Roles of Rad23 protein in yeast nucleotide excision repair

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ABSTRACT

Nucleotide excision repair (NER) removes many different types of DNA lesions. Most NER proteins are indispensable for repair. In contrast, the yeast Rad23 represents a class of accessory NER proteins, without which NER activity is reduced but not eliminated. In mammals, the complex of HR23B (Rad23 homolog) and XPC (yeast Rad4 homolog) has been suggested to function in the damage recognition step of NER. However, the precise function of Rad23 or HR23B in NER remains unknown. Recently, it was suggested that the primary function of RAD23 protein in NER is its stabilization of XPC protein. Here, we tested the significance of Rad23-mediated Rad4 stabilization in NER, and analyzed the repair and biochemical activities of purified yeast Rad23 protein. Cellular Rad4 was indeed stabilized by Rad23 in the absence of DNA damage. Persistent overexpression of Rad4 in rad23 mutant cells, however, largely failed to complement the ultraviolet sensitivity of the mutant. Consistently, deficient NER in rad23 mutant cell extracts could not be complemented by purified Rad4 protein in vitro. In contrast, partial complementation was observed with purified Rad23 protein. Specific complementation to the level of wild-type repair was achieved by adding purified Rad23 together with small amounts of Rad4 protein to rad23 mutant cell extracts. Purified Rad23 protein was unable to bind to DNA, but stimulated the binding activity of purified Rad4 protein to N-acetyl-2-aminofluorene-damaged DNA. These results support two roles of Rad23 protein in NER: (i) its direct participation in the repair biochemistry, possibly due to its stimulatory activity on Rad4-mediated damage binding/recognition; and (ii) its stabilization of cellular Rad4 protein.

INTRODUCTION

DNA is frequently damaged by many environmental agents. Nucleotide excision repair (NER) is an important mechanism for removing a wide spectrum of different DNA lesions. Many bulky DNA adducts are specifically repaired by NER in eukaryotes, such as N-acetyl-2-aminofluorene (AAF) adducts, cisplatin intra-strand crosslinks, polycyclic aromatic hydrocarbon adducts, and the major ultraviolet (UV) lesions cyclobutane pyrimidine dimers (CPDs) and (6-4) photoproducts (1–7). Therefore, NER constitutes a crucial defense mechanism against DNA damage-induced cytotoxicity, mutagenesis and carcinogenesis. Compromising NER activity will lead to increased cellular sensitivity to DNA-damaging agents, increased mutation frequency and a high risk of carcinogenesis, as exhibited by the human hereditary disease xeroderma pigmentosum (XP) (8).

Cellular NER consists of two subpathways: global genome repair and transcription-coupled repair (9,10). Transcription-coupled NER is specific to the repair of actively transcribed genes and specifically involves at least RNA polymerase II, CSA and CSB. On the other hand, global genome NER is responsible for the repair of the untranscribed regions of the genome and the untranscribed strand of an actively expressed protein-coding gene. Conceptually, NER can be divided into five distinct biochemical steps: damage recognition, incision, excision, repair synthesis and DNA ligation. In vivo genetics and in vitro biochemistry with cell-free repair systems and purified NER proteins have contributed a great deal to the understanding of eukaryotic NER. In vitro NER systems established to date in eukaryotes specifically reflect the global genome repair subpathway (1,5,11–13). Two categories of NER proteins are known. Most of the NER proteins are indispensable for repair, such as the repair/transcription factor TFIIH, and the yeast Rad4 and Rad14, which corresponds to the human XPC and XPA, respectively. The second category of NER proteins plays accessory roles in repair, without which cells exhibit moderate rather than severe sensitivity to DNA-damaging agents. Rad23 is such an accessory NER protein in yeast. Its mammalian homologs are HR23A and HR23B (14). Rad23 (HR23) strongly interacts with Rad4 (XPC) (14–16). Some studies have suggested that the XPC/HR23B complex functions in the damage recognition step of NER (17,18). However, the role of HR23B in the complex-mediated damage binding/recognition is not known.

