Yeast DNA Repair Proteins Rad6 and Rad18 Form a Heterodimer That Has Ubiquitin Conjugating, DNA Binding, and ATP Hydrolytic Activities

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The RAD6 and RAD18 genes of Saccharomyces cerevisiae are required for postreplicative bypass of ultraviolet (UV)-damaged DNA and for UV mutagenesis. The RAD6 encoded protein is a ubiquitin conjugating enzyme, and RAD18 encodes a protein containing a RING finger motif and a nucleotide binding motif. Rad18 can co-immunoprecipitated with Rad6, indicating that the two proteins exist in a complex in vivo. Here, we co-overproduce the two proteins using a yeast multicopy plasmid, purify the Rad6-Rad18 complex to near homogeneity, and show that the complex is heterodimeric. The Rad6-Rad18 heterodimer has ubiquitin conjugating activity, binds single-stranded DNA, and possesses single-stranded DNA-dependent ATPase activity. The Rad6-Rad18 complex provides the first example wherein a ubiquitin conjugating activity is physically associated with DNA binding and ATPase activities provided by an associated protein factor. The co-existence of these activities should provide the complex with the ability to recognize single-stranded DNA resulting from stalling of the replication machinery at DNA damage sites and to recognize the components of the DNA replication machinery for ubiquitination by Rad6.

Exposure of cells to ultraviolet (UV) light and to many other agents causes the formation of lesions in the DNA. During DNA replication, such lesions located in the template strand block the DNA replication machinery, resulting in a gap in the newly synthesized strand across from the damage site. A variety of postreplicative repair mechanisms have evolved to restore the continuity of the newly synthesized DNA strand (reviewed in Ref. 1).

Genetic studies in the yeast Saccharomyces cerevisiae have been instrumental in identifying the genes involved in postreplicative repair. RAD6 and RAD18, members of the RAD6 epistasis group, play a prominent role in this repair process. Mutations in RAD6 cause extreme sensitivity to UV light and to other DNA damaging agents; rad6 mutants are highly deficient in postreplicative repair of UV-damaged DNA (2) and they exhibit no mutation induction in response to UV (3). RAD6 encodes an ubiquitin conjugating enzyme of 172 residues (4, 5). The first 149 amino acids of Rad6 form a globular domain, while the distal 23 residues, which are predominantly acidic, constitute a freely extending tail domain (6). Mutational inactivation of the active site cysteine 88 residue in Rad6 has indicated that the ubiquitin conjugating activity is essential for all the biological functions of Rad6 (7). Mutants of RAD18 resemble those of RAD6 in their high degree of sensitivity to UV, defects in postreplication repair of UV-damaged DNA (2), and defects in UV mutagenesis (8, 9). However, unlike RAD6, which is indispensable for sporation, mutations in RAD18 do not affect sporation (10).

Other genes that belong to the RAD6 epistasis group include REV1, REV3, REV7, and RAD5. Although mutants of the REV genes show only a marginal increase in UV sensitivity, like rad6 and rad18 mutants, they are defective in UV mutagenesis (3, 11, 12). Rev3 and Rev7 together form a DNA polymerase activity (polζ) that can bypass a thymine-thymine cis-syn-cyclobutane dimer (13). Mutations in RAD5 enhance UV sensitivity to a greater degree than those in the REV genes; however, the incidence of UV mutagenesis at most loci is not affected (14). From these and other genetic observations, it has been suggested that REV genes and RAD5 function, respectively, in the mutagenic and nonmutagenic modes of RAD6, RAD18-dependent postreplication repair.

Rad18 can co-immunoprecipitated with Rad6, indicating physical interaction of the two proteins (15). For delineating the molecular functions of the Rad6-Rad18 complex in postreplicative repair processes, it is essential to purify this complex and to define its biochemical properties. Here, the Rad6-Rad18 complex is purified to near homogeneity from yeast cells genetically tailored to co-overproduce the two proteins. We show that the Rad6-Rad18 complex is heterodimeric and that the Rad6-Rad18 complex has ubiquitin conjugating activity, as well as single-stranded (ss) DNA binding and ssDNA-dependent ATPase activities.

