity was not as drastic as that of the Δrad51 strain (Fig. 1D). Measurement of the percentage of survivability of MMS-treated ig170Dhsp82 cells upon incubation for 4 h at 37°C showed a 62% decrement in survivability from that for cells grown at 25°C (Fig. 1E). In order to establish that the growth defect is due solely to MMS sensitivity and not to exposure to an increased temperature, the viability of isogenic wild-type cells (P82a) was monitored in parallel, where cells were preincubated at 37°C for 4 h prior to MMS treatment. We observed that such conditions were well tolerated by wild-type cells (data not shown). When the ig170Dhsp82 strain was grown at the restrictive temperature (37°C for 4 h), it showed significantly more sensitivity (measured by the percentage of viability) upon UV irradiation than when it was grown at 25°C (Fig. 1F). For wild-type cells, sensitivity to UV was also monitored after preincubation at 37°C (for 4 h). The percentage of survivability relative to that of cells grown at 30°C showed a similar trend (data not shown). Thus, our finding suggests that loss of both Hsp82 and Hsc82 functions makes cells hypersensitive to MMS- and UV-induced DNA damage. In contrast, single mutants behaved like the wild-type strain and were able to survive through both UV radiation and MMS treatment. Therefore, it is likely that the expression of either of the two isoforms of this chaperone (constitutively expressed Hsc82 and inducible Hsp82) is sufficient for the proper functioning of repair activity.

The reduced gene-targeting efficiency of the ig170Dhsp82 mutant at a nonpermissive temperature is correlated with decreased endogenous levels of Rad51 and Rad52. MMS treatment leads to single-strand breaks and subsequently results in DSBs during the S phase. UV radiation also causes DSBs, which are not restricted to any particular cell cycle phase. Thus, recovery from exposure to UV or MMS is a good measure of DNA repair activity. However, this method does not directly evaluate the efficiency of homologous recombination. On the other hand, gene targeting relies on the homologous-recombination machinery. Thus, we determined the gene-targeting efficiencies of the wild type and the Hsp82-inactivated mutant (the ig170Dhsp82 strain) at 37°C. To this end, we constructed an ADE2 cassette with flanking regions of *ADH4* that was targeted at the *ADH4* locus of chromosome VII-L. We measured the frequencies of targeted integration versus random integration by scoring the loss of the flanking *KANMX* marker (Fig. 2A). Gene-targeting efficiency was normalized by transforming equal amounts of an uncut replicating plasmid into the strains to account for any variation in the competence of DNA uptake between the strains. We observed that the gene-targeting
efficiency of the $iG170Dhsp82$ temperature-sensitive mutant was drastically lower at the nonpermissive temperature (37°C) than at the permissive temperature (25°C) (Fig. 2B). In order to rule out the possibility that the reduction did not result from exposure to a high temperature per se, we measured the gene-targeting efficiency of the isogenic wild-type (HSP82) control. For each strain, the upper row represents serial dilutions of untreated cells (−) and the lower row represents serial dilutions of cells treated with 0.03% MMS (+). (B) Percentages of surviability upon MMS treatment were plotted as the plating efficiencies of MMS-treated cells relative to that of untreated cells. Each treatment was repeated three times, and the mean value (±SD) was plotted. (C) Wild-type (HSP82), Δhsp82, and Δhscc82 cells were irradiated with increasing doses of UV radiation. Percentages of viability were plotted as the plating efficiencies of irradiated cells relative to that of control cells. Mean values (±SD) from three independent experiments were plotted. (D) The $iG170Dhsp82$ strain (Δhsp82 Δhscc82) at a permissive (25°C) or a restrictive (37°C) temperature (duration, 2 h or 4 h) was treated with 0.03% MMS, and return-to-growth analysis was performed. A Δrad51 strain served as a negative control. (E) Percentages of surviability upon MMS treatment were plotted for the $iG170Dhsp82$ strain grown at 25°C or 37°C for 4 h. $P$ values were calculated as 0.0032 using the two-tailed Student t test. (F) $iG170Dhsp82$ cells were grown at 25°C or 37°C for 4 h and were then exposed to increasing doses of UV radiation. Percentages of viability were calculated as described above. The experiment was repeated three times, and mean values (±SD) were plotted.