Yeast rad23 deletion mutant cells are moderately sensitive to DNA-damaging agents (19). Both transcription-coupled and global genomic NER in rad23 mutant cells are significantly reduced, but not totally abolished (20). Consistently, in vitro NER in rad23 deletion mutant extracts is also deficient (15). Clearly, proficient NER requires Rad23 function. Rad23 protein contains multiple functional domains: an N-terminal ubiquitin-like (UBL) domain, a Rad4-interaction domain and two ubiquitin-associated (UBA) domains (21–23). The

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UBA domains are not required for NER activity of Rad23 (23). Thus, in addition to its role in NER, this protein most likely plays other functions in cells, such as the suggested cell cycle progression (24,25). Consistent with multi-functionality of RAD23, knockout mice deleted of both the HR23A and the HR23B genes lead to embryonic lethality (26).

It has been shown that Rad23 interacts with the 26S proteasome and the UBL domain is required for this interaction (27,28). The 26S proteasome, consisting of a 20S core particle and two copies of a 19S regulatory complex, is a large protein complex involved in the degradation of proteins targeted by the ubiquitin pathway (29). It was further shown that the 19S regulatory complex negatively modulates NER in yeast cells (30,31). More recently, Lommel et al. (32) found that Rad4 protein transiently accumulates following DNA damage and that Rad23 enhances Rad4 stability following UV irradiation of yeast cells. It was suggested that Rad23 protects Rad4 from degradation by the proteasome in cells (32). A similar XPC stabilization effect by HR23A/B was reported by Ng et al. (26) most recently in cultured mouse cells even without UV radiation. These observations led Ng et al. (26) to conclude that the primary function of RAD23 protein in NER is its stabilization of XPC protein.

To better understand the role of Rad23 protein in NER, we have used the yeast model system to test the significance of Rad23-mediated Rad4 stabilization in NER and further analyzed the repair and biochemical activities of purified Rad23 protein. In this report, we show that (i) decreased Rad4 stability is not the major cause of deficient NER in rad23 mutant cells; (ii) Rad23 protein directly participates in NER; and (iii) Rad23 protein stimulates the binding activity of Rad4 on damaged DNA. These results support two roles of Rad23 protein in NER: its direct participation in the repair biochemistry, possibly due to its stimulatory activity on Rad4-mediated damage binding/recognition; and its stabilization of cellular Rad4 protein.

**MATERIALS AND METHODS**

**Materials**

Purified yeast Rad2 protein was obtained from Enzymax (Lexington, KY), which was purified from *Escherichia coli* cells overexpressing the yeast RAD2 gene. The *Pfu* DNA polymerase was from Stratagene (La Jolla, CA). Protease inhibitors, alkaline phosphatase-conjugated anti-mouse immunoglobulin G (IgG), alkaline phosphatase-conjugated anti-rat IgG, 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium were obtained from Sigma Chemicals (St. Louis, MO). A mouse monoclonal antibody against the His6 tag was purchased from Qiagen (Valencia, CA). A rat monoclonal anti-HA antibody was from Boehringer Mannheim (Indianapolis, IN). *N*-acetoxy-2-acetyl-aminofluorene (AAAF, the activated form of AAF) was obtained from the Midwest Research Institute (Kansas City, MO). All oligonucleotides were synthesized by Operon (Alameda, CA).

**Strains**

The yeast *Saccharomyces cerevisiae* wild-type strains used were SX46A (12) and W303-1B (15). The yeast *S.cerevisiae* mutant strains used were BJ2168Arad2 (MATa RAD2::URA3 leu2 trpl ura3-52 pep4-3 prbl-1122 prc1-407), SX46AArad4 (MATa RAD4::URA3 ade2 his3-52 ura3-52 trpl-289), SX46Aarad14 (15) and MGSC101 (rad23A::URA3) (15). While SX46Arad4 and SX46Aarad14 are isogenic to SX46A, MGSC101 is isogenic to W303-1B.

