Yeast Rad7-Rad16 Complex, Specific for the Nucleotide Excision Repair of the Nontranscribed DNA Strand, Is an ATP-dependent DNA Damage Sensor*

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In eukaryotes, nucleotide excision repair of ultraviolet light-damaged DNA is a highly intricate process that requires a large number of evolutionarily conserved protein factors. Genetic studies in the yeast Saccharomyces cerevisiae have indicated a specific role of the RAD7 and RAD16 genes in the repair of transcriptionally inactive DNA. Here we show that the RAD7- and RAD16-encoded products exist as a complex of 1:1 stoichiometry, exhibiting an apparent dissociation constant (Kd) of <4 × 10⁻¹⁰ M. The Rad7-Rad16 complex has been purified to near homogeneity in this study and is shown to bind, in an ATP-dependent manner and with high specificity, to DNA damaged by ultraviolet light. Importantly, inclusion of the Rad7-Rad16 complex in the in vitro nucleotide excision repair system that consists entirely of purified components results in a marked stimulation of damage specific incision. Thus, Rad7-Rad16 complex is the ATP-dependent DNA damage sensor that specifically functions with the ensemble of nucleotide excision repair factor (NEF) 1, NEF2, NEF3, and replication protein A in the repair of transcriptionally inactive DNA. We name this novel complex of Rad7 and Rad16 proteins NEF4.

In eukaryotes, nucleotide excision repair (NER) of ultraviolet light-damaged DNA occurs by dual incision of the DNA strand that contains the UV lesion, excising the damage in the form of a short DNA fragment ~30 nucleotides in length (1). Mutational inactivation of NER in humans results in the cancer-prone syndrome xeroderma pigmentosum, which under-scores the importance of this repair system in neutralizing the genotoxicity of UV light. The dual incision event in NER is accomplished by the concerted action of a large number of evolutionarily conserved proteins, and our studies in the yeast Saccharomyces cerevisiae have indicated an organization of these proteins into distinct subassemblies: nucleotide excision repair factor I, or NEF1, consisting of the damage recognition protein Rad14 and the Rad1-Rad10 endonuclease (2), NEF2, containing the Rad4 and Rad23 proteins (3), and NEF3, comprising the endonuclease Rad2 together with the RNA polymerase II transcription factor TFIIH (4). The combination of NEF1, NEF2, NEF3, and the heterotrimeric replication protein A (RPA) is sufficient for ATP-dependent dual incision to occur, indicating that the basic NER machinery is composed of these protein subassemblies (3). Dual incision of UV-damaged DNA can also be accomplished by combining the human equivalents of the aforementioned yeast repair proteins (5–7).

At the genomic level, two functionally distinct modes of NER have been described, the first concerns with the repair of the transcribed strand in transcriptionally active chromosomal DNA, which involves the stalling of RNA polymerase II at the DNA lesion, and requires the CSA and CSB gene products (8). The second mode of excision repair is specific for the nontranscribed strand and for genomic regions that are transcriptionally inactive. Interestingly, in the rad7 and rad16 mutants of S. cerevisiae, the nontranscribed strand is not repaired, while the repair of the transcribed strand is not affected (9, 10), and transcription-independent NER is impaired in rad7 and rad16 mutant extracts (11, 12). Thus, the repair of the nontranscribed strand has a specific dependence on the RAD7 and RAD16 genes.

Here we describe our biochemical studies that help elucidate the molecular function of the Rad7 and Rad16 proteins in the repair of the nontranscribed strand. We show by co-immunoprecipitation that Rad7 and Rad16 proteins exist as a stable complex in yeast cells. The Rad7-Rad16 complex, which we have named NEF4, has been purified to near homogeneity from extract of a yeast strain co-expressing the two proteins. Using a DNA mobility shift assay, we demonstrate that NEF4 binds with high specificity and avidity to UV lesions and that this damage binding reaction has a strong dependence on ATP. Importantly, the addition of NEF4 to the reconstituted NER reaction results in a marked stimulation of damage-specific incision. Taken together, our results suggest that NEF4 is an ATP-dependent DNA damage sensor that functions specifically to target the basic NER machinery (viz. NEF1, NEF2, NEF3, and RPA) to the repair of nontranscribed DNA.

MATERIALS AND METHODS

Overexpression of Rad7 and Rad16 Proteins in Yeast—The Rad7 protein coding frame from 20 nucleotides upstream of the ATG initiating codon to 130 nucleotides downstream of the TAG stop codon was placed under the control of the GAL-PGK promoter in plasmid pFM231, yielding plasmid pF7.2 (2μ, GAL-PGK-RAD7). To overproduce the RAD16-encoded product, the RAD16 gene from 30 nucleotides upstream of the ATG initiating codon until 130 nucleotides downstream of the TAG stop codon was fused to the ADC1 promoter in plasmid pSCW21, yielding plasmid pR16.15. Immunoblot analyses of yeast extracts with affinity-purified antibodies specific for Rad7 and Rad16 proteins revealed that these proteins are individually overexpressed at least 20-fold compared with the level seen in wild type extract.