FIG 1 Loss of Hsp82 function affects DSB repair activity. (A) Return-to-growth experiments comparing single mutant (Δhsp82 or Δhscc82) strains with their isogenic wild-type (HSP82) control. For each strain, the upper row represents serial dilutions of untreated cells (−) and the lower row represents serial dilutions of cells treated with 0.03% MMS (+). (B) Percentages of surviability upon MMS treatment were plotted as the plating efficiencies of MMS-treated cells relative to that of untreated cells. Each treatment was repeated three times, and the mean value (±SD) was plotted. (C) Wild-type (HSP82), Δhsp82, and Δhscc82 cells were irradiated with increasing doses of UV radiation. Percentages of viability were plotted as the plating efficiencies of irradiated cells relative to that of control cells. Mean values (±SD) from three independent experiments were plotted. (D) The $iG170Dhsp82$ strain (Δhsp82 Δhscc82) at a permissive (25°C) or a restrictive (37°C) temperature (duration, 2 h or 4 h) was treated with 0.03% MMS, and return-to-growth analysis was performed. A Δrad51 strain served as a negative control. (E) Percentages of surviability upon MMS treatment were plotted for the $iG170Dhsp82$ strain grown at 25°C or 37°C for 4 h. $P$ values were calculated as 0.0032 using the two-tailed Student t test. (F) $iG170Dhsp82$ cells were grown at 25°C or 37°C for 4 h and were then exposed to increasing doses of UV radiation. Percentages of viability were calculated as described above. The experiment was repeated three times, and mean values (±SD) were plotted.

efficiency of the $iG170Dhsp82$ temperature-sensitive mutant was drastically lower at the nonpermissive temperature (37°C) than at the permissive temperature (25°C) (Fig. 2B). In order to rule out the possibility that the reduction did not result from exposure to a high temperature per se, we measured the gene-targeting efficiency of the isogenic wild-type strain after preincubation at 37°C for 4 h. Our results showed that the high temperature (37°C) did not cause any significant reduction in gene-targeting efficiency (Fig. 2B). We sought to determine the steady-state levels of two key HR proteins, namely, Rad51p and Rad52p, in order to explore the reason behind the dramatic loss of gene-targeting efficiency in the mutant cells at the nonpermissive temperature. Figure 2C shows slight decreases in the levels of Rad51p and Rad52p upon incubation at 37°C for 4 h. On the other hand, drastic reductions in both Rad51p and Rad52p levels were observed upon overnight incubation at 37°C. However, the amounts of Rad51p and Rad52p were not reduced for the wild-type strain after 4 h of incubation at 37°C (Fig. 2C, lanes 4 and 5). Quantification of Rad51p and Rad52p levels after normalization with actin showed that there was about a 20% decrease in the Rad52p level after a 4-h incubation and that Rad52p was hardly visible after overnight incubation. Similarly, the level of Rad51p was also diminished by at least 80% after overnight incubation (Fig. 2D). In order to investigate whether the reductions in endogenous levels of Rad51p and Rad52p were occurring as part of a large transcriptional shift, we measured the transcript levels of $RAD51$ and $RAD52$ in the $iG170Dhsp82$ strain at both the permissive (25°C) and restrictive (overnight incubation at 37°C) temperatures. Our real-time RT-PCR data showed no significant change in the relative mRNA levels of the $RAD51$ or $RAD52$ transcript under those conditions (Fig. 2E). Thus, our work reveals that Hsp82 is involved in the maturation and stability of Rad52p and Rad51p.
Rad51 is a client of Hsp82. To date, there has been no report that establishes Rad51 as a client of Hsp82. One of the primary requisites for clientage is a physical interaction between Hsp82 and its client (39). A genomewide chemical genetic screen had identified Rad51 as a putative interactive partner of Hsp82 (28). However, a physical interaction between Rad51 and Hsp82 had never been established. We performed a coimmunoprecipitation experiment that showed strong association between Rad51p and Hsp82 (Fig. 3A). The fraction that was immunoprecipitated using an irrelevant antibody IgG did not cross-react with the anti-Rad51 antibody (Fig. 3A). Additionally, the anti-Hsp82 antibody did not cross-react with Rad51 on a Western blot (data not shown), suggesting that such an interaction is specific in nature. Also, the control strain lacking rad51 showed no detectable background, although Hsp82 was immunoprecipitated from the cellular extract. It had been demonstrated earlier that degradation of Hsp90 clients upon treatment with 17-AAG is mediated via proteasomal pathway (40, 41). We were interested in exploring whether Rad51p proceeded through proteasome-mediated degradation under conditions of hsp82 inhibition. For that purpose, we treated a wild-type strain with 17-AAG/H9262, which functionally inactivates HSP82. We used a pdr5 strain, since deletion of the PDR5 gene, which codes for a membrane-associated drug export pump, ensures the optimal entry of drugs (42). A fraction of that culture was supplemented with 50μM MG132 (a proteasome inhibitor) along with 17-AAG. Total protein was isolated from those strains and was probed with an anti-Rad51 antibody. Western blot analysis showed that treatment with 17-AAG led to a considerable reduction in the Rad51p level, while MG132 treatment resulted in the restoration of Rad51p abundance, indicating the inhibition of proteasomal degradation of Rad51p (Fig. 3B and C). This experiment demonstrates that under conditions of hsp82 inhibition, Rad51p is processed via proteasomal degradation, supporting the conclusion that Rad51p is a direct client of Hsp82.