**Plasmid constructions**

Yeast expression plasmid vectors pEGH6, pEGTh6 and pEGLha were derived from Yeplac195, Yeplac112 and Yeplac181 (33), respectively. All three vectors contained the 2 μm origin for multi-copy plasmid replication and the yeast-inducible GAL1/10 promoter. While both pEGH6 and pEGLh6 contained 6 His codons for protein N-terminal tagging, they contained the yeast URA3 gene and the yeast TRP1 gene, respectively, for plasmid selection. A sequence coding for the HA tag at the protein N-terminus and the yeast LEU2 gene for plasmid selection were contained in pEGLha. The yeast *RAD4* gene was cloned into the BamHI and PstI sites of the vector pEGTh6, yielding pEGTh6-RAD4. The yeast *RAD4* gene was amplified from yeast DNA by PCR with the *Pfu* DNA polymerase, using the primers 5′-CGGGATC- CATTGATGCTAATTAAAAATTTC-3′ and 5′-ACCGTCGACATCAAACCTGTGAAA-3′. The resulting 1.3 kb DNA fragment was cleaved with BamHI and SalI restriction endonucleases and cloned into the corresponding sites of pEGUb6 and pEGLha, generating pEGUb6-RAD23 and pEGLha-RAD23, respectively. The yeast *RAD4* gene was amplified from yeast DNA by PCR with the *Pfu* DNA polymerase, using the primers 5′-GAAGATCTGATGCAGAGGCCC- CATGTGGCC-3′ and 5′-GAACGTCAAGAGGGCCC- CATGTGGCC-3′. The resulting 1 kb DNA fragment was cleaved with BglII and PstI restriction endonucleases and cloned into the BamHI and PstI sites of pEGLh6, yielding pEGTh6-RAD14.

**Damaged DNA**

To prepare pUC18 DNA containing AAF adducts, the plasmid (100 μg) was incubated at 37°C for 3 h in 1 ml of TE buffer (10 mM Tris–HCl, pH 7.5 and 1 mM EDTA) containing 3 μM AAF and 20% ethanol. After adding NaCl to 0.5 M, the modified DNA was then purified by 5–20% sucrose gradient centrifugation at 28 000 r.p.m. for 17 h at 4°C in a Beckman SW41Ti rotor. Fractions of 0.5 ml each were collected from the bottom of the gradient and 5 μl aliquots were analyzed by electrophoresis on a 1% agarose gel to locate the DNA. Fractions containing supercoiled DNA were pooled, precipitated in ethanol, dissolved in TE buffer and stored at −20°C. To prepare AAF-adducted oligonucleotide DNA, 2 nmol of the 79mer oligonucleotide 5′-GGAATTCGCGAATTACA- GGCTCTAACCGAATTCGCTGTCGCCCATGGC-3′ was incubated with 200 nmol of N-AAAF at 37°C in the dark for 3 h in 100 μl of TE buffer containing 20% ethanol. Free AAF was removed by extracting the reaction mixture five times with water-saturated ether. Then, damaged and undamaged oligonucleotides were separated by electrophoresis on a 20% denaturing polyacrylamide gel. AAF-modified DNA migrated slower on the gel and was sliced out of the gel. The gel slices were soaked in 150 μl water at room temperature for 4 h.
Finally, AAF-damaged DNA was recovered using GenElute DNA spin column (Sigma). To obtain duplex oligonucleotide, equal molar amounts of undamaged or the AAF-damaged 79mer oligonucleotide and its 79mer complementary strand were mixed and annealed by incubating for 5 min at 85°C in TES (10 mM Tris–HCl, pH 7.5, 1 mM EDTA and 100 mM NaCl) buffer followed by cooling slowly to room temperature.

**Preparation of yeast cell-free extracts**

Yeast cell-free extracts used for *in vitro* NER were prepared according to our previously described method (12). Yeast whole cell extracts containing overexpressed Rad4 and/or Rad23 were prepared as follows. Yeast SX46Δrad4 cells containing pEgTh6-RAD23, or rad23 mutant cells (MGSC101) containing pEgTh6-RAD4 alone or both pEgTh6-RAD4 and pEGLha-RAD23 were grown at 30°C overnight in minimum medium containing 2% sucrose and the required amino acids at 30°C. The remaining culture was added to 100 ml of YP (2% Bacto-peptone, 1% yeast extract) medium supplemented with 2% galactose and 0.5% sucrose. After growing for 13 h at 30°C, the cells were collected by centrifugation and stored at 4°C.

**Analysis of Rad4 protein stability in yeast cells**

Yeast rad23 mutant cells (MGSC101) containing pEgTh6-RAD4 or both pEgTh6-RAD4 and pEGLha-RAD23 were grown in minimum medium containing 2% sucrose and the required amino acids at 30°C overnight. At an OD₆₀₀ of ~1, protein expression from the plasmids was induced by diluting the culture 10-fold in 100 ml of YP (2% Bacto-peptone, 1% yeast extract) medium supplemented with 2% galactose and 0.5% sucrose. After growing for 13 h at 30°C, the cells were collected by centrifugation and were resuspended in 800 μl of a buffer containing 20 mM Heps-KOH, pH 7.6, 10 mM MgSO₄, 10 mM EGTA, 20% glycerol, 5 mM DTT and protease inhibitors (34). The cells were homogenized at 4°C with Zirconium beads in a mini-Beadbeater, using 4 pulses of 1 min each with 2 min pause between pulses. Clear whole cell extracts were obtained after centrifugation at 14,000 r.p.m. for 15 min at 4°C in a microcentrifuge.