MATERIALS AND METHODS

**Plasmid Construct for Overexpressing Rad6 and Rad18 Proteins**—The DNA fragment with the ADC1 promoter and the RAD6 gene from pSCW242 (5) was cloned in a derivative of a 2-μm multicopy vector, which contains the GAL1 promoter and the URA3 gene. A DNA fragment containing the RAD18 gene from the ATG initiation codon to nucleotide 342 after the termination codon was cloned under the GAL1 promoter in the vector containing the ADC1-RAD6 insert. The plasmid obtained, pR18.36, is shown in Fig. 1A.

**Purification of the Rad6-Rad18 Complex**—Yeast strain LY2 (MATa leu2-3, leu2-112 gal1 reg1-501 pep4-3 ura3-52 trp1), harboring the Rad6-Rad18 overexpressing plasmid pR18.36 (2 μm, ADC1::RAD6 GAL1::RAD6), was grown at 30 °C to midlogarithmic
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phase (1 × 10^6 cells/ml) in synthetic complete medium lacking uracil and diluted with 10 volumes of YPD (yeast extract-peptone-dextrose) supplemented with 1% galactose. The culture was incubated in 10-liter batches in fermentors until a cell density of ∼2 × 10^6 cells/ml was reached (∼12 h). Cells were harvested by centrifugation, washed with ice-cold MgCl2, 2 mM ATP, 0.2 mM DTT, 100 µg/ml bovine serum albumin. The samples were run on a 11% polyacrylamide gel. The gel was dried and subjected to autoradiography.

DNA Binding Assays—DNA binding was examined using the nitrocellulose filter binding method. Nitrocellulose filters (Millipore, HAWP) were prewetted in 0.4 M KOH for 30 min, washed extensively in distilled water, and stored in filter buffer (FB) (25 mM HEPES-KOH, pH 7.0, 1.5 mM KCl). Filters prepared in this manner could be used over a period of at least 1 month. The buffer for DNA binding assays consisted of FB + 50 µg/ml bovine serum albumin and 0.5 mM DTT. Poly(dT) (Pharmacia) was dissolved in TE (10 mM Tris/HCl, pH 7.5, 0.5 mM EDTA, 1 mM DTT), and used as a control. Filters were prewetted at 16°C in buffer containing 0.5 M NaCl and washed after sample application with the same buffer. The flow-through and first 20 ml of the wash were discarded. The second 20 ml of wash was collected and analyzed by SDS-polyacrylamide gel electrophoresis. The resulting rates were calculated by linear regression. The reported values represent the average of at least two independent experiments.

Results

Heterodimer of Rad6 and Rad18 Proteins—To obtain co-overexpression of Rad6 and Rad18 proteins in yeast, we constructed plasmid pR18.36 in which the RAD6 gene is fused to the ADE1 promoter and the RAD18 gene is fused to the GAL1 promoter (Fig. 1A). Plasmid pR18.36 results in 10- and 50-fold overproduction of Rad6 and Rad18 proteins, respectively. Rad6 and Rad18 exist in a complex and were co-purified as such to near homogeneity by a combination of ammonium sulfate precipitation and four column chromatographic steps of Q-Sepharose, phenyl-Sepharose, Mono Q, and Mono Q (see “Materials and Methods” and Fig. 1B). The Rad6 and Rad18 proteins remain associated through all these purification steps (Fig. 1A and D), indicating a high degree of stability of the complex.
Western blot. For the Coomassie staining: extract of 4 different amounts were loaded for the Coomassie staining and for the anti-Rad6 antibodies (amide gel stained with Coomassie Blue. Rad6-Rad18 (was affected because of the association with Rad18 by testing (4, 5). We examined if the Rad6 ubiquitin conjugating activity—behavior of free Rad18 protein suggests that proper folding of the DNA agarose (data not shown). The poor chromatographic behavior of free Rad18 protein was subjected to fractionation in various chromatographic matrices, including DEAE-Sephacel, Q-Sepharose, Bio-Rex 70, and single-stranded DNA agarose (data not shown). The poor chromatographic behavior of free Rad18 protein suggests that proper folding of the protein may be affected via its association with Rad6 protein. The Rad6-Rad18 Heterodimer Has Ubiquitin Conjugating Activity—Rad6 protein has ubiquitin conjugating (E2) activity (4, 5). We examined if the Rad6 ubiquitin conjugating activity was affected because of the association with Rad18 by testing the ability of Rad18-bound Rad6 protein to form a thioester conjugate with ubiquitin (Fig. 2A) and to transfer the conjugated ubiquitin to a protein substrate (Fig. 2B). Since a thioester linkage can be easily disrupted by treatment with a thiol reducing agent such as dithiothreitol or β-mercaptoethanol, to detect formation of a thioester adduct between ubiquitin and Rad6, the results of two electrophoreses without and with prior boiling in the presence of dithiothreitol were compared (Fig. 2A, compare lanes 1–3 to lanes 4–6). In this assay, GST-ubiquitin was used because it could be easily radiolabeled with 32P (18). In the presence of ATP and the ubiquitin-activating (E1) enzyme Uba1, Rad6 forms a thioester adduct with GST-32P-ubiquitin (compare lanes 2 and 5, Fig. 2A). Rad18-associated Rad6 also forms a thioester with GST-32P-ubiquitin in the absence of Uba1 and ATP, and the reaction occurs to the same extent seen with free Rad6 (Fig. 2A, compare lanes 3 and 6). Thus, the binding of Rad18 to Rad6 does not prevent the latter from interacting with Uba1 and forming a thioester conjugate with ubiquitin.

