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Functional Analysis of *RAD4* Gene Required for Nucleotide Excision Repair of UV-induced DNA Damage in *Saccharomyces cerevisiae*

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The *RAD4* gene is essential for nucleotide excision repair in *Saccharomyces cerevisiae*. It has been known that the deduced amino acid sequence of Rad4 protein contains three DNA-dependent ATPase/helicase motifs. To determine the biochemical activities and functional role of *RAD4*, the Rad4 protein was expressed and purified. Immunoblot analysis showed a specific band of 21 kDa, which was well-matched with the size of open reading frame of the *RAD4* gene. The purified Rad4 protein had no detectable helicase activity. However, the protein could interact with double stranded oligonucleotides, as judged by mobility shift assay. This result suggests that the Rad4 protein is a DNA binding protein.

An intricate network of DNA repair systems protects the genetic information from continuous deterioration caused by environmental genotoxic agents and inherent chemical instability of DNA. Thus these systems prevent mutagenesis leading to inborn defects, cell death and neoplasia, and may counteract the process of aging. Nucleotide excision repair (NER) is one of the major cellular repair pathways. It removes a wide range of structurally unrelated lesions in a complex multistep reaction (Friedberg, 1988).

In order to analyse the mechanisms by which DNA repair occurs, a large number of putative DNA repair mutants have been isolated from different organisms by virtue of their sensitivity to DNA damaging agents. In *Saccharomyces cerevisiae* at least 30 complementation groups have been identified and subsequently classified into three groups, the *RAD3*, *RAD52* and *RAD6* epistasis groups (Phipps et al., 1985; Madura and Prakash, 1990; Praekelt and Macock, 1990), which are either sensitive to ultra-violet (UV) radiation, gamma-irradiation or both UV and gamma-irradiation. The *RAD3* group comprises genes required in an excision repair pathway, and has been studied extensively, while the other two groups comprise genes required for recombination repair (*RAD52*) and error-prone (*RAD6*) pathways (Reynolds and Friedberg, 1981; Weber et al., 1990; Weinert and Hartwel, 1990; Boothmann et al., 1993).

Complementation of the radiation-sensitive phenotypes has been used to isolate DNA repair genes from both yeast and mammalian cells. This has led to the identification of members of an excision repair pathway from *S. cerevisiae* and human. The *S. cerevisiae* genes *RAD3* and *RAD10* are homologoues of the human *ERCC2* and *ERCC1* genes, respectively (Tompson et al., 1988; Hoeijmakers and Bootsma, 1990; Murray et al., 1991).

The *RAD4* gene of *Saccharomyces cerevisiae* is one of the five genes (*RAD1, RAD2, RAD3, RAD4,* and *RAD10*) that are absolutely required for the incision step in nucleotide excision repair (Fenech et al., 1991). Mutation in any of these five genes renders yeast cells abnormally sensitive to UV light and other DNA-damaging agents (Miller et al., 1982; Perozzi and Prakash, 1986). These genes have been isolated by phenotypic complementation with appropriate rad mutants from a yeast genomic library (Higgins et al., 1983; Fleer et al., 1987; Choi et al., 1993; Reynolds et al., 1992). In our previous studies we have demonstrated that the cloned *RAD4* gene is composed of 2,190 nucleotides encoding a putative protein of 730 amino acids (Choi et al., 1990), and transcribed into 2.3 kb mRNA (Choi et al., 1991; 1993). We have further observed that the *RAD4* gene is neither essential for viability of the haploids under normal growth condition nor UV-inducible. At present, the functional role of this gene and its gene product has not been well characterized.

Therefore, we tried to address the structure and function of *RAD4* gene with the purified Rad4 protein.

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Materials and Methods

Strains, cell culture, and genetic methods

E. coli strains DH5α (*F- endA1, hsd17, (r-, mk-), supE44, thi-1, recA1, gyrA96, relA1, lacIqZ-M15*) and XL1 blue (Stratagene, USA) were used as a host for propagation of plasmids. Yeast *S. cerevisiae* RAD+ (a, *ura3-52 trp1-289)* and LP2693-4B rad4-4 mutant *(a, ura3-52 trp1-289 his3-1 leu2-212 leu2-2)* were used. *E. coli* strains were grown on LB media (1% tryptone, 1% NaCl, 0.5% yeast extract). Yeast strain was grown in YE (2% glucose, 0.5% yeast extract) medium supplemented with appropriate amino acids. Standard molecular biology techniques were employed (Sambrook and Russell, 2001). Yeast cells were transformed by the method of Ito et al. (1983) with a minor modification.