**Measurement of UV sensitivity**

Plasmids pEgTh6-RAD4, pEGLha-RAD23, pEgTh6-RAD14 and the vector pEgTh6 were used to individually transform various yeast strains as indicated. Yeast cells were grown at 30°C in minimum medium containing 2% sucrose and the required amino acids at 30°C. Protein expression from the plasmids was induced by diluting the culture 10-fold in 50 ml of YP medium supplemented with 2% galactose and 0.5% sucrose (YPg medium). After growing for 13 h at 30°C, the cells were collected by centrifugation. Appropriately diluted cells were plated onto YPG plates, and the uncovered plates were irradiated with short wave UV light from a germicidal UV lamp at the indicated doses. Surviving colonies were scored after 3–4 days of incubation in the dark at 30°C. UV survival was calculated by dividing surviving colonies after UV treatment by those without UV treatment.

**In vitro NER**

*In vitro* NER was performed as described by Wang et al. (1,12,35). Briefly, a standard NER reaction mixture (50 μl) contained 200 ng each of damaged pUC18 DNA and undamaged pGEM3Zf DNA, 45 mM Hepes-KOH (pH 7.8), 7.4 mM MgCl₂, 0.9 mM DTT, 0.4 mM EDTA, 2 mM ATP, 20 μM each dATP, dGTP, and dTTP, 4 μM dCTP, 1 μCi of [α-³²P]dCTP (3000 Ci/mmol), 40 mM phosphocreatine (disodium salt, 2.5 μg of creatine phosphokinase, 4% glycerol, 100 μg/ml BSA, 5% polyethylene glycol 8000 and 250 μg of yeast cell-free extracts. After incubation at 26°C for 2 h, EDTA and RNase A were added to 20 mM and 20 μg/ml, respectively, and incubated at 37°C for 10 min. SDS and protease inhibitors K were added to 0.5% and 200 μg/ml, respectively, and incubated at 37°C for 30 min. Plasmid DNA was purified by phenol and then by chloroform extractions, and linearized with the HindIII restriction endonuclease. DNA was separated by electrophoresis on a 1% agarose gel and repair synthesis was visualized by autoradiography.

**Electrophoretic mobility shift assay (EMSA)**

First, the AAF-damaged 79mer oligonucleotide was 5’-labeled with T4 polynucleotide kinase and [γ-³²P]ATP (3000 Ci/mmol). The labeled oligonucleotide was then mixed with its complementary strand (79mer) in equal molar amounts and annealed to form duplex DNA as described above. A standard DNA binding reaction mixture (10 μl) contained 10 fmol of the ³²P-labeled and damaged duplex DNA, various amounts of unlabeled competitor DNA as indicated, 20 mM Tris–HCl, pH 7.5, 5 mM MgCl₂, 1 mM EDTA, 100 μg/ml BSA, and purified yeast Rad4 or Rad23 protein or both as indicated. After incubation at 30°C for 30 min, 2 μl of a loading dye was added, which contained 100 mM Tris–HCl, pH 7.5, 0.05% bromophenol blue and 50% glycerol. DNA was separated by electrophoresis on a 4.5% non-denaturing polyacrylamide gel at 4°C and visualized by a phosphorImager.

**Protein purification**

Yeast SX46Δrad4 cells containing pEgTh6-RAD4 or pEg Uh6-RAD23 were grown in minimum medium containing 2% sucrose and the required amino acids at 30°C for 2 days. Overexpression of the protein was induced by diluting the culture 10-fold in 16 l of YP medium supplemented with 2% galactose and 0.5% sucrose. After growing for 12 h at 30°C, the cells were collected by centrifugation and washed with buffer. Then, the cells were resuspended in an extraction buffer containing 50 mM Tris–HCl, pH 7.5, 1 M KCl, 10% sucrose, 5 mM β-mercaptoethanol and protease inhibitors (34), and were homogenized on ice with Zirconium beads in a Beadbeater for 15 pulses of 30 s each with 2 min pause between pulses. After centrifugation at 33,000 r.p.m. for 2 h at 4°C in a Beckman type 50.2Ti rotor, the clarified extract (~100 ml) was loaded onto a HiTrap chelating column.
and the HA antibody did not show non-specific cross-reaction against the HA-tagged Rad23 and the His6-tagged Rad4, respectively (data not shown). Lane 5, protein size markers. In separate experiments, the His6 antibody was used to examine the Rad4 and Rad23 proteins in the whole cell extracts by western blot analysis as in (A). Lane 1, protein size markers.