**Mutational analysis of Hsp82 in DSB repair.** Six HSP82 point mutants with mutations (T22I, A41V, G81S, T101I, G313S, and T326A) were created, and their effects on DSB repair were studied. The results showed that these mutants exhibit varying degrees of sensitivity to 17-AAG, with the T22I and A41V mutants being the most sensitive. This suggests that Hsp82 plays a crucial role in DSB repair, and its inhibition leads to increased sensitivity to DNA damage. The study also highlights the importance of understanding the mechanisms of DSB repair in order to develop effective strategies for cancer treatment.
ia587T) spanning its three domains, as well as the charged linker deletion mutant HH1a-p2HG/Hsp82(Δ211-259), were monitored for DSB repair activity (Fig. 4A). The charged linker region connects the N-terminal domain with the middle domain. It was previously demonstrated that removal of the charged linker had a distinct effect on glucocorticoid receptor activity and Vsr kinase phosphorylation (20). However, neither of these two clients is naturally present in Saccharomyces cerevisiae. This particular region of Hsp82 is evolutionarily conserved in all eukaryotes. However, its importance in yeast biology has remained elusive. To determine whether the missense mutants mentioned above as well as deletion mutants were able to recover efficiently from MMS treatment, they were all treated with 0.03% MMS and were sub-

tered for DSB repair activity (Fig. 4A). Based on their DNA damage sensitivities and gene-targeting efficiencies, we categorized the mutants into three groups (Table 2). Group A consisted of the ia587T mutant, which had little effect on gene-
targeting efficiency (75% of activity retained). This correlates well
with the previous result showing a negligible effect on MMS and
UV survival (Fig. 4). Group B included the A41V, T22I, G81S, and G313S mutants, which showed moderate effects on gene-
targeting (about a 66% reduction). Group C comprised mutants that were severely defective in gene targeting. In our study, the T101I
mutant, the charged linker deletion mutant, and the Hsp82 tem-
perature-sensitive mutant at the nonpermissive temperature fall
into this category. Thus, mutational analysis revealed the specific
regions of Hsp82 that were vital for the proper functioning of
HR-mediated gene targeting. In order to understand the mechan-
ism underlying the loss of HR function in these mutants, we