Nucleotide sequence analysis

All sequencing reactions were performed by the double-stranded dideoxy method (Sanger et al., 1977) using kits from Pharmacia LKB or US Biochemicals. Homology searches comparing the amino acid sequence were performed using FASTA.

Preparation of anti-Rad4 antibody

To identify the gene product (Rad4), polyclonal antibody was produced against *RAD4* gene product. For expression of cloned *RAD4* gene in *E. coli, Bgl*II fragment of *RAD4* gene was inserted in-frame into pET3a plasmid (Kim et al., 1994). After expression and purification of protein with about 21 kDa molecular mass, the fusion proteins were injected into New Zealand white rabbit subcutaneously. In order to identify the *RAD4* gene product, immunoblot analysis was performed.

Western blotting

Cells were grown to 5×10^6 cell/mL and harvested. Total proteins were extracted in breakage buffer [100 mM Tris-HCl (pH 8.0), 20% glycerol, and 1 mM DTT]. About 50 μg of total protein was loaded in each lane on 8% SDS-polyacrylamide gel and subsequently wet-transferred to immobilon-P membrane (Millipore, USA). The blot was probed with a 1:1000 dilution of affinity purified anti-Rad4 antibodies (Jin et al., 1998). Immunodetection was performed by the ECL plus kit (Amersham).

Preparation of Rad4 protein

The yeast RAD^+ cells were harvested and washed three times with fresh media as described by Jang et al. (1993). After dilution to an OD_{595} of 0.1 into 10 liters of fresh media, cells were further cultured at 30 ℃ for 12 h, collected, and then washed with extraction buffer (20 mM Tris-Cl, pH 8.0, 1 mM EDTA, 1 mM DTT, and 10% glycerol). The cells were lysed in 150 mL of the extraction buffer using glass beads with a beadbeater (BioSpec Products) in the presence of some protease inhibitors (0.5 μg/μL leupeptin, 0.7 μg/μL pepstatin A, 35 μg/μL PMSF and 1 μg/μL aprotinin). The cell extracts were then clarified by centrifugation for 2 h at 43,000 rpm in a Beckman Ti70 rotor. The supernatant was bound batchwise with 20 mL of Ni^{2+}-NTA agarose (Qiagen) for 2.5 h at 4℃. The resin was poured into a column and washed sequentially with 300 mL of extraction buffer and 100 mL of extraction buffer containing 10 mM imidazole. The proteins were then eluted with 100 mL of extraction buffer containing 500 mM imidazole. Peak fractions were pooled and dialyzed aganist an equilibration buffer (20 mM Tris-Cl, pH8.0, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, and 10% glycerol) and loaded onto an DEAE Sepharose column (2 × 15 cm). After washing the column, bound proteins were eluted with 50 mL of linear salt gradient (100 mM to 400 mM NaCl) in equilibration buffer. The samples were applied onto a Sephacryl S-200 column (4 × 60 cm) at 0.2 mL/min and active fractions were pooled.

Preparation of dsDNA substrates for helicase assay

Standard ds DNA substrate used for DNA helicase activity assay was prepared by annealing two oligomers. Flush-ended duplex DNA was also prepared by annealing two oligomers. With two substrates, the lower DNA strands were end-labeled with [γ-^{32}P] ATP. In most cases, the specific activity of the labeled substrates was 1.5×10^3 cpm/fmol of dsDNA substrate. For annealing, two oligomers were mixed together in 0.2 mL of hybridization buffer [20 mM Hepes-KOH (pH 7.4), 0.5 M NaCl, 1 mM EDTA, and 0.1% SDS]. The mixture was heated at 100℃ for 5 min and then incubated at 65℃ for 30 min, followed by slow cooling for 12 h to room temperature (RT). The dsDNA was then separated by electrophoresis on a native 8% polyacrylamide gel and eluted from the gel. The gel slice containing dsDNA was ground with a micropestle in 0.4 mL of elution buffer (0.5 M ammonium acetate, pH 7.0, 0.1% SDS, and 10 mM EDTA) and incubated for 2 h at RT. After brief centrifugation, the supernatants were extracted with phenol/chloroform (1:1, v/v) and chloroform, and then treated with 2.5 volumes of cold absolute ethanol. After 1 h at -70℃, the substrate was collected by centrifugation at 4℃ for 15 min. The pellet was dried under vacuum. The purified dsDNA substrates were adjusted to a final concentration of 50 fmol/μL in a solution [20 mM Hepes-KOH (pH 7.4), 50 mM NaCl, 0.1 mM EDTA, 0.05% Nonidet P-40] and stored in 50 μL aliquots at -20℃.