RESULTS

Rad4 protein is stabilized by Rad23 in yeast cells

It was reported that yeast Rad4 protein is unstable in rad23 mutant cells following UV radiation (32). To determine whether Rad23 is able to stabilize Rad4 protein in the absence of DNA damage, we examined the stability of Rad4 under normal growth conditions. The rad23 deletion mutant cells were transformed with either a single plasmid expressing Rad4 or two plasmids expressing Rad4 and Rad23, respectively. To facilitate protein detection, Rad4 was tagged with His6 and Rad23 was tagged with the HA epitope at the protein N-terminus. Both RAD4 and RAD23 genes were under the control of the yeast GAL1/10 promoter. Gene expression from this promoter is suppressed by 2% glucose but induced by 2% galactose in the growth medium. Without induction, Rad4 and Rad23 expression from the plasmids were undetectable by western blot analysis in cell extracts (Figure 1A, lanes 1 and 3). Following galactose induction of the yeast culture, both Rad4 and Rad23 proteins were readily detected (Figure 1A, lanes 2 and 4). The steady state level of Rad4, which reflects the balance between protein synthesis and protein degradation, was slightly lower in the absence of Rad23 (compare Figure 1A, lanes 2 and 4). To directly examine the stability of Rad4 protein in yeast cells, we first induced gene expression with galactose. Then, Rad4 expression or Rad4 and Rad23 expressions were turned off by transferring yeast cells back to the medium without galactose but containing 2% glucose. Aliquots of the culture were removed at various times for cell extract preparation. Finally, Rad4 protein in the cell extracts were determined by western blot analysis. As shown in Figure 1B (lanes 2–7), in the presence of Rad23 protein, ~50% of Rad4 remained 5 h after turning off the gene expression from the GAL1/10 promoter. In contrast, Rad4 protein became non-detectable 3 h after turning off the gene expression from the GAL1/10 promoter in rad23 mutant cells (Figure 1B, lanes 8–13). These results show that Rad23 stabilizes Rad4 in yeast cells. This Rad4 stabilization by Rad23 does not require exogenously induced DNA damage.

Since Rad4 forms a tight complex with Rad23 (15,36), it is possible that Rad4 protein alone may be intrinsically unstable, thus contributing to the quick disappearance of Rad4 in rad23

Figure 1. Stability of Rad4 protein in yeast cells. (A) Yeast rad23 deletion mutant cells containing pEGTh6-RAD4 (lanes 1 and 2) or both pEGTh6-RAD4 and pEGLha-RAD23 (lanes 3 and 4) were grown at 30°C without (lanes 1 and 3) or with 13 h induction by 2% galactose (lanes 2 and 4). Equal amounts of the whole cell extracts (50 μg) were separated by electrophoresis on a 10% SDS-polyacrylamide gel. Expression of the His6-tagged Rad4 and HA-tagged Rad23 proteins were examined by western blot analysis using two monoclonal antibodies with each specific to the respective tag. In separate experiments, the His6 antibody and the HA antibody did not show non-specific cross-reaction against the HA-tagged Rad23 and the His6-tagged Rad4, respectively (data not shown). Lane 5, protein size markers. (B) Following 13 h induction of Rad4/Rad23 (lanes 2–7) or Rad4 (lanes 8–13), galactose was replaced by 2% glucose via medium change to repress expression of the tagged Rad4 or Rad4/Rad23 proteins. Aliquots of the culture were removed at various times as indicated 0–24 h after medium change. The tagged Rad4 and Rad23 proteins in the whole cell extracts were examined by western blot analysis as in (A). Lane 1, protein size markers.
mutant cells. To examine this possibility, we purified Rad4 (Figure 2A) and Rad23 (Figure 2B) proteins separately. To ensure that our Rad23 preparation was absolutely devoid of Rad4, Rad23 was purified from rad4 deletion mutant cells, following overexpression from pEGU6-RAD23. We did not notice any abnormal instability of either Rad4 or Rad23 protein during purification. We then added the purified Rad4 alone or the purified Rad4 and Rad23 together to cell-free extracts of rad23 mutant cells. Aliquots of the extracts were removed at various incubation times. The stability of the purified protein in the extracts was determined by western blot analysis. As shown in Figure 2C and compared to Figure 1B, purified Rad4 was relatively stable in the yeast cell-free extracts with or without Rad23. It should be noted that the small protein tags used in these experiments did not significantly affect the protein function, based on the following observations. First, the tagged Rad4 and Rad23 fully complemented UV sensitivity of the respective mutant strains (Figure 3A) (data not shown). Second, the tagged Rad4 and Rad23 proteins also formed a protein complex, as indicated by their co-purification sequentially through Ni-affinity and Resource Q columns following their co-expression in yeast cells (data not shown). These results show that Rad4 protein is not associated with an intrinsic instability. Thus, the quick disappearance of Rad4 in rad23 mutant cells is probably a result of active cellular protein degradation.