determined the endogenous levels of Rad51p and Rad52p in the
HR-dead T101I and hsp82(Δ211-259) mutants. To this end, for the
detection of Rad52p, we attempted to tag the C-terminal end of
RAD52 in these strains. Since the charged linker mutant is de-
fective in gene targeting, we swapped the hsp82(Δ211-259) plas-
mid in the hsp82 T101I point mutant (which was RAD52-MYC
tagged at the chromosomal locus) to create the RAD52-MYC-
tagged hsp82(Δ211-259) strain SLY65. Western blot analysis
showed that the levels of Rad51p and Rad52p were significantly
reduced in the T101I mutant, indicating the importance of
the ATPase domain in the maturation of Rad51p and Rad52p (Fig.
5C). This conclusion was further supported by real-time RT-PCR
analysis, which showed no change in the steady-state levels of
RAD51 and RAD52 transcripts in the T101I mutant background
(Fig. 5D). Additionally, we found that the levels of Rad51p and
Rad52p were unaltered in the hsp82(Δ211-259) mutant (Fig. 5E
and F). Quantification of band intensities showed a 4-fold reduc-
in the Rad51p level and a 2-fold reduction in the Rad52p level
in the T101I mutant, whereas the levels of these proteins in the
hsp82(Δ211-259) mutant remained unaltered (Fig. 5F). Taken

together, the loss of gene-targeting efficiency in the ATPase-dead
mutant can be correlated with the lower endogenous levels of
Rad51p and Rad52p. On the other hand, the apparent lack of

FIG 3 Rad51p is a client of Hsp82. (A) Western blot showing coimmunopre-
cipitation of Hsp82p and Rad51p from whole-cell extracts of a wild-type strain
(RAD51) and a strain lacking Rad51p (Δrad51). Immunoprecipitation (IP) was
performed using an anti-Hsp82 antibody or an irrelevant antibody (IgG).
An anti Rad51 antibody was used for Western blotting. Input, whole-cell ex-
tract; SN, supernatant; IP, pellet. (B) Western blot showing Rad51p levels in
wild-type cells (lane 1), in cells treated with 40 μM 17-AAG for 16 h (lane 2),
and in cells treated with 40 μM 17-AAG and 50 μM MG132 for 16 h (lane 3).
Actin is shown as a loading control. (C) Relative intensities of Rad51p bands in
the Western blot. Bar numbers correspond to lane numbers in panel B.

question: What are the ATPase domain point mutants discussed in the text? The ATPase domain point mutants discussed are A41V, G81S, and T101I. These mutants showed dramatic reductions in cell viability upon MMS treatment, indicating the importance of the ATPase domain in DNA repair.
correlation between HR function and the abundance of recombination proteins in the charged linker deletion mutant implies a complex interplay between the chaperone and these clients.

MMS-induced upregulation of Rad51p and Rad52p in the hsp82(Δ211-259) mutant. To understand the mechanism behind the drastic reduction in HR efficiency in the charged linker deletion mutant, we studied the MMS-induced upregulation of Rad51p and Rad52p. Wild-type and mutant strain were grown to an OD of 0.6 and were then separated into two groups, one of which was grown in the presence of MMS while the other was grown in its absence, and the Rad51p and Rad52p levels were estimated. Western blotting showed that the induction of Rad51p and Rad52p in the mutant strain was similar to that observed in the wild-type strain (Fig. 6A). Quantification of Rad51p from three independent harvests of cells showed a ~4-fold induction of Rad51p in the wild type and a ~2-fold induction in the charged linker deletion mutant (Fig. 6B). We wanted to establish further that there is no difference in the transcriptional upregulation of RAD51 and RAD52 in the mutant upon MMS treatment. For that purpose, we isolated the total RNAs of the wild-type and mutant strains that had been left untreated or treated with MMS, and we measured the relative abundances of the RAD51 and RAD52 transcripts. Semiquantitative RT-PCR showed upregulation of RAD51 and RAD52 upon MMS treatment in both strains (Fig. 6C). Real-time RT-PCR quantification showed almost equal induction (4.7-fold) of RAD51 and RAD52 transcripts (2-fold) in MMS-treated and untreated samples in the wild type as well as in the hsp82(Δ211-259) mutant (Fig. 6D and E). Thus, our results revealed that the MMS-induced transcriptional upregulation of RAD51 and RAD52 was unaltered in the charged linker deletion mutant.