Production of Antibodies—The portion of Rad7 protein encompassing amino acid residues 1–485 was expressed as a fusion with the 12
The Yeast Rad7-Rad16 Heterodimer

amino-terminal residues of LacZ. The portion of Rad16 protein from amino acid residues 273–534 was fused to the NH-terminal 48 residues of the Escherichia coli transcription terminator ρ. Both of these fusion proteins are insoluble in E. coli, and they were purified from inclusion bodies by preparative SDS-polyacrylamide gel electrophoresis. The purified Rad7 and Rad16 hybrid polypeptides were used as antigens for the production of polyclonal antiserum in rabbits. Antibodies were purified from rabbit sera using antigens cross-linked to cyanogen bromide-activated Sepharose 4B (Pharmacia Biotech Inc.).

Immunoprecipitation—Extract was prepared from yeast strain LY2 harboring pR7.8, pR16.15, or both of these plasmids, as described (13). Clarified extract (0.5 ml) was mixed gently at 4 °C for 2 h with 10 μl of protein A-agarose beads containing covalently conjugated, affinity-purified anti-Rad57 (13), anti-Rad7, and anti-Rad16 antibodies (2 mg of antibodies/ml of matrix). Immunoprecipitates were treated with 30 μl of 3% SDS to elute the bound proteins, and aliquots (10 μl) of the eluates were subjected to immunoblot analysis.

Purification of NEF4—Extract was prepared from 450 g of strain LY2 co-harboring pR7.8 and pR16.15 using a French press (14), clarified by centrifugation (100,000 × g, 90 min), and the Rad7-Rad16 complex was precipitated by the addition of ammonium sulfate to 0.22 g/ml. The ammonium sulfate pellet was dissolved in 200 ml of K buffer (20 mM KH2PO4, pH 7.4, 10% glycerol, 1 mM dithiothreitol, and 0.5 mM EDTA) and dialyzed against 2 liters of K buffer + 50 mM KCl for 12 h. The dialysate (Fraction I) was applied onto a column of Q-Sepharose (2.5 × 9 cm; 44-ml matrix) equilibrated in K buffer + 100 mM KCl. The flow-through from Q-Sepharose (Fraction II; 250 ml) was loaded directly onto a SP-Sepharose column (2.5 × 9 cm; 44-ml matrix), which was developed with a 400-ml gradient of 10–300 mM KCl in K buffer, collecting 50 fractions. The fractions containing the peak of the Rad7-Rad16 complex, eluting at 420 mM KCl, were identified by immunoblotting and pooled (Fraction III; 40 ml). The SP pool was dialyzed against K buffer for 2 h to lower the ionic strength to 50 mM KCl, before being fractionated in a column of Q-Sepharose (1.5 × 8 cm; 14-ml matrix) using a 150-ml gradient from 50 to 300 mM KCl in buffer K, collecting 50 fractions. The pool of Rad7-Rad16 complex (Fraction IV; 12 ml), eluting at about 160 mM KCl from the Q-Sepharose column, was applied onto a hydroxyapatite column (1 × 3 cm; 2.5 ml), which was eluted with a 20-ml gradient of 10–300 mM KH2PO4, pH 7.4, in buffer K, and 20 fractions were collected. The peak of the Rad7-Rad16 complex (Fraction V; 2 ml), eluting at about 280 mM KH2PO4 from hydroxyapatite, was diluted with 6 ml of K buffer and then fractionated in Mono S (HR5/5) with a 20-ml, 100–600 mM KCl gradient in buffer K, collecting 40 fractions. The Rad7-Rad16 complex elutes from Mono S at about 400 mM KCl, the pool of which (Fraction VI; 1.5 ml containing 65 μg of protein) was concentrated to 0.15 ml in a Centricon-20 microconcentrator (Amicon) and stored in small portions at −70 °C.

DNA Mobility Shift Assay—The 130-base pair HindIII-SalI fragment used in mobility shift experiments was isolated from plasmid pTB402, labeled with 32P, and irradiated with UV light (254 nm), as described (14). Fraction VI NEF4 was incubated with the 32P-labeled DNA fragment (1.2 ng) and 20 ng of unlabeled, unirradiated X174 dsDNA (linearized with Pst1) in 10 μl of reaction buffer (30 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 2 mM ATP, 100 μg/ml bovine serum albumin, and 1 mM dithiothreitol) at 30 °C for 10 min. Reaction mixtures were run in 3.6% polyacrylamide gels in TAE buffer (40 mM Tris-acetate, pH 7.0, 0.1 mM EDTA) at 30 mA and 4 °C for 1 h. Gels were dried onto Whatman No. 3MM paper and exposed to Kodak MS films. The autoradiograms were subjected to image analysis in a Bio-Rad GS870 densitometer to obtain data points for graphical representation of the results.