DNA helicase assay

DNA helicase activity was assayed according to Lee et al. (1997). The typical helicase activity assay was

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460  tataaatatgctatcgatgagaaagcattggacgaattgatgttcagtgtcttgaaagatgt
        I  C  Y  R  -  E  S  I  G  R  I  D  V  Q  C  L  E  R  C
520  accacaagcgattcaaaagatggcctacaagattaaaggcgcaaggaccttaccaaatgg
     T  T  S  D  S  K  D  G  L  Q  D  -  R  R  K  D  L  T  K  W
580  aggtaacgtctccagggtctcgtccattgtcaagtcgcatgctggctctttgttgatttt
     R  -  R  L  Q  G  L  V  H  C  Q  V  A  C  W  L  F  V  D  F
640  attgaatgatattactaatacggaaacattttagcagatgaaatgggtttgggtaaaacc
     I  E  -  Y  Y  -  Y  G  N  I  L  A  D  E  M  G  L  G  K  T
700  gtgcaaagcattagttcttcattctgtccttattgtcgtaccgctttccactgttaacga
     V  Q  S  I  S  S  S  F  C  P  Y  C  R  T  A  F  H  C  -  R
760  attgatgccttatcttttatcatacagaaggatcttaaaggagcttattaaatcaattgt
     I  D  A  L  S  F  I  I  Q  K  D  L  K  G  A  Y  -  I  N  C
820  tggagtatggtctacaacgagagaattacaaacccagcagattgcttcttttgccttttt
     W  S  M  V  Y  N  E  R  I  T  N  P  A  D  C  F  F  C  L  F
880  attcgctggcaatatcttgctatcgatgaagctcatcgactcgatcaataccaccaaaga
     I  R  W  Q  Y  L  A  I  D  E  A  H  R  L  D  Q  Y  H  Q  R
940  gttcaagaagtctatgctagaaacaaccttaaaacaacgtacctccacttttatcaaaag
     V  Q  E  V  Y  A  R  N  N  L  K  T  T  Y  L  H  F  Y  Q  K
1000 tagttgtcgtaaaacgacgaatatgcgttcattctccgtcgcttaaaaaaagctgtagag
     -  L  S  -  N  D  E  Y  A  F  I  L  R  R  L  K  K  A  V  E
1060 aaacgtaaaacgatgcgttctatgccactgctgatttttcaaaaatcggcagcgaattaa
     K  R  K  T  M  R  S  M  P  L  L  I  F  Q  K  S  A  A  N
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Fig. 1. Partial nucleotide sequence and predicted amino acid sequence of the *RAD4* gene. The number at left refers to adjacent nucleotides. The sequence in shaded boxes indicate the ATPase/helicase domain.

carried out in 20 μL of a reaction mixture composed of 20 mM HEPES-KOH (pH 7.4), 2 mM DTT, 5 mM $MgCl_2$, 1 mM ATP, 0.2 mg/mL BSA, 50 fmol of dsDNA substrate and various amounts of the Rad4 protein. Mixtures were incubated for 30 min at 30℃, and then stopped by the addition of 5 μL of a mixture containing 0.1 M Tris-Cl (pH 7.5), 20 mM EDTA, 0.5% SDS, 0.1% NP-40, 0.1% bromophenol blue, 0.1% xylene cyanol, and 50% glycerol. Aliquots (10 μL) of each reaction were loaded onto a native 8% polyacrylamide (30 : 1) gel and electrophoresed at 15 mA for 1.5 h. The gel was then vacuum-dried on Whatman DE81 paper, and the dsDNA substrate and the displaced single-stranded DNA product were visualized by autoradiography.