**Effect of Rad4 overexpression on UV sensitivity of rad23 mutant cells**

Recently, Ng et al. (26) concluded that the primary function of RAD23 in NER is to stabilize XPC protein (Rad4 homolog) in mammals. Since Rad23 indeed stabilizes Rad4, we then tested this possibility in yeast. Cells lacking Rad23 are sensitive to UV radiation due to deficient NER (19) (Figure 3A). If the primary function of Rad23 is to stabilize Rad4, it is predicted that persistent overexpression of Rad4 protein should lead to complementation of the UV-sensitive phenotype of rad23 mutant cells. The mutant cells were transformed with a RAD4 overexpression plasmid under the control of the strong GAL1/10 promoter. Rad4 overexpression was achieved by induction with 2% galactose in the culture medium (Figure 1A). Following Rad4 overexpression, the cells were irradiated with UV and grown in plates containing 2% galactose for continued Rad4 induction. As shown in Figure 3A, only a partial complementation for UV resistance was observed. The UV sensitivity of rad23 mutant cells remained largely unaffected by the overexpressed Rad4 protein. The small enhancement of UV resistance was specific to Rad4 overexpression in rad23 mutant cells, because this effect was not observed when Rad14 was overexpressed in rad23 mutant cells (Figure 3B), or when Rad4 was overexpressed in rad14 mutant cells (Figure 3C). Rad4 overexpression also had no effect on UV resistance of wild-type yeast cells (Figure 3A). These results are inconsistent with the notion that the primary function of Rad23 is to stabilize Rad4.

**Rad23 protein directly participates in NER**

To directly determine whether decreased Rad4 stability is the major cause of the deficient NER in rad23 mutant cells, we performed NER assays in yeast cell-free extracts, using plasmid DNA containing AAF adducts. We have previously shown that under the conditions used, AAF DNA adducts are repaired specifically by the NER pathway in yeast cell-free extracts (12,15). NER was monitored by radiolabeling the repair patch during DNA repair synthesis.

Consistent with our previous results (15), NER was deficient in rad23 mutant cell extracts (Figure 4A, lane 2), as compared to the proficient repair in wild-type cell extracts (Figure 4A, lane 1). We then attempted to complement the deficient repair in rad23 mutant cell extracts with purified Rad4 and Rad23 proteins. As shown in Figure 4A (lanes 3–6), purified Rad4 was unable to complement deficient NER of rad23 mutant extracts even at high protein concentrations. Purified Rad23 protein only partially complemented deficient repair of rad23 mutant extracts (Figure 4A, lanes 7–10). In contrast, addition of purified Rad23 and Rad4 together restored the deficient repair to the wild-type level (Figure 4A, lanes 11–14). Combining 10 ng of Rad4 with 50 ng of Rad23 was sufficient to achieve full complementation (Figure 4A, lane 11). The complementation activity of purified Rad4 and Rad23 was specific
to rad23 mutant extracts, because deficient NER in rad2
mutant extracts was complemented by purified Rad2
(Figure 4B, lanes 3–6), but could not be complemented by
purified Rad4 and Rad23 (Figure 4B, lanes 7–10).