The extent of MMS-induced Rad51 focus formation is less in hsp82(Δ211-259) cells than in the wild-type control.
induced Rad51p upregulation is unaffected in the hsp82 mutant, we sought to investigate whether the downstream function of Rad51 is affected in this mutant strain. For that purpose, we used an indirect immunofluorescence assay to investigate the ability to form MMS-induced Rad51 foci. We observed very bright fluorescent foci that were enriched with Rad51p in MMS-treated cells but not in untreated cells (Fig. 7A). We counted about 1,500 nuclei in each of the three independent harvests of cells in order to calculate the percentage of nuclei that had Rad51 foci. Our results demonstrated a 20% reduction in Rad51 focus formation in hsp82 cells from that in wild-type cells upon DNA damage (Fig. 7B). Next, we analyzed the distribution of foci in each nucleus. After analyzing a total of 4,500 MMS-treated nuclei of wild-type and mutant cells, we observed a striking, statistically significant difference in the distribution of foci. Our analysis revealed that mutant cells possessed primarily 1 focus per nucleus, and the total number was comparable to that for wild-type cells. However, the percentage of nuclei containing more than 1 focus was drastically lower in mutant cells than in wild-type cells. Our study showed 33% fewer nuclei with 2 foci, 42% fewer nuclei with 3 foci, and 77% fewer nuclei with 4 foci in mutant cells than in wild-type cells (Fig. 7C). Thus, we came to the conclusion that in the charged linker deletion mutant, the level of Rad51 focus formation, which is a prerequisite for repairing breaks in DNA, is significantly lower than that in the wild type, leading to greater MMS sensitivity.

TABLE 2 Effects of hsp82 mutants on DNA damage sensitivity and gene-targeting efficiency

| Group | hsp82 allele | Sensitivity* to: | Gene-targeting efficiency |
|-------|--------------|------------------|--------------------------|
|       |              | MMS              | UV                       |
| A     | hsp82 ts587T | +                | +                        | Little effect |
| B     | hsp82 T22I   | ++               | ++                       | Moderate effect |
|       | hsp82 A41V   |                  |                          |                |
|       | hsp82 G81S   |                  |                          |                |
|       | hsp82 G313S  |                  |                          |                |
| C     | hsp82(Δ211-259) | +++              | +++                      | Severe effect |
|       | iG170Dhsp82  |                  |                          |                |
|       | hsp82 T101I  |                  |                          |                |

*+, slightly sensitive; ++, moderately sensitive; ++++, highly sensitive.