Gel eletrophoresis mobility shift assay

The typical DNA binding assay was performed in 20 μL of a reaction mixture containing 20 mM HEPES-KOH (pH 7.4), 2 mM DTT, 0.2 mg/mL BSA, 100 mM NaCl, 5% glycerol, 50 fmol of ^{32}P-labelled-DNA probe and the indicated amount of Rad4. After 30 min of incubation at 30℃, the complexes formed were analyzed by 4% polyacrylamide gel electrophoresis in 0.5 TBE buffer.

Results

Purification of Rad4 protein

We have previously mapped the *RAD4* gene on chromosome V (Choi et al., 1990; 1993). Recently, we have determined the nucleotide sequence of this gene and also identified a *RAD4*-like gene in *Schizosaccharomyces pombe*. The Rad4 protein has a high content of basic residues (15.9% basic, 9.4% acidic, 31.6% polar, and 43.1% nonpolar amino acid residues). Analysis of the nucleotide sequence revealed that the *RAD4* gene has three ATPase/helicase domains (Fig. 1), implying that the Rad4 protein has a ATPase or helicae activity.

On the basis of this result, we tried to elucidate the biochemical activities and functional role of the *RAD4* gene. The Rad4 protein was purified near homogeneity by combination of affinity chromatography on Ni^{2+}-NTA agarose, DEAE-sepharose ion exchange and sephacryl S-200 gel filtration chromatography. Whole cell extract made from a 10 liter-culture of RAD^+ cells was bound with 20 mL of Ni^{2+}-NTA agarose (Qiagen) for 2.5 h at 4℃ and the Rad4 proteins were eluted as described in Materials and Methods.

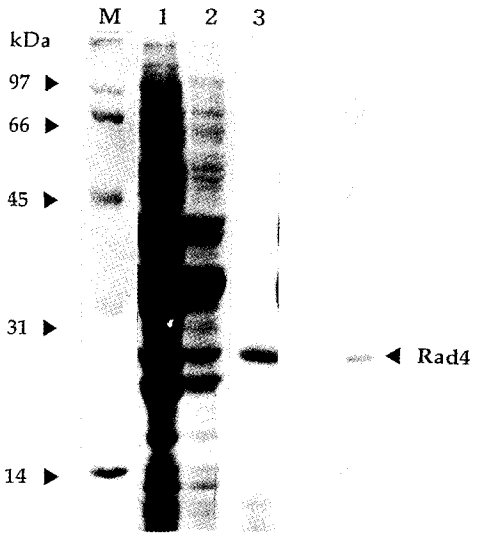

Fig. 2. Purification and immunoblot analysis of the Rad4 protein. Panel A. The peak fractions were pooled, concentrated and loaded onto a Sephacryl S-200 gel filtration column. Eluents were collected and each fraction was subjected to SDS-PAGE and the gel was stained with coomassie brilliant blue. M, standard marker; 1, total cell extract; 2, DEAE-Sepharose; 3, Sephacryl S-200. Panel B. Immunoblot analysis of Rad4 protein. The purified Rad4 protein was separated by SDS- PAGE and immunoblot analysis was carried out as described in **Materials and Methods** with anti-Rad4 antibodies.

As shown in Fig. 2B, a specific band of 21 kDa was identified, which was well-matched with the size of open reading frame of *RAD4* gene (Choi et al., 1993).

Rad4 has no helicase activity

Many DNA-dependent ATPase have an DNA helicase activity (Woodage et al., 1997). The presence of conserved helicase domains in Rad4 (Fig. 1) suggested that it might also have DNA helicase activity. We tested the possibility that the purified Rad4 protein has a helicase activity using an oligonucleotide release assay with two types of substrates. However, we were unable to detect any helicase activity associated with the Rad4 protein (Fig. 3), suggesting that the helicase motifs in Rad4 are merely indicators of a broader DNA-dependent ATPase activity of which helicase is a subset (Henihoff, 1993).