In separate experiments, we also attempted to complement
the deficient repair in rad23 mutant cell extracts with cell-free
extracts containing overexpressed Rad4 and/or Rad23. In
these experiments, Rad4 protein was overexpressed in
rad23 mutant cells, and Rad23 protein was overexpressed in
rad4 mutant cells. The respective cell-free extracts were
prepared and added to the rad23 mutant cell extracts for
in vitro NER. Again, neither overexpressed Rad4 cell extract
nor overexpressed Rad23 cell extract (10–100 μg) alone could
fully complement deficient NER of rad23 mutant extracts. In
contrast, addition of small amounts of an extract (3 μg) con-
taining both overexpressed Rad4 and Rad23 proteins fully
complemented deficient NER of rad23 mutant extracts
(data not shown).

Together, these results show that Rad23 protein directly
participates in NER, and that decreased Rad4 stability is
not the major cause of deficient NER in rad23 mutant cells.
Furthermore, these results suggest that Rad4 is the
only NER protein being significantly stabilized by Rad23 in
yeast cells.
Rad23 protein stimulates the DNA-binding activity of Rad4 protein

The Rad4–Rad23 protein complex is thought to play a major role in DNA damage recognition during NER, due to its DNA-binding activity (16–18,37). To understand what direct role Rad23 plays during NER, we examined the effect of Rad23 protein on the DNA-binding activity of Rad4. DNA binding was determined by an EMSA. Purified Rad4 protein alone was able to bind to AAF-damaged DNA (Figure 5, lane 2). In contrast, purified Rad23 protein alone was unable to bind to the damaged DNA (Figure 5, lanes 5–7). Rad23 protein was also unable to bind to undamaged duplex DNA and single-stranded DNA with or without AAF-adducts (data not shown). When Rad23 protein was added, Rad4 binding to the AAF-DNA was significantly enhanced, by as much as ~3-fold (Figure 5, lanes 3 and 4). These results show that Rad23 protein stimulates the DNA-binding activity of Rad4 protein.

To determine whether the DNA-binding activity of Rad4 is affected by DNA damage, we performed competition experiments in the presence of Rad23 protein. Standard EMSA was performed using purified Rad4, Rad23 and 32P-labeled duplex AAF-DNA (Figure 6A and B, lanes 2 and 9). In separate reactions, increasing amounts of unlabeled DNA were added as the competitor. As shown in Figure 6A (compare lanes 3–8 with lanes 10–14) and Figure 6C, AAF-damaged double-stranded DNA was a significantly better competitor than the corresponding undamaged DNA. Similarly, AAF-damaged single-stranded DNA was a significantly better competitor than the corresponding undamaged DNA (Figure 6B, compare lanes 3–8 with lanes 10–14 and Figure 6C). Furthermore, double-stranded DNA was a better competitor than single-stranded DNA, either in the absence of DNA damage (compare lanes 3–8 of Figure 6A and B; and Figure 6C) or in the presence of DNA damage (compare lanes 10–14 of Figure 6A and B; and Figure 6C). These competition experiments were also carried out with purified Rad4 protein alone. Similarly, Rad4 protein exhibited a stronger binding activity toward AAF-damaged DNA over the undamaged DNA, regardless of whether it was in double-stranded or single-stranded form (data not shown). These results show that Rad4 protein binds to damaged DNA stronger than undamaged DNA and binds to double-stranded DNA stronger than single-stranded DNA with or without Rad23 protein.

DISCUSSION

In this study, we have examined the role of Rad23 protein in yeast NER. Our results support two roles of Rad23 protein in NER: its direct participation in NER and its stabilization of Rad4 protein. Furthermore, we found that Rad23 protein stimulates Rad4 binding to damaged DNA. This stimulatory activity is probably responsible for the direct role of Rad23 in NER.

The stabilization effect of Rad23 on Rad4 has been observed by Lommel et al. (32) following UV radiation of...
yeast cells. Since transcription of the RAD23 gene is UV inducible (38), we asked whether Rad23 exerts a similar cellular effect on Rad4 in the absence of exogenous DNA damage. This proved to be the case. Apparently, the Rad23 function in stabilizing Rad4 is well conserved in mammals. It was shown recently that XPC protein, which is the homolog of Rad4, is also stabilized in mouse cells by HR23A and HR23B, two homologs of Rad23 (26). Lommel et al. (32) suggested that Rad23 stabilizes Rad4 by protecting it from proteasome-mediated degradation. Supporting this notion, Rad4 protein is stabilized in proteasome-deficient mutant cells (32), and removal of UV-induced CPDs was reported to be enhanced in these mutant cells (30). In our studies, we found that although Rad4 was unstable in rad23 mutant cells, the purified Rad4 protein was relatively stable in rad23 mutant cell extracts in vitro. Thus, Rad4 protein by itself is not associated with an intrinsic instability. Our results are consistent with the conclusion of Lommel et al. (32) that cellular Rad4 is actively degraded by the 26S proteasome, and its quick turnover is protected by Rad23 protein.