FIG 5 Gene-targeting efficiencies of hsp82 point mutants and charged linker deletion mutant. (A) Percentages of gene-targeting efficiency of six hsp82 point mutants and their wild-type control. The gene-targeting efficiency of the hsp82 T101I mutant is about 16-fold lower than that of the wild type strain P82a (HSP82). Error bars indicate SD; n = 3. P values were calculated using the two-tailed Student t test (**, P < 0.01; *, P < 0.05). (B) Gene-targeting efficiency of the hsp82(Δ211-259) strain compared to that of its isogenic wild-type control. Error bars indicate SD; n = 3. P values were calculated as 0.0010 (***). (C) Western blots showing reductions in endogenous levels of Rad51p and Rad52p in the hsp82 T101I mutant. Actin acts as the loading control. WT, wild type (HSP82). (D) Real-time RT-PCR shows the relative mRNA levels of RAD51 and RAD52 in the hsp82 T101I mutant and its isogenic WT control. (E) Immunoblot showing no difference in the endogenous levels of Rad51p and Rad52p between the wild-type strain (HH1a-p2HG/Hsp82) and the charged linker deletion mutant [HH1a-p2HG/Hsp82(Δ211-259)]. (F) Quantification of Western blots from three independent experiments for which results are shown in panels C and E. A 4-fold reduction in Rad51p levels and a 2-fold reduction in Rad52p levels were observed for the hsp82 T101I mutant. The band intensities in each lane were normalized against that of actin, and the mean densities ± SD are plotted.
Overexpression of Rad51 rescues MMS sensitivity and the defect in gene-targeting efficiency in the hsp82(Δ211-259) mutant. From Western blot analysis, it was apparent that the amount of Rad51p in the charged linker deletion mutant was similar to that in the wild type. However, the amount of active Rad51p may be limited, leading to decreased gene-targeting efficiency and increased MMS sensitivity. To establish that further, we overexpressed Rad51p in the mutant strain and investigated whether it could rescue MMS hypersensitivity and overcome the defect in gene-targeting efficiency. To this end, we transformed a 2μ expression vector harboring S. cerevisiae RAD51 into the hsp82(Δ211-259) and iG170Dhsp82 strains to generate strains TSY1 and SLY69, respectively. We exposed the cells to 0.03% MMS for 2 h and calculated the percentages of cell viability. We compared the viability of the strains with and without Rad51p overexpression (Fig. 8A) and observed a significant difference between the two alleles. In the iG170Dhsp82 strain grown at 37°C, overexpression of Rad51p could not rescue MMS sensitivity, as evidenced by the fact that the temperature-sensitive mutant at the restrictive temperature was unable to chaperone Rad51p folding. However, in the hsp82(Δ211-259) strain, overexpression of Rad51p can partially rescue MMS sensitivity. Our results showed that the percentage of cell viability increased 5-fold with Rad51p overexpression, but it was still 20% less than that of the wild type. In another assay, we studied the gene-targeting efficiency of mutant cells carrying pRAD51 and compared it with that of mutant cells carrying an empty vector. We observed that Rad51p overexpression resulted in ~2.3-fold increased gene-targeting efficiency in the hsp82(Δ211-259) strain; however, this level of efficiency was still significantly less than that of the wild type (Fig. 8B). Western blotting confirmed the overexpression of Rad51p in these mutant strains (Fig. 8C).

DISCUSSION

Our paper presents detailed work that helps in understanding whether the Hsp90 chaperone is responsible for the proper functioning of the DSB repair pathway in lower eukaryotes. Since these are unicellular organisms, failure to repair damaged DNA is lethal for them. Using various mutants with alterations throughout different domains of Hsp82, we have demonstrated that Hsp82 function is indispensable for the proper functioning of the HR pathway.

Although previous studies have suggested a possible link between Hsp90 and DSB repair, the involvement of Hsp90 in HR has remained inconclusive due to conflicting findings. First, for tumor cells, it has been reported that Brca2p and Rad51p are destabilized upon prolonged incubation with the Hsp90 inhibitor 17-AAG. However, in normal cells, Hsp90 inhibitors do not affect survivability under DNA-damaging conditions (32). Thus, it is not clear whether the phenotype observed in tumor cell lines is a direct or
an indirect effect of Hsp90. Second, although a high-throughput in vitro study (with tandem affinity purification tagging) revealed an interaction between the Hsc82 N-terminal domain and Rad52p (27), no direct interaction of Rad51p with Hsp82 has been reported. Thus, it has remained ambiguous whether Rad51p is a physiological client of Hsp90. We report here, for the first time, evidence of physical interaction between Rad51p and Hsp82p. Our work establishes that under conditions of Hsp82 inhibition, Rad51p levels are drastically reduced in cells via proteasomal degradation. Also, in the iG170Dhsp82 strain (upon prolonged incubation at a restrictive temperature), the steady-state levels of both Rad51p and Rad52p proteins decrease dramatically, with substantial loss in gene-targeting efficiency. However, there is no change in the transcript levels of RAD51 and RAD52 in the iG170Dhsp82 strain. Taken together, these findings establish Rad51p as a client of Hsp82.