Rad4 is a DNA binding protein

We examined whether the Rad4 protein has the activity to interact with ds oligonucleotides. As shown in Fig. 4, the Rad4 protein indeed bound to the DNA fragments,

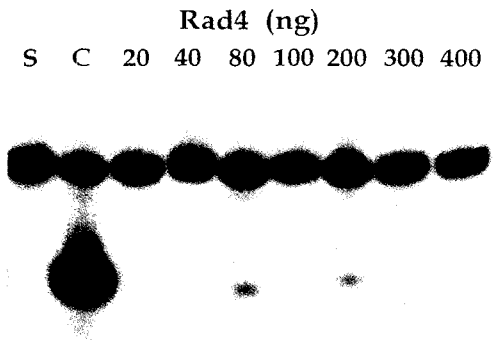

Fig. 3. DNA helicase activity assay. The standard assay was performed as described in Materials and Methods with amount of Rad4 protein indicated. The helicase protein (20 ng) was used as positive control (donated by Dr. Lee, University of New Jersey Medical School). C, positive control; S, substrate only.

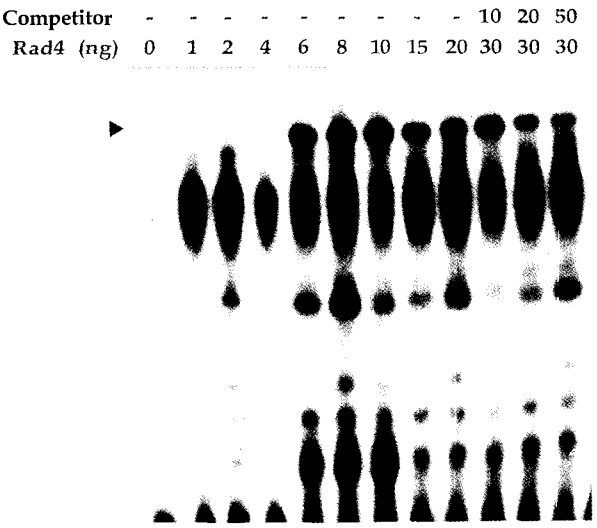

Fig. 4. DNA binding activity of the Rad4 protein. DNA probe was prepared as described in Materials and Methods. Probe was incubated with yeast cell extract in the presence of specific or non-specific competitors. The numbers at the top of figure indicate the fold molar excess of proteins to the DNA probe. The bound DNA-protein complex was indicated with an arrow.

as evidenced by the formation of a single retarded band. The amount of binding activity increased with increasing amounts of protein and the binding activity was sequentially eliminated by the addition of increasing amounts of unlabeled DNA fragment as a competitor.

Discussion

In this paper, we present purification and enzymatic activities of the Rad4 protein and discuss implication for their *in vivo* function. Until now, there has been no such reports although it is known that the *RAD4* gene might be involved in the incision step of nucleotide excision repair as judged by genetic analysis (Fenech et al., 1991)

We analyzed the nucleotide sequence of the *RAD4* gene and revealed that this gene has three ATPase/helicase domains. On the basis of this result, the functions of the *RAD4* gene were studied. The presence of the helicase motifs has been used to suggest that the helicase activity is conserved in the *RAD4* gene (Auble et al., 1994; Cote et al., 1994). To determine the functional role of the *RAD4* gene, we purified the Rad4 protein and detected a 21 kDa protein by immunoblot analysis, which was well-matched with the size of the *RAD4* gene (Choi et al., 1993).

On the other hand, the SW12/SNF family has a conserved region of ATPase/helicase domain (Delmas et al., 1993; Jin et al., 1998). The presence of the helicase motifs in the SNF2 domain has postulated that the helicase activity might be conserved. But helicase activity *in vitro* has never been detected in any of the proteins in the SNF2/SW12 family, even though it was proposed that this activity is needed for processes in which these proteins are known to be involved. We also failed to detect any helicase activity in the Rad4 protein. Therefore, we suggest a possible explanation for that as follows. Henikoff (1993) proposed that the SW12/SNF2 family proteins are not helicases and but a helicase motif indicator for DNA-dependent ATPase activity, of which helicase activity is a subset. Consistent with this proposal, the Rad4 protein has no detectable helicase activities. We concluded that the helicase motifs in Rad4 are merely indicators of a broader DNA-dependent ATPase activity. With these results, the biochemical activities and functional role of the *RAD4* gene will be further elucidated in our laboratory. The functional analysis may reveal informative structure-function correlations between the *RAD4* gene and its gene product.

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