In Vitro NER Reaction—The NER reaction was carried out as described (2–4, 15). Briefly, 50 ng of RPA, 30 ng of NEF1, 30 ng of NEF2, 120 ng of NEF3, and 30 ng of NEF4 (when added) were incubated in 10 μl of reaction buffer with 120 ng of M13mp18 DNA that had been irradiated under a UV germicidal lamp emitting at 254 nm and a fluence rate of 1 J/m2/s for 30 s. Reaction mixtures were incubated at 30 °C for varying times, deproteinized, and analyzed in 0.8% agarose gels, as described (3).

RESULTS AND DISCUSSION

The RAD7-encoded protein was overproduced in yeast by placing the RAD7 gene under the control of the GAL-PGK promoter-yielding plasmid pR7.8 (Fig. 1A). To overproduce the Rad16 protein in yeast, the RAD16 gene was fused to the alcohol dehydrogenase I (ADCl) promoter, yielding plasmid pR16.15 (Fig. 1A). When extract from the protease-deficient yeast strain LY2 co-harboring pR7.8 and pR16.15 was subjected to immunoprecipitation with protein A-agarose beads containing covalently conjugated anti-Rad7, anti-Rad16, and anti-Rad57 antibodies. After extensive washing of the immunoprecipitates, bound proteins were eluted with 2% SDS and analyzed by Western blotting, as indicated. B, purity analysis. Fraction VI Rad7-Rad16 complex, 1.5 μg, was run in a 7.5% denaturing polyacrylamide gel (lane 2) along with molecular mass markers (lane 1) and then stained with Coomassie Blue C. Nitrocellulose blot containing 15 ng of Fraction VI Rad7-Rad16 complex was probed with antibodies specific for Rad7 and Rad16 proteins.
protein concentration. When the Rad7-Rad16 protein complex from the last step of purification in Mono S (Fraction VI) was subjected to SDS-polyacrylamide gel electrophoresis and staining with Coomassie Blue, only the Rad7 and Rad16 bands were seen (Fig. 1, B and C), indicating a high degree of purity of the complex. Image analysis of the gel in Fig. 1B revealed a one to one stoichiometry of the Rad7 and Rad16 proteins in the complex.

We speculated that NEF4 might effect the repair of transcriptionally inactive DNA by sensing the DNA damage located in such regions and then recruiting the basic NER machinery to initiate the repair reaction. As a first test of this hypothesis, we examined whether NEF4 would bind UV-damaged DNA using a DNA mobility shift assay. The DNA fragment was labeled with $^{32}$P, irradiated with UV doses ranging from 1 to 12 kJ/m$^2$, and then incubated with NEF4 in the presence of ATP and a excess of unlabeled χX174 DNA at 30 °C. After running the reaction mixtures in polyacrylamide gels under nondenaturing conditions, the gels were dried and exposed to x-ray films to reveal the radiolabeled DNA species. As shown in Fig. 2, incubation of the UV-irradiated DNA probe with NEF4 resulted in the formation of slower migrating forms of DNA, indicating binding of the damaged DNA by NEF4; no binding of NEF4 to the undamaged DNA was seen. The level of nucleoprotein complex formation was proportional to the amount of NEF4 (Fig. 2, A and B) and to the UV dose (Fig. 2, C and D). Multiple nucleoprotein complexes were detected in these experiments, with the slower migrating nucleoprotein species being more prevalent at higher UV doses and with increasing concentrations of NEF4 (Fig. 2), suggesting that these species contained multiple NEF4 molecules bound to different damage sites in the DNA probe. Importantly, when ATP was omitted from the reaction, binding of the UV-damaged DNA decreased dramatically, such that only $\approx$6% of the UV-irradiated DNA was bound by NEF4 in the absence of the nucleotide, as compared with greater than 50% binding in its presence (Fig. 2E). Although NEF4 possesses an ATPase activity that is activated by either single-stranded or double-stranded DNA, ATP binding by NEF4 is apparently sufficient for damage recognition, because the nonhydrolyzable ATP analogue ATPγS is nearly as effective as ATP in promoting damage binding (Fig. 2E).

Having established that NEF4 has high affinity for UV-damaged DNA, we then tested if NEF4 might improve the

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2 S. N. Guzder, unpublished observations.
FIG. 3. NEF4 promotes incision of UV-damaged DNA. A, M13mp18 DNA not treated (−UV) or treated with 30 J/m² of UV (+UV) was incubated with NEF1, NEF2, and NEF3 (NEF1,2,3), with or without NEF4 at 30°C for different times; RPA was added to all the repair reactions except the no protein controls in lanes 1 and 4. Reaction mixtures were run in a 0.8% agarose gel and stained with ethidium bromide to visualize the supercoiled (SC) and open circular DNA (OC), which was generated as a result of the damage-specific incision of the supercoiled form by the NER factors. B, graphical representation of the results in A. DNA incised corresponds to the percent of the supercoiled form that had been converted to the open circular form. Reaction mixtures with (●) or without NEF4 (▲) are shown.

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