We performed detailed mutational analyses of several isogenic mutants of hsp82 to monitor survivability upon MMS treatment as a readout of repair activity. Our observations are that the Hsp82 temperature-sensitive mutant is hypersensitive to DNA damage at a nonpermissive temperature, while there is no significant reduction in cell survivability in either of the single knockout cells (the Δhsp82 or Δhsc82 mutant), implying that at least one copy of this chaperone is needed for survival under conditions of DNA damage. Our study reveals that most of the N-terminal domain mutants display severe defects in repairing MMS- and UV-induced DNA damage, suggesting the importance of the ATP hydrolysis function of Hsp82. The differential effects of many mutations across the three domains of Hsp82 suggest that in addition to the ATPase function, other functional domains of Hsp82 also contribute to the maturation/stability of DNA repair proteins. This claim is corroborated by the fact that the Hsp90-inactivated mutant (the iG170Dhsp82 strain at 37°C) shows a more dramatic phenotype than any of the point mutants. Since MMS sensitivity cannot be directly correlated with HR efficiency, we have measured the gene-targeting efficiencies of all the mutants. The results

FIG 7 The extent of MMS-induced Rad51 focus formation is less in hsp82(Δ211-259) cells than in the wild-type control. (A) Wild-type (HH1a-p2HG/Hsp82) and mutant [HH1a-p2HG/Hsp82(Δ211-259)] cells were treated with 0.03% MMS, and an indirect immunofluorescence assay was performed with anti-Rad51 to locate Rad51 foci. Nuclei were stained with DAPI. The arrows indicate Rad51 foci. The experiment was repeated three times, and representative data from one replicate are presented. (B) About 1,500 nuclei in each case were analyzed for the presence of Rad51 foci, and the percentages of nuclei with Rad51 foci were calculated for mutant and wild-type cells. The experiment was performed with three independent harvests of cells. The P value was calculated as 0.0103 using the two-tailed Student t test. (C) ImageJ software was used to analyze the image of each nucleus and to count the number of foci in it. A total of 4,500 wild-type and 4,500 mutant nuclei with 1, 2, 3, and 4 foci were counted, and the percentages of nuclei having 1, 2, 3, and 4 Rad51 foci were plotted for wild-type and mutant cells. An unpaired t test was performed using GraphPad Prism software, version 6. P values are indicated.
from the gene-targeting assay correspond well with the results from the DNA repair assay. Strikingly, three mutants exhibit severe reductions in HR-mediated gene targeting. One of them is the temperature-sensitive iG170Dhsp82 mutant, which was earlier reported to be nonfunctional at a restrictive temperature (35). The other two are the T101I and p2HG/Hsp82(Δ211-259) mutants. Previous work revealed that the hsp82 T101I mutation causes a 90% reduction in ATPase activity, whereas the iA587T mutant possesses wild-type-like ATPase activity (43). Our work implies that the T101I point mutation may affect the structural stability of Rad51p and Rad52p relative to that for the wild type and that this defect in stability is correlated with a lessening of HR efficiency. Thus, it is tempting to suggest that the ATP hydrolysis activity of Hsp82 is essential for Rad51p stability. On the other hand, the iA587T mutant, which possesses wild-type-like ATPase activity and AMP-PNP (adenylylimidodiphosphate) binding (43), exhibits wild-type-like sensitivity to MMS and has a marginal effect on gene-targeting efficiency. The other ATPase domain mutants, such as the A41V, T22I, and G81S mutants, also show moderate effects on gene-targeting efficiency. It was demonstrated previously that though the T22I mutant possesses 6 times more ATPase activity than the wild type, its affinity for AMP-PNP is less than that of the wild type. On the other hand, the A41V point mutant, with a mutation located at the rear end of the nucleotide binding pocket, also shows reduced affinity for AMP-PNP, which accounts for its moderate phenotype in HR efficiency.