Histone H2B (5) was used as the test substrate to examine the ability of Rad18-associated Rad6 to catalyze the formation of ubiquitin conjugates with protein substrates. The results in Fig. 2B show that H2B is a substrate for Rad18-associated Rad6, thus indicating that the Rad6-Rad18 complex retains ubiquitin conjugating activity.

Rad6-Rad18 Complex Binds DNA—We used nitrocellulose filter binding to examine the interaction between the Rad6-Rad18 heterodimer and 32P-labeled DNA. In this assay, protein bound DNA molecules are retained on the nitrocellulose filters,
whereas free DNA flows through the filters, thus allowing determination of the amount of radiolabeled DNA bound by Rad6-Rad18 complex by measuring the radioactivity associated with the filters. The data presented in Fig. 3 show that the Rad6-Rad18 complex binds the single-stranded polynucleotide substrate poly(dT) in a protein concentration-dependent manner (Fig. 3A). Binding of the polynucleotide reaches the maximal level at 0.25 μM Rad6-Rad18 complex.

We also determined the relative affinities of the Rad6-Rad18 complex for poly d(T) and double-stranded DNA. This was carried out by examining the ability of unlabeled linear double-stranded DNA to compete with radiolabeled poly(dT) for binding to the Rad6-Rad18 complex. The data presented in Fig. 3B were obtained from experiments wherein 0.1 μM Rad6-Rad18 complex was incubated with 2 μM 32P-labeled poly(dT) for 10 min before addition of increasing amounts of unlabeled competitor dsDNA (linearized pUC19) or poly(dT) and further incubation for another 10 min. Each aliquot was filtered and the amount of 32P-labeled poly(dT) remaining bound determined and expressed as a percentage of the binding observed in the absence of added competitor. Rad6-Rad18 complex (0.1 μM) was incubated with a 32P-labeled 300-base pair ds fragment as in B. Unlabeled poly(dT) was added as the competing polynucleotide.

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of dissociation from a radioactively labeled substrate. The presence of ATP has no effect on either the sensitivity to salt (Fig. 4A) or on the rate of dissociation from poly(dT) (Fig. 4B). Thus, we conclude that the DNA binding behavior of the Rad6-Rad18 complex is not modulated significantly by ATP.

Rad6-Rad18 Complex Has ssDNA-dependent ATPase Activity—The Rad18 protein sequence contains a Walker type A nucleotide binding motif GKS found in a variety of proteins that bind and hydrolyze ATP (10). Thus, it was important to determine whether the Rad6-Rad18 complex has ATPase activity. We found that purified Rad6-Rad18 complex hydrolyzes ATP with a dependence on a DNA cofactor, with single-stranded DNA being much more effective than double-stranded DNA in stimulating ATPase activity. For example, Rad6-Rad18 ATPase activity was stimulated 20-fold by M13 ssDNA whereas much less stimulation (2-fold) was observed by M13 dsDNA. The rates of ATP hydrolysis are linear over a 1 h time period, where the concentration of accumulated ADP reaches 50–60 \( \mu M \) (~25% of the total nucleotide concentration), suggesting there is no significant inhibition by ADP. Finally, there is no trace of pyrophosphate released, suggesting that the Rad6-Rad18 complex catalyzes the hydrolysis of ATP to ADP and inorganic phosphate.