Since purified Rad4 and Rad23 together are necessary and sufficient for full complementation of deficient NER of rad23 mutant cell extracts, it is clear that Rad4 is the only NER protein that is significantly stabilized by Rad23 in cells, at least for the global genome repair. It was observed that Rad4 and XPC proteins transiently accumulate in yeast and mammalian cells, respectively, following DNA damage (26,32). This has been proposed as an important mechanism of NER regulation (26). However, since persistent overexpression of Rad4 protein in wild-type yeast cells did not yield an effect on cellular resistance to UV radiation, it is unlikely that damage-induced Rad4 accumulation may represent a significant regulatory mechanism for repair. Rad4 accumulation may simply reflect its enhanced stabilization by higher levels of Rad23, as the RAD23 gene is damage inducible in yeast (38).

XPC stabilization by HR23A/B led Ng et al. (26) to conclude that this is the primary function of RAD23 protein. Such a mechanism predicts that deficient NER in rad23 mutant cells should be largely complemented by persistent high levels of Rad4 protein in the cell through gene overexpression. This prediction is not supported by our results in yeast. First, persistent overexpression of Rad4 protein only slightly enhanced the UV resistance of rad23 mutant cells, in contrast to full complementation by expressing the RAD23 gene. Thus,
deficient NER in rad23 mutant cells is not corrected to a large extent by high levels of Rad4 protein. Second, deficient in vitro NER in rad23 mutant cell extracts could not be effectively complemented by purified Rad4 protein. Consistent with our results, Lommel et al. (32) reported that overexpression of Rad4 or lack of the proteasome function could not suppress the deficient removal of UV-induced CPDs in rad23 mutant cells. On the other hand, we observed that Rad4 overexpression slightly enhanced the UV resistance of rad23 mutant cells, and that purified Rad23 protein alone could not fully complement deficient NER rad23 mutant cell extracts. Clearly, the Rad4 protein concentration in rad23 mutant cells is limiting relative to other NER proteins for repair. Hence, stabilizing Rad4 protein is an important but not the only function of Rad23 protein in yeast NER. Another important function of Rad23 protein is its direct participation in NER, as demonstrated by the requirement for both purified Rad23 and Rad4 proteins to fully complement the deficient NER of rad23 mutant cell extracts. Since rad23 mutant cells are only modestly sensitive to UV radiation unlike other mutant cells such as rad4 and rad14, Rad3 protein can only play an accessory role rather than an indispensable role in the biochemical pathway of NER. We found that while it is unable to bind DNA, Rad23 protein stimulates the DNA-binding activity of Rad4 protein. This is the only biochemical activity observed for Rad23 protein in NER. Thus, we postulate that stimulation on Rad4 binding to damaged DNA represents the major mechanism of the direct participation of Rad23 protein in NER. Definitive proof of this model awaits extensive structure-function studies of this protein.

Although the precise mechanism by which Rad23 stimulates DNA binding by Rad4 is not clear at present, physical interaction between these two proteins may be involved. Indeed, Rad23 strongly interacts with Rad4 in the yeast and the mammalian systems (14–16). Intriguingly, a protein complex consisting of XPC, HR23B and CEN4 was isolated from human cells, in which the CEN4 component directly interacts with XPC (39). The role of CEN4 in this complex and whether this protein contributes to NER remains unknown. Since the stimulatory activity of Rad23 on Rad4 DNA binding was observed with purified proteins in vitro, it is unlikely that the stimulatory mechanism involves protein modification during damage recognition by Rad4. In addition to Rad4, Rad23 also interacts with several other proteins, one of which is the 3-methyadenine DNA glycosylase involved in base excision repair (40). Consistent with our model that stimulating Rad4 binding to damaged DNA accounts for a major role of Rad23 in NER, the human RAD23 proteins additionally stimulate the activity of the 3-methyadenine DNA glycosylase in vitro (40). Our conclusions that yeast Rad23 plays dual functions by stimulating Rad4-mediated damage binding/recognition and by stabilizing Rad4 in cells may provide a general model for understanding RAD23 protein in NER of other eukaryotes.

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