The hsp82(Δ211-259) charged linker mutant presents an interesting scenario. It is the only mutant that shows a 8-fold reduction in cell survivability upon MMS treatment as well as a >80% reduction in gene-targeting efficiency even though the levels of Rad51p and Rad52p remain unchanged. This mutant is so severely defective in HR function that it remains refractory to gene tagging and gene knockout despite several attempts. Previously, it was reported that the hsp82(Δ211-259) mutant did not affect nucleotide binding to the N-terminal domain of Hsp90 and that its ATPase activity was also slightly higher (0.6 min⁻¹) than that of the wild type (0.5 min⁻¹) (20). Our work indicates that though the structural stabilities of Rad51p and Rad52p in this mutant probably remain unaltered, Rad51p-mediated gene-targeting activity is drastically reduced. To account for this discrepancy, we propose that the ATPase activity of Hsp82p is necessary but not sufficient for the maturation of active Rad51p. Our hypothesis is supported by the significant reduction in the number of nuclei with multiple Rad51 foci, which accounts for the enormous reduction in cell viability. Thus, it is likely that in the absence of the charged linker region, only a fraction of the total Rad51p is active. We propose that a dynamic equilibrium might exist between the active and inactive forms of Rad51p in the cell. In the charged linker deletion mutant, the equilibrium is probably shifted toward the inactive state of Rad51p. Based on this hypothesis, one may predict that if the amount of Rad51p were increased artificially, the abundance of active Rad51p would increase, and this might rescue the phenotype. Our work showing a partial rescue of MMS sensitivity and gene-targeting efficiency supports this hypothesis.

Earlier work also showed that the charged linker deletion mutant possessed diminished chaperone function toward Hsp90 clients such as glucocorticoid receptor (GR) and vSrc, although their structural stability was unaltered (20). However, none of the clients documented above are required for S. cerevisiae biology. Although the charged linker region (amino acids 211 to 259) is evolutionarily conserved, the significance of such sequence conservation was not known. Previously, this region was reported to be dispensable for steroid hormone receptor regulation and the phenome signaling pathway (19). Our work, for the first time, assigns a functional role in DSB repair to this charged linker region. Unlike steroid hormone signaling or phenome signaling,
which is restricted to certain organisms, DSBR repair pathways are conserved in all organisms. Thus, our finding that this evolutionarily conserved region of Hsp90 has an important role in a highly conserved cellular process such as DSBR repair makes perfect sense. Although yeast Hsp82 and its mammalian counterpart Hsp90 are widely viewed as cytoplasmic chaperones, more and more nuclear functions of Hsp82 have emerged in recent years. Its pivotal role in telomere capping and maintenance has been established (44). Recently, work on Sir2 protein dynamics as a function of Hsp82 activity has recognized a link between Hsp82 and telomere silencing (38). Two recent studies on Sba1 and Aha1, two important cochaperones of Hsp82, have unraveled an interesting connection with DNA repair proteins. In the first study, a high-throughput synthetic gene array (SGA) and network analysis revealed 11 DNA repair proteins (including Rad50) as putative clients of Sba1 (the yeast ortholog of p23). Overexpression of Sba1 has also been shown to enhance DNA repair activity (45). In another study, two major NHEJ proteins, Ku80 and DNA-PKcs, have been found to be associated with human Aha1 (46). Our current work, along the same line, establishes a compelling link between the Hsp82 chaperone and HR-mediated DNA repair. Thus, uncovering such a nuclear clientele of Hsp82 establishes new and distinct functions of the multifaceted chaperone Hsp82.

ACKNOWLEDGMENTS

We are indebted to Didier Picard (University of Geneva) and Susan Lindquist (MIT) for the DP533 and hsp82 mutant strains. We thank Meenu Babu and Meera Babu for critical reading of the manuscript. This work is partly supported by grants from the Department of Biotechnology, Government of India (BT/PR11174/MED/29/98/2008), to M.K.B. and from the Council of Scientific and Industrial Research, Government of India [37(1549)/12/EMR-II], to S.B.

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