We carried out a protein titration in the presence of a constant amount of ssDNA while monitoring the ATP hydrolysis rate. The data in Fig. 6B show that the rate of ATP hydrolysis increases linearly with added protein until an inflection point is reached where all of the available binding sites are occupied. Assuming that all of the available sites on the DNA co-factor are occupied, the site size (\( n \)) calculated for the Rad6-Rad18 complex (reflecting the binding site size) is four nucleotides/Rad6-Rad18 molecule. The \( k_{cat} \) calculated from these data (the maximum rate divided by the protein concentration at the inflection point) is in good agreement with the value calculated previously, where the amount of DNA is well in excess of what was needed to allow all the protein to bind. The pH dependence of the ATP hydrolysis activity shows that between pH 6.0 and pH 8.8 there is no significant difference in the observed rate of hydrolysis (data not shown).

**DISCUSSION**

In this study, we show that Rad18 exists in a stable complex with Rad6. The two proteins co-purify through sequential chromatographic fractionation in columns of Q-Sepharose, phenyl-Sepharose, Mono S, and Mono Q. As judged by Coomassie Blue staining, reverse phase chromatography, and glycerol gradient sedimentation, the purified complex contains an equimolar ratio of Rad6 and Rad18. The Rad6-Rad18 complex represents the first example wherein a ubiquitin conjugating enzyme is physically associated with a DNA-binding protein. This association provides an explanation for the very similar roles of RAD6 and RAD18 in DNA repair and damage-induced mutagenesis.
The Rad6-Rad18 complex forms a thioester adduct with ubiquitin and conjugates ubiquitin to histone H2B. Thus, the complex retains the ubiquitin conjugating activity of Rad6. In addition, we demonstrate that the Rad6-Rad18 complex binds preferentially to ssDNA. Since Rad6 contains no DNA binding motifs and displays no DNA binding ability, the ssDNA binding ability of the Rad6-Rad18 complex very likely derives from Rad18. Consistent with this, Rad18 contains a C3HC4 sequence motif (10), also known as the RING finger motif (20), as well as a CXXC motif (23), either or both of which could be utilized in DNA binding.

The Rad6-Rad18 complex exhibits an ssDNA-dependent ATPase activity with a $k_{cat}$ of 0.18 min$^{-1}$. The high degree of purity of the complex and the precise co-elution of the ATPase activity with the Rad6-Rad18 complex suggest that this activity is intrinsic to this complex. The presence of the “GKS” Walker type A nucleotide binding motif in Rad18 (10), and the absence of any nucleotide binding motif or of any NTPase activity in Rad6 implies that the ATPase activity of the complex resides in Rad18. The fact that the ATPase activity is relatively weak may indicate that the observed activity is a basal rate and only in the presence of an as yet unidentified partner is the rate increased, in a manner analogous to that seen with the stimulation of RF-C ATPase activity by proliferating cell nuclear antigen (21).

How might the DNA binding, ATPase, and ubiquitin conjugating activities of the Rad6-Rad18 complex function in the postreplicative bypass of damaged DNA? The DNA binding activity could target the complex to sites of ssDNA where the DNA replication machinery has been blocked by DNA lesions. As the ATPase activity seems to have no significant effect on the DNA binding activity of the Rad6-Rad18 complex, it is possible that this activity is utilized in Rad18’s role as a molecular matchmaker (22), wherein Rad18 recognizes the protein substrates for ubiquitination by Rad6, and ATP binding and hydrolysis modulate these protein-protein interactions.

The availability of the purified Rad6-Rad18 complex, and the identification of biochemical activities contained within it, now presents the opportunity to identify the components of the replication machinery that are ubiquitinated by the complex and to determine if ubiquitin-dependent degradation of some of the replicative proteins is required for the assembly of the postreplicative bypass DNA repair machinery.

Acknowledgment—We thank P. Sung for discussions.

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Fig. 6. The ATP hydrolysis activity displays Michaelis-Menten behavior. A, ATP hydrolysis experiments were performed as described under “Materials and Methods.” The concentration of poly(dT) and Rad6-Rad18 complex was 20 μM and 1 μM, respectively. Each data point is the average of at least two experiments. The data are fit to a hyperbola of the form $V_{max} = \frac{V_{max} \times [S]}{K_m + [S]}$. B, the stoichiometry of the ATPase activity was calculated as illustrated. ATP hydrolysis experiments were performed as described under “Materials and Methods.” The poly(dT) concentration was constant at 20 μM. The arrow denotes the inflection point of the binding isotherm.